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Review Article

Bioanalytical study by HPLC for Quantification of Drug in Plasma Blood Concentration

Vaishnavi A Shirsath, DR. Kaveri M Nannor, Pallavi k Danke, Rutuja D Khare, Sakshi B Vanjul

Pres's College of Pharmacy Chincholi, Nashik, Maharastra, India

A B S T R A C T

Bioanalytical quantification plays a vital role in pharmaceutical, clinical, and toxicological biological matrices such as plasma, serum, urine, and tissues. These measurements support the evaluation of pharmacokinetic (ADME) behavior, therapeutic effectiveness, and drug safety. However, biological matrices contain numerous endogenous substances that can introduce matrix effects, affecting extraction efficiency, chromatographic separation, and detector response, thereby reducing accuracy and sensitivity. High-Performance Liquid Chromatography (HPLC) is a highly reliable analytical technique widely used for the separation and quantification of analytes in complex samples due to its superior selectivity, precision, and reproducibility. Method development in HPLC involves careful selection of stationary and mobile phases, optimization of chromatographic conditions, and appropriate detection strategies such as UV, fluorescence, and LC–MS/MS. Robust sample preparation methods—including protein precipitation, liquid–liquid extraction, and solid-phase extraction—are essential to minimize matrix interference and enhance analytical performance. Regulatory validation ensures compliance with accuracy, precision, linearity, stability, and selectivity requirements for routine application in bioanalysis. Continuous advancements in UHPLC, hyphenated techniques, automation, and green analytical approaches are further improving throughput, sensitivity, and sustainability. Thus, HPLC-based bioanalytical quantification remains indispensable for drug development, therapeutic drug monitoring, and personalized medicine.

Keywords:(Bioanalytical quantification; High-Performance Liquid Chromatography (HPLC); Biological matrices; Matrix effects; Sample preparation; Method validation; LC–MS/MS; Pharmacokinetics; Therapeutic drug monitoring; Personalized medicine)

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*Address for Correspondence:
Vaishnavi A Shirsath, Pres's College of Pharmacy Chincholi, Nashik, Maharastra, India

INTRODUCTION

Bioanalytical quantification is a key part of pharmaceutical, clinical, and toxicological research. In simple terms, it's about measuring how much of a drug, its metabolites, or specific biomarkers are present in biological samples like blood, plasma, urine, or tissue. These measurements are essential for understanding how a drug behaves in the body — how it's absorbed, distributed, metabolized, and eliminated. They also help determine how effective and safe a drug is, which is critical for drug development and patient care.

In the context of drug development, accurate and reliable bioanalytical methods ensure that the data collected from both preclinical and clinical studies are valid, consistent, and meet regulatory standards. Beyond development, quantitative bioanalysis also supports therapeutic drug monitoring, toxicity studies, and personalized medicine, where precise measurement of drug levels can directly influence treatment decisions and patient outcomes.

In practice, bioanalysis involves analyzing biological matrices such as blood, plasma, serum, cerebrospinal fluid, urine, or saliva to determine the concentration of a drug or its metabolites [1–3]. This process covers everything from collecting and processing samples to storing and analyzing them under controlled conditions.

Before a bioanalytical method can be used routinely, it must be validated. Method validation confirms that the technique is accurate, precise, and suitable for its intended purpose. Key

validation parameters include selectivity, sensitivity, calibration range, accuracy, precision, stability, recovery, linearity, and limits of detection and quantification (LOD and LLOQ) [4–7]. Reliable, validated methods are crucial for quality control laboratories, which use them to confirm the identity, purity, potency, and bioavailability of drug substances[8,9]

Challenges of Analyte Estimation in Complex Biological Matrices

Biological matrices are inherently complex and contain numerous endogenous substances such as proteins, lipids, salts, and metabolites that can interfere with analyte detection. These matrix components may affect extraction efficiency, chromatographic separation, and detector response, leading to ion suppression or enhancement and inaccurate quantification. Moreover, analytes may exist in low concentrations, be unstable, or strongly bind to matrix components, further complicating their analysis. Sample variability among individuals, matrix effects, and the need for sensitive, selective, and high-throughput methods pose additional challenges..

1. **Complex Composition of Biological Matrices**

   Biological samples such as plasma, serum, urine, and tissues contain numerous endogenous substances (proteins, lipids, salts, and metabolites).These components can interfere with analyte detection and cause inaccuracies in quantification.

2. **Matrix Interference**

   Endogenous compounds may affect extraction efficiency, chromatographic separation, and detector response.Such interference can lead to ion suppression or enhancement, resulting in over- or underestimation of analyte concentration.

3. **Low Analyte Concentration**

   Many analytes exist in very low concentrations in biological matrices.Detecting and quantifying these trace levels requires highly sensitive analytical techniques.

4. **Analyte Instability**

   Some analytes are chemically unstable and may degrade during sample collection, storage, or processing.This instability can lead to inconsistent or inaccurate results.

5. **Binding to Matrix Components**

   Certain drugs or metabolites strongly bind to plasma proteins or other matrix components.Such binding reduces the availability of free analyte for detection, complicating extraction and measurement.

6. **Inter-Individual Variability**

   Biological matrices can vary greatly between individuals due to physiological differences, diet, disease state, or medication use.This variability can influence matrix effects and reproducibility of results. **s**

7. **Requirement for High Sensitivity and Selectivity**

   To overcome matrix effects, analytical methods must be highly sensitive, selective, and capable of distinguishing the analyte from background noise.

8. **Need for High-Throughput Analysis**

   Large-scale studies and clinical trials require methods that can process many samples quickly without compromising data quality.

9. **Ensuring Method Robustness and Reliability**

   Developing robust bioanalytical methods that minimize matrix interference while maintaining accuracy, precision, and reproducibility is essential for generating reliable data in both research and clinical environments.

10. **Method Robustness** – Reliable and validated methods must ensure accuracy, precision, and reproducibility.[9,10]

Measuring Plasma Drug Concentration In Therapeutic Drug Monitoring

The contribution of pharmacokinetic variability to differences in dose requirements can be identified by measuring the drug concentration at steady state and modifying the dose to attain a desired concentration known to be associated with efficacy. However, there is substantial inter-individual pharmacodynamic variability at a given plasma concentration [11,] hence a range of concentrations rather than a single level is usually targeted. For a limited number of drugs for which there is a better relationship between plasma or blood concentration-response than dose-response, the measurement of plasma or blood concentrations has become a valuable surrogate index of drug exposure in the body [12,13].

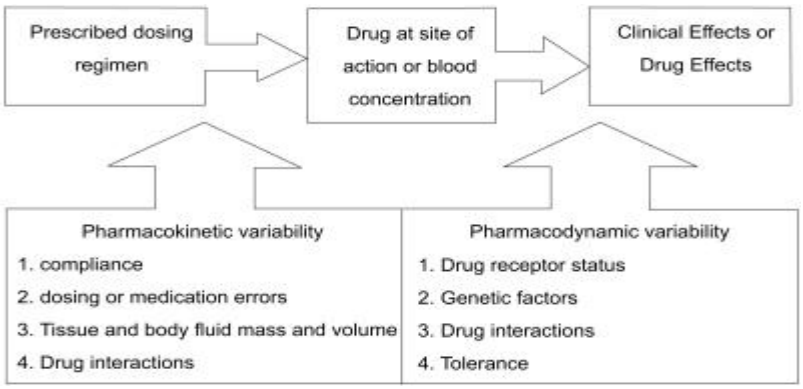

Figure 1: Sources of Variability in Drug Response: Pharmacokinetic and Pharmacodynamic Factors

Role of Chromatographic Techniques in Bioanalysis

High-Performance Liquid Chromatography (HPLC) is a fundamental technique in bioanalytical quantification, valued for its precision, reliability, and ability to separate, identify, and quantify analytes in complex biological matrices. By optimizing parameters such as stationary and mobile phases, flow rate, and detection wavelength, HPLC achieves high selectivity and sensitivity, effectively minimizing interference from endogenous substances.

The technique is highly versatile and compatible with various detectors, including UV, PDA, fluorescence, and mass spectrometry (LC–MS/MS), the latter providing superior specificity and ultra-low detection limits. Sample preparation techniques such as liquid–liquid extraction (LLE), solid-phase extraction (SPE), and protein precipitation further improve analytical efficiency and accuracy[14].

HPLC method development and validation focus on key parameters such as selectivity, linearity, precision, accuracy, and stability to ensure robust and reproducible results. Owing to its adaptability, HPLC is widely applied in pharmaceutical, clinical, environmental, food, and biotechnological analyses. Ongoing advancements—particularly in miniaturization and green analytical technologies—continue to enhance HPLC's performance, making it an indispensable tool in modern analytical and pharmaceutical sciences.[15-17].

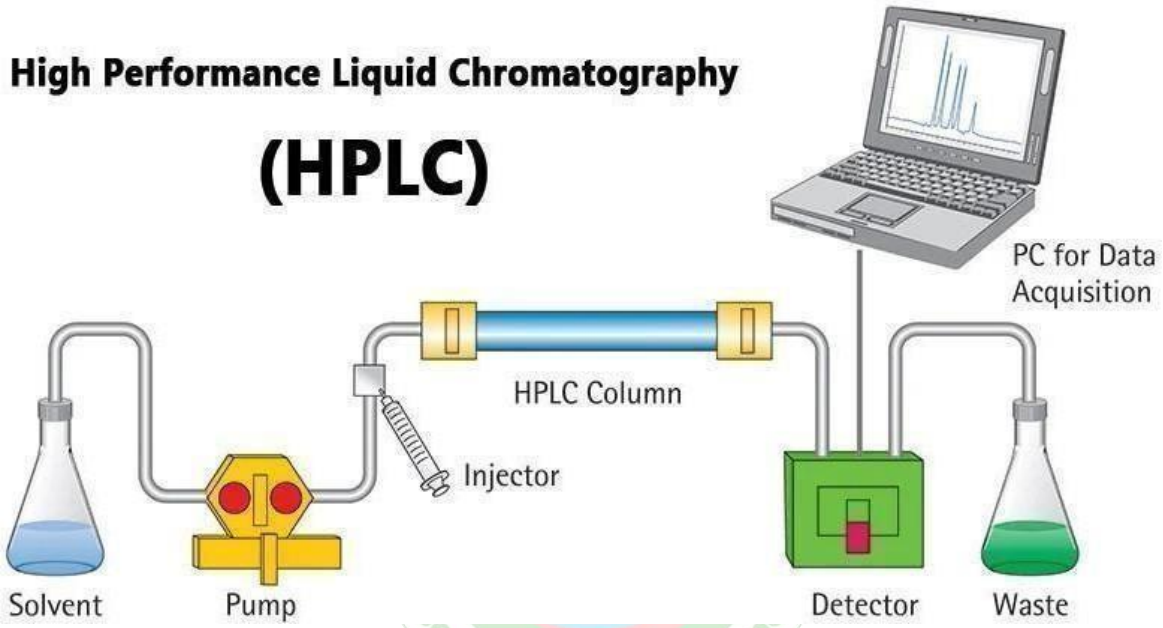

Figure 2: Schematic Diagram of High Performance Liquid Chromatography (HPLC) System

Aim and Scope of the Review

The present review aims to provide a comprehensive overview of the quantification of analytes from biological matrices using High-Performance Liquid Chromatography (HPLC). It highlights the principles and advancements of HPLC techniques, discusses the challenges associated with complex biological samples, and evaluates various sample preparation and method validation strategies. The review also examines recent developments, applications, and regulatory considerations in bioanalytical quantification. By summarizing current methodologies and identifying future trends, this review seeks to serve as a reference for researchers and analysts involved in pharmaceutical, clinical, and toxicological bioanalysis.

Biological Matrices in Bioanalysis

Bioanalysis involves the quantitative measurement of analytes—such as drugs, metabolites, or biomarkers—within biological systems. The biological matrix serves as the medium in which these analytes are present and provides critical information regarding absorption, distribution, metabolism, and excretion (ADME) processes. However, due to their complex composition, biological matrices pose significant analytical challenges that necessitate careful method development and validation.[18,19]

Biological Matrices in Bioanalytical Studies

In bioanalytical research, a wide range of biological matrices are used depending on the study objectives and the pharmacokinetic properties of the compound being analyzed. Each matrix offers unique advantages and challenges in terms of sample collection, preparation, and analysis[20].

Blood and Plasma

Plasma is the most commonly used biological matrix because it directly reflects the systemic exposure of a drug. It contains proteins, lipids, and electrolytes that can interact with analytes, making proper sample preparation crucial for accurate quantification. Whole blood is analyzed when the distribution of a drug within red blood cells is of particular interest[21,22].

Serum

Serum, similar to plasma but lacking clotting factors, is often chosen for clinical studies due to its stability and ease of handling. Its cleaner composition can simplify analysis compared to plasma.

Urine

Urine is widely used in pharmacokinetic and toxicological studies to monitor drug excretion and metabolite formation. It offers the advantage of noninvasive sampling, though factors like pH, dilution, and metabolite variability can affect results.

Tissue Sample

Tissue analysis provides valuable insight into how drugs distribute and accumulate at specific sites within the body. However, the heterogeneous nature of tissues and the requirement for homogenization make sample preparation and analysis more complex.

Saliva, Cerebrospinal Fluid and Other Matrices

Alternative matrices—such as saliva, cerebrospinal fluid (CSF), bile, and breast milk—are gaining attention for their potential in noninvasive or specialized studies. These matrices are particularly useful when blood sampling is difficult or when localized drug concentrations are of interest.

Blood and Plasma:

Plasma is the most commonly used biological matrix in bioanalysis, as it provides a direct reflection of systemic drug exposure. It contains proteins, lipids, and electrolytes that can interact with analytes, necessitating effective sample preparation techniques. Whole blood is used when drug partitioning into red blood cells is of interest.

- **Serum**
  Similar to plasma but devoid of clotting factors, serum is preferred in certain clinical studies due to its stability and ease of handling.
- **Urine**
  Urine analysis is widely used in pharmacokinetic and toxicological studies to assess drug excretion and metabolite formation. It offers noninvasive sampling but is subject to variability in pH, concentration, and metabolite composition.
- **Tissue Sample**

  Tissues provide valuable information on drug distribution and accumulation at the target site. However, their heterogeneous nature and the need for homogenization complicate analysis.
- **Saliva, Cerebrospinal Fluid, and Other Matrices**

  Alternative matrices such as saliva, cerebrospinal fluid (CSF), bile, and breast milk are increasingly explored for noninvasive or specialized analyses, particularly when direct measurement in blood is impractical.

Strategies to Minimize Matrix Interference

Several strategies are employed to mitigate matrix effects and ensure accurate quantification:

Effective Sample Preparation:(collection and extraction)

Sample collection

Serum and plasma samples were collected by local physicians in Kazakhstan using a patented dry plasma collection device (Chemcard; Chematics, Inc., North Webster, Ind.) which consists of a laminate of a semipermeable membrane, through which blood cellular and particulate matter cannot pass. This membrane is over a second membrane designed to absorb a measured amount of plasma. A hanging drop of blood obtained via fingerstick was touched to the test area of the commercial dry plasma collection device. The correct amount of blood applied was signified by a change in the color from white to red of the integrated control, indicating when an adequate volume of blood had been applied to the card. The top filter was removed after 3 min, and the card was air dried for 15 min. The resultant dried plasma sample was then stored in a desiccated zip-lock pouch at between 4 and 6°C for up to 2 months before being shipped to the United States by air at ambient temperature.

A venous blood sample was also obtained from each individual at the same time that the plasma sample was obtained. The blood was allowed to clot, and the serum was separated by centrifugation. Sera were stored at −20°C and then shipped frozen to the United States for analysis[22].

Sample extraction

These methods separate analytes from complex matrices before instrumental analysis.

Liquid–Liquid Extraction (LLE)

LLE is a traditional and widely used method that transfers analytes between immiscible liquid phases. It is simple, low-cost, and effective but requires large volumes of organic solvents and manual handling, increasing environmental and health risks. Recent improvements aim to reduce solvent use, waste, and operator exposure while maintaining efficiency [23].

Protein Precipitation (PP)

Protein precipitation removes proteins that interfere with analyte detection. Modern membrane-based PP plates eliminate the need for centrifugation, reducing processing time and improving solvent recovery compared to manual methods [24].

Solid-Phase Extraction (SPE)

SPE isolates analytes by passing a liquid sample through an adsorbent that retains the analyte, followed by elution with a suitable solvent. It is faster, uses less solvent, and offers better reproducibility than LLE, making it a preferred modern technique for sample preparation [25–28].

Microextraction Techniques (SPME, DLLME): Reduce solvent usage and enable miniaturized processing.

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe): Combines extraction and cleanup in one step.

Magnetic Solid-Phase Extraction: Uses magnetic nanoparticles for rapid analyte isolation.These innovations enhance sensitivity and environmental sustainability in bioanalytical workflows. [29]

Table 1: Comparison of Sample Preparation Techniques in Bioanalytical Methods

Technique	Principle	Advantages	Limitations	Typical Applications
Protein Precipitation	Precipitation of proteins by organic solvents	Simple, rapid, cost-effective	Co-precipitation of analytes, matrix effect	Small molecule drugs, routine analysis
Liquid-Liquid Extraction (LLE)	Separation based on solubility in immiscible solvent	Cleaner extracts, selective	Time-consuming, solvent-intensive	Lipophilic drugs, bioavailability studies
Solid-Phase Extraction (SPE)	Analyte binds to sorbent, impurities washed off	High recovery, reproducibility	Cartridge cost, requires optimization	Trace drug quantification, LC-MS/MS analysis
Microextraction (SPME/DLLME)	Adsorption or dispersive extraction in small solvent volumes	Eco-friendly, rapid, minimal solvent	Requires method optimization	Ultra-trace drugs, environmental monitoring[45]

1. **Chromatographic Optimization:**

   Appropriate selection of stationary and mobile phases, pH control, and gradient programming can enhance separation between analytes and matrix components.

2. **Use of Internal Standards**

   Isotopically labeled or structurally similar internal standards help compensate for matrix-induced variability in extraction and detection.

3. **Matrix-Matched Calibration**

   Preparing calibration standards and quality control samples in the same matrix as the

   study samples ensures that matrix effects are equally reflected in all measurements.

4. **Instrumental Approaches**

   Employing more selective detectors, such as tandem mass spectrometry (LC–MS/MS),

   improves specificity and reduces susceptibility to interference.

   Importance of Minimizing Matrix Effects (MEs)

Minimizing matrix effects is essential for achieving accurate, sensitive, and reproducible bioanalytical results. Simply compensating for MEs is not always sufficient, especially when high sensitivity is required.

- **Sensitivity Impact:** When analytes are present at very low concentrations, matrix components can suppress or enhance signals, reducing detection accuracy. Proper sample clean-up and optimized extraction can improve sensitivity by producing cleaner extracts.
- **Pre-concentration Considerations:** While pre-concentration increases analyte levels, it can also concentrate interfering substances, worsening matrix effects if not carefully optimized.
- **Calibration Limitations:** When calibration methods cannot fully compensate for MEs, extensive clean-up steps are necessary to ensure data accuracy.

  → **Goal:** The best approach is to reduce matrix effects at the source—through optimized sample preparation, chromatographic separation, and clean extraction methods—to ensure precise and reliable quantification.[30]

HPLC Method Development in Quantification of Analyte

Principle of HPLC

High-Performance Liquid Chromatography (HPLC) is an advanced separation technique used to identify, quantify, and purify components in complex mixtures. The principle of HPLC is based on the differential partitioning of analytes between a **stationary phase** (usually a packed column) and a **mobile phase** (a solvent or mixture of solvents). Compounds with greater affinity for the stationary phase elute more slowly, whereas those with higher solubility in the mobile phase elute faster. The separation is governed by factors such as polarity, molecular size, and ionic interactions. HPLC offers superior resolution, precision, and reproducibility compared to traditional column chromatography, making it indispensable in bioanalytical quantification.

Selection of stationary and mobile phases

Mobile Phase

Isocratic or gradient

Mobile phase preparation, in any method development or for any analysis, can be broadly classified into the techniques; whether to prepare an isocratic system or a gradient one. For isocratic mode of elution, the mobile phase comprises of a mixture of organic and aqueous phase. The contents of mobile and aqueous phases are in specific proportions. For example: the aqueous phase maybe a buffer, mixed with an organic solvent. Hence this mixture is used as the mobile phase for isocratic elution. For gradient elution, the mobile phase is separately prepared consisting of one aqueous mobile phase and another organic mobile phase.[31]

Selection of mobile phase

Mobile phase selection is basically dependent on the kind of drug we want to analyze. The nature of the drug implies to the polarity of the drug. Other than polarity, pH and pKa are amongst the most important criteria to look into when selecting a mobile phase, or developing a mobile phase.

Buffers as mobile phase

Buffers are often used as the aqueous part of the mobile phase for separation of analyte with acid-base properties. As we know there is a very interlinked relation between PH and

pKa. pka is basically the pH value at which a chemical entity will either accept or donate a proton. So, if the pKa of an acid is very low, it will have a greater ability to donate a proton in an aqueous solution[32].

pH of the solvent:

Talking of measuring the pH of solvent includes electrode[33]. pH has many scales on which it could be quantified. In pharmaceutical practice, pH is measured on molarity scale and requires a glass electrode and a reference electrode. Gradient elution technique provides a deciding factor whether the sample is suitable for isocratic elution or not. In case it is, it establishes the required ratio of the solvents to be used for effective separation[34]. In RPHPLC, the main factor which decides the retention time and resolution is the composition of the mobile phase. Initially, in case of every unknown development method, a gradient run is performed. This initial gradient run decides whether an isocratic run can be performed or not depending on peak and retention times[35-37].

Optimization of Chromatographic Parameters

Flow rate, temperature, gradient profile, and injection volume are adjusted to achieve optimal separation. Parameters are fine-tuned to reduce peak tailing, enhance reproducibility, and shorten analysis time.

Solvent Reservoirs: Contain mobile phases (aqueous and organic solvents).

Pump System: Delivers the mobile phase at a controlled, high pressure (up to 6000 psi).

Injector: Introduces the sample into the mobile phase stream with precision.

Column: The heart of the system, packed with stationary phase particles (commonly C18 silica).[38]

Table 2: Common HPLC Columns and Applications

Column Type	Stationary Phase	Particle Size	Typical Applications	Advantages
C18 Reversed-phase	Octadecyl silica	3–5 µm	Non-polar/moderately polar drugs	High retention, widely used
C8 Reversed-phase	Octyl silica	3–5 µm	Polar to moderately non-polar drugs	Faster elution than C18
Normal-phase	Silica	5 µm	Polar compounds, chiral drugs	Good for polar separation
Ion-exchange	Functionalized resin	5 µm	Charged drugs, peptides	Selective for ionic analytes
UHPLC columns	Sub-2 µm silica	1.7–2 µm	High-resolution, fast analysis	High efficiency, lower solvent use[46]

Detector: Monitors eluting compounds and converts their signals into chromatograms.

Comparison of Detection Methods for Plasma Drugs.

Table 3: Comparison of Detection Methods Used in Bioanalytical HPLC

Detection Method	Sensitivity	Specificity	Cost	Best Applications
UV Detection	Moderate	Moderate	Low	Drugs with chromophores, routine assays
Fluorescence	High	High	Medium	Trace drugs, fluorescent compounds
LC-MS/MS	Very High	Very High	High	Pharmacokinetics, metabolites, ultra-trace drugs
Electrochemical	High	High	Medium	

Data System: Software processes the detector signal, generating retention times and peak areas used for quantification.

Detectors Used in Bioanalytical HPLC

- **UV Detector:** Common, economical, and suitable for analytes with UV absorption.
- **Photodiode Array (PDA) Detector:** Captures a full spectral range, enabling peak purity analysis.
- **Fluorescence Detector:** Offers high sensitivity for compounds with native fluorescence or derivatized samples.
- **Mass Spectrometric (MS) Detector:** Provides superior selectivity and sensitivity; enables structural elucidation and ultra-trace quantification when coupled with LC (LC–MS/MS).[39]

Validation According To Regulatory Guidelines (FDA, EMA)

Method validation ensures reliability and compliance with bioanalytical guidelines from the U.S. FDA and EMA. Validation includes assessing accuracy, precision, linearity, selectivity, sensitivity, recovery, and stability under various conditions.[40,41]

Quantification and Validation Parameters.

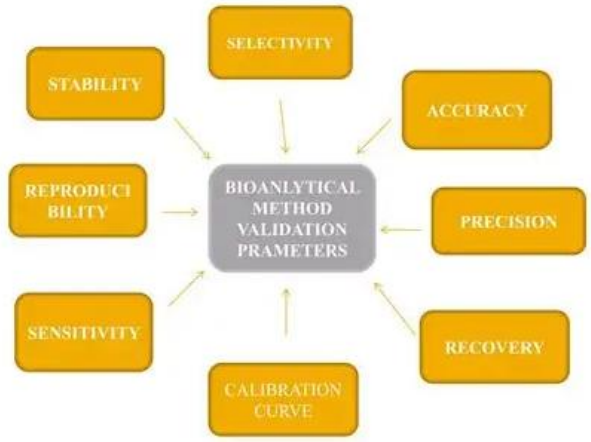

Figure 3: Bioanalytical Method Validation Parameters

- **Linearity, Accuracy, and Precision**
- Calibration curves must demonstrate linear response within the quantitation range. Accuracy reflects the closeness to true values, while precision measures repeatability.
- **Limit of Detection (LOD) and Limit of Quantification (LOQ)**
- Define the smallest detectable and quantifiable analyte concentrations, respectively, crucial for trace-level analysis.
- **Recovery, Selectivity, and Stability**
- Recovery measures extraction efficiency, selectivity ensures analyte identification without interference, and stability verifies analyte integrity during processing and storage.
- **System Suitability and Robustness**
- System suitability tests (SSTs) confirm consistent performance of the HPLC system, while robustness checks assess method reliability under small, deliberate variations.[42-49]

Applications of HPLC in Bioanalytical Quantification

Quantification of Drugs and Metabolites

HPLC is extensively used for quantifying drugs, metabolites, and biomarkers in biological samples, supporting therapeutic drug monitoring and pharmacokinetic profiling.

Pharmacokinetic and Bioequivalence Studies

HPLC methods enable precise concentration–time profiling of drugs, supporting dose optimization and bioequivalence evaluation between formulations.

Clinical and Toxicological Applications

HPLC aids in detecting toxins, endogenous metabolites, and therapeutic agents in clinical diagnostics, forensic science, and toxicology.

Recent Advances and Case Studies

Recent developments include the adoption of **UHPLC**, **LC–MS/MS**, and **green chromatography**, enabling faster, more sensitive, and environmentally friendly analyses. Numerous case studies demonstrate these improvements in drug monitoring, metabolomics, and clinical research.[51-52]

Challenges and Future Perspectives

1. Analytical Limitations and Matrix Effects
2. Despite its strengths, HPLC faces challenges such as matrix interference, analyte instability, and limited detection sensitivity for ultra-trace levels.
3. Integration with Hyphenated Techniques (LC–MS/MS, UHPLC)
4. Coupling HPLC with mass spectrometry and ultra-high performance systems has revolutionized bioanalysis, enhancing selectivity, sensitivity, and throughput.
5. Automation and Miniaturization Trends
6. Automation of sample preparation, robotic handling, and microfluidic HPLC systems are emerging to improve reproducibility and reduce analysis time and solvent usage.
7. Future Prospects in Bioanalytical Quantification
8. Future research focuses on **bioanalytical nanotechnology**, **biosensor integration**, and **artificial intelligence-assisted data interpretation** to further improve accuracy, efficiency, and sustainability in HPLC-based quantification.[53]

CONCLUSION

Bioanalytical quantification plays a crucial role in pharmaceutical research, clinical diagnostics, and therapeutic monitoring. The accuracy and reliability of results depend heavily on effective sample preparation, optimized chromatographic separation, and proper control of matrix effects. Techniques such as HPLC, LC–MS/MS, and advanced extraction methods (LLE, SPE, PP) have

significantly improved sensitivity, selectivity, and reproducibility in complex biological matrices.

Minimizing matrix interference and employing robust analytical validation ensure that generated data are both scientifically sound and regulatory compliant. With continuous advancements in chromatographic technology and greener, more efficient analytical approaches, bioanalysis continues to evolve as a cornerstone of modern drug development and personalized medicine.

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