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# BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION

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## Abstract

Bioanalytical methods are used for the quantitative analysis of drugs and their metabolites in the biological media like saliva, urine, plasma, serum. Development and validation of bioanalytical method is important to understand the pharmacokinetics of any drug and/or its metabolites. Bioanalytical method development consists of three essential interrelated parts sample preparation, chromatographic separation and detection by using proper analytical method. Validation of a Bioanalytical method is the process by which it is established that the performance characteristics of the method meet the requirements for the intended Bioanalytical application. The validation is further divided into 3 segments full validation, partial validation, and cross validation each of which has its own purpose. This review describes mainly the various aspects for development of bioanalytical method and for the validation of bioanalytical methods. Also the Bioanalytical method transfer is also described.

**Keywords:** Biological matrix, Method stability, Matrix effect, Extraction, Method transfer

## 1. Introduction

Bioanalytical Methods are widely employed for the Quantitative analysis of the drugs and their metabolites in the biological matrix or media like saliva, urine, plasma, serum, etc also plays a significant role in the evaluation of bioavailability, bioequivalence, pharmacokinetics studies[2]. Chromatographic techniques like Gas chromatography, HPLC, LC-MS, GC-MS, Ligand binding assay, immunological and microbiological procedures used for the above purpose[7]. Bioanalytical method development is a process of creating procedure which allows the drugs and their metabolites to be identified and quantified in biological matrix or media. A compound can often be measured by several methods and the choice of analytical method involves many considerations, such as chemical properties of the analyte, concentrations levels, sample matrix, cost of the analysis, and speed of the analysis, quantitative or qualitative measurement, and precision required and necessary equipment. A suitable protocol should be made for method development and validation technique. To create a relevant Bioanalytical method, the experts should initially understand the rationale of trial so as to understand which analytical information is going to be employed, prerequisite of the method, and outcome of the results after complete conclusion of investigation. Method development consists of three essential interrelated parts sample preparation, chromatographic separation and detection by using proper analytical method[4]. AProperly validated Bioanalytical method for quantitative determination of drugs and their metabolites (analytes) plays quite important role in the effective performance of bioequivalence, pharmacokinetic and toxicokinetic studies.Mathematical models could help to develop the robust and selective method[1]. Bioanalytical method is necessary to construct a concentration-time profile. Chemical techniques are employed to measure the concentration of drugs in biological matrix, most often plasma. Validation of a Bioanalytical method is the process by which it is established that the performance characteristics of the method meet the requirements for the intended Bioanalytical application. These performance characteristics are expressed in terms of Bioanalytical method validation parameters the fundamental Bioanalytical method validation parameters include precision and accuracy, sensitivity, reproducibility, recovery and stability. However, the stability of the method can be determined by several methods including freeze and thaw method, short-term temperature stability study, long term stability and bench top stability.

**1.1 Sample collection and Preparation[2]:** The biological media that contain the analyte are usually blood, plasma, urine, serum etc. Blood is usually collected from human subjects by vein puncture with a hypodermic syringe up to 5 to 7 ml (depending on the assay sensitivity and the total number of samples taken for a study being performed). The venous blood is withdrawn into tubes with an anticoagulant, e.g. EDTA, heparin etc. Plasma is obtained by centrifugation at 4000 rpm for 15 min. About 30 to 50% of the original volume is collected. The purpose of sample preparation is to clean up the sample before analysis and/or to concentrate the sample. Material in biological samples that can interfere with analysis, the chromatographic column or the detector includes proteins, salts, endogenous macromolecules, small molecules and metabolic

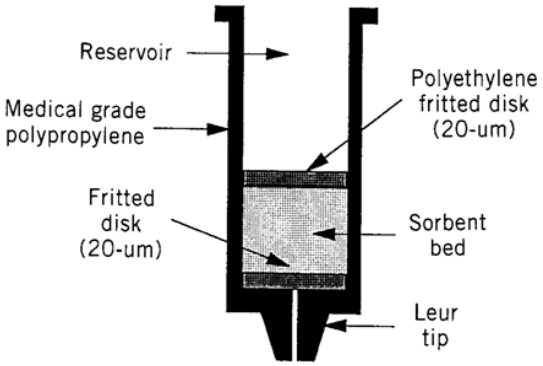
byproducts .A goal with the sample preparation is also to exchange the analyte from the biological matrix into a solvent suitable for injection into the chromatographic system. General procedures for sample preparation like liquid/liquid extraction, solid-phase extraction (SPE) and protein precipitation.

**a. Liquid/Liquid Extraction:** It is based on the principle of differential solubility and partitioning equilibrium of the analytes between the aqueous and organic phases. It generally involves the extraction of analyte from one phase into another phase and the distribution of the analyte molecules between two immiscible phases. After extraction of analyte the organic layer is separated from aqueous phase and it is evaporated in presence of nitrogen gas so as to get the dry form of sample. Now-a-days traditional LLE has beenreplaced with advanced and improved techniques like liquid phase microextraction (LPME), single drop-liquid phase micro extraction (DLPME) and supported membrane extraction (SME).[2,4]

**b. Solid –Phase Extraction:**This technique isolates the analytes of interest from wide variety of matrices includes urine, blood, animal tissue, soil, and beverages. The separation of desired analyte depends upon its affinity towards stationary phase. Analyte which is retained on the stationary phase can then be eluted from the solid phase extraction cartridge with the appropriate solvent. This technique is based on the principle of partitioning of the analytes between two phases. Now-a-days, SPE sorbent is packed between two fritted disks in the cartridge made up of polypropylene. The liquid phase is thus allowed to pass through the cartridge.

Commercial solid phase extraction cartridge is a short inert plastic tube packed with an adsorbent, usually a reversed phase or an ion exchange resin. The particle size of the packing is often significantly larger than that used in LC columns to ensure a reasonable permeability Silica-base with chemically bonded functional groups or highly cross-linked polymers such as styrene divinylbenzene and polymethacrylate are generally used materials for preparation of solid phase cartridges.The general procedure for SPE is loading of a sample onto a SPE cartridge after conditioning with water and methanol and then washing of the sample solution containing the analyte or sample matrix (commonly used solvent is HPLC grade water or MILLIQ water) for removing any undesired components and then eluting out the desired analyte into a test tube. Solid phase extraction uses the same type of stationary phase as it is used in liquid chromatography columns.SPE technique is modified by solid phase microextraction.[2, 4]

**Fig1: Solid-Phase extraction Assembly[9]**




**c. Protein Precipitation:** Protein precipitation can be applied to extraction of plasma and blood samples. Principle of PP is based on precipitation (denaturation) of the proteins by using various reagents like acid (trichloroacetic acid and perchloric acid), organic solvents (methanol, acetone and acetonitrile) or by salts (ammonium sulphate). After denaturation the sample is centrifuged, which results in extraction of analyte in the precipitating solvent. There are some benefits with the precipitation method as clean-up technique compared to SPE. It is less time consuming, smaller amounts of organic modifier or other solvents are used. The samples often contain protein residues and it is a non-selective sample cleanup method, there is a risk that endogenous compounds or other drugs may interfere in the LC-system. However the protein precipitation technique is often combined with SPE to produce clean extract. Methanol is generally preferred solvent amongst the organic solvent as it can produce clear supernatant which is appropriate for direct injection into LC-MS/MS. Salts are other alternatives to acid and organic solvent precipitation. This technique is called as salt-induced precipitation. As the salt concentration of a solution is increased, proteinsaggregate and precipitate from the solution.[2, 4]

**2. Basics considerations for method development[3]:**

1. The choice of given method involves several considerations such as analytical concentration range i.e. required for the method, sample matrix, whether the measurement is qualitative or quantitative, precision required, equipment requirements, place of method implementation and availability of qualified personnel.
2. The most important criteria during the method development is that the whole procedure, from sample collection to the analysis of the sample matrix, should be selective, simple and robust as possible so that the method could be implemented in an ordinary analytical lab without the purchase of highly expensive equipments.

3. Cost of analysis also plays an important role while developing a method for a particular analyte and also the method selected for the sample preparation.
4. Also choice of sampling media plays an important. The most common samples obtained for bioanalysis are blood, plasma and urine.
5. Separation or isolation of drugs and their metabolites is usually performed to purify a sample. This is done to obtain the selectivity and sensitivity to detect a particular compound and so as to reduce interference from the components of biological matrix.

## 3. Method Validation[2, 7, 5]:

The guidelines for bioanalytical method validation are published by the United States Food and DrugAdministration (USFDA) in May 2001. These guidelines are standard for validation parameters' evaluation and requirements. Bioanalytical method validation is the approach employed to indicate that the analytical method used to assess an analyte in biological matrix is reliable and also reproducible. There are three types of validations; full validation, partial validation, and cross validation.Method validation is a process used to verify/confirm that an analytic method developed is suitable for its intended purpose, that it provides reliable and valid data for a specific analyte. Typical parameters to validate are; include selectivity, accuracy, precision, linearity and range, limit of detection, limit of quantification, recovery, robustness and stability.

Need For Bioanalytical Method Validation:
1. for assurance of quality of products and formulations.
2. Achieving and enhancing of products by international agencies.
3. for compulsive requirement for registration of pharmaceutical product or formulations[5].

**3.1 Full Validation:** When carrying out bioanalytical method development of an analyte for the first time, fullmethod validation is employed. Full method validation is mandatory for any new drug entity. Also full method validation is recommended when metabolites are added to current assay of already existing analyte. The assay is required to be revised for all analytes measured.

**3.2 Partial Validation:** Partial validations are usually modifications of validated bioanalytical methods which do not essentially require complete revalidations. In partial validation either one intra-assay of precision and accuracy is carried out or "approximately" full validation is done. Partial validation can also be carried out whenthere is alteration in species within matrix (e.g. rat plasma to mouse plasma), changes in matrix withina 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.

**3.3 Cross Validation:** Cross validation is comparison of two bioanalytical methods. Cross validations are essential when two or more bioanalytical methods are applied to generate information within same study. Theevaluations should be done by considering an innovative validated bioanalytical method as the reference and the repeated bioanalytical method as the comparator and vice-versa.

**a. Selectivity:** Selectivity exercise is carried out to assess the ability of the bioanalytical method to differentiate and quantify the analyte(s) in presence of other components in the sample. For selectivity, analyses of blank samples of appropriate biological matrix (plasma, urine, or other matrix) obtained from at least six sources should be carried out. Each blank sample should be tested for interference and selectivity should be ensured at the lower limit of quantification (LLOQ).

**b. Accuracy:** The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy 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 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.

**c. 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, which measures precision with time and may involve different analysts, equipment, reagents and laboratories.

**d. Linearity and Range:** A calibration curve is the relationship between instrument response and known concentration of the analyte. The calibration curve should be prepared in the same biological matrix as the

samples and a calibration curve should be generated for each analyte. The range of the method is the concentration interval where accuracy, precision and linearity have been validated. The used calibration curve should be the simplest model that adequately describes the concentration-response relationship. The deviation should not exceed more than 20% from the nominal concentration of the LLOQ and not more than 15% from the other standards in the curve.

**e. Limit of Detection:** The limit of detection (LOD) is a characteristic for the limit test only. It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. 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).

**f. 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 concentration 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 concentration of a sample that can be quantified, with acceptable precision and accuracy. The ULOQ is identical with the concentration of the highest calibration standards.

**g. Recovery:** The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery. Recovery% = Extracted sample / Post-extraction spiked sample X 100 Recovery reflects the degree of extraction; in general recovery is impacted by the interaction of the analyte with endogenous and/or exogenous components of the matrix.

**h. Matrix Effect**: Matrix effect is investigated to ensure that selectivity and precision are not compromised within the matrix screened. Three blank samples from each of at least six batches of matrix under screening are extracted. For matrix effect LQC (lower quality control), MQC (middle quality control) and HQC (higher quality control) spiking dilutions and internal standard dilution are spiked in the above extracted blank samples. Recovery comparison sample at LQC, MQC and HQC concentration level along with internal standard are prepared and screened.

**i. Robustness:** According to ICH guidelines, The robustness 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.

**j. Stability:** The stability of the analyte under various conditions should also be studied during method validation. The conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The following stability conditions are required by FDA and are advisable to investigate;

**Stock solution stability:** The stability of the stock solution should be evaluated at room temperature for at least 6 hours.

**Short-term temperature stability:** The stability of the analyte in biological matrix at ambient temperature should be evaluated. Three aliquots of low and high concentration should be kept for at least 24 hours and then analysed.

**Long-term temperature stability:** The stability of the analyte in the matrix should exceed the time period from sample collection until the last day of analysis.

**Freeze and thaw stability:** The stability of the analyte should be determined, after three freeze and thaw cycles. Three aliquots of low and high concentration should be frozen for 24 hours and then thawed at ambient temperature.

**Post-preparative stability:** The stability of the analyte during stages of the analysis processshould be evaluated.

**Bench-Top Stability:** Bench top stabilityexperiments should be designed and conducted to cover the laboratory handling conditions that are expected for study samples.

**Processed Sample Stability**: The stability of processed samples, including the time until completion of analysis, 695 should be determined.

**Method transfer[10]:** Timely method transfer plays an important role in expediting drug candidates through development stages. Method transfer is not an easy task and requires careful planning and constant communication between the laboratory personnel involved in the transfer. Method transfer could occur within the same organization or between pharmaceutical companies and analytical service providers. To have a successful transfer, the bioanalytical method itself must be robust and the equipment differences between the delivering and receiving parties should be carefully evaluated. Use of standardized automation equipment has shown to be advantageous during the method transfer.

## 4. Conclusion

This review describes the various aspects for the bioanalytical method development and its validation. Bioanalytical method development consists of three essential interrelated parts sample preparation, chromatographic separation and detection by using proper analytical method. Validation of a Bioanalytical method is the process by which it is established that the performance characteristics of the method meet the requirements for the intended Bioanalytical application. Also the basic considerations for the method development are also discussed here. The development of bioanalytical method and its validation helps to determining the pharmacokinetics of drugs and their metabolites. For the method transfer the bioanalytical method must be robust and rugged also constant communication between the laboratory personnel involved in transfer is required.

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