International Journal of Medical and Pharmaceutical Excellence
VOLUME-1 ISSUE-1 | INAGURAL EDITION | January-March 2026 | MPE001

Paper Received: **15/01/2026** Paper Reviwed: **07/02/2026** Paper Published: **10/02/2026**

AN ANALYTICAL REVIEW OF BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION

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ABSTRACT: This research underscores the necessity of developing and validating bioanalytical processes for the precise assessment of medicines, metabolites, and biomarkers in biological matrices. Current procedures for sample preparation, chromatographic separation, and detection are examined, emphasizing LC-MS/MS. Selectivity, sensitivity, accuracy, precision, linearity, robustness, and stability are essential criteria for acceptable bioanalytical methods. Recent research examines common challenges, novel approaches, and best practices to improve the accuracy and reproducibility of bioanalytical assays. The project seeks to develop and evaluate procedures to guarantee bioanalytical precision in clinical trials, pharmacological investigations, and bioequivalence assessments.
Keywords: *Bioanalytical method development, Bioanalytical method validation, LC–MS/MS, Biological matrices, Regulatory guidelines, Accuracy and precision, Pharmaceutical analysis*

1. INTRODUCTION

Bioanalysis is essential for the advancement of innovative medications. It ensures precise pharmaceutical research data from the discovery phase to clinical development. Bioanalysis is crucial for pharmacokinetic, pharmacodynamic, and toxicological investigations as it clarifies the processes of pharmacological absorption, distribution, metabolism, and excretion. Superior bioanalytical data is crucial for assessing a medication's efficacy, safety, and quality.

Bioanalysis measures physiological components, metabolites, and medicinal substances in biological matrices. Typical matrices include whole blood, plasma, serum, cerebrospinal fluid, urine, and saliva. Utilize bioanalytical devices for the collection, processing, preservation, and evaluation of materials. Every procedure must be conducted with accuracy to preserve samples and yield precise results. owing to the difficulties inherent in manipulating and modifying biological matrices.

The importance of bioanalytical methods for evaluating bioavailability and bioequivalence (BA/BE) has increased with progress in pharmaceutical sciences. These tactics can improve pharmacokinetic research, clinical trials, and the development of generic pharmaceuticals. Minimal quantities of chemicals can be evaluated using accurate and meticulous analytical methods. This streamlines regulatory submissions and pharmacological assessments.

Bioanalysis uses extend beyond the creation of new medications. They provide substantial benefits for pharmacovigilance, fundamental biology, and pharmaceutical research. Improved analytical methods and tools make processes more accurate, dependable, and attainable. Bioanalysis is essential for ensuring patient safety and determining medication dosages.

Bioanalysis requires high-purity chemicals and solvents, sophisticated analytical tools, chromatographic apparatus, reference compounds or working standards, and a validated biological matrix source. Developing, evaluating, and refining effective bioanalytical procedures requires substantial knowledge and research. International standards, such as ICHQ2(R1), Q2(R2), and M10, are periodically updated to provide new insights and standardize bioanalytical techniques.

2. METHOD DEVELOPMENT

The bioanalytical approach elucidates its construction, limitations, optimal circumstances, and efficacy in achieving its objectives. This confirms the system's readiness for approval.

The following are the Steps involved in the development of the Method:

Method Selection and Complete Information Collection of the Sample: Review the literature on drug profiles and aggregate all accessible information. The chemical structure and properties of the analyte must correspond to select an RP-HPLC internal standard.

Selection of Initial Method Conditions: Select a diluent for the procedure depending on the drug's solubility, internal standards, metabolites, and the efficacy of the analytical method. Focus on the duration between peaks and the resolution.

Analyzing the Analytical Method in Aqueous Standards: Prior to employing bioanalytical procedures on biological fluids, they are evaluated using an aqueous standard. A calibration curve is generated from a minimum of four concentrations, arranged from lowest to highest. To derive the correlation coefficient, input each standard from the calibration curve into the computer. The range must not exceed 0.99.

Development and Optimization of Sample Processing Method: A matrix sample must be generated and compared to aqueous standards to maintain instrument parameters during validation.

Analyzing the Analytical Method in the Biological Matrix: When liquid-liquid extraction methods exhibit diminished recovery and reproducibility, solid-phase extraction provides superior sensitivity, accuracy, recovery, and minimum interference.

Pre-validation: Draft a sample preparation protocol that encompasses all requisite details, contributing factors, and methodological conditions following successful validation.

Sample Preparation Approaches:

Liquid-liquid Extraction (LLE): The concept is that "the organic immiscible solvent selectively extracts the analyte intended to be present in the liquid sample."

Diethyl ether, ethyl acetate, dichloromethane, and methyl tert-butyl ether are the predominant solvents used in liquid-liquid extraction (LLE). Solubility is employed in liquid-liquid extraction to segregate immiscible solutions. Organic and aqueous phases should not intermingle.

Solid Phase Extraction (SPE): Following a certain duration, analytes dissociate from a solid phase (adsorbent). The approach utilizes the analyte's preferential adsorption into the solid phase. Following adsorption, the target analyte can be extracted using the appropriate solvent. Owing to its efficacy and specificity, solid-phase extraction (SPE) is extensively employed. A multitude of cartridges is suitable with diverse sample matrices and analytes.

3. SPE PROCEDURES

Step 1: Preparation is the initial stage. It is essential to incorporate the needed solvents into each solid phase extraction tube prior to utilizing the sample. Solvents comprise methanol, tert-butyl methyl ether, and a minor quantity of dichloromethane. Alternative solvents, such as organic solvents, water, and buffers, may also be utilized. Figure 1 depicts an illustration of solid phase extraction.

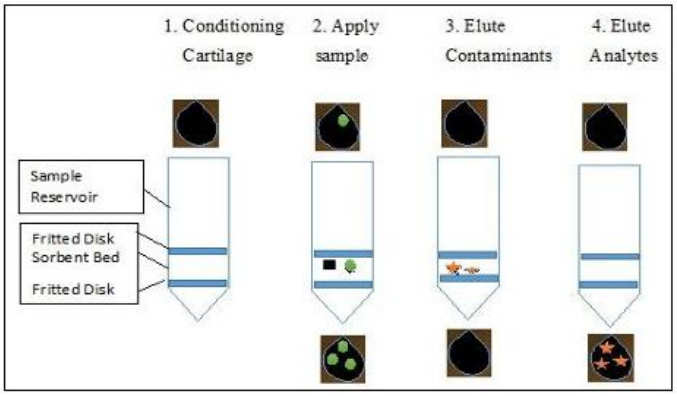

Fig. 1: Solid Phase Extraction

Step 2 Sampling: Ensure the sample does not contact the inner wall of the cartridge without traversing a passage as you meticulously place it into the container from above.

Step 3: Rinse or Wash: The analyst can remove weakly retained interferences by rinsing the cartridge with a poorly mixed solvent or buffer. This process will eliminate matrix components and other interferences while preserving the analyte.

Step 4: Drying: The vacuum pump may be utilized for the recommended drying duration, provided that the appropriate vacuum is employed. Allow it to dry for two to three minutes to eliminate any water that may hinder the elution process.

Step 5: Elution: The sequential passing of a solvent through a cartridge is essential to enhance the effectiveness of the extraction process.

The optimal solvents for elution include minimal quantities of methanol, acetonitrile, dichloromethane, and tert-butyl methyl ether. Acidic or alkaline methanol or acetonitrile, or a combination of these with other organic solvents, is utilized to facilitate efficient elution.

SEP's Advantages Versus LLE:

1. Comfortable method,
2. Application simplicity.
3. Accuracy and recovery improved.
4. The complete analyte fraction is more readily obtainable,
5. Automation is simpler.

The Technique of Solid-Phase Extraction (SPE): Analogous to liquid chromatography, the Solid-Phase Extraction (SPE) technique segregates analytes based on their interaction with a solid sorbent. Recent improvements in solid-phase extraction have resulted in the release of various novel formats, including:

1. Dispersive SPE,
2. Molecularly imprinted polymer SPE
3. Disposable pipette extraction
4. Micro-Extraction by packed sorbent
5. Solid-phase micro extraction
6. Stir bar sportive extraction

7. Online solid-phase extraction

Protein Precipitation: Analytes are chemical substances found in biological fluids, such as blood or serum, that are isolated when proteins undergo structural degradation. The most essential aspect of the procedure is the degradation of proteins utilizing organic solvents such as methanol or acetonitrile. Proteins may precipitate by modifying the sample's pH or by introducing organic solvents. Proteins may occasionally denature due to hydrophobic interactions on their surface.

This may also happen when inorganic salts are mixed with water-soluble organic solvents. This two-step technique, often termed "salt-induced phase separation" or "salting out," effectively extracts non-polar chemical compounds from water-miscible liquids. The predominant salts employed are calcium chloride (CaCl2), sodium chloride (NaCl), and magnesium sulfate. This is often performed as part of a salt-based liquid-liquid extraction (LLE) process.

High Performance Liquid Chromatography (HPLC): The active chemicals are categorized, positioned, and quantified using HPLC. The components of HPLC apparatus comprise a pump, an injector, columns, a detection system, a processor, and a monitoring system. Figure 2 contains an image of an HPLC machine. The principal element that separates the particles is the column. The stationary phase consists of small, porous particles measuring only a few microns, necessitating a high-pressure pump to propel the mobile phase through the column.

The mobile phase stream is augmented with a small amount of the substance for testing. The detector indicates the duration for which the substances will persist in the body. The duration required for a chemical to degrade or settle at the bottom of the column is referred to as the retention time.

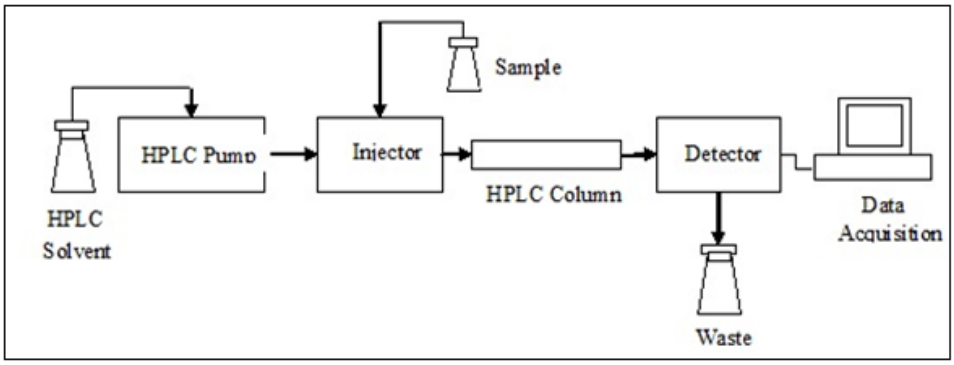

Fig. 2: HPLC Instrumentation

Injection of Sample: The sample solution can be introduced into the mobile phase during its movement via septum injectors. To attain reproducible results, an advanced loop injector and a specialized rotary valve may be employed.

4. HPLC METHOD DEVELOPMENT

The HPLC method design flowchart in Figure 3 illustrates the procedures.

Establish sampling and separation objectives, subsequently enhance sample preparation, select a detector, and execute test trials. Subsequently, optimize separation efficiency, resolve any complications, retrieve the purified analyte, and verify the technique's precision and reproducibility.

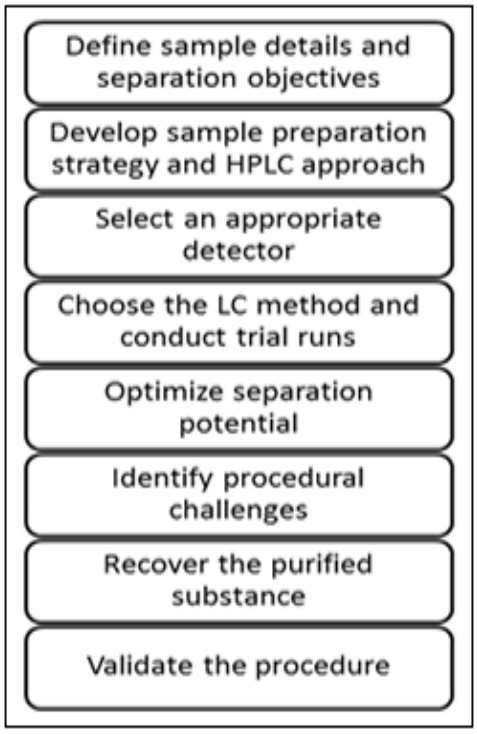

Fig. 3: Flowchart Of Hplc Method Development Process

Method Validation: The FDA's minimum criteria for stability, repeatability, accuracy, precision, selectivity, and sensitivity are met through technique validation of an analytical method.

The ICH guideline Q2 (R1) has been embraced by the pharmaceutical and medical industries. The European Medicines Agency and the FDA's guidelines for "bioanalytical method validation" are advantageous.

Bioanalytical Method Validation: The appropriateness of quantitative analysis for pharmaceutical and medical research is assessed through bioanalytical method validation (BMV).

Why Validate a Bioanalytical Method?: The precision, reliability, and consistency of results of a bioanalytical method are verified through validation. Validation establishes trust in data by verifying its adherence to scientific and legal standards. It is crucial to bear in mind that substantiation is not limited to the initial phase. In order to guarantee consistency and proper functionality, it is imperative to maintain ongoing supervision throughout the application procedure.

Need for Bioanalytical Method Validation: Accurate and comprehensible results are obtained through bioanalytical methods that are precisely defined and validated. Bioanalytical methodologies are regarded as innovative and are in a state of perpetual evolution. It is important to recognize that each bioanalytical approach has distinct characteristics that set it apart from other analytes. This suggests that it may be necessary to establish unique validation criteria for each analyte. The technique's effectiveness may also be contingent upon the inquiry's objective. It is imperative to assess the bioanalytical methods or processes at each site and provide relevant validity information in order to establish inter-laboratory reliability when a research's sample analysis is conducted across multiple locations.

5. TYPES OF BIOANALYTICAL METHOD VALIDATION:

Full Validation: Comprehensive validation transpires when all validation criteria are confirmed for application in the sample assessment of the bioanalytical process for each component. Comprehensive validation is necessary.

1. At the inception and initial use of a bioanalytical procedure.
2. Pertaining to a novel pharmaceutical agent.
3. The new assay must undergo comprehensive evaluation if compounds are incorporated into an existing quantification test.

Partial Validation: Partial validation refers to the modification of bioanalytical techniques that are either already validated or recognized as valid, without necessitating full revalidation. Typical alterations to bioanalytical methodologies encompass the following:

1. Transfer of bioanalytical methods between laboratories or analysts, including modifications to analytical techniques such as alterations to detecting systems.
2. Employing an alternative anticoagulant for the collection of biological fluid
3. A species-specific modification of the matrix (e.g., transforming human plasma into human urine)
4. Amendments to sample processing
5. Species transition inside the matrix
6. Adjusting the suitable range for concentration
7. Alterations to instruments and/or software systems
8. Small sample size
9. Uncommon matrices

Cross Validation: The process of confirming validation parameters while utilizing various bioanalytical techniques to collect data for a single research or numerous independent investigations is referred to as cross-validation. The approach utilizes a previously validated bioanalytical method as the comparator and a modified, sanctioned bioanalytical method as the reference. All essential elements are to be incorporated in the comparisons.

Common Parameters used in the Validation of Bioanalytical Methods: The recovery parameter assesses the biological matrix in the analysis and serves as a matrix effect for the validation of bioanalytical methods. The pharmacokinetic investigation also encompassed a comprehensive examination of the stability parameter.

Accuracy: Accuracy refers to the extent to which the measured concentration of an analyte aligns with its true or nominal concentration. It is commonly referred to as percentage bias or relative anticipated error (%RE). Precision and specificity can influence accuracy, which is a constant metric. It is commonly designated as "trueness." Each concentration level must be verified a minimum of five times to ascertain correctness. The specified range for concentrations in research samples must include at least three distinct concentrations. Excluding the lower limit of quantification (LLOQ), the mean for all concentrations must be within 15% of the nominal value. A 20% variation is permissible up to the lower limit of quantification (LLOQ). The most straightforward approach to validate correctness is to employ percentage bias, as detailed in the aforementioned calculation.

$$\text{Accuracy (\%)} = \text{Measured value - true value/ True value} \times 100$$

Precision: Precision refers to the extent of concordance across a series of measurements obtained using a bioanalytical procedure across multiple samples. It is a technique for quantifying stochastic error. Assessment of the dispersion for concentrations obtained from

repeated sampling of homogeneous specimens. The coefficient of variation (%CV) or relative standard deviation (R.S.D.) is commonly employed to represent repeated measurements.

Coefficient of correlation (٪) = Standard deviation / Mean

Each concentration level must exhibit a 15% coefficient of variation, whereas the limit of quantification must have a 20% coefficient of variation. Reproducibility, moderate precision, and repeatability are the three classifications of accuracy.

Repeatability: The notion of repeatability refers to the phenomenon wherein investigations conducted under identical conditions may not consistently produce the same results over a short duration (within-assay, intra-assay). A technique is deemed repeatable when it functions consistently throughout the day on a singular instrument within a single laboratory. Precision was assessed in optimal conditions.

Intermediate Precision: Intermediate precision refers to the variability observed when a process is conducted in a laboratory under differing conditions, including variations in days, analysts, equipment, and other factors. Besides fundamental repeatability, it is utilized to measure the influence of other random variables, such as inter-assay variances or daily run discrepancies. The metric evaluates the efficacy of a procedure in a laboratory environment, both broadly and in detail. The efficacy of a procedure in a laboratory setting can be assessed by intermediate precision, which quantitatively measures variations caused by different analysts, instruments, or time periods.

Reproducibility: The capacity to get consistent results across many laboratories or studies conducted in different facilities can be leveraged to enhance analytical processes, even if not required for submission.

The procedure's capability to yield consistent concentrations for a sample under diverse analytical conditions. Reproducibility refers to a method's capacity to perform consistently in both qualitative and quantitative terms across various laboratories, daily operations, analyst interactions, and instrument exchanges.

Linearity: Linearity refers to the capacity of an analytical procedure to yield results that are directly proportional to the analyte concentration within a defined range. The calibration curve must encompass the expected range of concentrations in the research sample. The accuracy and precision of the method may be compromised if a single calibration curve fails to encompass the full range, especially at the extremes. The predominant technique for assessing linearity involves the utilization of correlation coefficients, such as "r" or "r". They illustrate the efficacy of the data fit by a linear regression model.

Selectivity: Selectivity refers to a bioanalytical method's ability to reliably extract and quantify the analyte amidst other expected components. It involves proving that the analyte can be isolated from possibly interfering impurities through the analytical procedure. Selectivity refers to the method's capacity to accurately measure and isolate the analyte from other potential variables, and it is an essential validation criterion. To establish selectivity, it is essential to analyze blank samples from at least six different biological matrix sources, including plasma, urine, or other materials. The lower limit of quantification (LLOQ) for each blank sample must be assessed for interference. The FDA mandates that the accuracy of bioanalytical methods be validated by the assessment of at least six different matrix batches.

Limit of Detection: Adiscernible yet unmeasurable low analyte concentration. The LOD estimate may be imprecise due to certain bioanalytical laboratories analyzing only a limited

segment of a reference. The minimum detectable concentration of the target analyte is referred to as the LOD.

Limit of Quantification: The minimum concentration of an analyte that can be accurately and precisely measured with a high level of confidence is referred to as the limit of quantification (LOQ). It illustrates the method's sensitivity and is essential for accurately recognizing and detecting trace quantities of analytes in a sample. Analytical investigations can consistently report even minimal analyte concentrations when a limit of quantification (LOQ) is firmly established.

Recovery: Recovery refers to the percentage of analyte retrieved during the extraction and processing phases of the sample. Recovery assesses the efficacy of an analytical method in isolating substances. This illustrates the efficacy of the analyte's extraction from the biological matrix via the approach. While not always feasible, it is essential to retrieve the analyte and internal standard in a repeatable, precise, and consistent manner. The analytical outcomes of three tiers of extracted samples must be juxtaposed with the analytical results of standards exhibiting 100% recovery to evaluate recovery efficacy.

Stability: Stability refers to the capacity of an analyte to remain chemically unaltered within a specific biological matrix for a designated duration and under specified conditions. The objective of stability testing is to ascertain whether the target analyte or analytes deteriorate during particular phases of the technique, encompassing sample collection, processing, storage, preparation, and analysis. Stability testing is often conducted post-validation of the method, except for research assessing long-term stability. Long-term research may conclude years subsequent to the initiation of clinical trials. The criteria for evaluating stability differ based on the type of biological matrix, the analyte and its properties, and the anticipated storage period prior to analysis.

6. TYPES OF STABILITY TESTING

Short-term Stability: Prior to analysis, three aliquots of QC samples with low and high concentrations should be frozen and kept at room temperature for four to twenty-four hours. Analytes are stabilized by routine testing. Under specific circumstances, an analyte appears stable if it deviates by ±15% from nominal amounts.

Long-term Stability: You can freeze this for four to twenty-four hours at room temperature. Examine three samples of concentration. Project sample analyses should be outlived by storage. This test requires three analyses.

Freeze and Thaw Stability: Test samples with low and high concentrations using three freeze-thaw cycles. Aliquots should be kept at 70°C for one day per cycle. Allow it to melt at room temperature. For 12 to 24 hours, refreeze. Three times. After three rounds, examine the samples. SD ought to be less than 15%. Samples should be placed with new stock solutions in a sterile, interference-free biological matrix.

Stocked Solution Stability: To verify stability, test pharmaceutical stock and internal standard solutions for six hours at room temperature. They should be divided by less than 15%.

After freezing or chilling, the stability of the stock solution needs to be assessed. After storage, test stability by looking at how the gadget reacts to fresh solutions.

Post-Preparative Stability: The stability of the samples is tested by how long they remain in the auto sampler after preparation. The original calibration standard concentrations can be

used to determine the stability of the analyte and internal standard for the estimated run time for the batch size in validation samples. SOPs need to outline statistical techniques and approval criteria. Samples from drug users could provide proof.

Ruggedness: Throughout analysis, the robust technique can withstand daily variations in pH, temperature, and mobile phase. Transfer it to a different facility after confirming the stability of the analytical procedure. Although it can draw attention to problems and aid in their resolution, ruggedness does not imply truth.

7. CONCLUSION

To produce dependable, accurate, and repeatable results, develop and assess bioanalytical procedures for clinical trials, preclinical research, and drug discovery. Drugs and metabolites in biological matrices are analyzed using sound bioanalytical techniques. Stability, accuracy, precision, selectivity, and sensitivity are all confirmed by methodical validation. Bioanalytical techniques are becoming more widespread and investigations are becoming more precise because of ICH and other international regulations. Methods are now more dependable and feasible thanks to advancements in analysis and sample preparation. To develop and assess bioanalytical techniques and collect information for safe and efficient medications and regulations, you must put in a lot of effort.

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