IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
Volume 13, Issue 7 Series. I (July 2023), PP.01-12
www.iosrphr.org

Recent advances and strategies for successful bioanalytical method development and validation: A Comprehensive review

Ch. Nandini[1], M.V. Snehitha[1], A. Meghana[1], K. Sathvika[1], G. Anvesh[1], B. Shivani[1], Srinivasa Babu[1], P. Ravi Sankar*[1]

[1]*Department of pharmaceutical analysis, Vignan Pharmacy College, Vadlamudi, Guntur, 522213.*

Received 12 July 2023; Accepted 30 July 2023

Abstract:
Bioanalysis plays a crucial role in the processes of drug development and drug discovery. It serves as an essential component of toxicological evaluations, as well as pharmacokinetic and pharmacodynamic studies during the development of drugs. The bioanalysis procedure involves several stages, including sampling, sample preparation, analysis, calibration, and data evaluation and reporting. However, the development of bioanalytical methods presents a significant bottleneck in drug development. Furthermore, the validation of these methods is vital for accurately quantifying various analytes in biological samples. Method validation ensures the acceptability of assay performance and the reliability of analytical results. A bioanalytical method consists of a defined set of procedures used to measure analyte concentrations in biological samples. This review provides an overview of the parameters involved in bioanalytical method development and validation. It also explores the applications of bioanalytical methods in various areas, such as bioavailability (BA) and bioequivalence (BE) studies. The review discusses the importance of instrumentation in bioanalysis and presents various sample preparation methods. Additionally, the significance of different levels of validation is discussed, emphasizing their role in ensuring the accuracy and precision of bioanalytical methods.

Key points: Bioanalysis, Bioanalytical method development, validation, Drug development, Drug discovery.

I. Introduction

BIOANALYSIS

Bioanalytical methods are essential for quantifying drugs and their metabolites in biological samples, such as blood, plasma, serum, and urine. In modern drug development, highly sensitive and selective methods are required to accurately measure drug concentrations. Chromatographic techniques like high-performance liquid chromatography (HPLC) and gas chromatography (GC) have been extensively used for bioanalysis of small molecules. However, the most commonly employed technology is liquid chromatography-tandem mass spectrometry (LC-MS/MS). Once a method with the desired characteristics is developed, it needs to be validated to ensure accurate, precise, and reproducible data during sample analysis. Method validation is a process that demonstrates the method's ability to meet or exceed the minimum standards recommended by regulatory authorities like the FDA. These standards include accuracy, precision, selectivity, reproducibility, and stability.

Bioanalytical methods find application in various areas, including:

Bioavailability and Bioequivalence studies[1]: These studies assess the rate and extent of absorption of a drug from its dosage form. Bioavailability is determined by measuring drug/metabolite concentrations in the systemic circulation over a specific time period. Bioequivalence studies compare the relative rate and extent of drug absorption between two or more drug products of the same dosage form. These studies are crucial, especially when generic alternatives become available as drugs lose patent protection.

New Drug Development: Bioanalytical methods play a critical role in the development of new drugs. They help evaluate the pharmacokinetics and metabolism of drug candidates, providing valuable information on their absorption, distribution, metabolism, and excretion (ADME) profiles.

Clinical Pharmacokinetics and Metabolism[2]: Bioanalytical methods are utilized in clinical studies to understand the pharmacokinetic behaviour of drugs in humans. These studies investigate factors like drug absorption, distribution, metabolism, and elimination, aiding in dosage determination and optimization.

Therapeutic Drug Monitoring: In certain cases, therapeutic drug monitoring is required to maintain drug concentrations within a specific therapeutic range. Bioanalytical methods enable the measurement of drug levels

in patient samples, allowing healthcare professionals to adjust dosages as needed for optimal therapeutic outcomes.

Scope of Bioavailability studies:

In bioavailability studies, the systemic exposure profile of a drug or metabolite is obtained by measuring its concentration in the systemic circulation over a specific time period. These studies are important for:

1. Developing new formulations of existing drugs.
2. Assessing the effects of excipients, patient-related factors, and potential interactions on drug absorption.
3. Ensuring the quality of a drug product during the early stages of marketing, including evaluating the influence of manufacturing factors, storage conditions, and stability on drug absorption.

Bioequivalence studies, on the other hand, focus on comparing different drug products. They aim to demonstrate that the drug substances in multiple products with identical dosage forms reach the systemic circulation at the same relative rate and extent. For bioequivalence to be established, the plasma concentration-time profiles of the test and reference products should be statistically similar without any significant differences.

In summary, bioanalytical methods are crucial in drug development, clinical pharmacokinetics, therapeutic drug monitoring, and bioequivalence and bioavailability studies. They ensure accurate and reliable measurement of drug concentrations in biological matrices, supporting the evaluation and optimization of drug therapies.

The scope of bioequivalence studies includes:

Establishing relativity between different formulations used during the development of a new product: Bioequivalence studies compare the pharmacokinetic profiles of different formulations of the same drug to ensure they are therapeutically equivalent.

Demonstrating therapeutic equivalence of generic products: Bioequivalence studies compare the generic product to a reference product to show that they have similar pharmacokinetic equivalent therapeutic effects.

Development of modified release forms: Bioequivalence studies may be conducted when developing a modified release formulation of a drug that has already been approved as an immediate release formulation. These studies help determine if the modified release formulation achieves similar drug exposure as the immediate release formulation.

Development of alternative salt forms: Bioequivalence studies can be performed when developing alternative salt forms of pharmaceutically equivalent drugs. These studies assess whether different salt forms have comparable pharmacokinetic profiles.

Different types of studies can be conducted to determine bioequivalence, including:

Pharmacokinetic studies: These studies focus on the measurement of drug concentrations in blood or plasma over time to assess the rate and extent of drug absorption, distribution, metabolism, and elimination.

Pharmacodynamic studies: These studies evaluate the effects of drugs on the body and may involve measuring biomarkers, physiological responses, or clinical endpoints.

Clinical studies: Clinical studies involve administering the test and reference products to human subjects and monitoring their pharmacokinetic and/or pharmacodynamic responses.

In vitro studies: In vitro, studies assess the release characteristics of drug formulations using dissolution testing or other relevant techniques.

Bioanalytical method validation is crucial to ensure the suitability of the method for quantitating analytes/metabolites in biological matrices. The parameters considered during validation include:

Accuracy: The closeness of measured values to the true values.

Precision: The degree of agreement among repeated measurements.

Selectivity: The ability of the method to measure the analyte specifically in the presence of other components in the sample.

Sensitivity: The ability to detect and quantify low levels of analyte.

Reproducibility: The consistency of the method's performance when implemented by different operators, using different equipment, and at different times.

Stability: The ability of the method to maintain its performance characteristics over time.

Validation documentation includes specific laboratory investigations to demonstrate that the method meets the required validation parameters. The method is considered applicable only when the validation parameters fall within acceptable ranges.

It is important to note that while the information provided here is based on general knowledge, specific guidelines, and regulatory requirements may vary depending on the region and governing authorities involved in drug development and approval processes. It is recommended to refer to the applicable regulatory guidelines for detailed and up-to-date information.

II. Instrumentation

INSTRUMENTATION

Combined Liquid Chromatography/Mass Spectrometry:

LC/MS (liquid chromatography-mass spectrometry)[3,4] is a powerful analytical technique that combines the separation capability of HPLC (high-performance liquid chromatography) with the detection power of a mass spectrometer. It provides both qualitative and quantitative information about a compound.

Mass spectrometry (MS) is an analytical instrument that generates ions from neutral organic molecules, separates these ions based on their mass-to-charge ratio (m/z), and detects the resulting mass-separated ions to produce a mass spectrum. The mass spectrum contains valuable information about the molecular structure of organic and inorganic compounds.

A mass spectrometer consists of several key components:

1. Sample Inlet Unit: This unit allows the introduction of the sample into the mass spectrometer. In the case of LC/MS, the sample is typically introduced through an HPLC system.

2. Ionization Chamber: The ionization chamber is responsible for generating ions from the sample molecules. Various ionization techniques, such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), can be employed.

3. Mass Analyzer (Ion Separator): The mass analyzer is the central component of the mass spectrometer. It separates the ions based on their mass-to-charge ratio. Different types of mass analyzers are available, including quadrupole mass filters, magnetic sectors, quadrupole ion traps, and time-of-flight (TOF) analyzers.

4. Detector and Readout System: The detector captures the separated ions and converts them into measurable signals. Commonly used detectors include electron multipliers and photomultipliers. The readout system processes and displays the detected signals, allowing the construction of a mass spectrum.

5. Vacuum System: Mass spectrometers operate under high vacuum conditions to minimize the interference of gas molecules with the ionization and ion separation processes.

In LC/MS, the combination of HPLC and mass spectrometry offers several advantages. HPLC provides high-resolution separation capabilities, allowing the separation of complex mixtures into individual components. The mass spectrometer, on the other hand, offers highly sensitive and structure-specific detection, enabling the identification and quantification of specific compounds based on their mass spectra.

Overall, LC/MS is a versatile technique widely used in various fields, including pharmaceutical analysis, environmental monitoring, metabolomics, and proteomics, due to its ability to provide detailed molecular information about analytes in complex samples.

Table1: Ionisation Techniques

Ionization Techniques	
Electron impact ionization	EI
Chemical ionization	CI
Fast atom bombardment	FAB
Plasma desorption	PD
Laser desorption	LD
Field ionization	FI
Field desorption	FD
Secondary ion	SIMS
Thermospray	TSP
Electrospray	ESI
Atmospheric pressure chemical ionization	APCI
Atmospheric pressure photoionization	APPI

Table 2: Types of Mass Analysers

Mass Analysers	
Magnetic sector/electric sector	BE (or EB)
Quadrupole mass filter (hexapole/octapole)	Q
Triple quadrupole	QQQ

Time-of-flight	TOF
Quadrupole ion trap (cubic or linear)	Trap
Hybrid quadrupole/time-of-flight	QTOF
Hybrid time-of-flight	TOF/TOF
Hybrid quadrupole/ion trap	Q-trap

MS/MS:

Tandem mass spectrometry (MS/MS) involves the use of two or more mass analyzers, which can be either of different types (such as time-of-flight and quadrupole) or the same type (such as two quadrupoles). In MS/MS, the first mass spectrometer isolates an ion of interest, and the second stage is used to analyze the relationship between the selected ion and other ions generated from it. This technique provides valuable analytical information, improving the selectivity and sensitivity of quantitative methods.

There are several types of MS/MS experiments that can be performed, including:

1.Product-ion scan: In this experiment, the second mass spectrometer scans for fragments produced by the fragmentation of the selected ion. It provides information about the structural characteristics of the analyte.

2.Precursor-ion scan: Here, the second mass spectrometer scans for precursor ions that generate a specific fragment ion of interest. This technique is useful for identifying specific compounds or classes of compounds.

3.Constant-neutral-loss scan: In this experiment, the second mass spectrometer scans for ions that undergo a specific neutral loss during fragmentation. It is often used for identifying compounds with specific functional groups or modifications.

4. Selected decomposition monitoring: This approach involves monitoring specific fragment ions produced by the decomposition of a precursor ion. It can be used for structural characterization or identification of compounds.

When acquiring LC-MS/MS data, various modes can be employed, including:

1. Total ion current plot (TIC): The TIC is similar to the UV trace obtained in high-performance liquid chromatography (HPLC). However, the advantage of mass spectrometry is that it can detect UV-transparent components. The TIC represents the total ion current at each mass-to-charge ratio (m/z) plotted against intensity over time. Peaks appear in the TIC when small molecules elute from the chromatographic column. The main drawback of TIC is that many compounds can have the same mass, making it challenging to differentiate them. Therefore, TIC alone is not a unique identifier compared to other experiments like SIM.

2. Selected Ion Monitoring (SIM): In SIM, specific ions representing target analytes are monitored throughout the chromatographic run. This mode provides enhanced sensitivity for the selected ions of interest.

3. Selected Reaction Monitoring (SRM): SRM is similar to SIM but involves selecting a precursor ion for fragmentation in the first mass spectrometer, followed by monitoring specific fragment ions in the second mass spectrometer. SRM is commonly used for targeted quantitation and identification of specific compounds.

4. Multiple Reaction Monitoring (MRM): MRM is an extension of SRM and involves monitoring multiple transitions (precursor to fragment ion pairs) for multiple analytes simultaneously. This mode is frequently used in quantitative analysis, such as in pharmacokinetic studies.

In summary, MS/MS techniques in mass spectrometry employ multiple stages of analysis to improve selectivity and sensitivity. Various types of MS/MS experiments can be performed, and different modes of acquiring LC-MS/MS data offer advantages for specific analytical goals.

Selected Ion Monitoring (SIM) is a mass spectrometry technique that scans a very narrow mass range. It is commonly used in the analysis of serum or plasma samples. By selecting a specific mass range of interest, SIM can provide greater sensitivity compared to a full Total Ion Current (TIC) plot. This increased sensitivity is achieved by dwelling for a longer time over the narrow mass range, allowing for the detection of low-abundance analytes.

Selected Reaction Monitoring (SRM) is a mode of operation in tandem mass spectrometry where a specific precursor ion is selected and fragmented in the first mass analyzer (Q1), followed by monitoring a specific fragment ion in the second mass analyzer (Q3). SRM allows for the quantitation of the selected fragment ion, providing a simple and continuous peak in the resulting plot. The use of SRM enhances selectivity and enables specific quantification of targeted compounds or analytes.

Multiple Reaction Monitoring (MRM) is an extension of SRM, where multiple precursor-to-fragment ion transitions are monitored simultaneously. The first quadrupole (Q1) selects the parent ion, which is then fragmented in the collision cell (Q2), and the resulting fragment ions are focused to the third quadrupole (Q3) for

detection. MRM is widely used in quantitative analysis, such as in pharmacokinetic studies, where multiple analytes can be measured in a single run with high selectivity and sensitivity.
In summary, SIM, SRM, and MRM are powerful techniques in mass spectrometry that offer different advantages. SIM provides enhanced sensitivity by scanning a narrow mass range, SRM enables specific quantification of a single fragment ion, and MRM allows for simultaneous monitoring of multiple precursor-to-fragment ion transitions, facilitating multiplexed quantitative analysis.

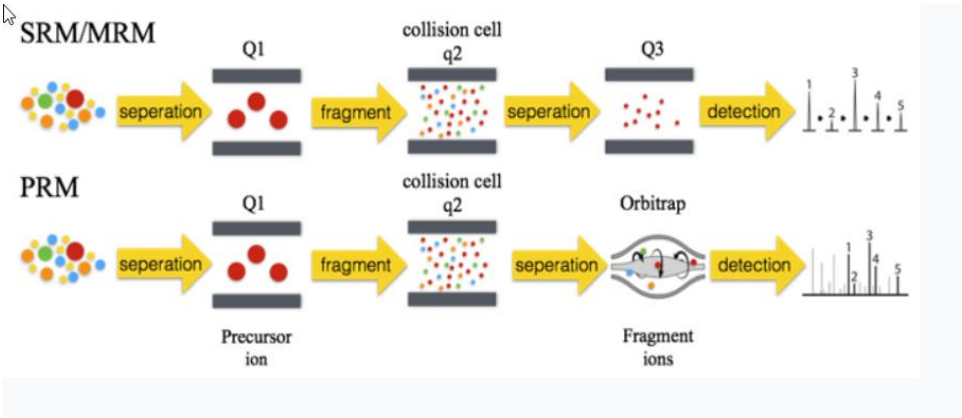

Figure1: Multiple reactions monitoring

Electrospray Interface:
The electrospray interface is a type of ionization process used in mass spectrometry to convert solution phase ions into gas phase ions. It involves several steps to generate and separate the ions in the mass analyzer. The steps involved in electrospray ionization are as follows:
Production of charged droplets: The liquid sample containing the analytes is passed through a capillary maintained at high voltage and atmospheric pressure. As the liquid stream emerges from the capillary, it breaks up into highly charged droplets due to the repulsion of like charges.
Droplet size reduction and fission: The charged droplets undergo a process of desolvation and reduction in size as they travel through the atmospheric-pressure region. The solvent molecules evaporate from the droplets, leading to a reduction in droplet size. Eventually, the droplets undergo fission, resulting in the formation of smaller droplets.
Gas phase ion formation: The smaller droplets continue to lose solvent molecules through evaporation until only gas-phase ions remain. These gas-phase ions are typically protonated or deprotonated species of the analyte molecules, depending on the pH and nature of the solvent used.
Ion transfer to the mass spectrometer: The gas-phase ions formed from the droplets are then transferred through two differentially pumped regions into the ion source of the mass spectrometer. The differential pumping ensures the efficient transfer of ions while maintaining the vacuum in the mass spectrometer.
In the electrospray interface, nitrogen gas is often introduced to aid in the desolvation of ions as they pass into the spraying region. The analyte ions produced through electrospray ionization are subsequently separated based on their mass-to-charge ratio (m/z) in the mass analyzer and detected by the mass spectrometer's detector.
The electrospray interface plays a crucial role in enabling the analysis of a wide range of compounds in mass spectrometry, including small molecules, peptides, proteins, and other biomolecules. Its versatility and ability to generate gas-phase ions from solution-phase analytes have made it a widely used technique in various fields, such as pharmaceutical analysis, proteomics, metabolomics and environment.
Electrospray ionization allows for the direct ionization of molecules from a solution, including thermally labile molecules that may be prone to degradation under other ionization techniques. This is a significant advantage as it enables the analysis of a wide range of compounds without the risk of thermal degradation.
One characteristic of electrospray ionization is the production of multiply charged ions. As the analyte molecules become ionized, they often acquire multiple positive or negative charges. This is due to the transfer of excess protons or electrons from the solvent or ambient environment to the analyte molecule during the ionization process. The formation of multiply charged ions is a common occurrence in electrospray ionization and has important implications for mass spectrometry analysis.
In mass spectrometry, the mass analyzer measures the m/z ratio of ions, which represents the mass-to-charge ratio. For multiply charged ions, the mass-to-charge ratio is divided by the charge state of the ion. As a result, the mass range effectively expands by a factor equivalent to the number of charges residing on the analyte molecule. This

allows for the detection and analysis of larger molecules or ions that would otherwise exceed the mass range limitations of the mass spectrometer.

The ability to measure fragment ions based on their m/z ratio and the extension of mass range provided by multiply charged ions is crucial for the identification and characterization of complex molecules. Fragmentation patterns can provide valuable structural information about the analyte, aiding in the determination of its chemical composition and arrangement. By analyzing the mass-to-charge ratios of fragment ions, researchers can gain insights into the structure, composition, and behaviour of the analyte molecule.

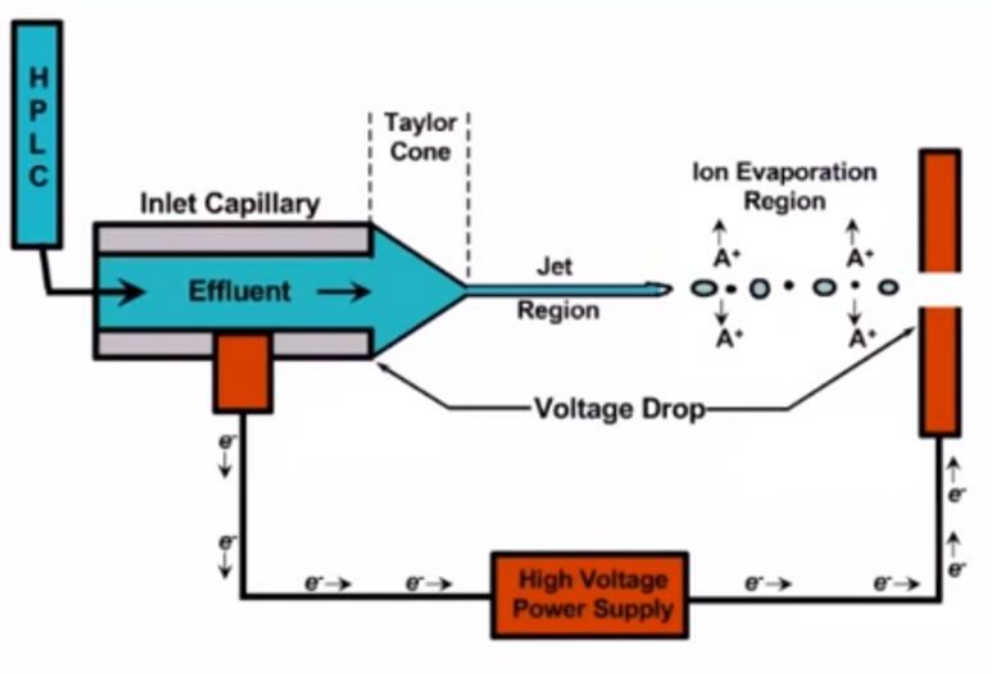

Figure 2: Schematic of an Electrospray LC-MS interface

In summary, electrospray ionization allows for direct ionization from solution, including thermally labile molecules, without degradation. It typically generates multiply charged ions, which extends the effective mass range of the mass spectrometer. This characteristic enables the measurement and analysis of fragment ions based on their m/z ratio, providing valuable information about the structure and composition of the analyte molecule.

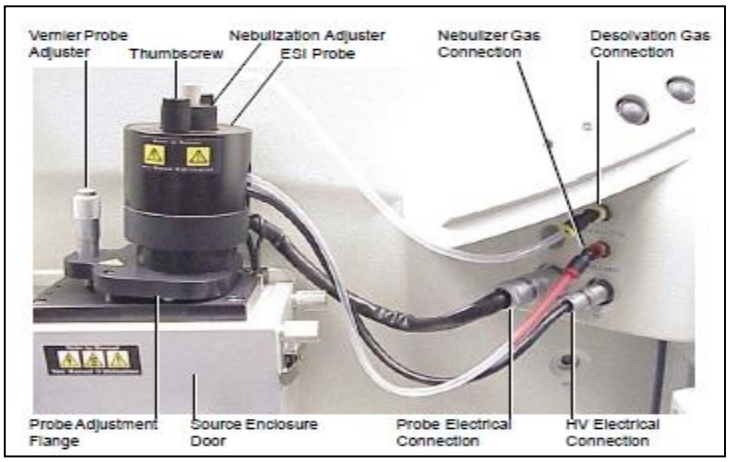

Figure 3: Electrospray ionization probe mounted on source enclosure

The Triple Quadrupole:

The triple quadrupole mass spectrometer is a widely used instrument in MS-MS (tandem mass spectrometry) analysis. It consists of three sets of quadrupole rods arranged in series. Each set of rods has a specific function in the MS-MS experiment.

The first set of rods (Q1): The primary role of the first set of quadrupole rods, Q1, is to act as a mass filter for the selection of the parent ion of interest. It allows ions of a specific mass-to-charge ratio (m/z) or a range of m/z values to pass through while filtering out other ions.

The second set of rods (Q2): The second set of rods in the triple quadrupole, Q2, is not used for mass separation. Instead, it serves as a collision cell. In this collision cell, the selected parent ions from Q1 are subjected to collisions with an inert gas, typically helium or nitrogen. These collisions cause the parent ions to fragment into smaller ions.

The third set of rods (Q3): The fragment ions generated in the collision cell (Q2) are then transmitted to the third set of quadrupole rods, Q3. Q3 acts as a mass filter, allowing specific fragment ions of interest to pass through while filtering out other ions. The filtered fragment ions are then detected by the mass spectrometer's detector.

The triple quadrupole mass spectrometer allows for precise control and manipulation of the ion transmission through the different sets of quadrupole rods. By selectively filtering parent ions and fragment ions based on their m/z ratios, the instrument can provide specific analytical information. This enables targeted analysis, such as the quantification and identification of specific compounds in complex mixtures.

The triple quadrupole mass spectrometer is widely used in various fields, including pharmaceutical analysis, environmental monitoring, metabolomics, and proteomics. Its versatility and ability to perform highly specific and sensitive MS-MS experiments make it a valuable tool for a wide range of applications.

III. Method development

Method development is a systematic process in analytical chemistry that involves several key steps. These steps are crucial in optimizing the method for accurate and reliable analysis. The main steps involved in method development include;

1. Literature review: Conducting a thorough literature review is essential before starting method development. This step helps in gaining knowledge about existing methods and techniques used for similar analytes or compounds. It provides valuable insights into the chromatographic conditions, mass spectrometry parameters, sample preparation techniques, and other relevant information that can guide the development of the method.

2. Tuning of Analyte/Metabolites/ISTD: The tuning of the mass spectrometer is an important step in method development. It involves optimizing various parameters such as voltages, currents, flows, and ion source settings to achieve maximum sensitivity and resolution in the mass spectra. Tuning ensures that the obtained spectra closely resemble previously established standards. Adjusting parameters like ion source voltage, mass filter settings, and detector parameters helps in optimizing the quality of the mass spectra.

3. Optimization of Mass Parameters: Optimizing mass parameters is crucial for obtaining accurate and reliable data. This includes selecting the appropriate mass range and setting the threshold. The mass range determines the range of masses to be analyzed, ensuring that the desired mass peaks are included in the spectra. Setting the threshold determines the minimum signal abundance that is recognized as a valid signal, preventing the exclusion of important mass abundance data.

4. Optimization of Chromatographic Conditions: Chromatographic conditions play a significant role in achieving efficient separation and resolution of analytes. Optimization of chromatographic conditions involves selecting the suitable stationary phase, mobile phase composition, flow rate, column temperature, and gradient program. These factors are adjusted systematically to achieve optimal separation and enhance the chromatographic performance.

5. Optimization of Extraction Procedure: The extraction procedure is crucial for obtaining accurate and representative results. Depending on the sample matrix and target analytes, the extraction procedure needs to be optimized. This includes selecting appropriate sample preparation techniques, optimizing extraction solvents, determining the optimal extraction time and temperature, and considering factors such as pH and matrix effects.

Method development is an iterative process, involving testing and refining different parameters until the desired analytical performance is achieved. It requires careful consideration of the sample characteristics, instrument capabilities, regulatory requirements, and specific analytical goals. Through systematic optimization of these parameters, a robust and reliable method can be developed for accurate analysis of the target analytes.

Sample preparation methods:

Sample preparation is a critical step in analytical chemistry that involves extracting and isolating target analytes from complex matrices for further analysis. Various sample preparation methods are employed, depending on the nature of the analytes, the sample matrix, and the specific analytical requirements. Some commonly used sample preparation methods include:

Protein precipitation method: This method is commonly used for the extraction of small molecules from biological samples, such as plasma or serum. In protein precipitation, a protein denaturant or an organic solvent is added to the sample, causing the proteins to precipitate. The supernatant containing the target analytes is then separated from the protein precipitate by centrifugation, and further purification steps may be applied if necessary.
Liquid-liquid extraction method: Liquid-liquid extraction, also known as solvent extraction, is a technique used to separate analytes from a liquid sample matrix. It involves the partitioning of analytes between two immiscible solvents, typically an organic solvent and an aqueous phase. By carefully selecting the solvents and adjusting pH if necessary, the target analytes can be selectively extracted into the organic phase. After extraction, the organic phase is separated and concentrated for further analysis.
Solid-Phase Extraction (SPE) method: SPE is a widely used sample preparation technique that utilizes a solid sorbent to extract and concentrate target analytes from a liquid sample. The sample is passed through a cartridge or disk containing a solid-phase sorbent with specific affinity to the analytes of interest. The analytes are retained on the sorbent, while interfering compounds are washed away. The retained analytes are then eluted with an appropriate solvent, resulting in a purified extract ready for analysis.
Hybrid Extraction method: Hybrid extraction methods combine different extraction techniques to improve the selectivity and efficiency of analyte extraction. For example, a combination of liquid-liquid extraction and solid-phase extraction can be used for complex samples. This approach allows for the removal of interfering compounds through liquid-liquid extraction followed by additional purification and concentration using solid-phase extraction.
The selection of a specific sample preparation method depends on factors such as the analyte properties, sample matrix complexity, required sensitivity, and instrumental analysis techniques. It is important to consider factors such as extraction efficiency, selectivity, reproducibility, and potential matrix effects when choosing a suitable sample preparation method.

IV. Bioanalytical method validation[5]

Method validation is an essential step in analytical chemistry to ensure that a specific method consistently produces accurate and reliable results. According to the ICH Q2B guidelines, method validation involves establishing documented evidence that a method will consistently meet predetermined specifications and quality characteristics. For the validation of analytical methods, there exists a guideline [ICH Q2(R1)][6-19] that determines the parameters of the validation procedure required by the pharmaceutical companies to follow. As per this guideline, analytical methods are categorized based on their application in purity, identity, content, and potency testing. These methods are tested for multiple parameters such as specificity, linearity, limit of detection and quantitation, trueness, precision, robustness, and range, depending on the category of the method. It is absolutely important to not mix the terms analytical and bioanalytical methods as they both serve different purposes and cover different parameters for their respective validation procedures. One of the biggest differences between the two is that in bioanalytical methods[20-33], the analyte is always tested in biological matrices (plasma, serum, urine).

Method validation is typically performed after method development and provides documented information regarding the performance of the method. It assesses parameters such as linearity, accuracy, specificity, and stability of the analyte. The validation process demonstrates that the method is suitable for quantifying the analyte in biological matrices and that the results are reproducible for intended and long-term use.

Validation parameters are established by considering various factors, including sample preparation, sample extraction procedure, and chromatographic parameters. The method must meet the acceptance criteria set by regulatory guidelines such as the USFDA, ANVISA, and ICH Q2B[34].

System suitability is an important aspect of method validation. It ensures that all the analyzing parameters of the method, including reagents, samples, columns, instruments, and glassware, are suitable and appropriate for the intended analysis. System suitability is typically assessed by performing multiple injections of a quality control sample to ensure consistency and reliability.

Auto-sampler carryover is another validation parameter that assesses the potential for sample contamination from previous injections. It is performed by injecting samples in a specific order and monitoring for any carryover effects.

Linearity is an important validation parameter that establishes the relationship between the experimental response values and the analytical response values. A calibration curve is constructed using standards with known concentrations to assess linearity. The curve is typically plotted as peak area ratio (analyte/metabolites to internal standard) against the nominal concentrations.

The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) are critical parameters that define the lowest and highest standard concentrations on the calibration curve, respectively. These limits help determine the analytical range and ensure accurate quantification within the specified concentration range.

Overall, method validation provides confidence in the accuracy, precision, and reliability of the analytical method. It ensures that the method is fit for its intended purpose and can consistently produce reliable results for the quantification of the analyte of interest.

Precision and accuracy are important parameters in method validation that assess the reliability and correctness of the analytical results.

Precision refers to the degree of reproducibility or repeatability of measurements or calculations. It measures the consistency of results obtained from multiple measurements. Precision is commonly represented by the coefficient of variation (% CV), which is calculated as the ratio of the standard deviation (SD) to the mean, multiplied by 100. The % CV indicates the variability of the measurements as a percentage of the mean.

Accuracy, on the other hand, measures the closeness of the experimental values to the true or reference values. It assesses the correctness of the measurement by comparing the obtained concentration of quality control (QC) samples to their nominal concentration. Accuracy is expressed as a percentage and can be calculated as the obtained concentration divided by the nominal concentration, multiplied by 100.

Both precision and accuracy can be determined within batches and between batches. Within batch precision/accuracy assesses the variability and correctness of measurements within a single batch, while between batch precision/accuracy evaluates the consistency and accuracy of measurements across different batches.

Acceptance criteria for precision and accuracy are typically set based on regulatory guidelines and the specific requirements of the analysis. For example, the % accuracy for standards (STD2-STD10) should generally be within 85.00-115.00 %, while the acceptance range for the lower limit of quantification (LLOQ) is 80.00-120.00 %. Additionally, a certain percentage of calibration curve standards (STD2-STD9) should meet the acceptance criteria, and the response of interfering peaks in the standard blank at the retention time of the internal standard (ISTD) should be ≤ 5.00 % of the LLOQ.

Selectivity is another important parameter in method validation, which is assessed through specificity and matrix effect experiments. Specificity ensures that the method can differentiate the targeted analyte in the presence of other interfering substances. Matrix effect determines whether any interference, either direct or indirect, may alter the analytical response of the analyte. Matrix effect experiments are typically performed by screening different plasma lots.

Other experiments conducted in method validation include reinjection reproducibility, evaluation of potential interfering drugs, recovery assessment, ruggedness evaluation (considering different analysts, columns, and equipment), and stability testing. These experiments help ensure the reliability, reproducibility, and robustness of the analytical method throughout the entire analytical process, including sample collection, extraction, storage, and analysis.

Stability parameters play a crucial role in method validation and ensure that the analyte and the sample remain stable throughout the analytical process. The stability of the analyte and the samples can be assessed in various ways, including:

Analyte stability: This parameter evaluates the stability of the analyte under different conditions such as sample collection, sample storage, and sample processing. It assesses whether the analyte remains stable and does not degrade or undergo any significant changes during these processes.

Stability in solutions:

Short-term solution stability: This assesses the stability of the analyte in solution over a short period, typically within 24 hours. It determines whether the analyte remains stable when stored in the solution before analysis.

Long-term solution stability: This evaluates the stability of the analyte in solution over an extended period, usually several weeks or months. It ensures that the analyte remains stable during long-term storage.

Matrix stability:

Benchtop stability: This parameter determines the stability of the analyte in the sample matrix when kept at room temperature for a specified period. It assesses whether the analyte remains stable under normal laboratory conditions.

Freeze-thaw stability: This evaluates the stability of the analyte in the sample matrix after subjecting it to multiple freeze-thaw cycles. It assesses whether the analyte remains stable after undergoing freezing and thawing.

Autosampler stability: This parameter assesses the stability of the analyte in the sample matrix when stored in an autosampler for a specified period. It ensures that the analyte remains stable during the automated sample preparation and analysis process.

Wet extract stability: This parameter determines the stability of the analyte in the sample matrix during the extraction process. It assesses whether the analyte remains stable in the presence of extraction solvents and other components.

Stability of analyte in blood: This parameter specifically assesses the stability of the analyte when present in blood samples. It ensures that the analyte remains stable in the blood matrix during sample collection, processing, and storage.

Long-term stock stability: This parameter evaluates the stability of the stock solution of the

Different levels of validation are defined based on the requirements of the analysis:
A. Full validation: This level of validation is performed when developing and implementing a bioanalytical method for the first time or for a new drug entity. It involves a complete validation process, including the assay of quantification.
B. Partial validation: Partial validation is conducted when modifying an already validated method. It can range from minor modifications to nearly full validation. Examples include method transfer, changes in detection systems, anti-coagulants, matrix, sample processing procedures, instruments, and software.
C. Cross-validation: Cross-validation involves comparing validated parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. It helps assess the reliability and comparability of the data generated using different analytical techniques.
In addition to stability parameters, basic definitions of key terms used in bioanalytical method validation are provided. These terms include analyte/metabolite, internal standard (ISTD), reference standard, working standard, calibrators, sample types (standard blank, quality control sample, standard zero, unknown sample), LLOQ, ULOQ, solvent blank/reconstitution solution (RS), selectivity/screening, and more.
These stability parameters and definitions help ensure the accuracy, reliability, and validity of the analytical results in bioanalytical method validation.

Quality control in the bioanalytical analysis
Precision and Accuracy: The variability of precision and accuracy should fall within acceptable tolerance limits. If they do not, duplicate or triplicate analyses should be performed to ensure reliable results.
Multilevel Calibrations: Calibration standards should cover the expected concentration range of the unknown samples, including a calibration sample at the lower limit of quantification (LLOQ). Extrapolating the standard curve below the LLOQ or above the highest standard is not recommended. Instead, the curve should be redefined or samples with higher concentrations should be diluted and re-assayed.
Analytical Batch: Each analytical batch should include a blank matrix, a zero standard (matrix with internal standard), and a minimum of six non-zero calibration standard points. This helps establish a reliable baseline and measure the concentration levels accurately.
Quality Control (QC) Samples: QC samples, which are matrix spiked with the analyte, should be included and analyzed alongside the test samples at intervals based on the total number of samples. The minimum number of QC samples should be 5% of the sample runs. They should be analyzed in duplicate at three concentrations: once at three times the LLOQ, once in the midrange, and once approaching the high end of the concentration range.
QC Acceptance Criteria: At least four out of every six QC samples must be within 15% of their respective nominal values. It is acceptable for two of the six QC samples to fall outside the 15% range, but not both at the same concentration. This ensures the reliability and consistency of the analytical method.
System Suitability: Qualified and properly maintained instruments should be used for implementing bioanalytical methods. System suitability tests (SST) should be defined with specific parameters and acceptance criteria to ensure the proper operation of the system. It's important to note that SSTs do not replace the required run acceptance criteria using QC samples.
By following these considerations, laboratories can maintain optimal conditions, monitor accuracy and precision, and ensure the defined quality of analytical data in the analysis of biological samples.

V. Conclusion

Bioanalytical method validation is a critical step in ensuring the accuracy, reliability, and acceptability of assay performance in the quantitative determination of analytes in biological samples. The validation process involves rigorous testing and evaluation of various parameters, including specificity, selectivity, sensitivity, accuracy, precision, linearity, range, robustness, and stability. By validating bioanalytical methods, researchers can confidently measure analyte concentrations in biological matrices, which is essential for making informed decisions in drug development and drug discovery.

The validation of bioanalytical methods provides assurance that the results obtained from these methods are trustworthy and reproducible. It allows for the comparison of data across different studies and laboratories, enabling reliable assessments of the safety, efficacy, pharmacokinetics, and pharmacodynamics of drug candidates. Additionally, validated methods facilitate regulatory compliance and support the submission of data to regulatory authorities for approval.

The level of validation required for a specific bioanalytical method depends on the intended use, the analyte of interest, the complexity of the matrix, and the regulatory guidelines. Therefore, it is crucial to carefully design validation experiments, establish appropriate acceptance criteria, and document all validation procedures and results.

Overall, bioanalytical method validation is an indispensable process in the field of drug development. By ensuring the reliability and accuracy of analytical results, validated methods contribute to the advancement of

pharmaceutical research, ultimately leading to the discovery of safe and effective drugs for the benefit of patients worldwide.

By understanding the intricacies of bioanalytical method development and validation, researchers and scientists can improve the efficiency and reliability of drug development processes. This review serves as a comprehensive guide, offering valuable insights into the essential aspects of bioanalytical methods in the pharmaceutical industry.

References:

[1]. Dighe S, Shah VP, Midha KK, McGilveray IJ, Skelly JP, Yacobi A,etal,Analytical methods validation: bioavailability, bioequivalence and pharmacokinetics studies, Conference Report, Eur J Drug Metabol Pharmacokinetics, 16, 1998, 249–55.

[2]. Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, et al. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. Conference report. Pharm Res. 2009;9:588–92.

[3]. Causon R. Validation of chromatographic methods in biomedical analysis viewpoint and discussion. J Chromatogr. 1998;689:175–80.

[4]. Raymond NX, Fan LR, Matthew J, Tawakol A. Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS. J Pharm Biomed Anal. 2007;44:342–55.

[5]. P. Ravi Sankar, A. Sai Geethika, G. Rachana, P. Srinivasa Babu, J. Bhargav, Bioanalytical Method Validation: A Comprehensive Review, Int. J. Pharm. Sci. Rev. Res.,2019, 56(1), 50-58.

[6]. ICH Q2 (R1), Validation of analytical procedures, Text and methodology, International Conference on Harmonization, Geneva, 2005, 1-17.

[7]. Lavanya Chowdary G, Ravisankar P, Akhil Kumar G, Mounika K, Srinivasa Babu, Analytical method validation parameters: An updated review. Int. J. Pharm. Sci. Rev.Res., 2020;61(2):1-7.

[8]. Ravisankar P, Naga Navya Ch, Pravallika D, Navya Sri D. A review on step-by-step analytical method validation. IOSR J Pharm. 2015;5(10):7-19.

[9]. Panchumarthy Ravisankar, Anusha S, Supriya K, Ajith Kumar U, Fundamental chromatographic parameters. Int. J. Pharm. Sci. Rev, Res., 2019; 55(2):46-50.

[10]. Ravi Sankar P, Madhuri B, Naga Lakshmi A, Pooja A, Bhargava Sai M, Suresh K, Srinivasa Babu P. Selected HPLC Applications - Quick Separation Guide: A Review. Int. J. Pharm. Sci. Rev.Res., 2020;60(2):13-20.

[11]. Ravisankar P, Gowthami S, Devala Rao G. A review on analytical method development, Indian Journal of Research in Pharmacy and Biotechnology. 2014;2(3):1183-1195.

[12]. Ramanlal N. Kachave, Komal P. Jadhav, Analytical method development and validation: A Review, International Journal of creative research thoughts, 2021;9(8):554-570.

[13]. Pathuri R, Muthukumaran M, Krishnamoorthy B, Nishat A. A review on analytical method development and validation of the pharmaceutical technology. Curr Pharm Res 2013;3:855-70.

[14]. Lavanya G, Sunil M, Eswarudu MM, Eswaraiah MC, Harisudha K, and Spandana BN: Analytical Method Validation: An Updated Review. Int J Pharm Sci Res 2013; 4(4); 1280-1286.

[15]. Araujo P. Key aspects of analytical method validation and linearity evaluation. J Chromatography B, 2009;877:2224-34.

[16]. Chauhan A, Mittu B, Chauhan P. Analytical method development and validation: a concise review. J Anal Bioanal Tech 2015;6:1.

[17]. Hema, Swati Reddy G. A review on new analytical method development and validation by RP-HPLC. Int Res J Pharm Biosci 2017;4:41-50.

[18]. Patil R, Deshmukh T, Patil V, Khandelwal K. Review on analytical method development and validation. Res Rev J Pharm Anal 2014;3:1-10.

[19]. Chauhan A, Mittu B, Chauhan P. Analytical method development and validation: a concise review. JAnal Bioanal Tech 2015;6:1.

[20]. Hartmann C, Smeyers VJ, Massart DL, Validation of bioanalytical chromatographic methods, J Pharm Biomed Anal, 17, 1999, 193–218.

[21]. Kelley M, DeSilva B, Key Elements of Bioanalytical Method Validation for Macromolecules, AAP J, 9, 2007, 156–63.

[22]. Dighe S, Shah VP, Midha KK, McGilveray IJ, Skelly JP, Yacobi A,etal, Analytical methods validation: bioavailability, bioequivalence and pharmacokinetics studies, Conference Report, Eur J Drug Metabol Pharmacokinetics, 16, 1998, 249–55.

[23]. Braggio S, Barnaby RJ, Grosi P, Cugola A, strategy for validation of bioanalytical methods, J Pharm Biomed Anal, 14, 1996, 375–88.

[24]. Viswanathan, CT, B Surendra, B Booth, AJ DeStefano, MJ Rose, J Sailstad, VP Shah, JP Skelly, PG Swann, R Weiner, 2007, Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays, Pharm Res, 24:1962-1973.
[25]. Nowatzke W, Woolf E. Best Practices during Bioanalytical Method Validation for the Characterization of Assay Reagents and the Evaluation of Analyte Stability in Assay Standards, Quality Controls, and Study Samples. AAPS J. 2007;9:E117–22.
[26]. Braggio S, Barnaby RJ, Grosi P, Cugola A strategy for validation of bioanalytical methods. J Pharm Biomed Anal. 1996;14:375–88.
[27]. James CA, Breda M, Frigerio E. Bioanalytical method validation: A risk-based approach. J Pharm Biomed Anal. 2004;35:887–9.
[28]. Dadgar D, Burnett PE. Issues in evaluation of bioanalytical method selectivity and drug stability. J Pharm Biomed Anal. 2004;14:23–31.
[29]. Dadgar D, Burnett PE, Choc MG, Gallicano K, Hooper JW. Application issues in bioanalytical method validation, sample analysis and data reporting. J Pharm Biomed Anal. 1995;13:89–97.
[30]. Hartmann C, Massart D, McDowall RD. An analysis of the Washington Conference Report on bioanalytical method validation. J Pharm Biomed Anal. 2005;12:1337–43.
[31]. Karnes HT, Shiu G, Shah VP. Validation of bioanalytical methods. Pharm Res. 2001;8:421–6.
[32]. Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, McGilvary IJ, et al. Bioanalytical method validation. A revisit with a decade of progress. Pharm Res. 2000;17:1551–7.
[33]. Miller KJ, Bowsher RR, Celniker A, Gibbons J, Gupta S, Lee JW, et al. Workshop on Bioanalytical Methods Validation for Macromolecules: Summary Report. Pharm Res. 2001;18:1373–83.
[34]. Validation of analytical procedure: Methodology Q2B, ICH Hormonized Tripartite Guidelines 1996:1-8.