IJRAR.ORG E-ISSN: 2348-1269, P-ISSN: 2349-5138

INTERNATIONAL JOURNAL OF RESEARCH AND ANALYTICAL REVIEWS (IJRAR) | IJRAR.ORG

An International Open Access, Peer-reviewed, Refereed Journal

"Bioanalytical Method Development and Validation for Estimation of Active Pharmaceutical Substance by LC-MS/MS: A General Review on Bioanalysis"

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ABSTRACT

In this review article, Bioanalytical method development is a process for quantitative estimation of a concentration of drug and their metabolites present in biological fluid such as a biological matrix like, serum, plasma matrices, blood, urine, saliva, etc. Bioanalysis of drugs in biological fluid has been grateful and determine by using Liquid chromatography with mass spectroscopy. This, methods should be useful to studies in research areas of preclinical and clinical study. Bioanalysis of drugs and their metabolites in a biological matrix is carried out using different extraction methods like solid phase extraction (SPE), liquid-liquid extraction (LLE), microextraction and protein precipitation from these extraction methods samples are spiked with reference standards and using quality control samples. A sophisticated bioanalytical development and its validation plays a crucial role in achieving the goals. Advance technique such as High-Pressure Liquid Chromatography (HPLC) and Liquid Chromatography coupled with double Mass Spectrometry (LCMS-MS) can be used for the bio analysis of drugs preclinical and clinical.

Keywords:

Bioanalytical method development, Validation parameters, LC-MS/MS bioanalysis, sample Preparation, extraction, Chromatography.

INTRODUCTION

Bioanalysis plays a pivotal role in drug development, ensuring the accurate measurement of drug concentrations in biological samples. LC-MS/MS has gained prominence in recent years, offering advantages such as improved sensitivity and specificity over traditional methods [1].

Bioanalytical method development and validation are important steps in pharmaceutical and medical research. They ensure that the tests used to measure drugs, their byproducts, and markers in the body are accurate and reliable. Method development means figuring out the best way to do these tests, making sure they can find tiny amounts of substances and only those substances. Validation checks if the tests work correctly and meet strict rules set by regulators. These steps are crucial for discovering drugs, understanding how they work in the body, and making sure they're safe and effective in patients.

BIOANALYTICAL METHOD DEVELOPMENT

a) Selection of Chromatographic Conditions:

Chromatographic conditions are critical for achieving efficient separation and accurate quantification of the active pharmaceutical substance (APS). The choice of column, mobile phase composition, and gradient elution significantly impacts the success of the method [2].

Column Choice:

The selection of an appropriate column is influenced by factors such as compound polarity, molecular weight, and sample matrix [2]. C18 columns are commonly used for hydrophobic compounds, while HILIC columns are suitable for more polar analytes. The column dimensions, such as length and particle size, also affect resolution and sensitivity.

Mobile Phase Composition:

The composition of the mobile phase plays a crucial role in achieving optimal separation. The choice between different solvent combinations and additives impacts peak shape, retention time, and selectivity. Factors like pH and buffer concentration should be carefully considered to prevent ionization or degradation of the APS[2].

Gradient Elution:

Gradient elution allows for better resolution of peaks by adjusting the solvent composition over time. It is essential to optimize the gradient profile to ensure efficient separation and prevent co-elution of interfering compounds. Balancing resolution and analysis time is crucial for method efficiency [3].

b) Sample Preparation Techniques:

The choice of sample preparation technique is crucial for extracting the APS from complex biological matrices while minimizing interference. Two common techniques are Solid-Phase Extraction (SPE) and protein precipitation.

- **Solid-Phase Extraction (SPE):**

  - Solid-Phase Extraction (SPE) is good for selectively pulling out and cleaning up APS from biological samples.

  - Various types of sorbents used in SPE have different preferences, meaning you can choose the one that works best for your compound.

- However, SPE can take a while to do, and you need to fine-tune the process for each substance you're testing.

➢ **Solid-Phase Microextraction (SPME):**

- SPME uses a special coated stick to pull out substances directly from the sample.
- Then, the stick is put into the testing machine to see what's in it.
- This method is great for capturing substances that easily turn into gas or are partly gas-like.

➢ **Liquid-Liquid Extraction (LLE):**

- LLE involves moving substances between two different liquids that don't mix together.
- Usually, a special liquid is used to grab the substances from the water-based sample.
- It works well for a wide range of substances but might need adjusting depending on what's being tested.

➢ **Protein Precipitation:**

- Protein precipitation is a quick and economical method.
- It may not be very specific.
- The process includes adding organic solvents or acids to make proteins form solid particles, which helps in extracting target substances (APS).
- Optimization is crucial to find the right balance between getting back enough of the substance you want and dealing with other unwanted stuff in the sample.

c) Optimization of Mass Spectrometric Parameters:

Ionization Source:

The choice of ionization source (e.g., electrospray ionization, atmospheric pressure chemical ionization) impacts ion formation and detection efficiency. It should be tailored to the physicochemical properties of the APS [4].

Collision Energy and Mass Transitions:

Optimizing collision energy and mass transitions enhances the specificity and sensitivity of the method. Collision-induced dissociation (CID) and selected reaction monitoring (SRM) are commonly used to improve signal-to-noise ratios and select the target analyte ions [4].

VALIDATION OF BIOANALYTICAL METHODS [5]

a. Specificity and Selectivity:

Ensuring specificity and selectivity is critical in distinguishing the APS from endogenous substances and metabolites. Strategies include using highly selective detection methods (e.g., tandem mass spectrometry) and optimizing chromatographic conditions to minimize interferences.

b. Sensitivity:

Sensitivity, expressed through the Lower Limit of Quantification (LLOQ), is pivotal for detecting low concentrations of APS. Sample pre-concentration techniques (e.g., solid-phase microextraction) and optimization of instrument parameters (e.g., dwell time) are employed to enhance sensitivity.

c. Precision and Accuracy:

Precision (reproducibility) and accuracy (closeness to true values) are paramount in method validation. Intra-day and inter-day variations are assessed, and acceptance criteria are established. Proper calibration curve fitting and statistical analysis are essential to ensure reliable results.

d. Linearity and Range:

Establishing linearity involves constructing a calibration curve to relate response to concentration. The calibration range should cover expected concentrations in the study. Selecting an appropriate range ensures accurate quantification and adherence to regulatory guidelines.

LC/MS TECHNIQUE OVERVIEW [6]

Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) is a powerful analytical technique that combines the separation capabilities of liquid chromatography with the mass analysis precision of tandem mass spectrometry. In LC-MS/MS, a sample is first separated by liquid chromatography, typically using a high-performance liquid chromatography (HPLC) system, which separates individual components based on their chemical properties. The eluted compounds are then directed into the mass spectrometer, where they undergo ionization and are subsequently fragmented. Tandem mass spectrometry involves the use of two mass analyzers separated by a collision cell. The first mass analyzer isolates the ion of interest, which is then fragmented in the collision cell. The second mass analyzer detects the resulting fragments, providing detailed structural information. This tandem approach enhances specificity and selectivity, making LC-MS/MS particularly suitable for the analysis of complex samples. LC-MS/MS offers high sensitivity, allowing for the detection of compounds at trace levels. Additionally, the technique is versatile, capable of analyzing a wide range of compounds, from small molecules to large biomolecules. The ability to quantify and identify substances with high accuracy and precision has made LC-MS/MS a cornerstone in bioanalytical research, particularly in pharmaceutical development and clinical studies.

BIOANALYTICAL METHOD DEVELOPMENT [7]

Bioanalytical method development is a crucial phase in the analytical process aimed at accurately quantifying active pharmaceutical substances (APS) within biological matrices. The first key step involves comprehensive planning, wherein researchers delineate the specific objectives and requirements of the analytical method. This initial phase includes defining the scope of the analysis, selecting suitable analytical techniques, and establishing performance criteria. Subsequently, selecting appropriate sample preparation techniques becomes paramount. Efficient extraction of the APS from complex biological matrices is crucial for achieving accurate and reproducible results. Various sample preparation methods, such as solid-phase

extraction or protein precipitation, are employed to isolate the target analyte while minimizing interference from endogenous substances.

The optimization of Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) parameters constitutes another pivotal aspect of bioanalytical method development. LC-MS/MS is chosen for its superior sensitivity, selectivity, and precision. Optimization involves fine-tuning chromatographic conditions, such as column selection and mobile phase composition, to achieve optimal separation of analytes. Additionally, mass spectrometric parameters, including ionization mode, collision energy, and detection settings, are meticulously adjusted to enhance sensitivity and selectivity. The interplay between these parameters is critical for achieving optimal performance, ensuring the accurate quantification of APS in biological samples. Overall, a systematic and well-executed bioanalytical method development process is indispensable for generating reliable data crucial for drug development and therapeutic monitoring.

BIOANALYTICAL VALIDATION PARAMETERS [8,9]

Bioanalytical validation parameters are crucial components in establishing the reliability and accuracy of analytical methods employed for the quantification of active pharmaceutical substances (APS) in biological matrices. Accuracy, the first parameter, denotes the closeness of measured values to the true values, illustrating the method's precision in quantifying the target analyte. Precision, the second parameter, measures the consistency of results under varying conditions, ensuring reproducibility and repeatability. Sensitivity, the third parameter, gauges the method's ability to detect low concentrations of the APS, highlighting its efficacy in capturing subtle changes [10].

Specificity, the fourth parameter, verifies the method's ability to differentiate the APS from other components in the sample, ensuring that the measured values truly represent the target analyte. Linearity, the fifth parameter, assesses the method's ability to produce results proportional to the concentration of the APS within a specified range. This is critical for accurately quantifying a wide spectrum of concentrations encountered in biological samples. Range, the sixth parameter, establishes the upper and lower concentration limits where the method maintains acceptable accuracy and precision, guiding the analyst on the method's practical applicability [11].

Stability, the final parameter, assesses the APS's stability throughout the analytical process, encompassing sample collection, storage, preparation, and analysis. Evaluating each of these validation parameters collectively ensures the robustness of the bioanalytical method, instilling confidence in the precision and accuracy of the results generated [12]. Complying with stringent validation criteria for these parameters not only meets regulatory standards but also enhances the overall quality and reliability of bioanalytical data, consequently contributing to the efficacy and safety assessment of pharmaceutical products [13].

METHODS FOR ASSESSING ACCURACY AND PRECISION:

1. Methods for Assessing Accuracy and Precision:

a. Accuracy:

- **Method Comparison:**

- Compare the results obtained from the bioanalytical method with a reference or established method.

- Use statistical tests like regression analysis or Bland-Altman plots to assess agreement [14].

- **Spiking Studies:**

- Spike known amounts of the analyte into the matrix (spiked samples) and compare the measured concentrations with the expected concentrations.

- Evaluate recovery and matrix effects during the spiking studies [15].

b. Precision [16]:

- **Repeatability (Intra-day Precision):**
  - Analyze multiple replicates of the same sample within a single day under the same conditions.
  - Calculate the standard deviation (SD) or relative standard deviation (RSD) for the results.
- **Intermediate Precision (Inter-day Precision):**
  - Assess precision by analyzing replicates of the same sample on different days or by different analysts.
  - Include variations such as different instruments, analysts, and days.
  - Calculate SD or RSD for the results [17].
- **Replicate Analysis:**
  - Conduct replicate analysis of samples to evaluate the precision of the method.
  - Analyze multiple aliquots of the same sample and calculate SD or RSD [18].

2. Acceptance Criteria for Accuracy and Precision in Bioanalytical Method Validation [19]:

i. Accuracy:

- **Acceptance Criteria:**
  - Typically expressed as a percentage of the true concentration.
  - Often set within ±15% of the nominal or true value for the majority of samples.
  - For higher accuracy, a ±20% acceptance criterion may be applied.
- **Criteria for Method Comparison:**
  - The correlation coefficient (r) in method comparison studies should be close to 1.
  - The intercept and slope in regression analysis should be close to 0 and 1, respectively.

ii. Precision [20]:

➢ **Acceptance Criteria:**

- Intra-day precision (RSD) is commonly set below 15%.
- Inter-day precision (RSD) is often set below 20%.
- For replicate analyses, RSD should be within acceptable limits.

➢ **Criteria for Replicate Analysis:**

- RSD of replicate analyses should be within predefined limits.
- Number of replicates should be sufficient to demonstrate precision adequately.

- **Additional Considerations:**
  - Matrix Effect Assessment:
    - Evaluate the impact of biological matrices on accuracy and precision.
    - Matrix-matched calibration standards and quality control samples are essential.
- **Internal Standards:**
  - Use internal standards to correct for variations in sample preparation and instrument response.
  - Monitor the precision and accuracy of internal standards.

SENSITIVITY AND SELECTIVITY[21]

1. **Sensitivity:**

Sensitivity in bioanalysis refers to the ability of an analytical method to detect and quantify low concentrations of the analyte accurately. It is a measure of how well the method can distinguish between small changes in analyte concentration. In the context of bioanalysis, particularly when dealing with pharmaceuticals, sensitivity is crucial for detecting trace amounts of active pharmaceutical substances (APS) in biological matrices.

Approaches to Enhance Sensitivity:

- Advanced MS Techniques:
- Utilization of High-Resolution Mass Spectrometry (HRMS): HRMS provides enhanced mass resolution, allowing for better differentiation between closely related analytes and reducing background interference.
- Selected Reaction Monitoring (SRM): SRM is a targeted MS approach that specifically monitors predefined transitions, increasing the method's sensitivity by focusing on relevant analyte signals.

Improved Sample Preparation:

- Optimization of extraction techniques: Enhanced sample extraction methods, such as solid-phase extraction (SPE) or liquid-liquid extraction (LLE), can improve analyte recovery and concentration, thereby increasing sensitivity.

- Reduction of matrix effects: Minimizing matrix effects through effective sample clean-up procedures contributes to improved sensitivity by reducing background noise.

Derivatization Strategies:

- Derivatization involves modifying the chemical structure of the analyte to enhance its detectability. Derivatization can increase sensitivity by improving the ionization efficiency of the analyte in the mass spectrometer.

Importance of Sensitivity:

Sensitivity is critical in bioanalysis for several reasons:

i. It allows for the detection and quantification of low concentrations of drugs or metabolites, which is essential in pharmacokinetic studies and therapeutic drug monitoring.
ii. Improved sensitivity increases the likelihood of detecting trace amounts of impurities or metabolites, contributing to a more comprehensive understanding of the drug's fate in the body.

2. Selectivity:

Selectivity in bioanalysis refers to the ability of an analytical method to accurately measure the analyte of interest in the presence of other components, such as endogenous substances, metabolites, or potential interfering compounds. Selectivity ensures that the method's response is specific to the target analyte and minimizes false positives or negatives.

Approaches to Ensure Selectivity [22]:

i. Optimized Chromatographic Separation:

- Proper column selection and tuning of chromatographic conditions help separate the analyte from interfering substances, reducing the likelihood of co-elution.

ii. Use of Internal Standards:

- Internal standards with similar chemical properties to the analyte can be added to the sample. This aids in correcting for variations in sample preparation and instrumental response, enhancing the accuracy and selectivity of the method.

iii. Isotopic Labelling:

- Isotopic labelling involves introducing stable isotopes into the analyte or internal standard. This technique enhances selectivity by creating mass differences between the analyte and potential interferences, facilitating their differentiation during mass spectrometric analysis.

Importance of Selectivity:

Selectivity is paramount in bioanalysis for the following reasons:

- Ensures that the measured signal is specific to the analyte, preventing false results.

- Mitigates the impact of endogenous substances or other matrix components that could interfere with the accurate quantification of the analyte.
- Facilitates reliable and reproducible results, critical for regulatory compliance and the overall success of bioanalytical studies.

LINEARITY AND RANGE [23]

1. Linearity:

Linearity refers to the ability of an analytical method to produce test results that are directly proportional to the concentration of the analyte in the sample over a certain range. In bioanalysis, this means that the relationship between the instrument response and the concentration of the analyte should be linear, allowing for accurate and reliable quantification across a specified concentration range.

Importance:

- Ensures that the analytical method can accurately quantify the analyte at different concentration levels.
- Demonstrates the reliability of the method across the intended range of concentrations encountered in biological samples.

Evaluation:

- Linearity is typically assessed by constructing a calibration curve using a series of standard solutions with known concentrations.
- The curve is plotted by correlating the instrument response (e.g., peak area in LC-MS/MS) against the corresponding concentrations of the standards.

Acceptance Criteria:

- The correlation coefficient (r) should be close to 1, indicating a strong linear relationship.
- Ideally, the method should demonstrate linearity over the entire intended range.

2. Range:

The range of an analytical method represents the interval between the lowest and highest concentrations of the analyte that have been demonstrated to be accurately quantified with acceptable precision and accuracy. It is a critical parameter that defines the applicability of the method to specific concentration levels.

Importance:

- Establishing a defined range ensures that the method is suitable for the intended purpose, covering the expected concentration levels in the study.
- Beyond the upper limit of the range, the response may saturate or become non-linear, leading to inaccurate quantification.

Determination:

- The range is determined during method validation by analyzing a set of standards at different concentrations that span the expected concentration range.
- The method's performance is evaluated, considering factors such as accuracy, precision, and linearity.

Acceptance Criteria:

- The method should exhibit acceptable accuracy and precision within the established range.
- The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) should be clearly defined.

Establishing a Linear Calibration Curve:

A. Preparation of Standards:

- Prepare a series of standard solutions covering the anticipated concentration range of the analyte.

B. Instrumental Analysis:

- Analyze each standard solution using the chosen analytical instrument (e.g., LC-MS/MS).

C. Data Analysis:

- Plot a calibration curve by correlating the instrument response (e.g., peak area) against the concentration of each standard.
- Use regression analysis to determine the equation of the calibration curve.

D. Evaluation:

- Assess the correlation coefficient (r) to ensure a linear relationship.
- Verify the goodness of fit and overall linearity of the curve.

E. Validation:

- Validate the linearity by repeating the process across multiple analytical runs.

STABLITY STUDIES [24,25]

I. Sample Storage Stability:

- Objective: Assess the stability of the APS in biological samples during storage conditions.
- Parameters to Consider:
  - Long-Term Stability: Evaluate the APS stability over an extended period, often at -20°C or -80°C.
  - Short-Term Stability: Assess the APS stability at room temperature or under refrigerated conditions over a shorter duration.

II. Sample Preparation Stability:

- Objective: Evaluate the impact of sample preparation methods on APS stability.
- Parameters to Consider:
  - Freeze-Thaw Stability: Assess the stability of APS through multiple freeze-thaw cycles.

- Auto-sampler Stability: Investigate APS stability during the auto-sampler waiting period.

III. Analysis Stability:

- Objective: Examine the stability of APS during the analytical process, including LC-MS/MS analysis.
- Parameters to Consider:
  - Instrument Stability: Evaluate the impact of instrument conditions on APS stability.
  - Matrix Stability: Assess the stability in different biological matrices used in the analysis.

IV. Forced Degradation Studies:

- Objective: Mimic extreme conditions to identify potential degradation pathways.
- Parameters to Consider:
  - Heat, Light, Acid/Base Stability: Expose APS to harsh conditions to induce degradation.

V. Data Analysis and Acceptance Criteria:

- Objective: Establish criteria for defining stability.
- Parameters to Consider:
  - Percentage Change: Define acceptable percentage change in APS concentration.
  - Identification of Degradation Products: Determine if any degradation products are within acceptable limits.

CONCLUSION

In conclusion, bioanalytical method development and validation are integral aspects of ensuring the accuracy, reliability, and regulatory compliance of analytical techniques in quantifying active pharmaceutical substances (APS) in biological matrices. The selection of appropriate chromatographic conditions, sample preparation techniques, and optimization of mass spectrometric parameters are critical steps in method development. The use of LC-MS/MS has proven to be highly advantageous in achieving precise and reliable results, particularly in the context of drug development.

Bioanalytical method validation involves assessing parameters such as specificity, sensitivity, precision, accuracy, linearity, range, and stability. Meeting stringent acceptance criteria for these parameters is essential to generate trustworthy data for pharmaceutical products. The methods for assessing accuracy and precision include method comparison, spiking studies, and various precision evaluations. Sensitivity and selectivity are crucial for detecting low concentrations of APS and ensuring accurate measurements in the presence of potential interferences.

The linear calibration curve and defined concentration range are pivotal elements in demonstrating the method's reliability and applicability. Stability studies encompass sample storage, preparation, and analysis, providing insights into the robustness of the analytical process. The conclusion of stability studies involves summarizing findings, recommending optimal conditions, and addressing regulatory implications to meet international guidelines.

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