GUIDELINES FOR THE ESTABLISHMENT, HANDLING, STORAGE AND USE OF ASEAN REFERENCE SUBSTANCES

Issued under ASEAN Working Group on Technical Cooperation in Pharmaceuticals Committee on ASEAN Reference Substances 2005
PREFACE

ASEAN Reference Substances are established as part of regional cooperation among the ASEAN countries to ensure the quality, safety and efficacy of pharmaceutical in the ASEAN region. These standards are validated in the national laboratories of the ASEAN member countries and are formally adopted after stringent qualitative and quantitative tests are carried out the candidate substances.

The establishment of reference substances is based on reports in which results of analytical testing have been evaluated. This guideline sets out the general requirement for the establishment of reference substances. It provides the guidance in conducting collaborative studies (chemical and microbiological) to demonstrate that the reference preparation is suitable for its intended use. Handling, storage and use of reference substances are also included.

The members of ASEAN reference substances project wish to express their appreciation to Dr. A.H. Thomas, Pharmaceutical Assessor, Medicine Control Agency, United Kingdom, (WHO Temporary Advisor to the 10th Meeting on the Production and Utilization of ASEAN Reference Substances) for his valuable tasks in revision of this guideline. Special thanks are also extended to JPMA for their financial support and to World Health Organization for the kind cooperation.

First revision of this guideline was endorsed in the 16th Meeting of the ASEAN Working Group on Technical Cooperation in Pharmaceuticals held in Singapore during 4-6 February, 1998. The guideline was revised in June 2005. Comments are invited from interested persons for future revision. Kindly address your comments to Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health, Tiwanon Road, Nonthaburi 11000, Thailand. Tel. (662) 5910000 ext. 99103, Fax (662) 5805733 or www.aseanrs.org

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Introduction

Pharmaceutical reference standards are needed to ensure accuracy and reproducibility of analytical results used for pharmacopoeial testing and pharmaceutical control. Reference standards are prepared for use as the standard in an assay, identification, and purity test, which should be considered for its intended use(s). Pharmaceutical reference standards are categorized into primary and secondary reference standards.

A primary reference standard is widely acknowledged as having appropriate qualities within a specified context and whose value is accepted without reliance on comparison to another reference material. Primary reference standards can be obtained from:

a) WHO Collaborating Centre for Chemical Reference Substances
b) European Pharmacopoeia Commission
c) British Pharmacopoeia Commission Laboratories
d) United States Pharmacopeial Convention Inc.

A secondary reference standard is a substance whose characteristics are assigned and/or calibrated by comparison with a primary reference standard. The evaluation of a secondary reference standard may be less extensive than for a primary reference standard. ASEAN Reference Substances (ARS) are classified as secondary reference standard.

Uses of Reference Standards

Analytical methods in current pharmacopoeias that may require the use of reference standards are:

a) Infrared (IR) spectrophotometry for identification or quantitation.
b) Ultraviolet (UV) absorption spectrophotometry for quantitation.
c) Colourimetric assays for quantitation.
d) Chromatographic methods for identification or quantitation.
e) Other separation techniques for quantitation.
f) Non-stoichiometric titrations for quantitation.
g) Assay methods based on measurement of optical rotation.
h) Microbiological assays.
i) Immunochemical tests.
j) Calibration of instruments.

Process for Establishment of ARS

1. Procurement of Bulk Material

The candidate substance should be obtained from a reliable source such as a major manufacturer. In general, a selected batch of good quality from the normal production process is satisfactory. The amount needed may vary from 20g to 500g depending on the expected demand for reference standards. The substances should be supplied with certificate of analysis which contains information needed e.g. assay value / potency and optimal storage conditions.

The purity requirements for a candidate substance depend upon the intended use of the standard. A reference standard proposed for assays should possess a high degree of purity, e.g. 99.5% on
the dried/anhydrous or volatiles free basis or higher. On the other hand, reference standards to be used for identification or other analytical method of low selectivity does not require excessive purification, since the presence of even several percent of impurities in a substance usually has no noticeable influence on the test.

2. Preliminary Assessments
Prior to start testing, the bulk material received in a single container or in a several vials should be mixed and shaken properly for completely homogeneous.

Preliminary assessments to confirm the identity, purity, content of active substances and potency of antibiotics using pharmacopoeial methods, which may include HPLC, thin-layer chromatography and other physicochemical methods should be performed by coordinating laboratory.

The reference standard for microbiological assay are first tested by the chemical methods of the monograph. If the results are satisfactory a microbiological assay is carried out, using primary reference standard.

3. Protocol for Collaborative Assay
The protocol for evaluation of proposed ARS (PARS) prepared by coordinating country should contain the appropriate pharmacopoeial methods in order to confirm the identity and to determine the purity of the substance, to certify values as needed for the intended use by comparison to the primary reference standard to which it is traceable and to demonstrate suitability for intended use.

The pharmacopoeial methods are;
1. For identification
   - Infrared spectroscopy: the IR spectrum should concordance with the primary reference standard.
   - Chromatographic techniques, HPLC, GC and TLC: the retention time, migration distance and migration time for PARS should corresponds to those of the primary reference standards within pre-defined tolerances.
   - Other methods; UV-absorption, melting point, differential scanning calorimeter.
2. For purity test
   - Chromatographic techniques, HPLC, GC and TLC: organic purities or related substances should be evaluated as limit test or quantified by comparing with primary reference standard.
3. For assay
   - PARS are assayed against the primary reference standard with an assigned content or potency. The content of reference standard for chemical assay should be determined by selective method (HPLC), including where possible, absolute method (volumetric titration). The content is expressed on the dried basis or on anhydrous basis.

The potency of reference standard for microbiological assay should be determined by large plate, latin square method as per BP or Ph. Eur. The potency are calculated from statistically valid results and expressed in International Units or microgram per milligram. Detail of collaborative trials of ARS use for microbiological assay is appeared in Appendix 1.

The number of independent replicate determinations to be performed and acceptance criteria to be applied must be pre-defined. The results must be shown to comply with the requirements of the monograph in selected pharmacopoeia.
4. Collaborative laboratories

Collaborative laboratories are National Laboratories of ASEAN member countries. PARS employed as chemical and microbiological assay standards should be tested in, at least, 3 collaborative laboratories.

A sufficient amount quantity of candidate substance for analytical examination should be packed in amber glass vials and distributed to collaborative laboratories with assay protocol and certificate of analysis. The rest of candidate substances should be stored in a suitable condition in a tight, light resistant container at a temperature about 5°C or below 0°C. Detail for filling of PARS is appeared in Appendix 2.

5. Evaluation of Results and Report

Reference standards for pharmaceutical quality control require careful evaluation by the issuing body to ensure that the substances are suitable for the intended use.

After complete the tests in protocol, collaborative laboratories should send full data of all tests to coordinating laboratory. The results of each assay should be submitted on the attached report sheets and should include full details of all weighing and dilution steps carried out and the numerical values of all responses measured (raw data). The IR spectra, UV-absorption spectra, TLC and HPLC chromatograms should also be attached. The data will be summarized and tabulated on the basis of the characteristic test. The table includes name of laboratory, test methods, individual result, laboratory mean and corresponding standard deviation/ relative standard deviation.

The assay results of chemical reference standard that passed the pharmacopoeial specification are evaluated by using Z-score. The assay value with Z-score less than 2 are acceptable then the average value with %RSD will be reported for consideration and adoption.

The procedures for combination of the results from collaborative trials of PARS for microbiological assay are detailed in Appendix 3.

A report containing the results of establishment study is prepared by coordinating country. The report is reviewed and approved by the relevant group of experts from member countries before submission to the ARS Meeting for adoption.

Storage and Distribution

ARS packed in tight, light resistance container are stored in refrigerator or cold room controlled at between 2°C and 8°C. Some substances and antibiotics are stored below 0°C.

Special packaging is employed to minimize the risk of damage during transport. Substances which are normally stored at about 4°C are dispatched by normal mail while substances stored below 0°C are packed on ice or solid carbon dioxide and dispatched by express courier.

Re-test Programme

ARS should be monitored by regular re-examination to ensure their stability during storage. A re-test programme is designed to detect any sign of decomposition using appropriate analytical techniques. The testing methods should be rapid and sensitive, consume small quantities of the existing stock and have been performed during the establishment phase. The monitoring programme include the following tests:
-determination of water, loss on drying;
-estimation of impurities by HPLC or TLC;
determination of content;
determination of purity by DSC, when appropriate.

It is recommended that re-testing be undertaken by coordinating laboratory in charge of initial examination of specified ARS. The maximum permitted variation from the assigned value should be pre-defined and the replacement should be made when a significant change in a property is observed. This monitoring programme made the expiry date for these substances unnecessary.

The frequency of re-testing depends on many factors; physico-chemical properties of each substance (e.g. physical form, hygroscopicity), container and closure system, storage conditions and intended use. ARS are recommended to re-test in a period of three years for reference standard used in chemical assay, two years for some antibiotics used in microbiological assay and one year for some highly sensitive substances.

**Information Supplied with Reference Standard**

Labels and certificate of analysis of adopted ARS will be prepared by Thailand, coordinator of ARS project, for all member countries. Labels of ARS should give the following information.

1. Name of reference standard, the international non-proprietary name (INN) should be used where ever possible.
2. Name and address of the issuing body
3. Approximate quantity of reference standard in the container
4. Batch or control number

The certificate of analysis should incorporate relevant items as listed in the label and should contain the following information, as necessary.

1. Description of the physical nature
2. Information about assigned value needed for calculation of the results of tests.
3. Intended use.
4. Direction for use (e.g. storage and handling)
5. Recommended storage conditions (if special conditions apply)
6. Health hazards information.

It is recommended that the data provided limited to what is necessary for the proper use of the substances in the tests and assays.
References

COLLABORATIVE TRIAL FOR MICROBIOLOGICAL ASSAYS

1. Weighing and Number of assays.
The number of assays performed should be six, over a period of three days, with two independent weighings of the standard per day and two independent weighings of the proposed standard per day. This leads to the following scheme of assays:

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<th>Assay</th>
<th>Day</th>
<th>Standard Weighting</th>
<th>Proposed Standard Weighting</th>
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If substance presents problems in weighing (e.g., hygroscopic) then special precautions should be taken. Specifically, the independent weighings should all be done on the one day and some assays should be done to verify that independent weighings do not differ by more than ± 5%. For instance weighings 1 and 2 from day 1 (standard) could be assayed, and weighings 1 and 2 from day 3 (proposed standard) could be assayed, giving an extra two assays performed.

If the standard is only available as a known quantity of international units per ampoule and the total contents are meant to be used, then two such ampoules should be used each day in place of the weighings.

2. Assay Design

Parallel line assays should be done with three doses equally spaced on a logarithmic scale. The doses must come from the linear of the dose-response curve which is to be established prior to setting up the assays. If large plates are available then a Latin square design should be used to randomize the application of the doses. The number of replicates of each of the three doses of standard and proposed standard should be six. A 6 x 6 Latin square will ensure the necessary six replicates per assay. If petri dishes are used then six inch dishes are recommended so that there is enough space on the dish to place the six doses (i.e., three standard doses and three proposed standard doses). The number of petri dishes (replicates) should be at least six and probably twelve per assay.

3. Reporting of Results

Each laboratory should submit full details of the experimental work performed. For each assay the results should be submitted on the attached report sheets and include full details of all weighings and dilution steps carried out, the dose levels employed and the numerical values of all responses measured (raw data).
4. Analysis of the Assays

Analysis of variance needs to be employed to investigate departures from linearity, parallelism and difference in curvature. If an assay shows either significant parallelism or significant difference in curvature then it should be declared invalid. Invalid assays should not be used when combining the results. The 5% level of significance is recommended for the analysis of variance, however it needs to be born in mind when using the 5% level that 1 in 20 assays can be expected to show a significant result and hence rejection of an assay may not always be appropriate. If the assay is significantly non-linear then a transformation should be applied to the responses to achieve linearity. Usually this transformation may sometimes give better linearity.

A further constraint on the individual assays is their precision. The precision of each single assay should be such that the fiducial limits of error are not less than 95% and not more than 105% of the estimated potency.
FILLING

Amber, neutral glass vials (4ml) fitted with bakelite screw caps and linked with silicone are used for packing ARS. Before use, the vials and caps must be washed thoroughly with liquid detergent, rinsed several times with distilled water and dried in an oven at 50°C. The dried vials are individually numbered and placed in special stainless steel trays and covered with aluminium foil.

The distribution and packing of the ARS is carried out in a glove-box. The interior surfaces of the glove-box must be thoroughly cleaned before use to avoid cross-contamination of other substances. The equipment, apparatus, vials and also the bottle containing the bulk material are introduced into the glove-box via the hatch. The glove-box is conditioned to achieve a relative humidity of less than 20%. Once this condition is achieved distribution may commence. Each vial is manually and carefully filled with approximately 100-200 mg of ARS, using a micro stainless steel spatula. The filling process follows the sequence of numbers indicated on the vials. When the filling process is completed, the caps are tightly secured. Powder spilt on the vials must be wiped clean. Labels are attached, indicating the name of ARS, the batch number, content / potency, amount and date of packing. To avoid moisture uptake, it is desirable to pack the filled vials in a box with dried silica gel and seal with tape.
**Combination of Results of Microbiological Assay**

The combination follows a two-stage procedure whereby the results of the individual assays are combined first to obtain laboratory estimates of potency and then the laboratory estimates are combined to obtain a final estimate.

The first stage in assay combination involves combining the individual assay results within each laboratory. It is recommended that each assay to given a weight \( W \) using:

\[
W = \frac{1}{V_i} \quad --------(1)
\]

Where \( V_i \) is the variance of the log potency of assay \( i \).

Supposing there are \( n \) assays in the laboratory. These \( n \) assays should be tested for homogeneity using the formula:

\[
\chi^2 = W(M - \bar{M})^2 \quad --------(2)
\]

and alternative formula being

\[
\chi^2 = \sum W M^2 - (\sum W M)^2 / \sum W \quad --------(2a)
\]

If the calculated \( \chi^2 \) is smaller than the tabulated value corresponding to \( (n-1) \) degrees of freedom \((P=0.95)\) the potencies are homogeneous and the mean log potency of the laboratory is calculated from:

\[
\bar{M} = \sum W M / \sum W \quad --------(3)
\]

The variance of the mean log potency of the laboratory is taken as the reciprocal of the sum of the individual assay weights:

\[
V(\bar{M}) = 1/W \quad --------(4)
\]

The degrees of freedom in the variance of the laboratory mean log potency is approximately equal to the sum of the degrees of freedom in the variance of the individual assays.

If the calculated \( \chi^2 \) is greater than the tabulated value the potencies are heterogeneous. This means the variation between individual estimates of \( M \) is greater than would have been predicted from the estimates of fiducial limits. In these circumstances the unweighted mean log potency and variance of the laboratory may be calculated:

\[
\bar{M} = \sum M / n \quad --------(5)
\]

\[
V(\bar{M}) = \frac{\sum(M - \bar{M})^2}{n-1}/n \quad --------(6)
\]

The variance of the laboratory log potency when calculated in this manner has degrees of freedom equal to \( n-1 \).

The second stage of the assay combination involves combining the individual laboratory results. A similar procedure applies, each laboratory is given a weight \( W \) calculated by:

\[
W_j = 1/V_j
\]

Where \( V_j \) is the variance of the log potency of the laboratory \( j \) (calculated by using equation (4) or equation (5) above). The log potencies form the \( k \) laboratories should then be tested for homogeneity using the formula

\[
\chi^2 = \sum W (M - \bar{M})^2
\]

or its alternative.
The calculated value should be compared with the tabulated value corresponding to (k-1) degrees of freedom P = 0.95. The laboratory’s values should be accepted as being homogeneous if the $\chi^2$ value is less than the tabulated value, otherwise the results should be considered heterogeneous. For homogeneous results the combined log potency and its variance are calculated as before. The degrees of freedom in this variance is approximately equal to sum of the degrees of freedom in the variances of the individual laboratory log potencies.

If the laboratories are significantly heterogeneous then the most discrepant individual assays should be removed. Plotting the distribution of the estimated potencies will assist in identifying the discrepant assays. These will be the ones which are furthest from the mean potency value. After the removal of each assay $\chi^2$ is calculated again.

Once the laboratory mean potencies are homogeneous then no further assays need be removed. Note, however, that a limit should be set on the number of assays removed. There should be a minimum of 20 assays remaining after removal of the discrepant assays. If after reaching the 20 assays limit the remaining potency estimates are still heterogeneous then an unweighted mean and variance should be calculated as before.

The fiducial limits of the final potency are then calculated. Provided these fiducial limits are within 3% of the final potency then the results may by accepted. If not then the collaborative assay should be repeated.