IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)
An International Open Access, Peer-reviewed, Refereed Journal

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION: A REVIEW

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Abstract:

In this study, medicines and their metabolites are often quantified in plasma matrices using bioanalytical techniques. These techniques should be applied to investigations in both nonhuman and human clinical research fields. A critical component of calculation and interpretation of bioequivalence, pharmacokinetic, and toxicokinetic research is the employment of the bioanalytical method for the quantitative assessment of medicines and their metabolites in biological medium. Method creation, method validation, and sample analysis are the three main responsibilities of bioanalysis. It is important to consider how environment, matrix, and procedural factors may affect the estimation of analyte in the matrix at each stage of the procedure, from setup to analysis. Each instrument has unique benefits and drawbacks. For the bioanalysis of small/large compounds, gas chromatography and HPLC have primarily been utilized, along with LC/MS/MS. In this review paper, it is advised that we add a few information on the development and validation of bioanalytical methods. We will be able to identify the medicine, its concentration, and its metabolite using these specifics, which will aid in quality control.

Keywords: Bioanalytical Method, Validation, Method Development, etc.

Introduction:

Bioanalytical procedures are necessary for the identification of drug concentration and their metabolites in a biological matrix (urine, plasma, saliva, serum, etc.). It is essential to a pharmaceutical product's development. The concentration of the biological matrix can be determined using chromatographic methods such as HPLC (High Performance Liquid Chromatography), LC-MS, etc. Bioanalysis is essential for the research of pharmacokinetics, pharmacodynamics, and toxicological evaluation during drug development. In addition to drugs and metabolites, the bioanalytical method analyses both small and large molecules, including proteins and peptides. The bioanalytical method is essential in many other research areas, such as forensic analysis, doping control, and the creation of biomarkers for the investigation of many diseases. The validation of bioanalytical methods is necessary for the quantitative measurement of many different analytes in biological and physiological matrices, and this methodology is highly beneficial in both non-human and human clinical pharmacology. [1]

Bioanalysis has an important role in drug development.

- ✓ Toxicological evaluation
- ✓ Pharmacokinetic studies
- ✓ Pharmacodynamics studies

Method development

Developing a Bioanalytical method is the process of designing a procedure to recognize and quantify a novel or unidentified component in a matrix. The chemical characteristics of the analyte, concentrations, sample matrix, cost of the analysis method and tools, speed and time of the analysis, quantitative or qualitative measurement, precision, and required equipment must all be taken into account when determining how to analyse a molecule. The procedure for developing a technique includes the following steps: sample preparation, sampling, separation, detection, evaluation of the results, and finally conclusion. [2]

Steps in the creation of a method

1. Method selection and thorough sample information conducting a literature study and gathering all the necessary information on drug profiles, as well as the pharmacokinetic and physicochemical properties of analytes and related compounds, is the initial step. Additionally, while selecting the internal standard in LC-MS/MS, the analyte's chemical makeup and structure must match exactly. The same molecule with various isotopes of deuterium, such as C13 and N15, must be used as the internal standard in LC-MS/MS. Today, HPLC-UV has replaced LC-MS/MS in many laboratories.

2.Initial technique setup involves selecting diluents depending on factors such drug solubility, internal standard, drug metabolites, and compatibility with the analytical method. In the aqueous solution, the analyte with the lowest concentration is measured. It's crucial to pay attention to the resolution and the separation between the peaks during this period.

3. Examining the analytical method in aqueous standards: Before moving on to a method in a biological matrix, the aqueous standard of the bioanalytical method is checked. The calibration curve is then plotted for the aqueous standard with at least four concentrations, with the lowest and highest. A preliminary study will identify the lowest standard concentration, and Cmax will determine the highest standard concentration. The correlation coefficient can be calculated using any calibration curve standard, but it shouldn't be greater than 0.99.

4. Improvement of the sample processing technique: A matrix sample must be created and compared to the aqueous standards in order to ensure that the instrument parameters remain constant throughout validation. An extensive investigation of the analyte, physicochemical parameters, and internal standards is carried out for method development in order to establish and enhance the bioanalytical technique.

5. Examining the analytical process in the context of the biological matrix Prior to validating the procedure for pre-validation, it is essential to check the accuracy, precision, and recovery of a well-established bioanalytical method for matrix materials. Due to its improved sensitivity, precision, recovery, and low interference, solid-phase extraction is favoured when there are issues with liquid-liquid extraction's reproducibility and recovery. At least three aliquots of each matrix sample from the Lower Quality Control (LQC), Higher Quality Control (HQC), and Lower Limit of Quantification (LLOQ) must be analysed with one set of the extracted calibration curve standards in order to compare the results for recovery with aqueous quality control (QC) samples of the same concentration.

6. Pre-validation: After determining the validity of this validation process, create a thorough sample preparation procedure with all the required data, contributing conditions, and method conditions. [5,6,7]

Sample collection and preparation

Typically, the analyte can be found in live media like blood, plasma, urine, serum, etc. Blood is commonly drawn from human volunteers or subjects by puncturing a vein and filling a hypodermic syringe with 5-7 ml of blood. The venous blood is drawn into tubes with an anticoagulant; frequently, heparin and ethylenediaminetetraacetic acid are combined. Plasma is obtained after 15 minutes of centrifugation at 4000 rpm. A third to half of the volume is collected. The aim of sample preparation is to clean the sample before to analysis. The components of biological samples that can affect analysis, the chromatographic column, or the detector include endogenous macromolecules, proteins, salts, small molecules, and metabolic wastes. The analyte from the biological matrix is also transported into a solvent that may be injected into the chromatographic device as part of the sample preparation process. Examples of general sample preparation methods include liquid/liquid extraction, solid-phase extraction (SPE), protein precipitation, chromatography, and ligand binding assay (LBA). [3,4]

Sample preparation technique in bioanalytical method development

Some of the following bioanalytical method:

- Extraction method
- Protein precipitation
- Chromatography method
- Ligand binding assay (LBA).

Extraction method

Liquid-Liquid Extraction (LLE): When processing biological and aqueous samples, the LLE method is most frequently used. In order to separate analytes from interferences, this approach separates them into two organic and aqueous immiscible phases. According to the partition coefficient, the analyte mixture separates into two immiscible solvents. Traditional LLE has been replaced by modern LLE due to its more advanced and improved techniques. The more sophisticated LLE approach includes liquid-phase microextraction (LPME), support membrane extraction (SME), and single drop liquid-phase microextraction (DLPME). It is easier to extract drugs from biological samples that are basic and acidic. The LLE process is helpful, especially for removing salts, but because it uses an excessive amount of hazardous solvents, it takes longer and has a negative impact on the environment. Additionally, it is frequently impossible to extract the required amount of the analyte from the same sample when it has a different polarity, such as a medication and its metabolites. [8,9]

SPE

Analytes are bound to solid supports using the selective sample preparation (SPE) method, interferences are removed, and the analytes are then selectively eluted. SPE is a relatively efficient method despite the wide range of sorbent choices. Four steps are involved in the solid phase: conditioning, sample loading, washing, and elution.

1.Conditioning

An organic solvent that also serves as a wetting agent for the packing material and solvates the functional groups of the sorbent is used to trigger the column. To activate the column for proper adsorption mechanisms, water or aqueous buffer is supplied.

2.Sample loading

Following pH correction, the sample is fed into the column by gravity, pump, or vacuum aspiration.

3. Cleaning

The analyte is kept while matrix interferences are eliminated.

4. Elimination

Using a suitable solvent and removing the fewest likely interferences, distribute the analyte-sorbent interactions. The sorbent used in SPE is typically silica gel with a 40 m diameter and 60 A0 pore sizes. This silica gel is chemically connected to functional groups. Syringe barrels, often referred to as packed columns, are the most widely used style. They have a 20 m frit at the bottom of the syringe with the sorbent material and another frit on top. Discs are placed in syringe barrels for extraction. These discs are constructed of inert matrix and 8–12 m packing material particles. Discs are used and maintained in a similar way to packed columns. Discs have the substantial advantage of merely implementing higher flow rates when compared to packed columns. Chemicals that are acidic, basic, neutral, and amphoteric are the four categories into which analytes can be categorised. Depending on the pH, especially the pH of 13, amphoteric analytes can act as cations, anions, or zwitterions. Both acidic and basic functional groups are present. [10,11,12]

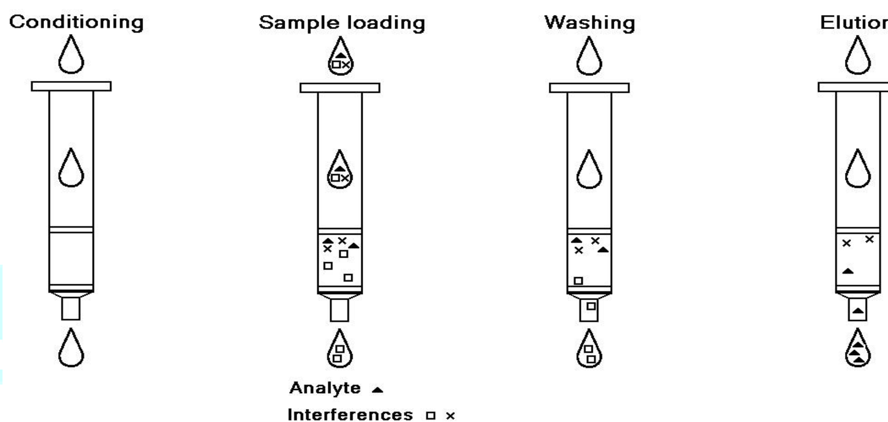

Figure 1: Steps in solid phase extraction

Precipitation of proteins

Protein precipitation is a common method for removing proteins in routine analysis. By altering the proteins' solubility, a salt, an organic modernizer, or a change in pH can all result in precipitation. The supernatant from centrifuging the samples can either be put to the HPLC system right away or dried off and then dissolved in the appropriate solvent. The sample is then concentrated after that. In comparison to SPE, the precipitation approach has a number of advantages.

It takes less time and uses less organic modifiers or other solvents. There are negative aspects as well, though. There is a potential that endogenous substances or other pharmaceuticals may hinder the reversed phase-HPLC method because it is a non-selective sample cleansing procedure and samples typically contain protein particles. However, protein precipitation is usually used with SPE to produce clean extract. Methanol is the most frequently utilised organic solvent because it can produce a clear supernatant that can be added right away to HPLC. Salts are an alternative to acid organic solvent precipitation. This technique is called salt-induced precipitation. As the concentration of salt increases, proteins congregate and precipitate out of a solution. [13,14]

Bioanalytical Method Validation (BMV)

The effectiveness and dependability of a bioanalytical approach should be demonstrated in order to raise the level of confidence that may be placed in the results. According to Shah et al., if the outcomes are to be used to support the registration of a novel drug or the reformulation of an existing one, all bioanalytical procedures must also be verified. In order to ensure that a method works as intended, it should be regularly monitored while being used, hence it should be emphasized that the first validation is merely the start. [15]

Need of Bioanalytical Method Validation

1. Adopting bioanalytical processes that have been well characterized and verified is essential for producing accurate results that can be comprehended properly.

2. It is known that bioanalytical techniques and methodologies are at the cutting edge of technology and are constantly being improved upon.

3. It is also very important to stress the fact that each bioanalytical method has distinct characteristics that rely on the analyte being employed. For each analyte, specific validation standards might therefore need to be developed.

4. In addition, whether the technique is appropriate may depend on the study's ultimate objective. It is necessary to assess the bioanalytical processes at each location and give the pertinent validation information for various locations in order to achieve inter-laboratory reliability when samples analysis for a particular study is carried out at numerous locations. [16]

Demand for Bioanalytical Method Validation

The nature of the analytes and the technology utilized in method development and validation determine the utility of a bio-analytical technique's validation or bio-analysis of a medicine. Dependable and reproducible methodologies and procedures are always required for the bioavailability (BA), bioequivalence (BE), and pharmacokinetic (PK) parameter assessments of drugs and their metabolites. [17,18,19]

Bioanalytical Method Validation

In bio-analytical method validation different types and levels are come which must be need to understand basic requirement in the process. Here all types are defined in very specific manner.

A. Full Validation

B. Partial Validation

C. Cross Validation

A. Full Validation: In accordance with ICH requirements, a new technique for a new drug must first undergo complete validation. It is vital to check whether metabolites are released along with the medication and the new substance.

B. Partial Validation: Modification of a bioanalytical method that could, but isn't required to, undergo full validation. the following modifications to traditional bioanalytical techniques are required:

- Bio-analytical method transfer between laboratories or analysts
- Change in analytical methodology
- Change in anticoagulant in harvesting biological fluid
- Change in matrix within species (e.g. human plasma to human urine)
- Change in sample processing procedure
- Change in species within matrix (e.g. rat plasma to mouse plasma)
- Change in relevant concentration range
- Change in instrument and /or software platforms
- Limited sample volume
- Rare matrices

C. Cross Validation: In this method of bioanalytical validation, two parameters are compared, either within one study or among studies. It is possible to cross-validate reference methodologies and contemporary bioanalytical techniques. It is ideal to evaluate utilising both methods. When data are generated using various analytical techniques (such as LC-MS-MS vs. ELISA), cross validation should also be done. [20]

Bioanalytical Validation Parameters

Validation in Bio-analytical method is including

1. Selectivity
2. Linearity
3. Accuracy, Precision, Recovery
4. Limit of detection (LOD)
5. Limit of quantification (LOQ)
6. Calibration Curve
7. Stability of analyte in spiked
8. Ruggedness (Robustness)

1. Specificity (Selectivity)

It will be expected that the ability of bioanalytical technology to distinguish and endure in the presence of the analytes and materials will be a gift. Degradants, pollutants, metabolites, or matrix components are a few examples of these.

Each step of development should be specifically addressed by the analytical procedure. The technique must be capable of making an accurate assessment of the target analyte in the presence of all predicted components, which may include degradants, excipients, sample blank peaks, and sample matrix. The sample blank peaks might also be attributed to elements like chemicals or filters used during sample preparation.[21]

2.Linearity

Analytical techniques have the potential to yield test results that are directly proportional to the concentration of the analyte in the sample. In the bioanalysis, at least five or six samples from the lower limit to the upper limit were spiked. It is recommended to perform specialised statistical computations like linear regression in addition to visually analysing the signal as a function of concentration. Additional parameters like slope and intercept, residual sum of squares, and the correlation coefficient should be provided to increase the method's linearity.

3. Recover, Accuracy, Precision

A minimum of six batches, ranging in concentration from the lowest detectable level to the highest concentration achievable, are used to achieve accuracy, precision, and recovery. Take nine samples in a single batch. Accuracy can be represented using the following techniques: Precision, linearity, and specificity are (a) inferred from them; (b) the results are compared to those of an independent, well-characterized process. Recovering the drug substance that was spiked with a placebo or drug product, recovering the drug substance that was spiked with the drug substance or drug product to check for impurities, and recovering the impurity that was spiked with the drug substance or drug product are all examples of ways to test for impurities. The mean value should be no more than 15% off from the actual value, with the exception of LLOQ, where it shouldn't vary by more than 20%.

The precision of an analytical method is defined as the similarity of individual measurements of an analyte when a procedure is repeated on several samples of a single homogeneous volume of biological matrix. To measure precision, a minimum of five determinations for each concentration should be employed. Within the estimated concentration range, it is recommended to sample a minimum of three concentrations. The precision determined at each level should not be more than 15% of the coefficient of variation (CV), with the exception of the LLOQ, where it should not be more than 20% of the CV.

Recovery is concerned with how well an analytical process extracts data while staying within the confines of variability. The analyte and the internal standard should both be recovered; however, the analyte recovery does not have to be 100%. It should instead be consistent, accurate, and reproducible. Recovery studies should be conducted by comparing the analytical results for extracted samples at three concentrations (Low, Medium, and High).

4.Limit of Detection (LOD)

The lowest sample concentration at which background noise and signal can be separated. Use a signal-to-noise ratio of 3.0 or higher for the analyte standard solution. The minimal sample concentration at which a quantity is sufficiently accurate and precise to be determined. A number of techniques are used to determine LOD, such as (a) visual definition (b) calculation from the signal-to-noise ratio (c) calculation from the standard deviation of the blank, and (d) calculation from the calibration line at low concentration.

5.Limit of Quantification (LOQ)

In addition to enabling the analyte to be reliably identified, it is the lowest concentration of analyte at which the preset goals for bias and imprecision are met.

6. Calibration Curve

An instrument response and analyte concentration are depicted by a standard curve, also known as a calibration curve. With eight different concentration values, a calibration curve spans a range from lower concentration to greater concentration. The number of standards required for the calibration curve will depend on the anticipated range of analytical results and the type of analyte response. The standard concentration should be selected based on the investigation's concentration range. A calibration curve should ideally consist of six to eight non-zero samples that span the expected range and LLOQ, as well as a blank sample (a matrix sample without an internal standard) and a zero sample (a matrix sample with an internal standard).

Lower Limit of Quantification (LLOQ): The standard analyte response for the calibration curve must be five times greater than the blank response to meet the lower limit of quantification (LLOQ) requirement. An analyte response should have an accuracy and precision of between 80 and 120 percent and 20 percent, respectively. The following conditions must be satisfied for a standard curve response: Both the LLOQ deviation and the other standard deviation must be less than 20%. If at least 80% of the sample falls within the allowed range, the standard curve will pass.

7.Ruggedness

A method's robustness is measured by how sensitive it is to small variations in parameters that could occur during normal analysis, such as temperature, the composition of the mobile phase, and pH levels. Although it is not necessary for comprehensive validation, robustness testing can be highly helpful during the method development/prevalidation phase since problems that may arise during validation are commonly detected in advance. A method's robustness should be assessed if it is intended to be transferred to another lab. [22,23]

8. Stability

During the method validation process, it is also important to look at the analyte's stability under varied conditions. Stability tests should be conducted under conditions that are similar to those that would actually be present when handling and analysing actual samples. FDA lists the following stability conditions as ones that should be looked into. [24]

Stock solution stability

It is necessary to assess the stock solution's stability throughout the course of six hours at room temperature.

Short-term temperature stability

The stability of the analyte in biological fluids at room temperature must be evaluated. Each concentration, low and high, was kept in three aliquots for at least 24 hours before being tested.

Long-term temperature stability

From the time the sample is collected until the very end of the analysis, the analyte in the matrix should stay stable.

Freeze and thaw stability

Three freeze-thaw cycles should be completed before determining the stability of the analyte. Three aliquots of each concentration, low and high, should be kept frozen for 24 hours before being defrosted at room temperature.

Post-preparative stability

The stability of the analyte during stages of process of analysis should be evaluated. [25,26]

Discussion:

The primary bioanalytical technique is an attempt to understand and explain the bioanalytical studies using a systematic approach that starts with data collection and progresses through method development and systematic validation from a basic point of view for determining selectivity, sensitivity, calibration curve, recovery, accuracy, precision, matrix effect, stability, and the robustness of chromatographic method to support pharmacokinetic, toxicokinetic, and bioavailability studies. Numerous significant sample preparation methods are also covered in bioanalysis using the detection methods HPLC, LC/MS/MS, and UPLC (Ultra Performance Liquid Chromatography).

Conclusion:

The pharmaceutical industry's methods for drug discovery and development heavily rely on bioanalysis and the production of pharmacokinetic, toxicokinetic, and metabolic data. The development and validation of bioanalytical methods have been attempted to understand and explain from the viewpoint of the quality assurance division. This article details some of the procedures and the numerous scenarios that were examined during the analysis of the research sample to validate the methodologies. To raise the standard and broaden the acceptance of this field of study, a number of key issues of developing and validating bioanalytical methodologies have been investigated.

Acknowledgments:

The authors are thankful to AICTE New Delhi for providing the financial support during M. Pharm tenure. Also, thankful to the Principle of Government College of Pharmacy, Karad for providing required facilities.

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