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Review Article

A REVIEW ON BIOANALYTICAL METHOD VALIDATION

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Abstract: Bioanalytical method validation is a controlled procedure that comprises all the vital steps to establish that a certain method is capable of producing accurate, dependable and reproducible results that are appropriate for a specific analytical application. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use. Bioanalytical method validation is necessary in an analytical laboratory to show its qualification and competency. This article highlights how highly reproducible validated bioanalytical methods for the quantitative determination of drugs and/or metabolites in biological matrices can be achieved by advancing analytical sensitivity.

Key**words:** Bioanalytical method validation, Bioanalysis, United States food and drug administration

INTRODUCTION

Bioanalytical method validation is crucial in minimizing random error and systematic bias, which ensures quality of analytical results. Validation is done to prove that the developed method produces reproducible and acceptable results. The guidelines for bioanalytical method validation are published by the United States Food and Drug Administration (USFDA) [1-2] in May 2001. The US Food and Drug Administration's guidance document for bioanalytical method validation, "Guidance for Industry, Bioanalytical Method Validation[3-4]," advocates extensive validation of the bioanalytical methods. Bioanalytical field deals with analysis of drugs, their metabolites and/or endogenous substances in the biological matrix. A properly validated bioanalytical method for quantitative determination of drugs and their metabolites (analytes) plays quite important role in the effective performance of bioequivalence, pharmacokinetic (pk) and toxicokinetic studies. What is the need of validation[5]?

Validation assures that-

1. Ensures that the analysis results are reliable, consistent and moreover there is reproducibility in the results.
2. It also provides an assurance that the method is suitable or fit for the intended purpose.
3. For assurance of quality of products and formulations.
4. Achieving and enhancing of products by international agencies.
5. For compulsive requirement for registration of pharmaceutical product or formulations.

Types of validation:

Validation is of three types depending upon the method employed. They are

1. Full validation
2. Partial validation
3. Cross validation

Full Validation

When carrying out bioanalytical method development of an analyte for the first time, full method validation is employed. Full method validation is mandatory for any new drug entity. It is recommended when metabolites are added to current assay of already existing analyte.

Partial Validation

Modification of validated bioanalytical methods that do not necessarily call for full revalidation. Partial Validation can range from as little as one intra-assay accuracy and precision determination to a "nearly" Full Validation. Partial validation can also be carried out when there is alteration in species within matrix (e.g. rat plasma to mouse plasma), changes in matrix within
a species (e.g., human plasma to human urine), change in analytical methodology (e.g., change in detection systems), change in sample processing procedure(s), change in anticoagulant in harvesting biological fluid (Shah et al, 2000; Shah, 2007).[6]

Cross Validation

Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. The evaluations should be done by considering an innovative validated bioanalytical method as the reference and the repeated bioanalytical method as the comparator and vice-versa. Cross validation with spiked matrix and subject samples should be carried out at each site or laboratory to create inter-laboratory reliability when sample analyses within a single study are carried out at more than one site, or more than one laboratory. Cross-validation

should also be considered when data generated using different analytical techniques (e.g., LC-MS-MS vs. ELISA) in different studies are included in a regulatory submission.

The vital parameters for validation include: carry over effect, selectivity, sensitivity, dilution integrity, matrix effect, accuracy & precision, stability, recovery, purity of working standard.

SELECTIVITY/SPECIFICITY

For every phase of product development, the analytical method must demonstrate specificity. The method must have the ability to unambiguously assess the analyte of interest while in the presence of all expected components, which may consist of degradants, excipients/sample matrix, and sample blank peaks. The terms selectivity and specificity are often used interchangeably. The term specific generally refers to a method that produces a response for a single analyte only, while the term selective refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate. Optimized sample preparation can eliminate most of the matrix components. The absence of matrix interferences for a quantitative method should be demonstrated by the analysis of at least five independent sources of control matrix. The method must be able to differentiate between the analyte of interest and compounds with a similar chemical structure that may be present.

ACCURACY:

Accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. This is sometimes termed as trueness. It is expressed as bias% or relative error%. The two most commonly used ways to determine the accuracy or method bias of an analytical method, are analyzing control samples spiked with analyte and by comparison of the analytical method with a reference method. It is determined by replicate analysis of samples containing known amounts of the analyte. It is measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

PRECISION:

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, interbatch precision or repeatability.

Repeatability expresses the precision under the same operating conditions over a short interval of time. It is sometimes also termed within-run or within-day precision. It shows how the method performs in one lab and on one instrument, within a given day. Repeatability can be determined by evaluating the precision from a minimum of nine determinations that encompass the specified range of the method. The nine determinations may be composed of triplicate determinations at each of three different concentration levels, one of which would represent the 100% test concentration.

Intermediate precision reflects within-laboratory variations such as different days, different analysts, and different equipment (between batches, different assay). Intermediate precision testing can consist of two different analysts, each preparing a total of six sample preparations, as per the analytical method. The analysts execute their testing on different days using separate instruments and analytical columns. It is also called as between-run, between-day, or inter-assay precision.

Reproducibility, i.e., the precision between laboratories (collaborative or interlaboratory studies), is not required for submission, but can be taken into account for standardization of analytical procedures.

LINEARITY

Linearity assesses the ability of the method to obtain test results that are directly proportional to the concentration of the analyte in the sample. ICH[7] guidelines recommend evaluating a minimum of five concentrations to assess linearity. The five concentration levels should bracket the upper and lower concentration levels evaluated during the accuracy study. If the total range cannot be described by a single calibration curve, two calibration ranges can be validated. A calibration curve should be generated for each analyte in the sample. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of

i) A blank sample (matrix sample processed without internal standard)

ii) A zero sample (matrix sample processed with internal standard)

iii) Six to eight non-zero samples covering the expected range, including LLOQ.

MATRIX EFFECT

The recent 3rd bioanalytical workshop proposed determination of matrix factor as a way of assessing the matrix effect. Since ionization of analytes will be affected

by presence of endogenous components in biological matrix, it could be either suppression or enhancement.

Matrix Factor (MF) can be calculated as

$$= \frac{\text{Peak response in presence of matrix ions}}{\text{Peak response in absence of matrix ions}}$$

MFs for an analyte and its stable isotope labeled IS are usually similar. It is recommended that matrix factor or IS normalized MF being determined in six different lots of matrices. The variability in matrix factors as measured by coefficient of variation (%CV) should be less than 15%.

CARRY OVER EFFECT

Carry over effect in the assay is used to study the rinsing cycles or wash program of auto sampler required to wash the injection needle properly so as not to get any interference from the previous injection. For carry over recovery comparison sample of 1.5 to 1.8 times of non-extracted standard H (highest calibration curve standard) containing internal standard, extracted sample containing 1.5 to 1.8 times concentration of standard H with internal standard, LLOQ sample in duplicate with internal standard from biological matrix and extracted blank samples from the same matrix lots are used.

STABILITY

Stability is defined as the chemical stability of an analyte in a given matrix under specific conditions for given time intervals. Stability of the analyte during the whole analytical procedure is a prerequisite for reliable quantification. Therefore, full validation of a method must include stability experiments for the various stages of analysis including storage prior to analysis[8].

Long-term stability

The stability in the sample matrix should be established under storage conditions, i.e. in the same vessels, at the same temperature and over a period at least as long as the one expected for authentic samples.

Freeze/thaw stability

As samples are often frozen and thawed, e.g. for reanalyis, the stability of analyte during several freeze/thaw cycles should also be evaluated. It requires a minimum of three cycles at two concentrations in triplicate.

In-process stability

The stability of analyte under the conditions of sample preparation (e.g. ambient temperature over time needed for sample preparation) is evaluated here. There is general agreement, that this type of stability should be evaluated to find out, if preservative have to be added to prevent degradation of analyte during sample preparation.

Processed sample stability

Instability cannot only occur in the sample matrix, but also in prepared samples. It is therefore important to also test the stability of an analyte in the prepared samples under conditions of analysis (e.g. auto sampler conditions for the expected maximum time of an analytical run). One should also test the stability in prepared samples under storage conditions, e.g. refrigerator, in case prepared samples have to be stored prior to analysis.

ROBUSTNESS/ RUGGEDNESS

According to ICH guidelines, the robustness/ ruggedness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness can be described as the ability to reproduce the (analytical) method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained result(s), and a robustness test as an experimental set-up to evaluate the robustness of a method.

DETECTION LIMIT:

The limit of detection (LOD) is the lowest concentration of analyte in the sample that can be detected but not quantified under the stated experimental conditions. It is also defined as the lowest concentration that can be distinguished from the background noise with a certain degree of confidence. There is an overall agreement that the LOD should represent the smallest detectable amount or concentration of the analyte of interest. The detection is usually expressed as the concentration of the analyte in the sample, for example, percentage, parts per million (ppm), or parts per billion (ppb).

LIMIT OF QUANTIFICATION

Lower limit of quantification: LLOQ is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. Determining LLOQ on the basis of precision and accuracy is probably the most practical approach and defines the LLOQ as the lowest conc. of the sample that can still be quantified with acceptable precision and accuracy. LLOQ based on signal and noise ratio (s/n) can only be applied only when there is baseline noise, for example to chromatographic methods. Upper limit of quantification: ULOQ is the maximum analyte conc. of a sample that can be quantified, with acceptable precision and accuracy. The ULOQ is identical with the conc. of the highest calibration.

CONCLUSION

This review summarizes the validation parameters that are required according; to the requirements of ICH. The method validation process and the minimum requirements to be included in a regulatory method are discussed. The objective of this paper is to provide approaches for determining selectivity, limit of detection, lower limit of quantitation, linearity, accuracy, precision, carry over effect, stability, robustness of liquid chromatographic methods to support pharmacokinetic, toxicokinetic, bioavailability, and bioequivalence studies. An attempt has been made to make validation parameters to be understood by every analyst.

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