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REVIEW ARTICLE

A Review on Bio-Analytical Methods and its Validation by RP-HPLC

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ABSTRACT

In the process of drug discovery and development, establishing reliable bioanalytical methods is critical. These methods are essential for quantifying drugs and their metabolites in plasma matrices, both in preclinical and clinical research settings. Various physicochemical and biological techniques, such as mass spectrometry, chromatography, and immunoassay, are employed to achieve this goal. Liquid chromatography-mass spectrometry (LC-MS) is a prominent technology utilized for qualitative and quantitative analysis of pharmacological compounds and metabolites. The review discusses the use of RP-HPLC and liquid chromatography in this context. Additionally, extraction techniques like protein precipitation, solid-phase extraction, and liquid-liquid extraction are explored for preparing materials for Reverse Phase-HPLC analysis. The focus lies on validating bioanalytical methods to ensure accuracy and repeatability. Validation involves demonstrating the suitability of a technique for quantitatively measuring analytes in biological matrices like blood, plasma, serum, or urine. Parameters such as accuracy, sensitivity, selectivity, standard curve, quantification limits, range, recovery stability, among others, are crucially evaluated. Trustworthy bioanalytical methods are indispensable for various stages of drug development and clinical research. Through rigorous validation, these methods can reliably quantify drugs and their metabolites, aiding in bioequivalence, pharmacokinetic, and toxicokinetic assessments. Techniques like LC-MS, coupled with appropriate extraction methods, play a pivotal role in achieving accurate and reproducible results, thereby enhancing the reliability of drug development processes.

Keywords: *Bioanalytical methods, Validation parameters, liquid chromatography, extraction methods and bioanalysis of samples.*

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INTRODUCTION

An essential part of drug development, production, and discovery is the creation and verification of analytical procedures. In quality control laboratories, formal test techniques resulting from these processes are used to guarantee drug items' identity, purity, potency, and effectiveness. A set of procedures for collecting, handling, storing and analyzing a biological matrix such as saliva, urine, plasma or serum is called a bioanalytical method [6]. A biological matrix, like saliva, urine, plasma, or serum, can be collected, treated, preserved, and examined using a series of procedures known as a bioanalytical method. The suitability of quantitative analysis techniques for biochemical applications is assessed through the Bioanalytical Method Validation [BMV] process. Medication dosages are calculated using bioanalytical techniques on a biological matrix, such as whole blood, serum, plasma, or urine. Medication dosages are calculated using bioanalytical techniques on a biological matrix, such as whole blood, serum, plasma, or urine. The pharmacological concentration would be determined through bioanalysis, it is essential for the creation of novel medications and dosage forms [7]. This information, based on biology, pharmacokinetics, hazard kinetics and other areas, can be employed to preserve the medication's safety and effectiveness. The quality of the underlying bioanalytical data is predominantly determined by these studies. Consequently, it is essential to define and inform the pharmaceutical industry of the guiding principles for confirming these analytical procedures. RP-HPLC and LCMS-MS both find application in the bioanalysis of drugs in plasma [14]. Each instrument has different advantages. When combined with a UV, PDA or fluorescence detector, A large variety of compounds can be estimated using RP-HPLC. One of the primary advantages of these chromatographic principles is their low detection limits and capacity to cover a broad range of analytes with varying polarity, providing structural information, requiring little sample preparation, and having low detection limits. A bioanalytical method validation method is any method that proves that a particular method was employed to determine the quantitative concentrations of an analyte. Selectivity, accuracy, precision, linearity, detection limit, quantification limit, recovery, resilience, stability, and range are important validation criteria. Before bioanalytical methods are used, it is important to demonstrate that they are suitable for the purpose for which they were developed. The widely accepted standards for method validation are the ICH standards Q2 (R1), which are relevant to both pharmaceutical and medical sciences [6]. Other standards, aimed specifically at bioanalysis, are much more specific, demand for more rigorous validation, and also define hard limitations for the majority of determined parameters [1,2]. Other bioanalytical standards are more specific, require more stringent validation, and also set hard limits for most of the parameters determined [12]. Food and Drug Administration (FDA) guidelines for commercial bioanalytical technique verification and recommendations from the European Medicines Agency (EMA) for bioanalytical method validation [16]. New parameters such as matrix effect, carryover, dilution integrity etc. must also be determined during the validation process as discussed in previous years. A key characteristic of bioanalytical method development is the rigorous testing of the stability of the analytes under a wide range of conditions during method application [20]. When generating bioanalytical method (BAM) validation data for clinical pharmacology and bioavailability studies that necessitate pharmacokinetic evaluation or bio equivalency assays, sponsors of New Drug Applications (NDAs), Abbreviated New Drug Applications (ANDAs), and Investigational New Drug Applications (INDAs) can utilize these guidelines as a guide [19].

Development of method:

Many steps are involved in developing the approach, some of which are as follows [11]:

- Step 1- Selecting a methodology and gaining knowledge of the characteristics of the sample.
- Step 2- Selecting the first method condition
- Step 3- Validation of the analytical technique in an aqueous standard
- Step 4- Development and improvement of sample processing technology
- Step 5- Examination of the biological matrix to confirm the analytical procedure
- Step 6- When developing bioanalytical methods, these stages are used for pre-validation [16].

Step 1- Approach selection and sample details data on the review is used to compile the physicochemical characteristics of the drug analyte and related compounds, the relevant literature to establish the analytical procedure. Choose an internal reference whose polarity, molecular size, structure, shape, and functional groups—as well as its partition coefficient, dissociation constant, and other characteristics—are comparable to those of the analyte.

Step 2- The solubility of the drug analyte, drug metabolite, internal standard, and compatibility with the analytical method are taken into consideration when choosing diluents. In this phase, transit time and peak-to-peak resolution are measured. The minimum amount of analyte must be determined using the aqueous solution.

Step 3- Examining analytical methods for aqueous standards Prior to examining the process in a biological matrix, review the analytical techniques in aqueous standards [15]. It is best to use the minimum produced aqueous calibration curve standards at their highest and lowest concentrations. The highest concentration level should be determined by C_{max}, while the lowest concentration standard should be determined by preliminary studies. Determine the correlation coefficient by injecting each calibration curve standard. The suggested value for the correlation coefficient (r) is 0.999 or above. To obtain the required clarity and sensitivity, the mass spectral parameters, mobile phase, buffer strength, ratio, pH, flow rate, wavelength, and other chromatographic parameters (column, column oven temperature, mobile phase materials, etc should be adjusted as needed [8].

Step 4- Aqueous standards should be used to prepare the matrix sample before the instrumental method is finished. This will help to develop and improve the sample processing procedure. The structure, functional groups, pH, partition coefficients, dissociation constant, polarity, and solubility of the analyte and internal standard are among the physicochemical characteristics that should be taken into account while deciding on and refining the sample preparation method. Among these are protein precipitation, liquid-liquid extraction, and solid phase extraction.

Sample collection and preparation: The analyte is typically found in biological media like blood, plasma, urine, and serum. In order to extract blood from human volunteers, a hypodermic needle is typically used to puncture veins up to 5-7 milliliters (the amount necessary for the study and the test's sensitivity will determine how many samples are needed). A tube containing an anticoagulant, such as EDTA, heparin, etc., is filled with taken blood from the veins. Centrifugation is used to make plasma for 15 minutes at 4000 rpm. Amounts collected range from thirty to fifty percent. Prior to testing, sample preparation aims to concentrate and purify the sample. Proteins, salts, endogenous macromolecules, tiny molecules, and metabolic wastes are a few examples of substances in biological samples that can hinder analysis or cause chromatography column or detector malfunction. A solvent is also substituted for the analyte from the biological matrix to prepare the sample for introduction into the chromatographic apparatus. SPE, protein precipitation, as well as additional generic sample preparation methods such liquid/liquid extraction [6].

Precipitation of proteins, solid-phase extraction (SPE), and liquid/liquid extraction are common techniques for sample preparation [9].

Liquid/Liquid Extraction: The basis for this method is the concept of different solubility and balance of distribution between the analyte's organic and aqueous phases. The analyte is typically extracted from one phase to another after being partitioned into two immiscible phases. After the analyte has been extracted, the organic layer is separated from the aqueous phase and evaporated in the presence of nitrogen gas to make the sample dry. Modern LLE has been replaced by Liquid Phase Extraction (LPME), Single Drop Liquid Phase Extraction (DLPME), and Assisted Membrane Extraction (SME).

Solid Phase Extraction (SPE): The technique of Solid phase extraction helps for eliminating interference and selectively eluting analytes from samples after they have been bound to a solid substrate. Because there are so many different sorption alternatives, it is especially successful to use solid phase extraction. Elution, washing, conditioning, and sample loading are the four stages that make up the solid phase technique.

Conditioning: The packing material gets moist and the sorbent's functional groups dissolve when an organic solvent is used to activate the column. The column is activated for efficient adsorption mechanisms by adding water or an aqueous buffer.

Sample Loading: Material is supplied into the column by gravity, pumps, or vacuum suction after the pH has been adjusted.

Washing: While matrix interferences are removed, the analyte is retained.

Elution: Elution involves breaking down the analyte-sorbent interactions using the appropriate solvent and removing as few interferences as possible.

A typical sorbent in SPE is silica gel with a pore size of 60 A^0 and a diameter of 40 µm. Chemically functional groups are bound to this silica gel, which enable a variety of mechanisms of action. Packing columns, often known as syringe barrels, are the most used design. The sorption material is contained in a 20 m long frit at the bottom of the syringe and another frit is located at the top. The discs are put inside barrels of syringes for extraction. Eight-to-twelve-millimeter packing material particles encased in an inert matrix make up these discs. Discs are used and stored in a same way as packed columns. Using discs instead of packed columns has the primary advantage of allowing for larger flow rates.

Amphoteric, basic, acidic, and neutral substances are the four categories into which analytes can be separated. Depending on the pH, amphoteric analytes can act as cations, anions or zwitterions because their functional groups are both basic and acidic.

Protein Precipitation: Blood and plasma samples can be obtained using protein precipitation. A range of reagents, including acids (like trichloroacetic acid and perchloric acid), organic solvents (like acetone, acetone, and methanol), and salts (like ammonium sulfate) as a base PP, are used to precipitate (denature) proteins. After the sample is denatured, it is centrifuged, which extracts the analyte and precipitates it into the solvent. As a purification method, the precipitation method has several advantages over SPE. It takes less time and does not require as many organic modifiers or other solvents. There is a possibility that additional drugs or endogenous compounds may interact with the LC system since the sample is not selected purification process and samples typically contain protein residues. Still, to obtain a pure extract, SPE is frequently coupled with the protein precipitation approach. Because it can yield a clear supernatant that is appropriate for direct infusion into LC, methanol is a frequently utilized organic solvent+MS/MS. Organic solvents and acids that precipitate precipitate salts instead. This process is known as precipitation produced by salt. Proteins build up and precipitate out of solutions with higher salt contents [14].

Solid phase micro extraction: Blood and plasma samples can be obtained using protein precipitation. Proteins can be precipitated (denatured) using a variety of reagents, such as acids (such as trichloroacetic acid and perchloric acid), organic solvents (such as acetone, acetone, and methanol), and salts (such as ammonium sulfate) as a base PP. After the sample is denatured, it is centrifuged, which extracts the analyte and precipitates it into the solvent. As a purification method, the precipitation method has several advantages over SPE. It takes less time and does not require as many organic modifiers or other solvents. There is a possibility that additional drugs or endogenous compounds may interact with the LC system as it is a non-selective sample purification process and samples typically contain protein residues. This simple solvent-free method combines sampling, extrapolation and sample introduction in a single step. This method concentrates and isolates the analyte within a specific region of the coating material by coating quartz glass fibers with a modified bonded phase, contacting them with the sample, subjecting them to steam, or immersing them in a stream of gaseous sample. Removal of the Analytes from these fibers and measurement with analytical devices such as a GC or GC-MS. The SPME is a seemingly simple device that uses a syringe modified to contain a fiber holder and fiber array. Using an extraction fiber, the analyte in the sample was immediately removed and concentrated [14]. To examine thermally adhesive or low-volatility substances that GC or GC/MS cannot identify, these SPME are directly coupled with HPLC and HPLC/MS.

Matrix-solid phase dispersion: Sample preparation involves the use of solid matrices. Because less than 1g of sample and less solvent are needed, the process is known as microscale extraction.

Supercritical fluid extraction:

In order to recover nonpolar to moderately polar analytes from matrices, supercritical fluid extraction is frequently utilized. Legal requirements require it to be an environmentally friendly substitute for organic solvents. Supercritical fluids are gaseous and liquid in terms of viscosity and diffusivity, yet they have the same density as liquids. Ten to sixty minutes is the average duration of SFE. Supercritical fluids have a lower sensitivity to temperature and pressure variations in their dissolving power. Under normal circumstances, many supercritical fluids are gases. Compared to bodily fluids, this facilitates analyte recovery.

Step 5- Protein precipitation and tests for recovery, accuracy, and interference are preferred analytical techniques in the biological matrix under research when drug sensitivity is higher. Liquid-liquid extraction and testing for interference, accuracy, and recovery should be considered when drug sensitivity is decreased. Due to its higher sensitivity, recovery, precision and minimal interference, solid phase extraction is preferred when the repeatability and recovery of liquid-liquid extraction are lower. In this pre-validation phase of the bioanalytical methods, the validation parameter is then searched for.

Step 6- Before validation when the reliability of the approach has been established, proceed with pre-validation by developing a brief protocol that describes the sample preparation, instrument conditions, and method conditions. The pre-validation phase should include assessment of selectivity, accuracy, precision and recovery factors.

Basics considerations for method development [17]:

1. When choosing a particular method, a number of considerations must be made, such as the analytical concentration range or what is needed for the method, the sample matrix, the kind of measurement (qualitative or quantitative), the required level of precision, the equipment requirements, etc., the location where the method is applied, and the capacity of qualified personnel.
2. To enable the method to be used in a regular analytical laboratory without the need to buy a lot of expensive equipment, every stage of method development process—from sample collection to sample matrix evaluation—must be as precise, straightforward, and dependable as feasible.
3. Apart from the sample preparation process, an additional crucial consideration when formulating a method for a particular analyte is the analytical cost.
4. Furthermore crucial is the kind of sample medium that is employed. Urine, plasma, and blood are the most often obtained samples for bioanalysis.
5. Drugs and their metabolites must frequently be extracted or isolated from a sample in order to facilitate purification. This procedure aims to decrease interference from biological matrix components and boost a compound's selectivity and sensitivity for detection.

Bioanalytical Method Validation (BMV):

The process of verifying the efficacy of a particular analytical methodology for the quantitative analysis of the analyte in a biological matrix in order to achieve the intended method validation is known as bioanalytical applications. The validity of these bioanalytical methods depends on the analyte or techniques used for method development and validation. These methods are extremely difficult for studying the bioavailability, bioequivalence and pharmacokinetics of drugs. To meet the basic requirements of the validation process, several phases must be understood [3].

Need of Bioanalytical Method Validation:

1. The use of carefully characterized and verified bioanalytical methods is critical to achieving accurate and understandable results.
2. It is well known that cutting edge bioanalytical techniques and protocols are always being updated and enhanced [4].
3. Additionally, it is crucial to highlight the distinct characteristics of each bioanalytical method that vary according to the analyte being used. It might therefore be necessary to create unique validation standards for every analyte.
4. The end goal of the study may also affect how applicable the technique is. To guarantee interlaboratory reliability when analyzing samples for a specific study at many locations, it is crucial to validate the bioanalytical methods at each site and provide pertinent multi-site validation information [18].

Validation Parameters:

a. **Selectivity:** The aim of the selectivity exercises is to assess how well bioanalytical techniques can identify and quantify the analyte(s) when additional sample components are present. Blank samples of the relevant biological matrix—plasma, urine, or another matrix—must be assessed using samples derived from at least six different sources in order to demonstrate selectivity. Ensuring the selectivity of the lower limit of quantification (LLOQ) and examining each blank sample for interference are crucial [5].

b. **Accuracy:** The degree to which the mean test results generated by the procedure properly represent the true value (concentration) of the analyte is known as the analytical method's accuracy. Accuracy is assessed via repeated examination of samples with known analyte concentrations. Each concentration should be determined at least five times in order to assess accuracy. Using a minimum of three concentrations is advised, and each concentration should fall within the anticipated concentration range. The mean for LLOQ should be within 15% of the true value unless there is a variation of greater than 20%. The accuracy can be assessed by utilizing the standard deviation of the mean from the actual value [19].

c. **Precision:** When analyte measurements are this close, they are considered precise. This is achieved by applying a technology to many aliquots of a single homogenous quantity of biological matrix. Five determinations per concentration are required at minimum to quantify precision. It takes three concentrations within the expected concentration range to satisfy the minimal requirement. For every concentration level, an accuracy of 15% of CV or less should be calculated; the LLOQ, on the other hand, requires a precision of 20% of CV. Furthermore, there are two ways in which accuracy can be measured: inter-run precision or repeatability, also known as inter-batch, which assesses accuracy over time measurements and may use a variety of techniques, and intra-run precision or repeatability, also known as intra-batch, which assesses accuracy inside a single analytical run.

d. **Linearity and Range:** The relationship between an analyte concentration and an instrument response is called a calibration curve. Every analyte must be treated with the identical biological matrix that was used to produce the sample calibration curves. The term method range refers to the concentration range for which linearity, accuracy, and precision have all been confirmed. The calibration curve should be based on the simplest model that explains the concentration-response relationship accurately. 15% and 20%, respectively, are the maximum deviations allowed from the other curve requirements and the nominal concentration of the LLOQ [18].

e. **Limit of Detection:** The limit test is the only one with the limit of detection (LOD) function. It is the smallest amount of analyte in a sample—which may or may not be quantifiable—that can be recognized under the given experimental conditions. The analyte concentration in the sample is often expressed as a percentage, parts per million (ppm), or parts per billion (ppb) upon detection.

f. **Limit of Quantification:** The lower limit of quantification, or LLOQ, is the smallest amount of analyte in a sample that can be quantitatively identified with sufficient precision and accuracy. The lowest concentration of the sample that can still be quantified with a reasonable level of precision and accuracy is the LLOQ, according to the most practical technique, which establishes the LLOQ based on precision and accuracy. Applications where baseline noise is present, such as chromatographic techniques, are the only ones where signal-to-noise ratio (S/N)-based LLOQ is applicable. The maximum analyte concentration in a sample that can be reliably and precisely quantified is known as the upper limit of quantification, or ULOQ. The concentration of the highest calibration standard and the ULOQ are identical [19].

g. **Recovery:** The distinction between the detector response obtained for a quantity of the analyte injected into and extracted from the biological matrix and the detector response obtained for the precise concentration of the pure authentic standard is known as the analyte recovery in an experiment. Analytical findings for extracted samples at three concentrations (low, medium, and high) should be compared to unextracted standards that indicate 100% recovery in order to conduct recovery testing.

Recovery % = extracted sample / post-extraction spiked sample x 100

Recovery, which is typically based on the analyte's interactions with internal and/or external matrix components, serves as a proxy for the degree of extraction.

h. **Matrix Effect:** To ensure that the tested matrix has no effect on selectivity or precision, the matrix effect is examined. Every one of the minimum of six sieve matrix batches yields three blank samples. The previously mentioned extracted blank samples are spiked with internal standard dilutions and spiking dilutions for matrix impact on lower quality control (LQC), intermediate quality control (MQC), and higher quality control (HQC) [8].

i. **Robustness:** According to ICH guidelines, an analytical technique's robustness is determined by its ability to withstand minor but intentional modifications in method parameters, which also serves as a gauge of the technique's reliability in normal scenarios. A robustness test is a type of experimental setup designed to assess an approach's robustness. Robustness is the capacity to replicate the (analytical) process across many labs or surroundings without unanticipated alterations in the outcomes.

j. **Stability:** Validating a method requires analyzing the stability of the analytes under different circumstances. Tests for stability must be carried out in environments similar to those that are probably going to be encountered when handling and analyzing samples in real life. The following stability conditions must be followed, as advised by the FDA; It is recommended to assess the stability of the stock solution after a minimum of six hours at room temperature [10].

Short-term temperature stability: It is essential to assess the analytes' thermal stability within the biological matrix. It is recommended to wait at least 24 hours before comparing three aliquots of low and high concentration.

Long-term temperature stability: The stability of the analyte in the matrix must be maintained for a period of time greater than the interval between the final analysis day and sampling. Analyzing the analyte's stability during three cycles of freezing and thawing is crucial. Three aliquots of the low, high, and medium concentrations should be frozen for a full day before being thawed at room temperature [11].

Post-preparative stability: Throughout the analytical procedure, it is critical to evaluate the analyte's stability.

Bench-Top Stability: When planning and conducting benchtop stability tests, consideration should be given to the laboratory conditions expected for handling research samples.

Processed Sample Stability: To determine how long the analysis will take, it is crucial to evaluate the stability of the processed samples.

Method transfer: Drug candidates can advance more swiftly through the development stages with timely technology transfer. Careful preparation and continuous communication between the involved laboratory staff are essential for the intricate process of technique transfer. Method transfer can take place between pharmaceutical corporations and analysis service providers or it might happen inside the same business. Successful transfer necessitates a reliable bioanalytical plan, and it is important to carefully evaluate the equipment variations between the parties providing and receiving the information. Method transfers are now more successful when standardized automation devices are used [13].

CONCLUSION

The generation of pharmacokinetic, toxic kinetic, and metabolic data through bioanalysis is a critical component of pharmaceutical research and development, which is incorporated into the drug discovery and development process. For small molecule bioanalysis, RP-HPLC has been the preferred technique. However, more recent developments and enhancements in a number of areas (sample preparation, separation, matrix effect reduction, and specific recommendations for validation of bioanalytical methods) covered in this review confirm this. In addition to reducing the matrix effect brought on by unwanted analytes or other interfering chemicals in the sample, discussion of novel ideas and suggestions can aid in the development of bioanalytical RP-HPLC procedures.

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