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A MINI REVIEW ON BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION

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ABSTRACT: This review emphasizes the effectiveness of bioanalytical techniques in pharmacokinetic, pharmacodynamics, and toxicological evaluations, highlighting their essential role in drug development. The quantitative assessment of pharmaceuticals, metabolites, and endogenous chemicals in biological matrices such blood, plasma, urine, and cerebrospinal fluid was the focus of a thorough examination of bioanalysis procedures. Sample collection, preparation, analysis, and reporting are among the essential processes in the bioanalytical process that are described in the review. Particular focus is placed on the phases of method development, from pre-validation to literature evaluation, as well as sample preparation methods such as protein precipitation, liquid-liquid extraction, and solid-phase extraction. Method validation procedures were based on international norms like ICH Q2 and M10. The outcomes show that data correctness, precision, selectivity, and dependability are guaranteed by established bioanalytical techniques. Different stages of drug development require different forms of validation, such as cross-, partial, and full validation. Effective method validation is essential for both clinical monitoring and medication approval because it reduces variability and promotes regulatory compliance. For drug development initiatives to be successful, reliable bioanalytical methods must be established and validated. Following internationally accepted validation guidelines guarantees high-quality data, allowing for well-informed choices to be made throughout therapeutic evaluation and regulatory filing. The significance of scientifically competent bioanalytical methods in producing trustworthy data for drug safety and efficacy evaluations is emphasized by this review.

INTRODUCTION: A significant part of drug development involves bioanalysis. Bioanalysis plays an essential role in pharmacokinetic and pharmacodynamics investigations as well as toxicological review during drug development [1].

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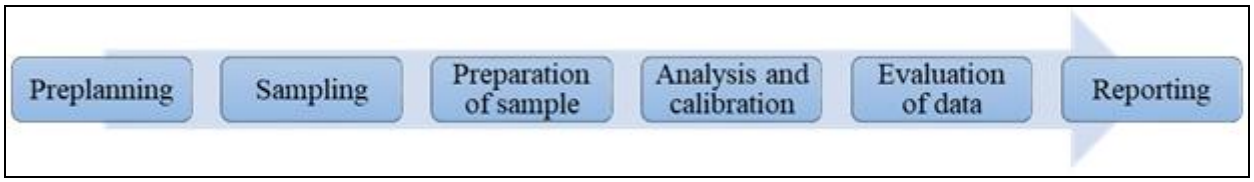
Bioanalysis: Bioanalysis is used to determine the concentrations of drugs, their metabolites, and/or naturally occurring chemicals within various biological matrices, including urine, saliva, serum, plasma, blood, and spinal fluid. The procedure involves collecting, processing, storing, and analyzing a drug's biological matrix [2].

These techniques for identifying drugs in biological fluids are becoming increasingly crucial for research on bioavailability, bioequivalence (BE), pharmacokinetics (PK), and quantitative drug evaluation.

Bioanalytical techniques play a vital role in new drug development, fundamental pharmaceutical and biological research and, therapeutic drug monitoring, *etc* [3].

Bioanalysis Procedure [3]:



Requirements: 1) A verified source for the biological matrix is required. 2) Working standards or references. 3) Chemicals and solvents. 4) Instruments and chromatographic devices. 5) Skilled manpower 6) Books and literature survey [1].

TABLE 1: GUIDELINES

Codification	History	Date
Q2(R1)	Since the methodology guideline Q2B has been added to the parent guideline, it is now known as Q2 (R1). "Validation of Analytical Procedures: Text and Methodology" is the new title [4].	November 2005
Q2(R2)	Complete update of the guidelines to align material with Q14 and incorporate more modern analytical technique applications. Approval by the ICH members in Step 2 and publication for public review [5].	24 March 2022
M10	Approval by the ICH Assembly members in the second step and publication for people review (document date 15 January 2019) [6].	26 February 2019
M10	The title was changed from "Bioanalytical Method Validation" to "Bioanalytical Method Validation and Study Sample Analysis" upon approval by the ICH Assembly Regulatory Members in Step-4 [6].	24 May 2022

Method Development: To ensure that the system is optimized for approval, the bioanalytical technique explains its design, limitations, effective conditions, and compatibility for the intended determination [7].

The following are the Steps involved in the development of the Method:

Method Selection and Complete Information Collection of the Sample: Begin by conducting the literature survey and gathering all relevant data on drug profiles. Additionally, the analyte's chemical structure and characteristics must be comparable for the internal standard to be selected in RP-HPLC.

Selection of Initial Method Conditions: Selecting a diluent based on drug solubility, internal standards, drug metabolites, and comparability with the analytical method are all part of the initial method setting. During this phase, focus must be placed on resolution and run time between the peaks.

Analyzing the Analytical Method in Aqueous Standards: Before developing a method in a biological fluid, the bioanalytical method is first assessed using aqueous standard, and the calibration curve is plotted for a minimum of four concentrations, ranging from the lowest to the maximum. By injecting each calibration curve standard, the correlation coefficient can be found; the range shouldn't be greater than 0.99.

Development and Optimization of Sample Processing Method: To make sure the instrument parameters stay consistent during validation, a matrix sample must be prepared and compared to the aqueous standards

Analyzing the Analytical Method in the Biological Matrix: When liquid-liquid extraction techniques have lower recovery and reproducibility, solid-phase extraction is the preferred method to improve sensitivity, precision, recovery, and low interference.

Pre-validation: Create a comprehensive sample preparation procedure with all the necessary information, contributing factors, and method conditions once the validation process has been determined to be reliable [7].

Sample Preparation Approaches:

Liquid-liquid Extraction (LLE): Principle: "It's grounded on selective extraction by the organic immiscible solvent of the intended analyte present in the liquid sample."

Texans, diethyl ether, ethyl acetate, dichloromethane, and methyl tert-butyl ether are the common solvents used in LLE. Liquid-liquid extraction involves the separation of two immiscible fluids based on varying solubility. Generally, there must be an aqueous phase and immiscible organic phase.

Solid Phase Extraction (SPE): Under specific conditions, it involves delayed partitioning of an analyte on solid phase (adsorbent). The process relies upon selective adsorption, where the analyte becomes adsorbed onto the solid phase. Upon adsorption, the target analyte may then be eluted with a solvent that is selected to suit its purpose. SPE is widely utilized owing to the selectivity and efficiency of its processed, and cartridges of varied type exist which are suited for varying analytes and sample matrices.

SPE Procedures:

Step 1: The first step is conditioning. Before using the sample, each solid phase extraction tube must be packaged with the appropriate solvents. Tertiary butyl methyl ether, methanol, and trace amounts of dichloro methane are solvents; additional solvents, such as organic, water, and buffers, can also be utilized. The diagramatic representation of solid phase extraction is shown in **Fig. 1.**

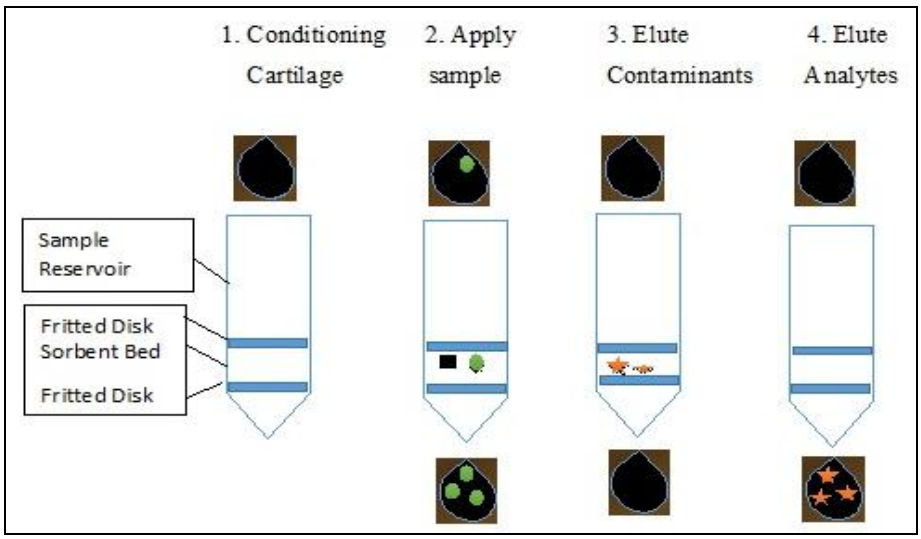


FIG. 1: SOLID PHASE EXTRACTION

Step 2: Sampling: Apply the sample gradually from above the container, making sure that no sample falls on the interior wall of the cartridge without channeling.

Step 3: Rinse or Wash: The analyst can filter out weakly retained interferences by rinsing the cartridge with a weakly diluted solvent or buffer, which will eliminate matrix components and other interferences without removing the analyte.

Step 4: Drying: By applying an appropriate vacuum, the vacuum pump can be utilized for the suggested drying period. Two to three minutes is the suggested drying time, removes the minute traces of water, which may interfere with the elution process.

Step 5: Elution: To increase the overall extraction efficiency, a solvent must be slowly transferred through a cartridge.

The best elution solvents are small volumes of dichloromethane, tertiary butyl methyl ether, methanol, and acetonitrile, acidic or alkaline methanol or acetonitrile or their combinations with other organic solvents employed are determined by the chemical nature of the analyte and facilitate effective elution.

SEP's Advantages Versus LLE: Comfortable method, 2. Application simplicity. 3. Accuracy and recovery improved. 4. The complete analyte fraction is more readily obtainable, 5. Automation is simpler [8].

The Technique of Solid-Phase Extraction (SPE): It is based on a principle analogous to liquid chromatography, where analytes are separated according to their interactions with a solid sorbent material. Recently, new developments in SPE have seen the introduction of several novel formats such as;

1. Dispersive SPE,
2. Molecularly imprinted polymer SPE
3. Disposable pipette extraction
4. Micro-Extraction by packed sorbent
5. Solid-phase micro extraction
6. Stir bar sportive extraction
7. Online solid-phase extraction [9]

Protein Precipitation: The working principle of protein is the separation of analytes, particularly analytes present in biological fluids like blood or serum, by protein structure disruption. The technique mainly involves the denaturation of proteins through the application of organic solvents like acetonitrile or methanol. Protein precipitation is possible by using several methods, such as adding organic solvents or changing pH of the sample. In other instances, denaturation is caused by hydrophobic interactions on the protein surface. Another mechanism associated with it is the presence of inorganic salts in a solution of water and water-miscible organic solvents. This biphasic process, also known as "salt-induced phase separation" or "salting out", is useful for extracting non-polar organic compounds and separating water-miscible solvents. the salts used are usually magnesium sulfate, sodium chloride (NaCl), and calcium chloride(CaCl2), commonly as part of a salt-assisted liquid-liquid extraction(LLE) process [8].

High Performance Liquid Chromatography (HPLC): The activated chemicals are separated, identified, and quantified using HPLC. The pump, injector device, columns, detection system, processor, and monitoring system make up HPLC equipment. The diagrammatic representation of HPLC instrument is depicted in **Fig. 2**. The column is the main part where differentiation of particles occurs. Since the stationary phase is made up of porous particles that are micron in size, a high-pressure pump is needed to transfer the movable phase across the column.

The mobile phase stream is supplemented with a small volume of the sample to be analyzed. The retention durations of the chemicals are shown by the detector. The period during which a certain substance dissolves, or leaves at the bottom of the column, is known as the retention time [7].

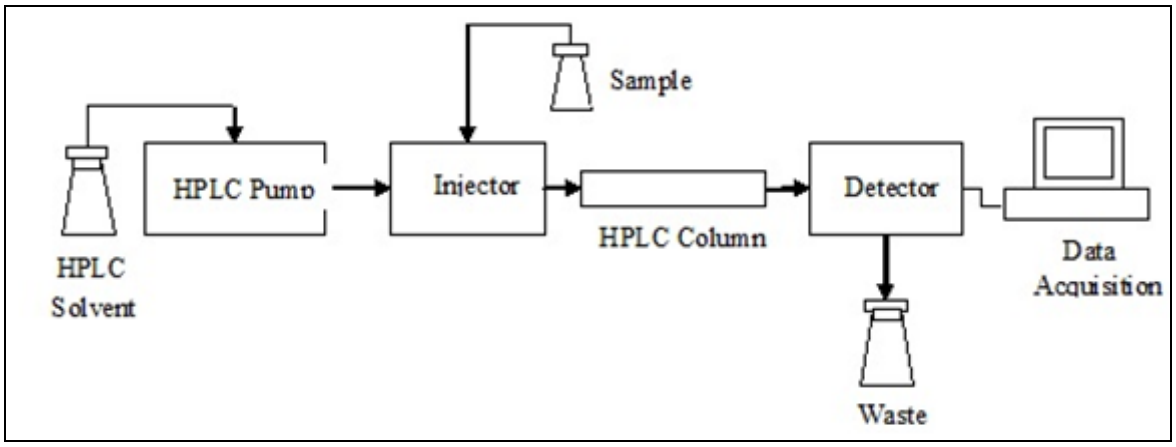


FIG. 2: HPLC INSTRUMENTATION

Injection of Sample: The sample solution can be injected using septum injectors when the mobile phase is flowing. To get repeatable results, a creative superior loop injector and rotary valve might be utilized [2].

HPLC Method Development [7]: The diagram Fig. 3. Flowchart of HPLC Method Development Process outlines the systematic steps involved in developing a High-Performance Liquid Chromatography (HPLC) method.

It begins with defining sample and separation goals, followed by optimizing sample preparation, selecting the appropriate detector, and conducting trial runs. Subsequent steps include improving separation efficiency, troubleshooting procedural issues, recovering the purified analyte, and finally validating the method for accuracy and reproducibility.

FIG. 3: FLOWCHART OF HPLC METHOD DEVELOPMENT PROCESS

Method Validation: The procedure of proving that an analytical technique constantly satisfies the minimum standards for accuracy, precision, selectivity, sensitivity, repeatability, and stability as described in Food and Drug Administration (FDA) guidelines is referred to as method validation.

The medical and pharmaceutical sciences utilize ICH guideline Q2 (R1) to a large extent. The European Medicines Agency's guidelines on “bioanalytical method validation" and the FDA are valuable sources” [10].

Bioanalytical Method Validation: Bioanalytical method validation, or BMV, involves assessing whether a quantitative analysis technique is appropriate for medical and pharmaceutical research [11].

Why Validate a Bioanalytical Method?: The purpose of the validation of a bioanalytical procedure is to demonstrate its accuracy, reliability and overall suitability for generating consistent results. Validation is responsible for the establishment of trust in the resultant data and ensuring that it adheres to the regulatory and scientific requirements. An important observation to make here is that the first step only represents initial validation, and constant monitoring needs to be carried out in the process during application to reaffirm continuous working and consistency [10].

Need for Bioanalytical Method Validation: To generate accurate and well- understood results, well-characterized and validated bioanalytical procedures must be used. It is widely accepted that bioanalytical approaches and procedures are at the forefront of technology and are constantly developing and getting better. It's also critical to stress that every bioanalytical method has unique properties that differ from one analyte to the next; hence, it can be necessary to create particular validation standards for every analyte. In addition, the study's final goal may also have an impact on the technique's suitability. When a study's sample analysis is carried out in multiple locations, it is required to achieve inter-laboratory dependability by validating the bioanalytical method or methods at every site and providing pertinent details about validity for various sites [11].

Types of Bioanalytical Method Validation:

Full Validation: It is called full validation when all the criteria for validation are established to be used in the sample evaluation for the bioanalytical process for each constituent. It is required to fully validate.

1. During the initial stages of creating and applying a bioanalytical technique.
2. For a brand-new medication.
3. It is necessary that the revised assay be thoroughly verified if chemicals are introduced to an already-existing test for quantification [10].

Partial Validation: Partial validation is the process of making adjustments to previously approved bioanalytical methods or to recognized bioanalytical methods that don't necessarily need full revalidation.

The following are typical bioanalytical method modifications:

1. Transfers of bioanalytical techniques between analysts or laboratories Changes into analytical techniques (such as adjustments to detection systems)
2. Modification of the anticoagulant used to collect biological fluid
3. A species-specific matrix change (human plasma to human urine, for example)
4. Modifications to sample processing methods
5. Species shift inside the matrix
6. Modification of the pertinent concentration range
7. Modifications to software systems and/or instruments
8. Small sample size
9. Uncommon matrices [10]

Cross Validation: When multiple bioanalytical methods are employed to gather data for a single study or for many independent studies, the process of evaluating validation parameters is referred to as cross-validation. The process employs a validated bioanalytical method with modifications as the reference and a previously validated bioanalytical method as the comparator. The comparisons are designed to encompass all relevant aspects [10].

Common Parameters used in the Validation of Bioanalytical Methods: For the bioanalytical method validation uses the recovery parameter is used as a matrix effect to evaluate the biological matrix on the analysis. Also, the stability parameter was studied in detail for the pharmacokinetic study.

Accuracy: Accuracy is the extent to which the measured concentration of an analyte is an agreement with its nominal or true concentration. It is usually stated as relative expected error (%RE) or percentage bias. Accuracy is an absolute measurement and may be affected by specificity and precision. It is often referred to as trueness. A minimum of five replicates for each level of concentration should be evaluated in order to quantify accuracy. At least three concentrations should exist within the range expected for study sample concentrations. The mean should be within 15% of the nominal for all concentrations except the lower limit of quantification (LLOQ). A 20% variation is acceptable up to the LLOQ. The most direct way of representing accuracy is through percentage bias as obtained from the given formula [10].

Accuracy (٪) = Measured value - true value/ True value × 100

Precision: Precision, which is a measure of random error, is the degree of agreement between a set of measurements made from several samples using a bioanalytical method. Measurement of scatter for concentrations acquired from homogeneous samples, repeated samplings. Usually, it is presented as the relative standard deviation (R.S.D.) or coefficient of variation (%CV) of the repeated measurements.

Coefficient of correlation (٪) = Standard deviation / Mean

The precision at the LOQ should remain within a 20% CV, while at each concentration level, it must not exceed a 15% CV. There are three levels of accuracy: repeatability, moderate precision, and reproducibility [10].

Repeatability: Repeatability is a concept used to describe the experimental inconsistency over a short time period (within-assay, intra-assay) under the same working conditions. Repeatability refers to the method's performance throughout a day in a single lab and on a single instrument. Precision was measured in the most ideal circumstances [10].

Intermediate Precision: Intermediate precision means the variation present when a process is carried out in one lab under varying circumstances, e.g., alterations by days, analysts, equipment, and other changes. It is used to quantify the effect of other random variables beyond simple repeatability, e.g., variations between assays (inter-assay) or between runs per day. The parameter measures the qualitative and quantitative performance of an approach under general laboratory variations. By quantitatively measuring differences resulting from different analysts, equipment, or times, intermediate precision gains insight into a procedure's robustness in a laboratory environment [10].

Reproducibility: Although it is not necessary for submission, the capacity to obtain consistent results between laboratories or between inter-laboratory studies can be considered for the consolidation of analytical methods.

The method's capacity to produce comparable concentrations for a sample when examined at various times. Reproducibility describes the method's performance in both qualitative and quantitative term across labs, daily operations, analyst-to-analyst interactions, and instrument-to-instrument interaction [10].

Linearity: The ability of an analytical technique to produce results proportional to analyte concentration to a specific degree within an interval is called linearity. The calibration curve must span at least the study sample's expected concentration range. If a single calibration curve cannot adequately encompass the entire range, substantially beyond that needed can have detrimental effects on the method's accuracy and precision, especially at the extremes of high and low values. Linearity is most routinely tested using correlation coefficients (e.g., "r" or "r") to quantify the extent how well data are represented in a linear regression model [10].

Selectivity: The capacity of a bioanalytical technique to consistently separate and measure the analyte in the presence of other anticipated components is known as selectivity. It entails proving that the analytical process can successfully isolate the analyte from interfering contaminants. Selectivity is a crucial validation criterion that is commonly defined as the method's ability to precisely measure and isolate the analyte in the presence of potentially co-existing variables. Analyzing blank samples from a minimum of six separate sources of the biological matrix (such as plasma, urine, or other materials) is necessary to demonstrate selectivity. Every blank sample needs to have its lower limit of quantification (LLOQ) assessed for interference. FDA bioanalytical technique validation standards state that in order to validate the method's accuracy, at least six distinct matrix batches must be studied [10].

Limit of Detection: A minimal analyte concentration that is recognizable but not measurable. Because some bioanalytical labs only analyze the smallest amount of a reference, the LOD estimate may be erroneous. Everyone agrees that the LOD should be the lowest concentration or amount of the target analyte that can be detected [10].

Limit of Quantification: The smallest concentration of an analyte that can be consistently measured with a satisfactory level of accuracy and precision using a particular analytical technique is known as the limit of Quantification (LOQ). It is essential for identifying and precisely measuring low levels of analytes in a sample and represents the sensitivity of the method. Even very low analyte concentrations can be reliably reported in analytical investigations when a LOQ is well-established [10].

Recovery: Recovery is the percentage recovery of a known quantity of analyte during the sample processing and extraction step. Recovery is used to measure the extraction efficiency of an analytical procedure. It is an indicator of how well the analyte is extracted from the biological matrix by using the procedure. The analyte and internal standard should be recovered reproducibly, accurately, and consistently, although not 100% recovery. Analytical results of three levels of extracted samples should be compared to analytical results of standards reporting 100% recovery in testing for recovery [10].

Stability: Stability refers to the chemical stability of an analyte in a definite biological matrix under specified conditions over a definite period. Stability testing is carried out to identify any degradation of the target analyte(s) during different stages, such as sample collection, sample processing, storage, preparation, and analysis. Stability testing is generally done at method validation, except for long-term stability studies. Long-term studies can sometimes be finished several years after clinical trial initiation. The conditions for assessing stability vary according to the analyte and characteristics, the nature of the biological matrix, and the expected duration of storage before analysis [3, 10].

Types of Stability Testing:

Short-term Stability: Before analysis, three aliquots of both the low and high concentration

quality control (QC) samples should be thawed and incubated in room temperature for four to twenty-four hours. This tests the stability of the analyte under normal handling conditions. The results should indicate that the analyte is stable under those conditions if the deviation from nominal concentrations remains within ±15% [3, 10].

Long-term Stability: Freeze at room temperature (4–24 hours) and analyze at least three aliquots of low and high concentrations. The overall storage time should be longer than the duration between the study's initial sample collection and last sample analysis. Three different analytical runs must be used to replicate this test [3, 10].

Freeze and Thaw Stability: Assess the impact of at least three cycles of freeze-thaw on low and high concentration samples. For each cycle, maintain aliquots at the target storage temperature (e.g., -70°C) for 24 hours. Thaw out completely at room temperature. Refreeze for 12–24 hours. Repeat this three times. Following the third cycle, examine the samples. The acceptable standard deviation should be below 15%. Samples must be compared to a new, freshly prepared stock solution in a blank, interference-free biological matrix [3, 10].

Stocked Solution Stability: It is recommended that the stability of the drug stock and internal standard solutions be assessed at least for six hours at room temperature. The deviation should not exceed 15%.

The stock solution stability has to be noted if the stock solutions are stored frozen or chilled for the adequate period of time. Stability must be evaluated following the specified storage period by comparing the device's response with that of freshly prepared solutions [3, 10].

Post-Preparative Stability: Post-preparative stability entails evaluating the stability of samples based on the duration they spend in the auto sampler. To determine stability, concentrations need to be calculated from the original calibration standards, and the stability of the analyte and internal standard needs to be evaluated for the anticipated run time for the batch size in validation samples. Standard Operating Procedures (SOPs) need to detail the statistical methodology and acceptance criteria. In addition, analysis of samples from dosed subjects may give additional confirmation [3, 10].

Ruggedness: Ruggedness is the ability of the method to be unaffected by minor, day-to-day variations that can happen during regular analysis, including temperature, pH, and mobile phase composition fluctuations. If an analytical method is supposed to be transferred to another lab, its ruggedness should be tested. Though ruggedness is not a prerequisite for full validation, it may prove to be a useful component of method development, as it informs about prospective problems during the validation phase [3, 10].

TABLE 2: FOLLOWING ARE SOME REPORTED METHODS OF BIOANALYTICAL DRUG ANALYSIS

Analyte	Biomatrix	Sample volume	Sample preparation	Column	Extraction solvent
Favipiravir [12]	Human plasma	10µl	PPT	Kromasil C_{18}	Ethyl acetate
Resveratrol [13]	Rat plasma	10µl	LLE	Phenomenex kinetics C_{18}	Methyl tert-butyl ether
Docetaxel [14]	Human plasma	100µl	LLE	C8 Column	Acetonitrile
Kaempferol [15]	Rat plasma	50µl	LLE	Lichrocart C18 column	Acetonitrile
Teneligliptin [16]	Rabbit plasma	20µl	LLE	Thermo C18 column	Ethyl acetate
Remogliflozin [17]	Rat plasma	20µl	PPT	Phenomenex C18	Methanol
Verapamil [18]	Rat plasma	20µl	PPT	Thermo hypersil GOLD C18	Perchloric acid: acetonitrile(8:2)
Ellagic acid [19]	Rat plasma	5µl	PPT	Ascentis C18	Acetonitrile
Sofosbovir [20]	Human plasma	10µl	LLE	Kromasil C18	Acetonitrile
Clobazam [21]	Rat plasma	5µl	LLE	Phenomenex Luna C18	Acetonitrile
Ponazurin [22]	Cat plasma	100µl	SPE	Symmetry Shield RP 18	Acetonitrile
Gallic acid [23]	Rat plasma	5µl	PPT	Zorbax SB C18	Acetonitrile
Naringenin [24]	Rat plasma human plasma	20µl	PPT	Analytical column RP18	Methanol
Itopride Hydrochloride [25]	Rabbit plasma	2µl	PPT	Atlantis HILIC column	Acetonitrile
Dapagliflozin [26]	Rat plasma	20µl	LPE	C18 column	Ethyl acetate

CONCLUSION: Bioanalysis is a critical component of drug development, involving the determination of drug concentrations and metabolites accurately in various biological matrices. Rigorous method development and validation, and adherence to predefined guidelines and standards such as Q2 (R1) and Q2 (R2) are required during the process of bioanalysis. The process of method validation allows for accuracy, precision, selectivity, sensitivity, and stability by FDA and EMA guidelines. Overall, bioanalytical method validation serves to ensure the reliability and dependability of quantitative analytical methods, thus rendering them successful in biomedical applications.

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CONFLICTS OF INTEREST: The Authors declare that they have no conflicts of interest regarding the publication of this review.

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