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Review Article

Bioanalytical Method Validation: A Quality Assurance Auditor View Point

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ABSTRACT

In the development of medicinal products, bioanalytical methods are used in clinical and non-clinical pharmacokinetic studies to evaluate the efficacy and safety of drugs and their metabolites. Drug concentrations determined in biological samples are used for the assessment of characteristics such as in vivo pharmacokinetics (adsorption, distribution, metabolism, and excretion), bioavailability, bioequivalence, and drug-drug interaction. Bioanalytical methods, based on a variety of physico-chemical and biological techniques such as chromatography, immunoassay and mass spectrometry, must be validated prior to and during use to give confidence in the results generated. It is the process used to establish that a quantitative analytical method is suitable for biomedical applications. Any method developed for the analysis of analytes in biological fluids must yield consistent results despite the variations in conditions during the course of a project. An ideal bioanalytical method should include all of the probable effects that are going to occur during the routine analysis of study samples.

The present manuscript focuses on the consistent evaluation of the key bioanalytical validation parameters is discussed accuracy, precision, sensitivity, selectivity, limits ofquantification, range, linearity, ruggedness, robustness, and stability. Some of the proposals were made to the validation procedure to encounter the possible situations in the routine study sample analysis. An attempt has been made to understand and explain the bioanalytical method validation for chromatographic assays from the quality assurance auditor viewpoint. A good understanding of the background and principles of the bioanalytical method validation will help the quality assurance personnel to perform their duties in a most effective and focused manner.

Key-words: Bioanalytical method,Biomedical applications, Validation parameters, Quality assurance auditor.

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Introduction[1,2,3,4,5,6]

Bioanalytical method validation (BMV) employed for the quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic study data. These studies generally support regulatory filings. The quality of these studies is directly related to the quality of the underlying bioanalyticaldata. It is therefore important that guiding principles for the validation of these analytical methods be established and disseminated to the pharmaceutical community. 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.

As per the Guidance for Industry, "Bioanalytical Method Validation" guidelines form FDA, the analytical laboratory conducting pharmaceutical/toxicology and other preclinical studies for regulatory submissions should adhere to FDA's Good Laboratory Practices and to sound principles of quality assurance throughout the testing process. In this article an attempt has been made to understand and explain the issues and concepts of BMV.

This guideline serves as a general guidance recommended for the validation of bioanalytical methods to ensure adequate reproducibility and reliability. It also provides a framework for analyses of study samples by using validated methods to evaluate study results supporting applications for drug marketing authorization.

Method validation is a process that demonstrates that the method will successfully meet or exceed the minimum standards recommended in the Food and Drug Administration (FDA) Guidance for accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Chromatographic methods (high-performance liquid chromatography [HPLC] or gas chromatography [GC]) have been widely used for the bioanalysis of small molecules, with liquid chromatography coupled to triple quadrapole mass spectrometry (LC/MS/MS) being the single most commonly used technology.

The objective of validation of bioanalytical procedure is to demonstrate that it is suitable for its intended purpose. The most widely accepted guideline for method validation is the ICH guideline Q2 (R1), which is used both in pharmaceutical and medical science. Other guidelines, which are much more detailed, which require more extensive validation and which also have defied strict limits for the most of determined parameters are focused directly toward bioanalysis. They are represented by a "Guideline on Bioanalytical Method Validation" by EMA and "Guidance for Industry, Bioanalytical Method Validation" by FDA.

Quality assurance department plays an important role in the flow chart of the bioanlytical lab, indeed bioequivalence centre. Quality assurance personnel have to assure the management and/or regulatory agencies that the validation of the bioanalytical method has been done as per the standard operating procedures (SOPs) of the organization and as per applicable regulatory guidelines. To do so, quality assurance personnel must understand the basic principles and underlying concepts of a bioanalytical method validation.

Need of Bioanalytical Method Validation

1. It is also important to emphasize that each bioanalytical technique has its own characteristics, which will vary from analyte to analyte, specific validation criteria may need to be developed for each analyte.
2. It is essential to used well-characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactorily interpreted.
3. It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements they are at the cutting edge of the technology.

Types of Bioanalytical Method Validation[7,8,9,10]

Bioanalytical method validation is having following three types

1) Full validation
2) Partial validation
3) Cross validation

Full validation

A full validation should be performed when establishing a new bioanalytical method for quantification of an analyte. A full validation should also be considered when a new analyte, such as a metabolite is added to an existing fully validated analytical method.

Full validation is important:

1. When developing and implementing a bioanalytical method for the first time.

2. For a new drug entity.
3. A full validation of the revised assay is important if metabolites are added to an existing assay for quantification

Partial validation

Partial validation may be performed when minor changes are made to an analytical method that has already been fully validated. A set of parameters to be evaluated in a partial validation are determined according to the extent and nature of the changes made to the method.

Partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation. Typical bioanalytical method changes that fall into this category include, but are not limited to:

1) Change in analytical methodology
2) Change in sample processing procedures
3) Change in relevant concentration range
4) Limited sample volume (e.g., paediatric study)
5) Bioanalytical method transfers between laboratories or analysts
6) Change in anticoagulant in harvesting biological fluid
7) Changes in instruments and/or software platforms

Cross validation

Cross validation is primarily conducted when data are generated in multiple laboratories within the same study or when comparing analytical methods used in different studies. In the cross validation conducted after full or partial validation in each laboratory or for each analytical method to be compared, the same set of QC samples spiked with the analyte or the same set of study samples is analyzed at both laboratories or by both analytical methods, and the mean accuracy at each concentration level or the assay variability is evaluated. Cross-validation should also be considered when data generated using different analytical techniques (e.g., LC-MSMS vs. ELISA) in different studies are included in a regulatory submission.

Common Parameter used in Bioanalytical MethodsValidation

The common terms used in bioanalytical method validation is given as follows, these are available in FDA guidance or draft guideline on bioanalytical method validation, but are provided here for convenience.

Accuracy[11,12,13]

The Accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. It is typically measured as relative error (%RE). Accuracy is an absolute measurement and an accurate method depends on several factors such as specificity and precision.

Accuracy is best reported as percentage bias which is calculated from the expression

$$\text{Abso\% Bias} = \frac{\text{Measured value} - \text{True value}}{\text{True value}} \times 100$$

The mean value should be within 15% of the nominal value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the nominal value serves as the measure of accuracy. The two most commonly used ways to determine the accuracy or method bias of an analytical method are (I) analysing control samples spiked with analyte and (II) by comparison of the analytical method with a reference method.

Precision[9,14,15]

The precision of an analytical procedure expresses the closeness of agreement between a series of Measurements obtained from multiple sampling of the same homogeneous sample under the prescribed condition.Precision should be investigated using homogeneous, authentic sample. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. It is typically measured as coefficient of variation (%CV) or relative standard deviation (R.S.D.) of the replicate measurements. 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% coefficient of variation (CV) except for the LOQ where it should not exceed 20% CV.

Intermediate precision[16,17]
It includes the influence of additional random effects within laboratories, according to the intended use of the procedure for example different days, analysts or equipment, etc. Intermediate precision refers to how the method performs, both qualitatively and quantitatively, within one lab, but now from instrument-to-instrument and from day-to-day Precision measure of the within laboratory variation due to different days, analysts, equipment's, etc.

Repeatability[9,12]
Repeatability expresses the analytical variability under the same operating conditions over a short interval of time(within-assay, intraassay). Repeatability means how the method performs in one lab and on one instrument, within a given day. Precision measured under the best condition possible (short period, one analyst etc.).

Selectivity[18,19,20,21]
Selectivity or specificity should be evaluated to assess the interference at the retention time (RT) of the analyte and internal standard (IS) with predetermined method conditions. If the single method assesses one or more two analyte simultaneously, the interference should be evaluated separately for each analyte individually. These could include metabolites, impurities, degradants, or matrix components. Selectivity is the documented demonstration of the ability of the bioanalytical procedure to discriminate the analyte from interfering components. It is usually defied as "the ability of the bioanalytical method to measure unequivocally and to differentiate the analytes in the presence of components, which may be expected to be present".Analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantifiation (LLOQ). Process six samples of LOQ with the addition of internal standard as per the method and inject. Then assess the interference in all the lots of blank batches against the mean response of the LOQ at the RT of the analyte and IS.
The acceptance criteria followed should be as per the internal SOP. For chromatographic assays, the peak response in blank matrix at the retention time of analyte should be not more than 20% of the mean response of the LOQ samples and the peak response at the RT of the IS should be no more than 5% of the mean response of the IS of the LOQ. QA Auditor at this stage should ensure that only blank matrix lots which were showed no significant interference will only be used for further usage in the validation. An acceptance criteria should be set in the SOP for the number of batches, like out of all screened lots, 80 % should be acceptable. These interferences may arise from the constituent of the biological matrix under study. Thy may depend on characteristics of the individual under study, be it an animal (age, sex, race, ethnicity, etc.) or a plant (development stage, variety, nature of the soil, etc.), or they could also depend on environmental exposure (climatic conditions such as UV-light, temperature and relative humidity). The actual FDA guidance for bioanalytical method validation requires the use of at least six independent sources of matrix to demonstrate methods selectivity.

Reproducibility[5,13,17]
Reproducibility is the precision between laboratories (collaborative or interlaboratorystudies), is not required for submission, but can be taken into account forstandardisation of analytical procedures. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedure in pharmacopeias. Ability of the method to yield similar concentration for a sample when measured on diffrent occasions [27]. Reproducibility refers to how that method performs from lab-to-lab, from day-to-day, from analyst-to-analyst, and from instrument-to-instrument, again in both qualitative and quantitative terms.

Limitof Detection (LOD)[11,13]
The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The calculation of the LOD is open to misinterpretation as some bioanalytical laboratories just measure the lowest amount of a reference solution that can be detected and others the lowest concentration that can be detected in the biological sample. There is an overall agreement that the LOD should represent the smallest detectable amount or concentration of the analyte of interest.

Limit of Quantitation (LOQ)[9,12]

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compound in sample matrices, and is used particularly for the determination of impurities and /or degradation products.

Lower limit of quantification (LLOQ)[18,21]

The lowest concentration of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy.

Upper limit of quantification (ULOQ)[18,21]

The highest amount of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy.

Several approaches exist in order to estimate the lower limit of quantification (LLOQ). A fist approach is based on the well-known signal-to-noise (S/N) ratio approach. A 10:1 S/N is considered to be sufficient to discriminate the analyte from the background noise [11].The other approaches are based on the "Standard Deviation of the Response and the Slope".

The computation for LLOQ is:

$$LLOQ=10\sigma/S$$

Where,σ is the standard deviation of the response and S = the slope of the calibration curve. Another approach to estimate the LLOQ is to plot the RSD versus concentrations close to the expected LLOQ.

Linearity [22,23]

The linearity of an analytical procedure is its ability (with a given range) to obtain test result which are directly proportional to the concentration (amount) of analyte in the sample. The concentration range of the calibration curve should at least span those concentrations expected to be measured in the study samples. If the total range cannot be described by a single calibration curve, two calibration ranges canbe validated. It should be kept in mind that the accuracy and precisionof themethod will be negatively affected at the extremes of the range by extensively expanding the range beyond necessity. Correlation coefficients were most widely used to test linearity.

Range[24]

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analytein the sample (including these concentration) for which it has been demonstrate that the analytical procedure has suitable level of precision, accuracy and linearity.

Robustness [23,24]

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variation in method parameters and provides an indication of its reliability during normal usage.It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurement are susceptible to variations in analytical conditions, the analytical conditions should be suitable controlled or a precautionary statement should be include in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (eg.-resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Ruggedness [25]

These includedifferent analysts, laboratories, columns, instruments, sources of reagents, chemicals, solvents. Ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test condition. The ruggedness of the method was studied by changing the experimental condition such as-

a. Changing to another column of similar type
b. Different operation in the same laboratory

Stability[7,9,14,26,27]

Drug stability is a function of the chemical properties of the analyte, the storage conditions, the matrix/solutions in which it is stored, and the container system. The aim of a stability test is to detect any degradation of the analytes of interest during the entire period of samplecollection, processing, storing, preparing, and analysis. The condition under which the stability is determined is largely dependent on the nature of the analyte, the biological matrix, and the anticipated time period of storage (before analysis). Thy also include the evaluation of the analyte stability in the biological matrix through several freeze–thaw cycles, bench-top stability (i.e. under the conditions of sample preparation), long term stability at for example -20°C or -70°C (i.e. during storage conditions of the samples) and stability of samples on the auto-sampler.

Generally, stability should be evaluated at least at two concentration levels, using blank biological matrix matched samples spiked at a low and high concentration level. It should be assessed in each matrix and species in which the analyte will be quantified. Also the stability of the analyte must be investigated under various conditions: in the standard solutions used to prepare calibration curves, in any biological matrix stored at -20°C and at room temperature prior to analysis and also in the final extract awaiting analysis. There may also be the need toinvestigate the stability of the analyte between the sample being taken and stored: some compounds are metabolized by esterase's in the blood and have very shorthalf-lives, therefore to stabilize the compound an inhibitor should be added, the effectiveness of which will need to be assessed and validated.

Percent stability could be calculated as follows-

$$\% \text{ stability} = \frac{\text{Mean response of stability samples}}{\text{Mean response of comparison samples}} \times 100$$

Short-term stability

The stability of the analyte in biological matrix at ambient temperature should be evaluated. Thee aliquots of low and high concentration should be kept for at least 24 hours and then analysed.

Long-term stability

The stability of the analyte in the matrix should equal or exceed the time period between the date of fist sample collection and the date of last sample analysis.

Freeze and Thaw Stability

During freeze/thaw stability evaluations, the freezing and thawing of stability samples should mimic the intended sample handling conditions to be used during sample analysis. Stability should be assessed for a minimum of three freeze-thaw cycles.

Stock solution stability

The stability of stock solutions of drug should be evaluated. When the stock solution exists in a different state (solutions vs. solid) or in a different buffer composition (generally the case for macromolecules) from the certified reference standard, the stability data on this stock solution should be generated to justify the duration of stock solution storage stability.

Matrix Effect (28)

The recent 3rd bionalytical 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,

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

Where peak response could be peak area, peak height, peak area ration or peak height ratio according to the method. Matrix Factor equal to 1 indicates no matrix effect, matrix factor less than 1 indicates suppression and greater than one indicates enhancement.

Specific Proposal for Bioanalytical MethodValidation[7,29]

1. Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported.
2. For validation of the bioanalytical method, accuracy and precision should be determined using a minimum of five determinations per concentration level (excluding blank samples). The mean value should be within 15% of the theoretical value. Other methods of assessing accuracy and precision that meet these limits may be equally acceptable.
3. The stability of the analyte in biological matrix at intends storage temperatures should be established.
4. The specificity of the assay methodology should be established using a minimum of six independent sources of the same matrix.
5. The accuracy and precision with which known concentrations of analyte in biological matrix can be determined should be demonstrated. The can be accomplished by analysis of replicate sets of analyte samples of known concentrations QC samples from an equivalent biological matrix.
6. The stability of the analyte in matrix at ambient temperature should be evaluated over a time period equal to the typical sample preparation, sample handling, and analytical run times.
7. Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalysed in the case of instrument failure.

Application of Validated Method to Routine DrugAnalysis[7,27,30,31]

In general, biological samples can be analyzed with a single determination without duplicate or replicate analysis if the assay method has acceptable variability as defied by validation data. Theseis true for procedures where precision and accuracy variability's routinely fall within acceptable tolerance limits.

The following recommendations should be noted in applying a bioanalytical method to routine drug analysis.

1. Any required sample dilutions should use like matrix (e.g.,human to human) obviating the need to incorporate actual within-study dilution matrix QC sample.
2. The QC samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.
3. Sample Data Reintegration: An SOP or guideline for sample data reintegration should be established. These SOP or guideline should explain the reasons for reintegration and how the reintegration is to be performed.
4. System suitability: Based on the analyte and technique, a specific SOP (or sample) should be identified to ensure optimum operation of the system used.
5. A matrix-based standard curve should consist of a minimum of six standard points, excluding blanks (either single or replicate), covering the entire range.

Conclusion

Bioanalysis and the production of pharmacokinetic, toxicokinetic and metabolic data plays a fundamental role in pharmaceutical research and development involved in the drug discovery and development process. An attempt has been made to understand and explain the bioanalytical method validation from a quality assurance auditor view point. Therefore the data must be produced to the acceptable scientific standards and specifications lay by the different regulatory agencies across the globe. Bioanalytical methods must be validated to objectively demonstrate the fitness for their intended use. Some of the proposals were described for inclusion in the validation parameters for the different situations encountered in the study sample analysis. This article highlights the Specific Recommendation Applications of bioanalytical method in routine drug analysis for drug discovery and development.

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