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**Conference Reports**

# Analytical methods validation: Bioavailability, bioequivalence and pharmacokinetic studies

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This is a summary report of the conference on 'Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies.' The conference was held from December 3 to 5, 1990, in the Washington, DC area and was sponsored by the American Association of Pharmaceutical Scientists, U.S. Food and Drug Administration, Fédération Internationale Pharmaceutique, Health Protection Branch (Canada) and Association of Official Analytical Chemists. The purpose of the report is to represent our assessment of the major agreements and issues discussed at the conference. This report is also intended to provide guiding principles for validation of analytical methods employed in bioavailability, bioequivalence and pharmacokinetic studies in man and animals. The objectives of the conference were: **(1)** to reach a consensus on what should be required in analytical methods validation and the procedures to establish validation; **(2)** to determine processes of application of the validation procedures in the bioavailability, bioequivalence and pharmacokinetic studies; and **(3)** to develop a report on analytical methods validation (which may be referred to in developing future formal guidelines). Acceptable standards for documenting and validating analytical methods with regard to processes, parameters or data treatments were discussed because of their importance in assessment of pharmacokinetic, bioavailability, and bioequivalence studies. Other topics which were considered essential in the conduct of pharmacokinetic studies or in establishing bioequivalency criteria, including measurement of drug metabolites and stereoselective determinations, were also deliberated.

## Introduction

Analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples play a significant role in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. It is essential to employ well characterized and fully validated analytical methods to yield reliable results which can be satisfactorily interpreted. It is recognized that analytical methods and techniques are constantly being changed and improved; and, in many instances, they are at the cutting edge of the technology. It is also important to emphasize that each analytical technique has its own characteristics which will vary from drug to drug. Moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study. Specific validation criteria are needed for methods intended for analysis of each analyte (drug and/or metabolite). While validation of each method will stand on its own, there may be situations where comparison of the methods will be necessary, e.g., when more than one method has been employed in a long-term study. When sample analysis is conducted at more than one site, it is necessary to validate the analytical method(s) at each site and provide appropriate validation information for different sites to establish inter-laboratory reliability. Unless a method is used on a regular basis that provides confidence in its continued validity, it is essential to document that the method is still valid prior to analysis of samples in the study. Adequate validation for the above purpose often consists of running a standard curve with new quality control

samples to show that the responses, relationship and general characteristics of the method are similar to previous validation results.

## Analytical Method Validation

Method validation includes all of the procedures required to demonstrate that a particular method for the quantitative determination of the concentration of an analyte (or series of analytes) in a particular biological matrix is reliable for the intended application. Some of the more commonly employed bioanalytical techniques include: (1) chemical methods, such as chromatography (GC, HPLC), a variety of procedures using mass spectrometry (MS) methods (such as direct MS, MS-MS, and combination techniques, for example, GC-MS and LC-MS); and (2) biological methods such as those based on immunoassay procedures (RIA, EMIT and ELISA) and microbiological methods. Many of the principles, procedures and requirements are common to all types of analytical methodologies.

The parameters essential to ensure the acceptability of the performance of an analytical method are stability of the drug in the matrix under study storage conditions, accuracy, precision, sensitivity, specificity (selectivity), response function and reproducibility. Although there are various stages in development and validation of an analytical procedure, the validation of an analytical method can be envisaged as consisting of two distinct phases: (1) the development phase of the analytical method in which the assay is defined; and (2) application to actual analysis of samples from pharmacokinetic, bioavailability and bioequivalence studies.

## Analytical Methods: Development and Establishment of Methods (Chemical Assays)

The following principles of analytical method validation provide steps for the development of a new method or establishing an existing method in a particular laboratory for the first time. Any modification of an analytical method would require revalidation of the procedures. Analytical method validation should be performed to support pharmacokinetic, bioequivalence and related studies in a new drug application (NDA) or an abbreviated new drug application (ANDA). Full method validation may not be necessary in conducting exploratory pharmacokinetic studies. It is suggested that validation include investigation of samples from dosed subjects.

## Principles of Method Validation – Method Establishment

A specific, detailed description and protocol of the method should be written, (standard operating procedure).

Each step in the method should be investigated to determine the extent to which environmental, matrix, material or procedural variables, from the time of collection of the material up to analysis and including the time of analysis, may affect the estimation of analyte in the matrix. Variability of the matrix due to a particular physiological state may need to be considered.

A method should be validated for the intended use, employing an acceptable protocol. All experiments used to make claims or draw conclusions about the validity of the method should be presented in a report (method validation report).

Whenever possible, the same biological matrix as that in the intended samples should be used for validation purposes (for tissues of limited availability, such as bone marrow, physiologically appropriate proxy matrices may suffice). The stability of the analyte (drug and/or metabolite) in the matrix during the collection process and the sample storage period should be assessed, preferably prior to sample analysis. It is recommended that the stability of the analyte in the matrix from dosed subjects be confirmed. Accuracy, precision, reproducibility, response function and the specificity of the method with respect to endogenous substances, metabolite(s) and known degradation products should be established with reference to the biological matrix. In regard to specificity, there should be evidence that the substance being quantitated is the intended analyte.

The concentration range over which the analyte will be determined must be defined in the method, based on evaluation of actual standard samples over the range, including their statistical variation. This defines the *standard curve*.

It is necessary to use a sufficient number of standards to adequately define the relationship between concentration and response. The relationship between response and concentration must be demonstrated to be continuous and reproducible. The number of standards to be used will be a function of the dynamic range and nature of the concentration response relationship. In many cases, five to eight concentrations (excluding blank values) may define the standard curve. More standard concentrations may be necessary for nonlinear than for linear relationships.

The accuracy and precision with which known concentrations of analyte in biological matrix can be determined must be demonstrated. Within and between run accuracy and precision should be calculated using commonly accepted statistical procedures. This can be accomplished by analysis of replicate sets of analyte samples of known concentrations from an equivalent biological matrix. At a minimum, three concentrations representing the entire range of the calibration curve should be studied: one near the lower limit of quantitation (LOQ); one near the center; and one near the upper boundary of the standard curve. For a method to be considered valid, specific criteria must be set for accuracy and precision over the range of the standard curve.

The LOQ is the lowest concentration on the standard curve which can be measured with acceptable accuracy, precision, and variability. The LOQ should be determined using at least five samples independent of standards and determining the coefficient of variation and/or appropriate confidence interval. The LOQ should serve as the lowest concentration on the standard curve and should not be confused with the limit of detection (LOD, see Glossary, p. 7).

### Specific Recommendations for Method Validation

The stability of the analyte in the biological matrix at the intended storage temperature(s) should be established. In addition, the influence of freeze/thaw cycles (a minimum of two cycles at two concentrations in duplicate) should be studied.

The specificity of the assay methodology should be established using six independent sources of the same matrix.

The accuracy and precision should be determined using a minimum of five (excluding blank sample) determinations per concentration. The mean value should be within $\pm 15\%$ of the actual value except at the LOQ where it should not deviate by more than $\pm 20\%$. The precision around the mean value should not exceed 15% of the coefficient of variation (CV), except for the LOQ where it should not exceed 20% CV. Other methods of determining accuracy and precision which meet these limits may be equally acceptable.

The standard curve should consist of five to eight standard points, excluding blank, using single or replicate samples. The standard curve should cover the entire range of expected concentrations.

Response function: the simplest relationship for response vs concentration should be determined and the fit should be statistically tested. The function should be represented using an appropriate algorithm or graphical technique.

### Analytical Validation: Application to Routine Drug Analysis

Many of the above principles (see Principles of Method Validation – Method Establishment) are relevant to pre-study validation. This section will emphasize the validation parameters that should be performed during routine application of a method to a particular study.

In general, with acceptable variability as defined by validation data, analysis of biological samples can be conducted by single determination without the need for duplicate or replicate analysis. The need for duplicate analysis should be assessed on a case-by-case basis. For example, for a robust procedure of low variability with accuracy and precision routinely well within toler-

ances, single analysis would suffice. For a difficult procedure with a labile analyte when the precision and accuracy tolerances are difficult to achieve, duplicates may be essential. A procedure should be developed that documents the reasons for re-analysis.

A standard curve should be generated for every analytical run for each analyte and should be used for calculating the concentration of analyte in the unknown samples assayed with that run. It is important to use a standard curve that will cover the entire range of concentrations in the unknown samples. Estimation of unknowns by extrapolation of the standard curve below the low standard or above the high standard is not recommended. Instead, it is suggested that the standard curve be redetermined or samples be reassayed after dilution. The quality control (QC) samples should be used to accept or reject the run. These QC samples consist of matrix spiked with analyte.

A standard curve should consist of five to eight standard points, excluding blank (either single or replicate) covering the entire range.

Response function: this is determined by appropriate statistical tests based on the actual standard points during each run in the validation.

System suitability: based on the analyte and technique, a specific procedure (or sample) can be identified in order to ensure the optimum operation of the system employed.

## Acceptance Criteria for the Run

*Accuracy and precision*: The acceptance criteria are not greater than 15% CV for precision and not more than 15% deviation from the nominal value for accuracy. However, at LOQ 20% is acceptable for both precision and accuracy. It is desirable that these tolerances be provided both for intra-day and inter-day or inter-run experiments.

*Quality control samples*: QC samples in duplicate at three concentrations (one near the LOQ, one in the middle range and one approaching the high end of the range) should be incorporated into each run. The results of the QC samples provide the basis of accepting or rejecting the run.

At least four of the six QC samples must be within 20% of their respective nominal values; two of the six QC samples (not both at the same concentration) may be outside the $\pm 20\%$ respective nominal value. A confidence interval approach yielding comparable accuracy and precision is an acceptable alternative.

*Repeat analysis*: The protocol for repeat analysis should be established a priori. Some aberrant values can be identified which can be attributed to processing errors, equipment failure, poor chromatography or QC samples outside predefined tolerance. Cautious use of a 'pharmacokinetic fit' such as a double peak may call for repeat analysis of some samples in the study, but the reasoning should be clearly documented.

## Analytical Method Validation – Immuno- and Microbiological Assays

Many of the analytical validation parameters and principles discussed above are also applicable to immuno- and microbiological methods, but there are some specific differences. In immuno- and microbiological assays, the response must be shown to relate to the concentration of the analyte in question.

## Selectivity Issues

As with chromatographic methods, it must be demonstrated that the bioassay is selective for the analyte. An alternative method, if rigorously established, may be used to compare the results of the bioassay.

For bioassay, an appropriate combination of other techniques may be used to show selectivity including the following.

Comparison of standards in biological fluids with standards in buffer to detect matrix effects.

Parallelism of diluted clinical samples with diluted standards to detect the presence of closely related compounds.

Serial separation techniques, e.g., extraction, and chromatography, with the bioassay as detector, to demonstrate that the response is due only to the analyte in question.

Metabolite (or endogenous compound) cross-reaction may be initially assessed by comparison of displacement curves, but in critical cases should also be evaluated by addition of metabolite to analyte. Similar criteria will be applicable when the drug is concomitantly administered with other drugs.

## Quantitation Issues

Criteria for precision and accuracy of immuno- and microbiological assays should be based on the requirements of the study and should match those of chromatographic methods. Any decision to run the sample analysis in single/duplicate/triplicate should be based on variability.

Immunoassay standard curves are essentially nonlinear, and in general require more concentration points to define the fit over the range claimed.

It should be established that an acceptable curve fitting model is being used by examining statistics for goodness of fit, back-calculation of standards and control sample results.

Both upper as well as lower limit of quantitation must be defined by acceptable accuracy, precision or confidence interval criteria based on the study requirements.

For all assays it is the accuracy of the *reported results* which is the key factor. This accuracy may be improved by use of replicate samples. In the case where replicate samples need to be measured during the validation to improve accuracy, the same procedure must be followed for unknown samples.

If there are intermediate steps between the plasma (or other biological matrices) and the final assay (such as extraction of biological sample followed by immunoassay) and if parallel processed standards in biological matrix are not being used, it is necessary to establish recovery and to use it in determining results. Possible approaches to assess efficiency and reproducibility of recovery are: (1) use of radiolabeled tracer analyte (quantity too small to affect the assay); (2) advance establishment of reproducible recovery; and (3) use of an internal standard which is not recognized by the antibody, but can be measured by another technique.

Correction for nonspecific matrix effects: separation techniques may be used to remove the effect or the matrix may be utilized in defining the standard curve, in controls and samples. The use of standards in the matrix is recommended. This approach will obviate many of the above concerns.

## Other Issues

*Commercial kits*

These are available for both immuno- and microbiological assays and the analytical methods based on such kits should be validated. The validation assures that the bioassay kit is applicable to the study problem and for ensuring that subsequent batches or lots of kits have performance characteristics similar to those of the original validated kit or the test. Any modifications and extensions of assays from one kit (or test) to another must be validated.

*Measurement of metabolite(s)*

The complex area of determination of drug metabolites in bioavailability studies to support drug submissions was discussed. The questions differed somewhat according to the objective of the application of the bioanalytical measurement, e.g., bioequivalence vs pharmacokinetic profiling. Some situations exist in bioavailability/bioequivalence studies where: (1) the parent drug cannot be assayed in biological samples and only metabolite can be measured; (2) the parent drug along with active and/or inactive major metabolite(s) can be measured; (3) more than one metabolite is present; and (4) the accumulation of metabolite is augmented, e.g., in the case of renal impairment. Under such situations should one measure the metabolite(s)? Can decision criteria be developed for measuring the metabolite in such situations? From the discussions, it was suggested that:

All methods applied for measuring drug and metabolite(s) should be validated for that particular study matrix, with the same general parame-

ters listed above (accuracy, precision, specificity, recovery and reproducibility).

Pharmacokinetic, bioavailability, and bioequivalence studies should be based upon the moieties that contribute significantly to the *pharmacologic* or therapeutic effect.

*Stereoisomer assays*

The need for stereoselective determination in bioavailability/bioequivalence studies was another issue which was discussed. There are many drugs which are administered as racemic mixtures, and they may undergo stereoselective metabolism and/or elimination. One isomer may be more active than the other. Under what circumstances should one measure individual drug isomers and/or metabolite(s) isomers from a biological matrix? It was suggested that:

All methods used for measurement of stereoisomer should be validated (with emphasis on stereospecificity).

For bioequivalence studies of an existing racemic product, a stereospecific assay is not required if the rate and extent profiles are superimposable (within the usual statistical boundaries).

For new chemical entities, the pharmacokinetic profiles for the stereoisomer should be characterized in normal subjects.

*Pharmacodynamic measurements*

The final difficult issue identified was the area of pharmacodynamic measurements. The suggestions were:

All pharmacodynamic procedures used for definitive bioequivalence or related studies must be fully validated under controlled conditions and should include a placebo.

The pharmacodynamic effect measured for bioequivalence studies should be related to the actual pharmacologic (therapeutic) end point of the drug's activity.

## Glossary

**Accuracy** Closeness of determined value to the true value. Generally, recovery of added analyte over an appropriate range of concentrations is taken as an indication of accuracy. Whenever possible, the concentration range chosen should encompass the concentration of interest.

**Analyte** An analyte is a specific, unique chemical moiety in the form(s) that would be found in a biologic matrix.

**Biological matrix** A biological matrix is a unique material of biological origin which can be prepared in a reproducible manner. Examples are: blood, serum, plasma, urine, feces, saliva, sputum and various discrete tissues.

**Limit of detection** The lowest concentration of an analyte that the analytical process can reliably differentiate from background levels.

**Limit of quantitation** The lowest concentration of an analyte that can be measured with a stated level of confidence.

**Linear range** Generally taken as the range over which the procedure has been demonstrated to give a linear detector response. A reproducible nonlinear response curve, however, can also be acceptable. Nonlinearity is certainly the case with immunological procedures.

**Method** A method is a set of all of the procedures involved in the collection, processing, storage and analysis of a biological matrix for an analyte.

**Precision** This describes the closeness of replicate determinations of an analyte by an assay. Precision can be further subdivided into within-day precision or intra-assay precision and between-day precision or inter-assay precision.

**Specificity** Ability of a method to measure only what it is intended to measure.

**Standard curve** The relationship between the experimental response value and analytical concentration is commonly referred to as a *standard curve* or a calibration curve.