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A COMPREHENSIVE REVIEW ON BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR PHARMACEUTICALS

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ABSTRACT: Bioanalysis is most widely used for drug discovery and development. Bioanalytical methods have been working for the quantitative estimation of drugs, or its metabolites and biomarkers in its biological samples. The steps involved in bioanalysis comprise of many steps that are from sample collection to sample analysis and then data reporting. The first step involves sample collection from clinical or preclinical studies. After that, the analysis of the sample is done in the laboratory. The second step involves the sample cleanup the cleanup process should be carried out carefully to eliminate the interferences present in the sample matrix. And it increases the performance of the analytical system. The last step involves sample analysis and detection of a separated compound and detection of techniques used are HPLC and Liquid chromatography coupled with double mass spectrometry (LCMS-MS) and are used in the bioanalysis of drugs in the body. Each one of these has its advantage and disadvantage. For bioanalysis of small or large molecules the chromatographic methods used are HPLC, and Gas Chromatography with LC/MS/MS. The review focuses on an overview of the bioanalytical method, development, validation, and the quality of sample preparations.

INTRODUCTION: Bioanalytical methods play a crucial role in measuring drug concentrations and their metabolites within biological matrices such as urine, plasma, saliva, and serum. This process is vital for the development of medicinal products. Techniques like High-Performance Liquid Chromatography (HPLC) and LC-MS are commonly employed to determine the concentration of substances in these matrices [1]. Bioanalysis is essential for understanding pharmacokinetics, pharmacodynamics, and toxicological assessments during drug development.

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It encompasses the measurement of both small molecules, like drugs and their metabolites, as well as larger molecules, such as proteins and peptides. Additionally, bioanalytical methods are significant in various research fields, including doping control, forensic analysis, and the identification of biomarkers for numerous diseases [2]. The validation of bioanalytical methods is critical for accurately quantifying different analytes in biological and physiological matrices. This technique is beneficial for both human clinical pharmacology and studies involving non-human subjects.

Research efforts focus on clinical trial outcomes and toxicokinetics, including bioequivalence, to draw essential conclusions regarding the safety and efficacy of medicinal products consequently, the bioanalytical methods employed must be thoroughly characterized, validated, and documented to ensure reliable results.

For analyzing various drugs, HPLC or LC/MS/MS techniques are typically utilized. While HPLC, combined with UV, PDA, or fluorescence detectors, is a cost-effective option for identifying many drugs and metabolites, it tends to have lower sensitivity compared to LC/MS/MS. Therefore, the rapid, straightforward, and highly sensitive LC/MS/MS technique is preferred for identifying analytes and impurities, making it a common choice in many laboratories for both quantitative and qualitative analysis [3].

Method of Development: The development of bioanalytical techniques involves outlining the design, identifying limitations, establishing effective conditions, and assessing the appropriateness of the process for its intended purpose. This ensures that the system is fine-tuned for validation.

Steps Involved in Method Development:

Method Selection and Complete Information of the Sample: The initial task involves conducting a thorough literature review to gather all essential information regarding the drug profile, as well as the pharmacokinetic and physicochemical characteristics of the analytes and their related compounds. Additionally, selecting the appropriate internal standard for LC-MS/MS should be based on the chemical structure and properties of the analyte, ensuring they are comparable. When choosing an internal standard, it is essential to ensure that the same molecule is present. Isotopes such as deuterium, C13, and N15 are utilized in LC-MS/MS. However, many laboratories have transitioned to using HPLC-UV instead of LC-MS/MS [4].

Selection of Initial Method Condition: The setting of initial methods includes diluent selection depending on drug solubility, internal standard and drug metabolites, and comparability with the analytical method. Using the aqueous solution the analyte is quantified and is the lowest concentration. Resolution and run time between the peaks must be taken attention during this phase

Analyzing the Analytical Method in Aqueous Standard: Initially, it is essential to verify the aqueous standard of the bioanalytical method before developing a technique within a biological matrix. Following this, a calibration curve is created using the aqueous standard, which should include at least four different concentrations, encompassing both the lowest and highest values. The maximum concentration is determined by $C_{max,}$ while the minimum concentration is based on findings from a preliminary study. To assess the correlation coefficient, each standard from the calibration curve must be injected, ensuring that the range does not surpass 0.99.

Development and Optimization of Sample Processing Method: The instrument settings need to be verified using aqueous standards to confirm stability throughout the validation process, and a matrix sample should be prepared. For method development, a thorough literature review is conducted to gather information on the analyte, its physicochemical properties, and internal standards, which helps in establishing and refining the bioanalytical technique

Analyzing the Analytical Method in the Biological Matrix: In situations where liquid-liquid extraction shows lower recovery and reproducibility, solid-phase extraction becomes the preferred method. This approach improves sensitivity, precision, recovery, and minimizes interference. Before moving forward with pre-validation, it is crucial to verify the established bioanalytical method using matrix samples to ensure accuracy, precision, and recovery. For this verification, at least three aliquots from each of the Lower Quality Control, Higher Quality Control, and Lower Limit of Quantification (LLOQ) must be analysed alongside a set of extracted calibration curve standards, which includes a matrix blank and a zero standard (the blank containing only the internal standard). The results obtained should then be compared to the recovery rates of aqueous quality control (QC) samples at the same concentration levels [5].

Pre-validation: To ensure the reliability of the validation process, develop a comprehensive procedure for sample preparation. This should include all necessary details, contributing factors, and method conditions to proceed with the pre-validation phase [6].

Methods used for Sample Preparation: Before beginning the analysis, it is crucial to prepare the sample properly. Sample preparation plays a vital role in the overall analysis process. Its main goal is to create a sample aliquot that is free from any interference.

Liquid-Liquid Extraction (LLE): Liquid-liquid extraction (LLE) is the most commonly employed technique for preparing aqueous and biological samples. This method effectively isolates analytes from interfering substances by partitioning them between two immiscible phases: aqueous and organic. The separation of the analyte mixture occurs based on the partition coefficient, allowing for distinct separation in the two solvents [3]. In recent years, more advanced LLE techniques have emerged, replacing traditional methods. These include liquid-phase micro extraction (LPME), support membrane extraction (SME), and single drop-liquid-phase micro extraction (DLPME). These advanced methods enhance the extraction efficiency of both basic and acidic drugs from biological samples [6]. While LLE is particularly effective for removing salts, it can be time-consuming and environmentally taxing due to the excessive use of toxic solvents. Additionally, in many instances, analytes with differing polarities, such as drugs and their metabolites, may not be suitable for extraction from the same sample [7].

Solid phase Extraction (SPE): Solid Phase Extraction (SPE) is widely regarded as one of the most effective and popular methods in drug analysis. It is designed to be highly efficient, reproducible, and cost-effective. The technique operates on the principle of partitioning analytes between two distinct phases [3]. SPE is primarily utilized to isolate target analytes from a solution by utilizing sorption on various sample matrices. By selecting an appropriate solvent, the analyte can either be strongly retained (K>1) or not retained at all (K=0) on the stationary phase, with retention depending on the analytes affinity for that phase [6]. Once the desired analyte is retained, any interfering substances are washed away from the cartridge, resulting in a purified analyte. The cartridge format remains the most popular choice, typically employing a sorbent of 50-200 mg to effectively separate key analytes from complex matrices. In recent years, innovative designs such as micro-SPE and flat disks have also emerged, expanding the options available for this essential technique [7].

SPE Types:

Reversed-phase: In this context, the sample matrix is either polar or moderately polar, while the analytes are mid to non-polar, utilizing a non-polar sorbent as the stationary phase. Carbon-based materials, such as C4, C8, C18, and phenyl bonded phases, are commonly employed for packing in reversed-phase chromatography. The reversed-phase setup typically incorporates polymer-based sorbents, which require the conditioning of an organic solvent, like methanol, alongside an aqueous solvent, such as water [8].

Normal Phase: Analyzing polar analytes in non-polar matrices can be effectively achieved using polar sorbents. Commonly utilized polar sorbents include silica, amino, and alumina, which serve as the foundation for various bonded phases. In this process, the packing may require conditioning with a non-polar solvent, followed by the elution of compounds using polar solvents. Silica plays a crucial role in bonding components with basic pH and functional groups. However, it is important to note that polar materials can become irreversibly bonded to the silica surface, making amino sorbents a viable alternative in such cases [9].

Ion Exchange: Ion-exchange processes are effective in holding onto charged substances or eliminating ionic interferences. The way anions and cations are retained primarily involves the exchange of these ions with those present in the sample, facilitated by the resin. Typically, these resins are made from silica-based materials, which maintain a stable pH range. However, it's important to note that their exchange capacity diminishes as the amount of organic solvent in the sample increases [10].

TABLE 1: EVALUATION OF SAMPLE TECHNIQUES

Parameter	PPT	LLE	SPE
Workability	Less	More	More
Selectivity	Bad	Good	Very good
Ion suppression	High	Low	Low
Automation	Low	Low	High
Analyte Suitability	Hydrophilic	Lipophilic	Hydrophilic and lipophilic
Cost requirement	Less	More	More

Protein Precipitation (PPT): This extraction method is relatively straightforward when compared to liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Protein precipitation is particularly useful for samples with high analyte concentrations. The process involves adding a specific amount of a water-miscible organic solvent, such as acetonitrile, acetone, or methanol, to precipitate proteins. Additionally, certain salts like aluminium chloride or metal ions such as zinc sulfate can be used, along with adjustments to the pH, which can influence protein solubility through acids like trichloroacetic, Perchloric, meta phosphoric, and tungstic acids .One of the key benefits of this method is its ability to remove unwanted plasma proteins from plasma fluid, serving as an effective cleanup technique compared to SPE. It requires smaller quantities of organic modifiers and solvents, making it less time-consuming and allowing for lower temperature conditions to achieve better purification [9]. For both hydrophilic and hydrophobic compounds, this simple extraction technique can be utilized, although in some instances, it may be beneficial to follow up with LLE and SPE for enhanced efficiency [12].

Bioanalytical Method Validation: The validation of a method is an essential component of Good Laboratory Practice (GLP) studies, ensuring that the analytical technique meets high-quality standards.

Types of Method Validation:

Full Validation: When a bioanalytical method is developed and introduced for the first time, especially for a new drug entity, it holds significant importance. Additionally, if metabolites are incorporated into an existing assay for quantification, it becomes crucial to revise the assay accordingly [6].

Partial Validation: This involves an update to the bioanalytical procedures that have already been approved. The necessary adjustments for improving standard bioanalytical methods include:

- Moving bioanalytical methods from one laboratory or analyst to another.
- Modifications to the analytical method are permissible.
- Changes can be made to the matrix within species.
- Sample processing procedures are adaptable.
- Variations can be introduced within the species matrix.
- The relevant concentration range can be adjusted.
- The instrument platform can also be altered.

Cross Validation: This process requires an assessment of validation parameters to generate documents either within a single study or across multiple studies when utilizing more than two bioanalytical techniques. For instance, cross-validation should be performed using reference methods alongside the updated bioanalytical method, and this evaluation needs to be conducted in both directions.

Parameters for method validation:

Selectivity: The analytical approach is capable of detecting and distinguishing the analyte even when other substances are present in the sample. These substances may include metabolites, impurities, or degradation products that are typically found in the matrix. It is essential to analyse blank samples from at least six different sources of the relevant biological matrix, such as plasma or urine.

Each blank sample must be evaluated for potential interference, and selectivity should be confirmed at the lower limit of quantification (LLOQ) [11].

Sensitivity: Sensitivity is assessed by determining the Lower Limit of Quantification (LLOQ), which represents the minimum concentration of the standard curve that can be accurately and precisely measured.

Calibration Curve: To establish the relationship between the experimental response value and a known concentration from the standard curve, it is essential to utilize the simplest model that ensures acceptable accuracy and precision. A calibration curve should be created by introducing a known concentration of the analyte into the same biological matrix. This study may also involve multiple analytes [6].

If obtaining the matrix proves challenging, an appropriate calibration matrix should be chosen, and a comparison of the responses from both matrices should be conducted. The selection of standard concentrations should be based on the estimated concentration range relevant to the specific study. The calibration curve must include a blank sample (a matrix sample processed without the internal standard), a zero sample (a matrix sample with the internal standard), and 5-9 non-zero samples that span the expected range, including the lower limit of quantification (LLOQ)

Recovery: The recovery of an analyte in an assay refers to the detector response generated from a specific amount of the analyte that has been added to and extracted from a biological matrix. This response is then compared to the detector response obtained from the true concentration of a pure authentic standard. Recovery is an important aspect of extraction efficiency in analytical methods, acknowledging that it may not always reach 100%. However, it is essential that the recovery levels for both the analyte and the internal standard remain consistent, precise, and reproducible. To assess recovery, experiments should involve comparing the analytical results of extracted samples at three different concentrations (low, medium, and high) with un-extracted standards that indicate 100% recovery [13].

Accuracy: The accuracy of an analytical method refers to how closely the average results obtained align with the true value, or nominal concentration, of the analyte. To assess accuracy, it is essential to conduct at least five determinations for each concentration level. It is advisable to include at least three different concentrations within the expected range. The average result should fall within 15% of the actual value, except at the lower limit of quantification (LLOQ), where the deviation should not exceed 20%. Essentially, accuracy serves as a gauge for how much the mean result diverges from the true value [14].

Precision: Precision refers to how closely individual measurements of an analyte align with one another. This is assessed by examining a series of measurements taken from different aliquots of the same uniform sample, all under consistent conditions. For each concentration level, the criteria for precision should not surpass 15% of the coefficient of variation (CV). Additionally, the acceptable deviation for the limit of quantification (LOQ) is set at 20% [15].

Matrix Effect: There can be challenges arising from the suppression or enhancement of ionization of analytes due to the influence of matrix components in biological samples. This can lead to variations in analyte response, either increasing or decreasing it. To accurately assess each analyte and the internal standard (IS), it is essential to quantitatively measure the matrix factor for each distinct batch of matrices. This involves calculating the ratio of the analyte peak area in the presence of the matrix-determined by analyzing a blank matrix spiked with the analyte after extraction-to the peak area obtained from a pure analyte solution. The IS-normalized matrix factor is derived by dividing the matrix factor of the analyte by that of the IS. The coefficient of variation for the IS-normalized matrix factor, measured across six different matrix lots, should not exceed 15%. This assessment should be conducted at both low and high concentration levels, specifically up to three times the lower limit of quantification (LLOQ) and near the upper limit of quantification (ULOQ) [16].

Stability: The stability of an analyte in a biological fluid depends on several factors that must be carefully managed throughout the storage process. It is crucial to ensure that the chemical properties of the drug, the matrix or solution in which it is stored, and the type of container used do not alter the analyte's concentration. To accurately assess the stability of analytes, it is important to monitor every stage, including sample preparation, collection, analysis, and handling. This includes evaluating both long-term storage at freezing temperatures and short-term storage at room temperature. Additionally, the effects of freeze-thaw cycles and the analytical process on analyte concentration must be considered. The conditions used in stability experiments should mimic those encountered during actual sample handling and analysis. Furthermore, the method should include an assessment of analyte stability in the stock solution [16].

Ruggedness: It is essential to assess the reliability of a method by utilizing a different instrument and

column that share the same dimensions, make, and material. This approach can be beneficial during the method development and pre-validation stages. In one of the batches focused on precision and accuracy, evaluations can be conducted by injecting samples with an alternative column. Additionally, to thoroughly assess the robustness of the extraction method, it is important that different analysts handle the precision and accuracy batch [17].

Quantitative Application of Bioanalytical Method:

TABLE 2: THE UTILIZATION OF VARIOUS SAMPLE PREPARATION TECHNIQUES IN THE ANALYSIS OF DRUGS THROUGH LC-MS/MS APPLICATIONS

S. no.	Drug	Matrix	LC column	Sample preparation	LLOQ	Ref.
1	Dapoxetine	Human plasma	ACE C8 (50 mm × 4.6 mm × 5 µm)	LLE	5 ng/ml	16
2	Fisetin	Rat plasma	Nucleoden C18 (250 mm × 4.6 mm × 5 µm)	PPT	3.18 ng/mL	17
3	Chlorogenic acid	Human plasma	Zorbax C18 (50 mm ×2.1 mm,1.8 µm)	PPT	10 ng/ml	18
4	Rufinamide	Mouse plasma	LichroCART purospher star pre column C18 (55 mm × 4mm,3 µm)	PPT and LLE	0.1 µg/ml	19

DISCUSSION: The primary bioanalytical technique aims to enhance our understanding of bioanalytical studies through a structured approach. This process begins with gathering relevant information, followed by the development of methods and thorough validation. It focuses on key aspects such as selectivity, sensitivity, calibration curves, recovery, accuracy, precision, matrix effects, stability, and the robustness of chromatographic methods. These elements are crucial for supporting studies related to pharmacokinetics, toxicokinetics, bioavailability, and bioequivalence. Additionally, the text explores various important sample preparation techniques utilized in bioanalysis, employing detection methods like HPLC, LC/MS/MS, and UPLC (Ultra Performance Liquid Chromatography).

CONCLUSION: This review explores the latest concepts and advancements across various domains, particularly in sample preparation tools and separation techniques. Solid Phase Extraction (SPE) and Liquid-Liquid Extraction (LLE) are highlighted as the most widely utilized sample preparation methods. Significant developments in SPE technology, including new formats and sorbent selections, are discussed. The focus is primarily on LC-MS/MS, emphasizing its application in high-quality bioanalysis of small molecules. Additionally, the article addresses innovative ideas and emerging technologies that have the potential to enhance LC-MS/MS capabilities.

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