IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Review Article
September 2019 Vol.:16, Issue:2

Bioanalytical Method Development and Validation - An Overview

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

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Submission: 29 August 2019
Accepted: 5 September 2019
Published: 30 September 2019


www.ijppr.humanjournals.com

Keywords: Bioavailability, Bioequivalence, Method Development, Method Validation

ABSTRACT

Bioanalytical methods are widely used in quantification of drugs and their metabolites in plasma matrices. Bioanalytical method employed for the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic and toxicokinetic studies plays a significant role in quantitative estimation of drugs and their metabolites in biological matrix. The crucial bioanalytical role is method development, method validation and sample analysis. Bioanalytical method development is essential during the process of drug discovery and development for marketing approval. Bioanalytical method validation includes all the approaches which explain that a specific procedure used for a quantitative measurement of analytes in a given biological matrix is selective, sensitive, reliable and reproducible for intended use. Techniques such as High Performance Liquid Chromatography (HPLC) and liquid chromatography coupled with double mass spectroscopy(LC/MS/MS) can be used for bioanalysis of drugs in body. This review article focuses on consistent evaluation of bioanalytical parameters which include accuracy, precision, sensitivity, selectivity, calibration curve, limits of quantification, range, recovery and stability which are crucial in determining the concentration of drug and its metabolite.

INTRODUCTION

A bioanalytical method is a set of procedures involved in the collection, processing, storage and analysis of a biological matrix for a chemical compound or a xenobiotic. The availability of selective and sensitive bioanalytical methods is essential for the generation of reliable data on pharmacokinetics, bioavailability and bioequivalence of drugs. These methods should allow quantification of drugs and their metabolites in biological matrices(e.g. plasma, urine and cerebrospinal fluid) and must be validated with respect to reliability for the intended use. Bioanalytical method validation comprises all criteria determining data quality such as selectivity, sensitivity, accuracy, precision, recovery and stability. Bioanalysis of drugs and its metabolites study is very important for the drug efficacy, side effects and bioavailability of drug. Bioanalysis is affected by many factors which includes variation in matrix, presence of endogenous biochemicals or chemicals, differences in chromatographic techniques. By performing the validation of bioanalytical method, it assures that the method will yield reliable and reproducible results over a period of time.

Validation is a basic requirement to ensure quality and reliability of method developed in analytical and bioanalytical process. Bioanalytical Method Validation (BMV) involved in quantitative determination of drugs and drug-metabolites in biological matrix has a significant role in evaluating bioavailability, bioequivalence, pharmacokinetic parameters.

BMV plays a pivotal role not only in terms of regulatory submission but also for assuring generation of high quality data during drug discovery and development. It assures that the quantification of analyte in biological fluids is reproducible, reliable and suitable for the application.

The objective of validation of bioanalytical procedure is to demonstrate that it is suitable for the intended purpose. The most widely accepted guideline for method validation is the ICH guidelineQ2 (R1), which is used both in pharmaceutical and medical science.

BIOANALYTICAL METHOD DEVELOPMENT

Bioanalytical method development is the process of making a procedure to an unknown compound to be identified in matrix. Before development of bioanalytical method, the analyte of intrest should be understood (i.e, the physicochemical properties of drug, *in-vitro* and *in-vivo* metabolism and protein binding) and any prior analytical methods that may be

applicable should be considered. The choice of analytical method involves chemical properties of the analyte, concentrations, sample matrix, cost of analysis method and instruments, speed and time of analysis, quantitative or qualitative measurement, precision and the necessary equipment. Method development is important for new drug discovery and drug development, preformulation studies, formulation studies, validation of product, analysis of compound, bioanalytical research purpose. Method development involves sample preparation, sampling, separation, detection and evaluation of results and conclusions.

Screening of Biological Matrix and Sample Preparation:

The biological media which contain the analyte are usually blood, plasma, urine, serum, etc. Blood is usually collected from human volunteers or subjects by vein puncture with a hypodermic syringe up to 5 to 7ml. The venous blood is withdrawn into tubes with an anticoagulant, generally ethylene diamine tetraacetic acid (EDTA), heparin is used. Plasma is obtained by centrifugation at 4000rpm for 15minutes.

The purpose of sample preparation is to clean up the sample prior analysis. Material in biological media that can affect with analysis and chromatographic column includes endogenous macromolecules, proteins, salts and metabolic byproducts. The sample preparation is also for conversion of analyte from the biological matrix into a solvent suitable for instillation into chromatographic system. General methods for sample preparation include extraction method, protein precipitation, chromatography and ligand binding assay.

Bioanalytical Techniques:

Some of Bioanalytical techniques include:

1. Liquid-Liquid Extraction.
2. Solid phase Extraction.
3. Protein Precipitation.

Techniques such as High Performance Liquid Chromatography (HPLC) and liquid chromatography combined with Double Mass Spectroscopy(LCMS-MS) can be used for bioanalysis of drugs.

Liquid-liquid Extraction:

Liquid-liquid Extraction, also known as solvent extraction, is a technique used to separate analytes from interferences in sample matrix by partitioning the analyte between two immiscible liquids or phases (aqueous and organic). It is based on principle of difference in solubility and partitioning equilibrium of analyte molecules between aqueous(sample) and organic phases. Organic solvent generally used in are hexane, diethyl ether, tert-butyl methyl ether, ethyl acetate. Extraction of analyte occurs from aqueous phase into organic phase, when analytes are unionized and soluble in organic solvent. Analytes get extracted into organic phase and are easily recovered by evaporation of solvent in presence of nitrogen gas so as to get the dry form of sample which is reconstituted with mobile phase prior to chromatographic analysis, while analytes extracted into aqueous phase can often be injected directly into reverse a phase-HPLC column. Liquid extraction has been replaced by advanced and improved methods like liquid phase microextraction, supported membrane extraction and single drop liquid phase microextraction.

Solid Phase Extraction

Solid phase extraction is based on the principle of partitioning of analytes between two phases. The analyte of interest should have higher affinity to solid phase than the matrix components. It consists of disposable column containing sorbent. Solid phase extraction involves four main steps including conditioning the packing, sample application (loading), washing the packing (removal of interferances), recovery of analyte. This technique is preferred for sample preparation where analyte is bound onto a solid support, interferences are washed off and the analyte is selectively eluted. Advantages of this technique is low concentrations of drug can be detected, effective in selective removal of interferences. Extraction is difficult for high density materials and it is a time consuming process.

Protein Precipitation

Protein precipitation is widely used in routine analysis to remove proteins. Precipitation can be induced by the addition of an organic modernizer, a salt or by changing the pH which influences the solubility of proteins. The samples are centrifuged and the supernatant can be inserted into the HPLC system or be evaporated to dryness and dissolved in a suitable solvent. A concentration of sample is then achieved. As it is a no-selective sample cleanup method, there is a risk that endogenous compounds may restrict in reverse phase HPLC

system. Hence, protein precipitation technique is often combined with solid phase extraction to produce clean extract. Methanol is generally favored solvent among the organic solvents as it produces clear supernatant which is appropriate for direct addition into HPLC.

Method development involves optimizing the techniques and conditions involved with extracting and detecting the analyte. The development of sound bioanalytical method is of paramount significance during the process of drug discovery and development, culminating in marketing approval.

BIOANALYTICAL METHOD VALIDATION

Once the method has been developed, bioanalytical method validation proves the optimized method is suitable for the analysis of the study samples. Method validation is the process used to establish that a quantitative analytical method is reliable for biomedical applications. Bioanalytical procedure has to be validated because it is necessary to use well characterized and fully validated bioanalytical methods to yield results that are reliable.

Types of Bioanalytical Method Validation:

Method Validation is classified into three types:

1. Full Validation.
2. Partial Validation.
3. Cross Validation.

Full Validation:

After development of new method, all validation parameters are applied to sample analysis for the bioanalytical method of each analyte.

Full validation is required:

a. If the method is developed and implemented for the first time.

b. If a new drug entity is analysed.

c. If added metabolites are present in an existing assay for quantification.

Partial Validation:

A partial validation is performed if the validated bioanalytical methods have been modified or modification of validated bioanalytical methods that do not necessarily call for full validation. It can range from as minimum of one intra-assay accuracy and precision determination to a nearly full validation. Typical situations for a partial validation are:

a. Method transfers between laboratories and analysts.

b. Instrument and/or software platform changes.

c. Changes in species within the same matrix.

d. Change in analytical methodology.

e. Change in sample processing procedures.

Cross Validation:

In a cross validation two bioanalytical methods for the same analyte are compared. Cross validations are required when two or more bioanalytical methods are used to generate data within the same study. They should be conducted with spiked matrix standards and subject samples.

A cross validation is considered when:

a. Sample analysis within a single study are conducted in more than one laboratory.

b. Data generated using different analytical techniques in different studies are included in a regulatory submission.

BIOANALYTICAL VALIDATION PARAMETERS:

The method developed is validated on the basis of the following method validation parameters:

1. Selectivity.

2. Specificity.

3. Accuracy
4. Precision.
5. Matrix effect.
6. Linearity and range.
7. Limit of Detection (LOD).
8. Limit of Quantification (LOQ).
9. Calibration Curve.
10. Recovery.
11. Robustness.
12. Ruggedness.
13. Stability.

Selectivity:

Selectivity is the ability of a analytical method to differentiate and quantify the analyte in a sample in presence of other components in the sample. For selectivity, analysis of blank samples of the respective biological matrix should be obtained from at least six sources. Each blank sample should be tested for interference which could originate from endogenous matrix components, metabolites, decomposition products and concomitant medication.

Specificity:

Specificity is the ability to evaluate the analyte in presence of components that may be expected to be present, like impurities and matrix components. Specificity is confirmed by obtaining positive results from samples containing analyte, coupled with negative results from samples which do not contain the analyte.

Accuracy:

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value of analyte. Accuracy is determined by replicate analysis of samples containing known amounts of analyte. Accuracy should be measured using a minimum of 3 concentrations and 5 determinations per concentration. The minimum value should be within 15% of the true value except at lower limit of quantification(LLOQ), where it should not deviate by more than 20%. The deviation of the mean from true value serves as measure of accuracy.

Bias: It is the difference between the expectation of test results and an accepted reference value. It may contain more than one systematic error component. Bias can be measured as percent deviation from the accepted reference value.

Accuracy is reported as percentage bias which is calculated as:

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

Precision:

Precision of an analytical method describes the closeness of individual measures of an analyte when the method is applied repeatedly to multiple aliquots of a single uniform volume of biological media. Precision should be measured using a minimum of 3 concentrations and 5 determinations per concentration. The imprecision determined as coefficient of variation (CV) at each concentration level should not exceed 15% except for the LLOQ, where it should not exceed 20%.

$$\% \text{ CV} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

Precision is subdivided into:

a. Within-day precision, which assesses precision during a single analytical run.

b. Between-day precision, which measures precision with time, and may involve different analysts, equipment, reagents and laboratories.

Matrix effect:

Matrix co-extracted with analytes can alter the signal response, causing either suppression or enhancement resulting in poor analytical accuracy, linearity and reproducibility.

Matrix effect can be determined by Matrix Factor which is calculated as,

$$\text{Matrix Factor (MF)} = \frac{\text{Peak response in presence of matrix ions}}{\text{Peak response in absence of matrix ions.}}$$

Matrix Factor equal to 1 indicates no matrix effect, Matrix Factor less than 1 indicates suppression and greater than 1 indicates enhancement. The variability in matrix factor is measured by coefficient of variation (%CV) which should be less than 15%.

Linearity and Range:

Linearity of a bioanalytical method is its ability to elicit test results that are directly proportional to the concentration of analyte within the range of standard curve. Range of the method is the concentration interval at which accuracy, precision and linearity have been validated. The used calibration curve should be the simplest model that appropriately describes the concentration-response relationship. The deviation should not exceed more than 20% from the nominal concentration of the Lower Limit of Quantification (LLOQ) and not more than 15% from the other standards in the curve.

Limit of Detection (LOD):

It is the lowest concentration of sample which can quantitatively be determined with suitable accuracy and precision. LOD is by Visual definition, calculation from single to noise ratio, calculation from standard deviation of the blank, Calculation from the calibration line at low concentration.

Sensitivity or Limit of Quantification (LOQ):

It is the lowest concentration of analyte at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met.

Calibration Curve:

The relationship between the experimental response value and known concentrations of analyte is referred to as calibration curve. The concentration of standards should be chosen on the basis of concentration range expected in a particular study. A calibration curve should comprise of a blank sample(matrix sample processed without internal standard), a zero sample(matrix sample processed with internal standard), and five to eight non zero samples covering the expected range including the LLOQ.

The calculated concentrations of the calibration standards should be within 15% of the nominal value except for LLOQ for which it should be within 20%. At least 75% of the calibration standards, with a minimum of six calibration standard levels, must fulfill this criteria.

Lower limit of Quantification (LLOQ): The LLOQ is the lowest concentration of an analyte that can be measured with acceptable accuracy and precision.

The lowest standard on calibration curve should be accepted as LLOQ if the following conditions are met:

a. The analyte response should be at least 5times the response compared to blank response.

b. The analyte should be reproducible with imprecision of maximum 20% and an accuracy of 80-100%.

Recovery:

The recovery of an analyte assay is the response of detector obtained from an amount of the analyte added to and extracted from the biological fluids, compared to the detector response obtained from true concentration of the pure authentic standard. Recovery of the analyte is not necessary to be 100%, but they are the extent to recovery of an analyte and of the IS should be precise, consistent and reproducible.

Robustness:

It is the measure of capacity of an analytical procedure to remain unaltered by small, but deliberate variations in method parameters and provides an indication of its reliability. Robustness is ability to replicate the bioanalytical method in different laboratories or under different environment without the episodes of unexpected differences in the obtained result.

Ruggedness:

Ruggedness is the measure for the susceptibility of a method to small changes that might occur during routine analysis like small changes of pH values, mobile phase composition, temperature etc. Full validation must not necessarily include ruggedness testing, however, it can be helpful during method development or prevalidation phase, as problems that may occur during validation are often detected in advance. Ruggedness should be tested if a method is expected to be transferred to another laboratory.

Stability:

Stability of analyte in biological matrix should be determined to detect any degradation of the analytes of interest during the entire period of sample collection, processing, storing, preparing and analysis. Moreover, stability should be investigated at ambient temperature over a time period that encompasses the duration of typical sample preparation, sample handling and analytical run time. The FDA guidelines addresses analyte stability as a separate validation parameter.

It includes the following types of stability tests:

1. Short term stability:

The stability of analyte in biological matrix at ambient temperature should be evaluated. Three aliquots (of low, medium, high concentrations) should be kept for at least 24 hours and analysed.

2. Long term stability:

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

3. Freeze Thaw Stability:

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

4. Bench Top Stability:

Benchtop stability evaluation will be performed to evaluate the stability of the samples, which were kept on bench during the extraction process. The anticipated time for benchtop stability (usually 4 to 24 hours) should cover the duration of the time, it takes while extraction process.

5. Stock Solution stability:

The stability of stock solution and the internal standard should be evaluated at room temperature of at least six hours. After completion of the desired storage time the stability should be evaluated by comparing the instrument response with that of freshly prepared solution.

6. Processed Sample Stability:

The stability of the processed samples, including the time until the completion of analysis should be determined.

7. Post Preparative Stability or Reinjection stability:

The stability of analyte during stages of process of analysis should be evaluated.

CONCLUSION

Bioanalysis and production of pharmacokinetic, toxicokinetic and metabolic data plays a fundamental role in pharmaceutical research and development involved in drug discovery and development process. Hence the data should be produced to the acceptable scientific standards and specifications laid by different regulatory agencies across the globe. Bioanalytical methods should be validated to objectively demonstrate the fitness for their intended use.

ACKNOWLEDGEMENT

I would wholeheartedly express my gratitude to Mr.Raghavendhar of Bioanalytical Quality Control Department of Clinsync Clinical Research Pvt Ltd who in spite of being busy with his duties, took time out to patiently hear, guide me with the article. His guidance was extremely valuable both theoretically and practically. I truly acknowledge his contribution gratefully.

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