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5.12. REFERENCE STANDARDS

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1. INTRODUCTION

'Reference standard' is used in this chapter as a general term covering reference substances, reference preparations and reference spectra.

Reference standards are frequently necessary to achieve adequate quality control of substances for pharmaceutical use and pharmaceutical preparations.

Reference standards are established using suitable procedures and their continued suitability for use is monitored according to a predefined programme. Where a reference standard is needed, it is an integral part of the pharmacopoeial monograph or the manufacturer's specification. Where a European Pharmacopoeia reference standard is referred to in a monograph or general chapter, it represents the official standard that is alone authoritative in case of doubt or dispute.

Reference materials and certified reference materials are defined below but are not otherwise dealt with in this chapter.

In several parts of the chapter, detailed information is given for chemical reference substances but not for biological reference preparations. The general principles given apply to the latter, but in view of their heterogeneous nature and frequently their complexity compared to chemical reference substances, detailed information on their use, establishment and the re-test programmes applied is not included. For peptide and protein reference standards, a specific approach is used for certain aspects, notably the establishment of an assigned content; this chapter does not deal with that approach.

2. TERMINOLOGY

Primary standard. A standard shown to have suitable properties for the intended use, the demonstration of suitability being made without comparison to an existing standard.

Secondary standard. A standard established by comparison with a primary standard.

International standard. An international standard is a primary standard that defines an International Unit. The equivalence in International Units of an international standard is stated by the World Health Organisation.

European Pharmacopoeia reference standard. A reference standard established under the aegis of and approved by the European Pharmacopoeia Commission.

European Pharmacopoeia Chemical Reference Substance (CRS). A substance or mixture of substances intended for use as stated in a monograph or general chapter of the European Pharmacopoeia. European Pharmacopoeia Chemical Reference Substances are primary standards, except for those (notably antibiotics) that are calibrated in International Units. The latter are secondary standards traceable to the international standard.

European Pharmacopoeia Biological Reference Preparation (BRP). A substance or mixture of substances intended for use as stated in a monograph or general chapter of the European Pharmacopoeia. European Pharmacopoeia Biological Reference Preparations are either secondary standards calibrated in International Units or primary standards, which may be used to define a European Pharmacopoeia Unit. Other assigned values may also be used, for example, virus titre, or number of bacteria.

Reference Material (RM). A material or substance, one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.

Certified Reference Material (CRM). A reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure that establishes its traceability to an accurate realisation of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence.

NOTE: pharmacopoeial reference standards are to be distinguished from reference materials and certified reference materials, which may be used in several contexts using a variety of analytical techniques for quantitative purposes. The use of reference materials is required or recommended in a number of monographs and general chapters of the pharmacopoeia, notably for calibration or verification of satisfactory performance of instruments.

The specificity of pharmacopoeial reference standards has been officially recognised in the introduction of ISO Guide 34 - *General requirements for the competence of reference material producers - (Second Edition 2000)*: "Pharmacopoeia standards and substances are established and distributed by pharmacopoeia authorities following the general principle of this guide. It should be noted, however, that a different approach is used by the pharmacopoeia authorities to give the user the information provided by the certificate of analysis and the expiration dates. Also, the uncertainty of their assigned values is not stated since it is negligible in relation to the defined limits of the method-specific assays of the pharmacopoeias for which they are used."

3. USE OF REFERENCE STANDARDS

Reference standards are employed in the identification, purity testing and assay of substances for pharmaceutical use and pharmaceutical preparations. Reference standards are shown to be suitable for their intended purpose; they are not necessarily suitable for other purposes. If a reference standard is to be used for any purpose other than that for which it has been established, its suitability for the new use has to be fully demonstrated. Any value assigned to a reference standard is valid for the intended use and not necessarily for other uses.

A European Pharmacopoeia reference standard with an assigned content/potency for use in the assay of a substance for pharmaceutical use may be suitable to determine the content of that substance in a pharmaceutical preparation where all the following conditions are fulfilled:

- the chromatographic assay method described in the active substance monograph is employed;
- the user verifies the applicability of the method to the particular pharmaceutical preparation (absence of interference);
- any pre-treatment of the sample (e.g. extraction) is validated for the particular pharmaceutical preparation;
- the use is approved by the competent authority.

Reference standards are also established for the determination of the content of components of herbal drugs and herbal drug preparations. These may be: the active principles themselves; marker constituents used for quantification; or extracts. Reference standards consisting of extracts are established using well-characterised samples of active principles or marker constituents.

It is the policy of the European Pharmacopoeia to supply reference standards in adequate quantities for immediate use after opening of the container. Use in other conditions is the responsibility of the analyst. If an unopened container is stored in the recommended conditions, it remains suitable for use as long as it is of the current batch. Information on current batch numbers is provided in the reference standards catalogue available from the European Directorate for the Quality of Medicines & HealthCare (EDQM). Storage of solutions of reference standards is not recommended unless suitability has been demonstrated by the user.

Secondary standards. A secondary standard may be used for routine quality control purposes for any of the uses described above for primary standards, provided that it is established with reference to the primary standard. A secondary standard is established and employed to reduce the use of the primary

standard, which requires more extensive characterisation and evaluation and may be available only in a limited quantity. A secondary standard is used only for the same purpose as the primary standard with reference to which it has been established.

4. ESTABLISHMENT OF REFERENCE STANDARDS

4-1. *PRIMARY STANDARD*

A substance or preparation to be established as a primary standard is characterised by a variety of analytical techniques chosen to demonstrate its suitability for use.

For substances for pharmaceutical use and their impurities, relevant parts of the following test programme are usually applied.

- Characterisation of the substance (structural elucidation) by appropriate chemical attributes such as structural formula, empirical formula and molecular weight. A number of techniques may be used including:
  - nuclear magnetic resonance spectrometry;
  - mass spectrometry;
  - infrared spectrophotometry;
  - elemental analysis.
- Determination of the purity:
  - determination of the content of organic impurities by an appropriate separation technique or spectrometric method, where applicable;
  - quantitative determination of water;
  - determination of the content of residual solvents;
  - determination of loss on drying, which may in certain circumstances replace the determinations of water and residual solvents;
  - determination of inorganic impurities (test for heavy metals, sulfated ash, atomic spectrometry, inductively coupled plasma spectrometry, X-ray fluorescence); the results are not used in determining an assigned content, except where they would have an appreciable impact upon it;
  - determination of the purity by an absolute method (e.g. differential scanning calorimetry or phase solubility analysis where appropriate; the results of these determinations are used to support and confirm the results obtained from separation techniques; they are not used in the calculation of the assigned value).

For a primary chemical reference substance to be established for assay purposes, the assigned content is generally calculated from the values obtained from the analyses performed for the determination of impurities (organic, inorganic, water and solvents) by applying the principle of mass balance; other suitable methods are also used.

An establishment report for the reference standard is prepared and approved by the qualified person.

4-2. *EUROPEAN PHARMACOPOEIA REFERENCE STANDARDS*

The candidate standards are tested against a wide variety of analytical methods. The extent of testing and the number of laboratories involved depends on the use of the reference standard. Compliance with the relevant monograph is usually required, unless otherwise justified.

Where a collaborative trial is carried out during establishment, a protocol is provided for each participant and only valid results derived according to the protocol are used for establishing an assigned value or otherwise confirming suitability.

For chemical reference substances, relevant parts of the following programme are typically applied.

4-2-1. **Identification**. In general, a batch selected from the normal production of the substance is satisfactory. It is shown to comply with the requirements of the monograph; full structural elucidation is carried out for the first batch.

4-2-2. **Related substances test**. A reference standard corresponding to an impurity is characterised for identity and purity. Where a reference standard is used to determine the content of a given impurity, the preferred minimum content is 95.0 per cent; where this is achieved no assigned value is given, the content being considered as 100.0 per cent; this approximation is acceptable since there will be no appreciable effect on the determination of impurities. When this minimum content cannot be obtained, the standard has an assigned content.

If an impurity is not available in a sufficient quantity to establish a reference standard, a number of other options exist:

- preparation of a reference standard that contains a mixture of the compound(s) and the impurity or impurities;
- preparation of a reference standard containing a mixture of specified impurities.

Where such a mixture is also used to determine the content of a given impurity, the content of the impurity in the reference standard is determined by appropriate separation methods and a value assigned to the reference standard.

4-2-3. **Assay**

4-2-3-1. *Chemical assay.* When a reference standard is to be used for quantitative determination of an active substance or an excipient (assay standard), the extent of testing is greater. In general, several collaborating laboratories examine the proposed substance, following a detailed protocol that describes the procedures to be followed. The results obtained are used to assign a content. It is particularly important to quantify the impurities if a selective assay is employed. In such a case, it is best to examine the proposed substance by additional analytical procedures that are scientifically justified, including, where possible, absolute methods.

If a reference standard is required for a non-chromatographic assay method (e.g. colorimetry or ultraviolet spectrophotometry), the relative reactivity or relative absorbance of the impurities present in a substance must be checked to ensure that they are not markedly different from those of the substance.

A protocol is prepared and must be strictly followed by the participants of the collaborative trial to assign the content. The protocol usually requires:

- determination of water (or loss on drying);
- estimation of the organic impurities (including residual solvents when appropriate) using the prescribed separation techniques;
- and possibly, determination of the content of the substance by an absolute method; this would be a confirmatory determination not necessarily performed by all participants and the results would not be used in the calculation of the assigned value.

The protocol also indicates the system suitability tests and acceptance criteria for each of the tests performed.

Unless otherwise stated, an assigned value is given for the substance or preparation as presented in the container ('as is'), and the contents are not to be dried before use. For assay standards prepared by lyophilisation the content of the pure substance is indicated in milligrams or International Units per vial.

4-2-3-2. *Microbiological assay.* A reference standard for the microbiological assay is first shown to comply with the monograph. If the results are satisfactory a collaborative microbiological assay is carried out, using the international standard. The potency is expressed in International Units. If an international standard does not exist, European Pharmacopoeia Units are used. The assigned potency is calculated from the results of a collaborative trial. Various validity criteria are applied including parallelism, linearity, and quadratic fit, according to the usual statistical procedures (*5.3*). The assigned potency with the confidence limits is calculated from statistically valid results.

4-2-3-3. *Assay of components of herbal drugs and herbal drug preparations.* Reference standards used in monographs of herbal drugs vary in the extent of testing depending on the type of reference standard.

- An active component or marker constituent is characterised and evaluated for identity and purity; a value for content is assigned irrespective of the purity.
- An extract is used as a reference standard when insufficient active principle or marker constituent is available. The assigned content of the extract is established by means of a collaborative trial using a well-characterised sample of the active principle or marker component for which a value is to be assigned.

4-2-4. **Establishment report**. A report containing the results of the establishment study as well as information concerning the use of the reference standard is prepared. The report for a chemical assay standard has a value assigned to the substance with the rationale for attributing that value. The estimated uncertainty of the assigned value is calculated, and where it is less than a predefined value, which is considered to be negligible in relation to the acceptance criteria for the assay, then the study is accepted. Otherwise, the trial may be repeated, in whole or in part, or the limits defined for the pharmaceutical substance may be widened. The uncertainty of the assigned value is not given as part of the information provided with the reference standard, since the precision of the method and the uncertainty of the value attributed to the reference standard are taken into account when setting the limit(s) in a monograph.

4-3. *SECONDARY STANDARD*

A secondary standard should exhibit the same property or properties as the primary standard, relevant for the test(s) for which it is established. The extent of testing is not so great as is required for the establishment of a primary standard. The secondary standard is established by comparison with the primary standard to which it is traceable. An official primary standard is used wherever possible for establishment of secondary standards.

Identification

- For use in infrared spectrophotometry: the absorbance bands correspond in position and relative size to the absorbance bands of the primary standard.
- For use in separation techniques: the migration distance, migration time and retention time of the secondary standard are the same as those of the primary standard for thin-layer chromatography or electrophoresis, capillary electrophoresis and gas or liquid chromatography respectively.

Purity test. For use in separation techniques: as for identification but when used for quantification, a content relative to the signal from the primary standard is to be established.

Assay. Secondary standards are assayed against a primary standard with an assigned content or potency. The property for which a value is to be assigned for the secondary standard is similar in magnitude to that of the primary reference standard with which it is compared. Both the number of independent replicate determinations to be performed and the acceptance criteria to be applied are predefined.

5. PRODUCTION, LABELLING, STORAGE AND DISTRIBUTION

5-1. *PRODUCTION*

All operations are carried out according to the relevant norms of best practice to ensure the traceability and integrity of the reference standard. The production record includes information regarding filling, labelling and storage. Reference standards are dispensed into containers under appropriate filling and closure conditions, to ensure the integrity of the reference standard. The containers employed may be multi-use or single use, but the latter is preferred to minimise the risk of decomposition, contamination, or water uptake.

5-2. *LABELLING*

The labelling bears the name of the reference standard, the name of the supplier, the batch number, and any other information necessary to the proper use of the reference standard. If used as an assay standard the following information is also given:

- the assigned percentage content;
- or, the content in milligrams or millilitres of the chemical entity in the container;
- or, the assigned potency (for biological assays or microbiological assays) in units either per milligram or per vial.

For a manufacturer's reference standard, the label indicates a re-test or expiry date. For European Pharmacopoeia reference standards, no re-test or expiry date is given since the re-test programme (see below) monitors continued fitness for use.

Leaflets. An accompanying explanatory leaflet may also be provided giving information needed for correct use of the reference standard. An explanatory leaflet is considered as part of the labelling. Where stated in a monograph, a chromatogram is included in the leaflet.

5-3. *STORAGE AND DISTRIBUTION*

Reference standards are to be stored and distributed in conditions suitable to ensure optimal stability.

European Pharmacopoeia reference standards. European Pharmacopoeia reference standards are mostly stored in temperature-controlled rooms at 5 ± 3 °C. However, a number of reference standards that are relatively unstable are stored at – 20 ± 5 °C or, in a few cases (e.g. live virus preparations), at – 80 ± 10 °C, and for cell cultures, under liquid nitrogen (– 180 °C).

Special packaging is employed to minimise the risk of damage during transport.

Reference standards that are normally stored at 5 ± 3 °C are dispatched by normal mail since short excursions from the long-term storage temperature are not deleterious to the reference standard. Reference standards stored at – 20 °C are packed on ice and dispatched by express courier. Reference standards stored at – 80 °C or stored under liquid nitrogen are packed on solid carbon dioxide and dispatched by express courier.

6. RE-TEST PROGRAMME

A system is established and implemented to ensure the continued fitness-for-use of the reference standards. Normally, a re-test programme is applied, taking account of the known physico-chemical properties and stability data for the reference standard. Reference standards are periodically tested for stability during storage. A monitoring programme is applied that is designed to detect at an early stage any sign of decomposition using appropriate analytical techniques. The methods employed should be chosen from amongst those performed during establishment so that baseline data are available.

The periodicity and extent of re-testing reference standards depends on a number of factors including:

- stability;
- container and closure system;
- storage conditions;
- hygroscopicity;
- physical form;
- intended use;
- presentation (single use/multiple use).

Most reference standards are presented in powder form but some are prepared as solutions. Preferably, reference standards are presented as single-use units. However, if the standard is presented in multi-use containers then re-testing may be more frequent for hygroscopic or oxygen-sensitive substances. The testing methods include the determination of water and decomposition products (where known). The re-test period may be lengthened with the support of sufficient data. The

maximum permitted variation from the assigned value should be pre-defined, and if exceeded, the batch should be re-established or replaced.

European Pharmacopoeia reference standards. The monitoring programme of the EDQM includes a selection of the following tests, chosen for their rapidity, sensitivity and applicability to small quantities:

- determination of water, loss on drying and/or thermogravimetric analysis;
- estimation of impurities by stability-indicating separation techniques;
- where appropriate, determination of the molar purity by differential scanning calorimetry;
- application of other specific tests for detecting impurities.

Any significant differences observed compared with the last examination will lead to more extensive examination of the batch and, if necessary, to the establishment of a replacement batch.