Journal of Food and Drug Analysis
1993, 1(1): 1 - 6

藥物食品分析
第一卷 第一期

The Validation of Analytical Assays for Biopharmaceutical Studies

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ABSTRACT

The information required for an adequate analytical method validation for either *in vitro* or *in vivo* biopharmaceutical studies is discussed in this article. The most commonly applied assay parameters: accuracy, precision, detection limit, specificity, selectivity, calibration linearity or nonlinearity, stability, recovery, reproducibility and repeatability are carefully defined along with some recommendations required for assessing data acceptability arrived at the conference on Analytical Methods Validation. Though more specific analytical issues concerning microbiological assay radioimmunoassay and stereoisomer assay were not examined, this article should provide good guidance to pharmaceutical chemists to properly perform analytical method validation and carry out routine analysis.

Key words: analytical method, validation, quality control, calibration.

INTRODUCTION

Validation of an analytical assay method to ensure the method's suitability and to generate accurate and precise results is an absolute requirement if the method is to be used in *in vitro* or *in vivo* biopharmaceutical studies. Failure to adequately validate the analytical method can be sufficient grounds for rejection of a study by the regulatory authorities.

Several papers, including one by this author, have been recently published on bioanalytical methods validation[1-5]. In addition, a special Conference on Analytical Methods Validation was held in December 1990[6] whose goal was to try to reach a consensus on what should be required in analytical method validation and procedures to establish validation. The author had the opportunity to participate in the conference and was involved in panel discussions on various analytical issues. It is a pleasure and an honor for the author to share some of his opinions and experience with you here in the pages of the first issue of the Journal of Analysis for Foods and Drugs.

ASSAY PARAMETERS

Regulations and guidelines promulgated by the U.S. Food and Drug Administration (FDA) often describe assay requirements quite briefly. In the Code of Federal Regulations, it is clearly stated that "bioavailability testing shall be conducted using the most accurate, sensitive and reproducible approach..." (CFR21:320.24b) and "the analytical method...shall be demonstrated to be accurate and of sufficient sensitivity to measure, with appropriate precision, the actual concentration of the active drug ingredient or therapeutic moiety, or its metabolite(s), achieved in the body." (CFR21:320:29a). In practice, the actual application of these guidelines rests on their interpretation by FDA's reviewing staff at the Division level. Quite often

the essential information required as part of an assay validation is dependent on the type of study performed i.e. should always include accuracy and precision, sensitivity and detection limit, specificity and selectivity, calibration, linearity and nonlinearity, stability and recovery, repeatability and reproducibility. In this report, I will briefly discuss the general procedures used to determine these parameters and the more specific recommendations arrived at by the Conference.

ANALYTICAL METHOD VALIDATION

Accuracy and Precision

Accuracy refers to the bias of the method while precision refers to the repeatability of the method. Together they determine the total quality of the method. Statistically, accuracy is determined by calculating the difference between an observed(or calculated) value and a true (or actual/spiked value) value. The degree of variation is most frequently calculated as percent error or mean relative per cent error. Precision, on the other hand, is a calculation of variability within a set of measurements of the same quantity. It is sometimes referred to as "reproducibility" although it is somewhat different from the reproducibility that we will discuss in a later section. Precision is reported as the coefficient of variation (%CV) or relative standard deviation (%RSD).

Accuracy and precision are basically interdependent in assessing the adequacy of the analytical method. Usually accuracy is difficult to achieve without good precision but precision does not always ensure accuracy. During method validation, some analysts determine the accuracy by calculating the difference of the mean of observed values (analyses of numbers of N samples) and the actual values. This practice is misleading especially when dealing with bioassay since only one measurement is carried out for each sample during routine drug analysis. The author suggests that the mean of the absolute sum of the differences between each observed value and actual value should be used in calculating mean relative per cent error. In this way, good accuracy will always comply with good precision.

Acceptance limits for accuracy and precision vary among analysts from different disciplines. The Conference recommended that in bioanalysis, the mean value for accuracy should be within 15% of the actual value and the precision less than or equal to 15% coefficient of variation (%CV) except at the limit of quantitation where 20% error and 20% CV are acceptable. Accuracy and precision should be based on at least 5 determinations.

During routine drug analysis, it is essential (the author's view versus "desirable"--the Conference's recommendation) that these criteria be applied for both intraday (or intra-batch) and interday (or inter-batch) experiments.

Sensitivity and Detection Limit

Sensitivity, a term which defines the ability to determine low concentrations of an analyte (generally accepted to be 3 times the signal to noise ratio) is meaningless in an analytical method validation. On the contrary, minimal quantifiable level (MQL), or limit of quantitation (LOQ), terms which include a preset level of certainty, i.e. both accuracy and precision within 20%, are more adequate in defining the detection limit.

The MQL or LOQ of a method is taken as the lowest standard concentration used in the validation, provided that accuracy and precision at this level are acceptable. It is our common practice as well as the Conference's recommendation that no data extrapolated beyond this point should be accepted.

Specificity and Selectivity

Many bioanalytical chemists use the terms specificity and selectivity interchangeably. Actually, specificity refers to a method which produces a single response for only a single analyte. Selectivity, however, refers to a method which provides responses for a number of chemical entities but the response of the chemical entity of interest can be distinguished from all other responses[7]. Since most methods, especially chromatographic methods, have varing responses due to the presence of metabolites, endogenous compounds, etc., the term selectivity is more appropriate in the majority

of cases.

An analytical method should be validated using the same biological matrix as that in the samples which are to be analyzed. Any effect from the matrix and/or other interferences can then be compensated for by making up calibration standards in the same matrix as found in the samples.

In chromatographic studies, matrix interference (or lack of interference) can be easily demonstrated by recording the chromatogram of the blank biological matrix taken through the method. The recently developed photo-diode-array detector used in liquid chromatographic assay is one of the most effective ways to confirm selectivity of an assay for analytes and metabolites alike. For competitive binding assays, lack of interference is generally indicated by a lack of measurable response in the blank along with an evaluation of cross-reactivity to structurally related or concomitant substances. Lack of cross-reactivity is generally accepted to be no response when the substance is present at 1000 times the lower limit of quantitation for the analyte or less than 0.1% cross-reactivity[1].

Calibration: Linear and Non-linear

Basically, concentrations of sample analytes are calculated by comparison of responses of standards spiked at known concentrations to responses of unknown samples. Since the same analytical method is used to assay both samples and standards, the same accuracy and precision applies to the samples and standards. It is important to use a sufficient number of spiked standards to define the relationship between response and concentration. When the concentration-response relationship is linear, four or five concentrations in addition to a blank sample may be sufficient to cover the range of samples analyzed.

However, if the concentration-response relationship is non-linear, more standard concentrations will be needed to establish an adequate standard curve. The MDL(or LOQ) standard must always be included in the validation.

To cover a large dynamic range of calibration, the use of weighting factors is common. However, while the use of a weighting factor improves accuracy at one end of the calibration curve, there is always some sacrifice in accuracy at the other end. A plot of logarithmic values of concentration and response, on the other hand, provides an ideal linear model that theoretically yields a slope equal to one. This unique slope value can be used as a criterion of acceptance for linear data. Any mathematically justified manipulations of calibration data may be applied in this author's opinion. However, to avoid complexity, the simplest adequate procedures should be applied in method validation whenever possible.

As discussed in the accuracy and precision section, the validation should include a minimum of five measurements at each concentration in the calibration range. During routine drug analysis, five to eight standard concentrations, either single or replicate, is adequate.

Prior to any sample analysis, the stability of the drug analyte should be established. It is particularly important to assess the possible instability of the drugs and/or metabolites in the biological matrix during sample collection and storage since, in most cases, the samples will be stored for a period of time before analysis. The stability of the drugs should be determined for the anticipated storage period. When the integrity of the drug is affected by freezing and thawing, the spiked samples should be assayed repeatedly under several freeze/thaw cycles. During routine analysis, pooled clinical samples subjected to the same storage procedure should be included in the stability study. In the event instability is detected, addition of appropriate stabilizing agents such as antioxidants, enzyme inhibitors, etc. may be needed to minimize degradation of the analytes.

The measurement of the response of a spiked matrix standard which has been subjected to the entire procedure, expressed as a percent of the response of pure standard which has not been taken through the method, is referred to as absolute recovery. When derivatization is part of the procedure, the response measured from the matrix is generally compared to the

response from the pure solvent, both subjected to the entire experimental procedure. In this case, the recovery is referred to as relative recovery. In fact, both recoveries can be treated as a test of matrix effect. Although it is desirable to achieve a recovery as close to 100% as possible, it is by no means absolutely necessary for good accuracy and precision. Lower recovery values, i.e. 50,70%,etc.can all be accepted as long as the recovery is shown to be consistent and the accuracy and precision are within acceptable limits.

Reproducibility and Repeatability

Reproducibility is used to describe the closeness of agreement between results obtained with the same method under different conditions (a test of ruggedness) whereas repeatability refers to agreement between successive measurements on the same sample using the same method and conditions. In other words, reproducibility can be considered as the measurement of precision between laboratories (inter-laboratory) when studies are performed in more than one laboratory, and repeatability is the measurement of precision within the same laboratory different analysts, or the analysis resumed after a long period of time. Under these situations, the method must be re-validated and the reproducibility and repeatability data be determined.

A common way to test the ruggedness of a method is to analyze quality-control (QC) samples over time or under different conditions in order to observe any changes which occur. The establishment of QC charts (Levy Jennings charts[8]) is very helpful to determine if up/down trends or shifts of data occur. These data can then be statistically analyzed to determine a criterion for acceptance of results generated daily.

The Conference recommended that during routine analysis, three QC samples in duplicate at different concentrations should be incorporated into the unknown samples to determine the acceptability of the batch of samples analyzed. The Conference further elaborated that:"At least four of the six QC samples must be within 20% of their respective nominal values; two of the six QC samples (both not at the same concentration) may be outside the range +/- 20% of their respective nominal values. A confidence interval approach yielding comparable accuracy and precision is an acceptable alternative"[6]. It is the sequence of sample placement, the order in which QC concentrations are run, and the criterion for acceptance of sample data based on QC data should be carefully chosen and justified by the analyst.

CONCLUSION

The general information essential for an adequate analytical method validation for either *in vitro* or *in vivo* biopharmaceutical studies have been briefly discussed in this article. The author does not claim to have covered all aspects of analytical validation. Specific analytical methods such as microbiological assay, radioimmunoassay, stereoisomer assay, etc. require that additional data be determined for an adequate validation. Documentation on method validation, preset criteria on reanalyzing samples, and many other analytical issues related to good laboratory practice (GLP) also need to be properly addressed. This article, however, in addition to the references, should provide good guidance to pharmaceutical chemists wishing to properly validate an analytical method.

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(*Accepted for Publication: Aug. 15. 1992*)

生物藥劑學試驗分析方法之認證

蕭　高　興

摘　　要

本文將討論生物藥劑學體內、體外試驗分析方法論證的必要依據。美國分析方法認證會議曾推薦用分析參數(準確度,精密度,偵測限度,專屬性、選擇性、校準線性或非線性、穩定性、回收率、重演性與重覆性)作爲判斷實驗數據的可採納性。作者將爲從事分析方法認證的工作者提供指南,但對專屬分析方法的認證如微生物分析,同位素免疫分析及立體異構體分析未及論述。