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REVIEW ON: BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF SOME ANTIHYPERTENSIVE DRUGS BY HPLC METHOD

Sanket S.Dandge[1], Prof. Monika P. Jadhao[2]

[1] Department of Quality Assurance,Vidyabharti College of Pharmacy, Amravati, 444602
[2] Department of Quality Assurance,Vidyabharti College of Pharmacy, Amravati, 444602

A B S T R A C T :

Hypertension is a prevalent condition characterized by elevated blood pressure, posing significant risks for cardiovascular, cerebrovascular, and renal complications, particularly in individuals over 60 years of age. Antihypertensive agents, essential in clinical management, operate through diverse mechanisms, including natriuresis promotion, vascular resistance reduction, and cardiac output modulation. Due to variations in therapeutic responses and tolerability, combination therapies are often employed for optimal blood pressure control. This paper provides a comprehensive overview of bioanalytical methods essential for the quantification of antihypertensive drugs and their metabolites in biological matrices, emphasizing the critical role of bioanalysis in pharmacokinetics, toxicokinetics, and regulatory submissions. The evolution of bioanalytical techniques is discussed, highlighting advancements from early colorimetric methods to modern high-throughput technologies such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) ELISA, and HPLC. The validation of bioanalytical methods is presented as a foundational step to ensure accuracy, precision, specificity, and reliability of data, which is crucial for informed clinical decision-making. Furthermore, the paper addresses sample preparation techniques, including protein precipitation, liquid-liquid extraction, and solid-phase extraction, which are essential for achieving reliable analytical outcomes. Overall, this research underscores the importance of robust bioanalytical methodologies in enhancing the efficacy and safety of antihypertensive therapies.

Keywords: Cardiovascular complications, Renal complications, Vascular resistance, Biological matrices, Colorimetric methods, Clinical decision-making.

1.Introduction:

Hypertension, a habitual elevation of blood pressure, is a major threat factor for cardiovascular, cerebrovascular, and renal complications. Affecting over 50 of entities above 60, it remains a leading cause of morbidity and mortality encyclopedically.

Antihypertensive agents, critical in clinical practice, lower blood pressure via various mechanisms—promoting natriuresis, reducing vascular resistance, or modulating cardiac output. Therapeutic response and tolerability vary, often necessitating combination therapy for optimal control.

These drugs fall into seven primary classes: diuretics, sympatholytics, calcium channel blockers, ACE inhibitors, ARBs, direct renin inhibitors, and vasodilators. While generally safe, drug-induced liver injury is rare, with methyldopa and hydralazine being notable exceptions.

The precise quantification of drug and endogenous composites in natural matrices is a foundation of multitudinous scientific and nonsupervisory operations. Within the pharmaceutical assiduity, bioanalytical ways are native to measuring attention of active medicine substances and their metabolites, thereby easing crucial examinations in pharmacokinetics, toxicokinetics, bioequivalence, and exposure – reaction connections encompassing pharmacokinetic/ pharmacodynamic(PK/ PD) estimations. Beyond medicine evolution, bioanalysis serves overcritical places in the discovery of lawless substances, forensic toxicology, anti-doping duties in competitive derisions, and the monitoring of environmental pollutants, where accurate dimension is essential for compliance, security, and public health guidance.

Historically, bioanalysis primarily concentrated on the quantification of fragile- patch rectifiers. Over the once two decades, still, there has been a significant ascent in the evolution of biopharmaceuticals similar as remedial proteins and peptides aimed to target numerous of the same pathological conditions as their fragile- patch equals. The structural complication, advanced molecular cargo, and different physicochemical parcels of these voluminous biomolecules have acquainted distinct logical expostulations, challenging the enhancement of technical bioanalytical styles and platforms for accurate quantification.

In the early stages of bioanalytical science, drug quantification in biological fluids relied on nonspecific assays that lacked the ability to distinguish between parent compounds and their metabolites. For instance, early drugs such as aspirin (circa 1900) and sulfonamides (introduced in the 1930s) were measured using rudimentary colorimetric techniques. Antibiotics, in particular, were often quantified based on their capacity to inhibit microbial growth in bioassays. The emergence of pharmacokinetics in the 1930s highlighted the need for more selective and reliable analytical methodologies. As drug

development progressed, particularly with the advent of highly potent therapeutic agents, the demand for greater assay sensitivity increased, necessitating the evolution of sophisticated bioanalytical technologies capable of detecting trace concentrations with high specificity and accuracy.

Bioanalysis refers to the qualitative and quantitative assessment of drugs, metabolites, and biomarkers within biological matrices such as plasma, serum, whole blood, urine, saliva, and tissues. It constitutes a critical component of the drug development continuum, beginning with in vitro and in situ evaluations, extending through preclinical studies, and continuing into clinical trials. In the context of modern high-throughput drug discovery and development, bioanalytical laboratories face increasing demands for rapid turnaround times and high precision. Given that bioanalytical outcomes directly influence clinical decision-making and therapeutic evaluations, these processes are integral to regulatory submissions. To ensure data reliability, consistency, and scientific integrity, global regulatory authorities have established rigorous guidelines and standardized protocols governing bioanalytical method validation and performance.

The elaboration of bioanalytical sample medication ways has faced adding complication due to the perpetual demand for meliorated perceptivity, delicacy, and rapid-fire dissection of elaborate natural fluids similar as race, tube, serum, slaver, feces, and urine. given away that prey analytes frequently live at trace situations, preconcentration way are constantly necessary to achieve sensible attention. still, similar way can in advertently co-extract snooping substances, involving fragile motes(e.g., medicines, mariners, and metabolites) as well as macromolecules(e.g., nucleic acids, proteins, and peptides). As a result, largely picky and effective sample remittal procedures are essential to insure logical particularity and perfection, especially in brace of nonsupervisory cessions similar as Investigational New Drug(IND) operations, New Drug Applications(NDAs), and shortened New Drug Applications(ANDAs). Thorough confirmation of sample medication methodologies is thus obligatory before their perpetration in routine dissection. Biological matrices innately contain plentiful endogenous ingredients similar as carbohydrates, proteins, lipids, and electrolytes — that can beget matrix goods, thereby sophisticating trace- position analyte discovery. The primary ideal of sample medication is to alleviate these goods and insulate the analyte of interest. In this composition, we present a complete review of current literature pertaining to coincidental sample medication strategies in bioanalysis.

1.2 Bioanalysis Study :-

Bioanalysis is the scientific discipline concentrated on the quantitative dimension of drugs, metabolites, and biomarkers in natural systems similar as blood, tube, urine, or apkins. It plays a critical part in pharmacokinetics, bioequivalence, toxicology, and medicine development studies. Bioanalysis involves ways like liquid chromatography- tandem mass spectrometry(LC- MS/ MS), ELISA, and HPLC. These styles insure delicacy, perfection, selectivity, and perceptivity in detecting analytes at veritably low attention. Bioanalytical studies must follow nonsupervisory guidelines(e.g., FDA or EMA) to insure data integrity. The process includes system development, confirmation, and sample analysis under Good Laboratory Practices(GLP). Eventually, bioanalysis provides essential data for assessing medicine safety, efficacity, and dosing in humans and creatures.

Bioanalysis has an important role in drug development.

- Toxicological evaluation,
- Pharmacokinetic studies
- Pharmacodynamics studies

Bioanalytical Method Validation(BMV), especially within the compass of guidelines 13- 22, plays a vital portion in the quantitative determination of a wide range of analytes in natural and physiological matrices. These validated styles are workable to both mortal clinical pharmacology inquiries and non-mortal inquiries. BMV is essential for the accurate evaluation and interpretation of bioequivalence, pharmacokinetic(PK), and toxicokinetic inquiries.

The bioanalysis process encompasses several critical steps, including preplanning, sampling, sample preparation, analysis, calibration, data evaluation, and reporting. In modern bioanalytical practices, effective sample preparation techniques and the use of advanced hyphenated instrumentation (such as LC-MS/MS) are key to achieving reliable and reproducible results.

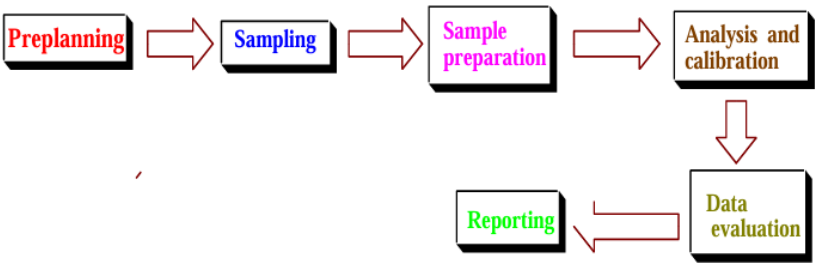

Figure 1:-Bio-Analytical Work Flow

1.3 Requirements:

1) Authenticated source for Biological Matrix.
2) Reference or working Standards.
3) Solvents and Chemicals.

4) Chromatographic Devices- Instruments, Columns.
5) Well trained Man Power
6) Literature.

2. PREPARATION AND PRESERVATION OF BIOLOGICAL SAMPLES

Sample Medication is a vital element of bioanalytical studies, aimed at segregating analytes from complex natural matrices similar as tube, serum, and urine while minimizing hindrance from proteins and other endogenous or exogenous substances. For analytes susceptible to enzymatic declination, the prompt addition of esterase impediments(e.g., sodium fluoride)post-collection is essential. also, proper selection of storehouse holders is critical, as accoutrements like plastics and hype factors can strain pollutants(e.g., bis(2- ethylhexyl) phthalate,tri-butoxyethyl phosphate) that may compromise logical delicacy. icing clean sample running and storehouse preserves analyte integrity and supports dependable data generation.

2.1 Protein precipitation

Protein precipitation is a widely employed technique in bioanalytical sample preparation, particularly for plasma and serum clean-up. It exploits the interaction between precipitation reagents and protein functional groups. Organic solvents like methanol or acetonitrile disrupt hydrophobic interactions within protein structures, leading to denaturation and subsequent precipitation. The precipitated proteins are then efficiently removed by centrifugation, facilitating clearer sample matrices for analysis.
The resulting supernatant, largely free of protein interference, contains the analyte of interest and is suitable for chromatographic analysis. Owing to its simplicity, speed, and cost-effectiveness, protein precipitation is widely utilized in the preparation of biological samples for drug quantification.

2.2 Liquid Extraction

Liquid–Liquid Extraction (LLE)
Liquid – Liquid birth(LLE) is a extensively assumed fashion in bioanalytical chemistry for segregating analytes from fluid natural matrices. The principle of LLE involves the partitioning of an analyte between two immiscible liquid phases, generally an waterless phase(frequently thick) and an organic phase(generally less thick). The effectiveness of birth relies on the discriminational solubility of analytes in the two detergents — hydrophilic composites preferentially dissolve in the waterless subcaste, whereas hydrophobic composites favor the organic detergent.
The immiscibility of the two phases is critical to the success of the method, as it ensures clear separation after mixing. The technique is particularly effective in separating analytes from interfering substances, enabling better downstream detection.
The theoretical basis of LLE is governed by the Nernst Distribution Law, which states that a solute will distribute itself between two immiscible solvents such that the ratio of its concentrations in each solvent remains constant at a given temperature, provided the solute exists in the same chemical form in both phases. This ratio is referred to as the partition coefficient (P), commonly expressed as Log P, where:

$$\log P = Log \frac{Solute(Octanol)}{Solute(Water)}$$

Log P values help predict the affinity of a compound for lipophilic versus hydrophilic environments, which in turn guides solvent selection for optimal analyte recovery.

2.3 Solid Phase Extraction (SPE)

Logical Phase birth(SPE) is one of the most considerably exercised and effective sample drug ways in bioanalytical chemistry. A check of scientific literature reveals its extensive operation a quest for the term" SPE" on ScienceDirect yielded 242,964 goods, with 7,547 publications in the time 2021 alone. This underscores the fashion's significance in modern logical workflows.
SPE is a logical- fluid phase birth fashion in which analytes are extensively separated from a natural matrix through discriminative commerce with a logical- phase sorbent substance. The fashion enables the sequestration and sanctification of the analyte by retaining poking substances on the sorbent or allowing them to pass through, depending on the birth mode exercised.
Sorbents exercised in SPE are generally plugged into disposable charges or discs and may parade-polar, or ionic characteristics, named predicated on the physicochemical parcels of the analyte and matrix factors.

3. VALIDATION

Confirmation is the process of establishing proved substantiation that provides a high place of confidence that a special process, system, or system will constantly produce a asked result or product that meets destined specifications and quality attributes. confirmation is a overcritical and mandatory step following system evolution. It's essential to demonstrate that the logical system or process constantly performs as intended and produces dependable, accurate, and reproducible effects. Without proper confirmation, there's no confidence that the system is able for its willed purpose, especially in restrained surroundings like pharmaceutical quality control.

3.1 Bioanalytical method validation (BMV)

Bioanalytical Method Validation (BMV) is a critical process that ensures analytical methods are fit for purpose in the quantitative assessment of analytes within biological matrices. By confirming reliability and reproducibility through structured laboratory investigations, BMV provides essential documentation that the method meets the performance standards required for its intended biomedical application. This validation is fundamental not only for regulatory compliance but also for maintaining the integrity of data throughout drug development, clinical research, and therapeutic monitoring, *BMV is indispensable for generating high-quality, credible data, thereby supporting sound scientific and regulatory decision-making.*

A) Full Validation:

Full validation entails the comprehensive assessment of all relevant parameters necessary for the reliable application of a bioanalytical method to a specific analyte. It is essential when a method is initially developed and implemented for a new drug entity, ensuring its accuracy, precision, and overall suitability for biological sample analysis. Additionally, full validation is required when new metabolites are incorporated into an existing assay to confirm that the method remains robust and reliable. *In conclusion, full validation is a foundational step in establishing method credibility and ensuring the integrity of bioanalytical data in both new and modified applications.*

B) Partial Validation:

Certain modifications to bioanalytical methods do not warrant full validation but may require partial validation or method verification to maintain analytical reliability. Such changes include matrix shifts within the same species (e.g., human urine to plasma), species changes within the same matrix (e.g., rat to mouse plasma), alterations in laboratory settings, analyst personnel, instrumentation, or minor procedural adjustments, including detector substitution. *while full validation is not always necessary, appropriate verification ensures method integrity is preserved under modified conditions.*

C) Cross Validation:

In method comparison, two bioanalytical methods are evaluated: the *"reference" method*, the original validated method, and the *"comparator" method***,** the revised or alternative approach. This comparison ensures that both methods yield equivalent and consistent results. It is typically employed when introducing a new or modified method, when a different laboratory, analyst, or equipment is involved, or when data from both methods will be used interchangeably within the same study.

3.2 Accuracy:

Delicacy refers to the closeness of the measured effects to the true attention of the analyte, frequently appertained to as facticity. It's assessed by performing replicate breakdowns on slices with known analyte attention. The most common or garden path to estimate delicacy is through reclamation inquiries, where the measured value is assimilated to the known(true) value. effects are considered respectable if they fall within ± 10 of the anticipated attention. In accession inquiries, delicacy was determined utilizing the standard-issue extension system, where a known quantum of drug was appended topre-analyzed slices at 80, 100, and 120 of the labeled claim. Percent reclamation and relative standard-issue divagation(RSD) were calculated to charge the delicacy. The delicacy was ventilated utilizing the formula reclamation = (quantum set up – initial quantum)/ quantum appended) × 100. delicacy is considered respectable when the percent reclamation falls within the range of 90 to 110, and the RSD is generally lower than or equal to 2, indicating both high delicacy and perfection.

$$\%RE = (E - T) \times (100/T),$$

where E is the experimentally determined concentration, and T is the theoretical concentration.

3.3 Precision :

Precision reflects the consistency and reliability of an analytical method and is typically evaluated at three distinct levels. The first level is Repeatability (Intra-assay precision), which assesses the precision under the same operating conditions over a short time interval, involving the same analyst, equipment, and laboratory. The second level is Intermediate Precision (Within-lab reproducibility), which examines the method's performance under varied conditions within the same laboratory, such as different days, analysts, or equipment. The third level is Reproducibility (Inter-lab precision), which measures the method's precision across different laboratories and is particularly important when methods are transferred or intended for use in multiple locations. These evaluations help ensure that the method delivers reliable and consistent results across different settings and conditions.

Precision was evaluated through intraday and interday studies, with two replicate concentrations. The %RSD for intraday precision was 0.47%, and for interday precision, it was 0.84%. Since both values were below the 2% threshold, the method was deemed precise and satisfactory.

3.4 Linearity:

Linearity, as outlined by the International Council for Harmonisation (ICH) guidelines, is a critical parameter in analytical method validation that describes the ability of a method to produce results that are directly proportional to the concentration of an analyte within a defined range. This characteristic is fundamental for achieving accurate quantification in bioanalytical assays. To evaluate linearity, a series of standard solutions at varying concentrations of the analyte are prepared and analyzed. The analytical response, typically expressed as peak area or peak height, is plotted against the corresponding analyte concentrations to generate a calibration curve. Linear regression analysis is then applied using the least squares method to determine the best-fit line. The quality of the linear relationship is assessed by calculating the correlation coefficient (r) or the coefficient of determination (r^2), with values

approaching 1.000 indicating strong linearity. The resulting calibration curve is described by the linear regression equation, y = bx + a, where y represents the instrument response, x is the analyte concentration, b is the slope, and a is the intercept.

3.5 Specificity/ Selectivity

A method is considered *specific* when it generates a measurable response exclusively for the intended analyte, without interference from impurities, degradation products, excipients, or matrix components. Specificity is a critical validation parameter, particularly for complex biological samples or pharmaceutical formulations. To demonstrate specificity, several approaches are employed**:** *Placebo or Blank Interference* is assessed by analyzing samples without the active drug to ensure no interference at the analyte's retention time; *Forced Degradation Studies* subject the sample to stress conditions to identify potential degradation products; and *Impurity Analysis* evaluates known impurities to assess system suitability parameters, including *retention factor (k'), tailing factor (Tf),* and *resolution (Rs). Peak purity* analysis using HPLC with PDA or MS detectors can confirm that a peak represents a single compound, ensuring no co-elution of impurities.

3.6 Limit of detection (LOO):

The Limit of Detection (LOD) is the lowest concentration of an analyte in a sample that can be reliably detected, though not necessarily quantified, under specified experimental conditions. It represents the smallest amount that produces a response significantly different from background noise, typically with a signal-to-noise ratio (S/N) ≥ 3. To determine LOD, the following procedure is employed: 1) Blank Injections involve preparing a blank solution and injecting it six times to assess baseline noise; 2) Linearity Solutions are prepared across a concentration range to establish the linearity curve; 3) LOD Calculation is done using the S/N method (S/N ≥ 3) or by applying the formula:

LOD = 3.3 × (Standard deviation of the blank response) / (Slope of the calibration curve),

where σ is the standard deviation and S is the slope from the calibration curve.

3.7 Limit of quantification (LOQ):

The Limit of Quantification (LOQ) is the lowest concentration of an analyte in a sample that can be reliably quantified with acceptable accuracy and precision under the given experimental conditions. It represents the lowest point on the calibration curve meeting pre-established criteria for accuracy (closeness to the true value) and precision (reproducibility). The LOQ is expressed as:

LOQ = 10 × (Standard deviation of the blank response) / (Slope of the calibration curve),

where σ is the standard deviation (typically from the blank or low concentration sample) and S is the slope of the calibration curve. While the Limit of Detection (LOD) represents the lowest amount detectable but not quantifiable, the LOQ is the lowest analyte concentration that can be determined quantitatively with reliable accuracy and precision. Both values are expressed in terms of concentration and are calculated using the slope of the linearity curve.

3.8 Robustness:

It's a overcritical aspect of system confirmation, as it ensures that the system maintains its delicacy, perfection, and common interpretation despite minor diversions from the optimized conditions. true variations assessed during robustness testing carry slight changes in the pH of the movable phase, oscillations in string temperature, minor adaptations in inflow rate, use of nonidentical lines of the same type, and fragile changes in discovery wavelength. assessing these procurators helps confirm that the system can produce harmonious and dependable effects during regular use, buttressing its felicity for long- tenure operation in quality control and routine dissection.

3.9 System Suitability :-

The system suitability parameters, including column efficiency (theoretical plates), resolution factor, peak asymmetry factor, tailing factor, and the lower limit of quantification (LLOQ), were evaluated to ensure the adequacy of the optimized analytical methods. These parameters are critical for assessing the performance and reliability of the chromatographic system. The results obtained from system suitability studies in plasma samples demonstrated that all measured values were within the predefined acceptable limits. This indicates that the chromatographic conditions were appropriate and the method was capable of producing reliable and reproducible results. Overall, the system suitability parameters of the optimized methods were found to be satisfactory.

4. Application of a validated bioanalytical method to routine

Biological samples are generally suitable for analysis using a single determination, without requiring duplicate or replicate measurements, provided the assay method exhibits acceptable variability as demonstrated by validation data. This strategy is appropriate when the method reliably operates within the established precision and accuracy tolerance limits, ensuring that the results obtained are both consistent and dependable. When such criteria are met, single measurements are sufficient to maintain the integrity and reliability of the analytical process.

Bioanalytical method validation, used for the quantitative determination of drugs and their metabolites in biological fluids, plays a critical role in the evaluation and interpretation of *bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic study data*. These studies are essential components of regulatory submissions. The *quality and reliability of these studies* are directly dependent on the *quality of the underlying*

*bioanalytical data***.** Therefore, it is imperative that *guiding principles for the validation of these analytical methods* are well-established and effectively communicated to the pharmaceutical and scientific community.

Method validation is a process that demonstrates a method's ability to consistently meet or exceed the *minimum standards outlined by the U.S. Food and Drug Administration (FDA)* for key performance parameters such as *accuracy, precision, selectivity, sensitivity, reproducibility, and stability***.** Among analytical techniques, *chromatographic methods*—notably *High-Performance Liquid Chromatography (HPLC)* and *Gas Chromatography (GC)*—have been extensively employed for the *bioanalysis of small molecules***.** In particular, *Liquid Chromatography coupled with tandem Mass Spectrometry (LC-MS/MS)* has emerged as the *most widely adopted and powerful technology* for quantitative bioanalytical applications, owing to its high sensitivity, specificity, and robustness.

5. Objectives :-

- The primary objective is to develop an analytical procedure that is not only reliable, ensuring consistent and reproducible results under varied conditions, but also accurate, providing measurements that closely align with the true values of the analyte, and sensitive, capable of detecting and quantifying low concentrations of the analyte with a high degree of precision and minimal interference from matrix components.
- It is employed for the quantitative and/or qualitative determination of various substances, including *drugs***,** *metabolites*, and *biomarkers*, enabling precise analysis of these compounds in biological samples for research, clinical, and pharmaceutical applications.
- The method is applied to a range of biological matrices, including *blood***,** *plasma***,** *serum***,** *urine*, and *tissue samples*, facilitating the analysis of analytes within these complex biological environments.
- It plays a critical role in various areas, including *drug discovery***,** *pharmacokinetics***,** *bioequivalence studies*, and *therapeutic drug monitoring*, supporting the development, evaluation, and optimization of pharmaceutical therapies.
- The method must consistently produce valid results under defined conditions.
- It should clearly distinguish the analyte from other matrix components.
- The method must ensure *precision*, delivering consistent results under repeated conditions; *accuracy*, providing measurements that closely match the true analyte concentration; *selectivity*, distinguishing the target analyte from other components in the sample; and *reproducibility*, ensuring the method yields reliable results across different conditions, analysts, or laboratories.
- A robust method supports confident decision-making in both clinical and regulatory settings.
- It must meet validation standards as per regulatory guidelines (e.g., FDA, EMA).

6. Conclusion :-

Bioanalysis, encompassing the generation of pharmacokinetic, toxicokinetic, and metabolic data, is a critical component of pharmaceutical research and development. To ensure the reliability and regulatory compliance of such data, bioanalytical methods must be appropriately validated and documented in accordance with established scientific standards. Despite the availability of various bioanalytical techniques, particularly for low molecular weight drug candidates, a lack of clear experimental and statistical guidelines for method validation has posed significant challenges. In response, researchers have proposed practical strategies aimed at assessing the reliability of chromatographic methods commonly employed in bioanalysis. This article presents scientifically grounded, user-friendly approaches designed to improve the overall quality, consistency, and acceptance of bioanalytical method development and validation. By addressing key validation parameters, these approaches contribute to the advancement of methodological standards in the bioanalytical field.

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