



Review Article

Bioanalytical Method Development And Validation For The Estimation Of Active Pharmaceuticals In Dosage Forms

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ABSTRACT

In this review article, bioanalytical techniques are often employed to quantify pharmaceuticals and their metabolites in plasma matrices, and the techniques should be used in both human clinical investigations and nonhuman research. A key component of estimate and interpretation of bioequivalence, pharmacokinetic, and toxicokinetic investigations is the use of the bioanalytical technique for the quantitative measurement of medicines and their metabolites in biological medium. Method creation, method validation, and sample analysis are the three main responsibilities of bioanalysis. To determine the amount to which environment, matrix, or procedural factors might affect the estimation of analyte in the matrix from the time of set up to the time of analysis, each step in the technique must be examined. Techniques such as high-pressure liquid chromatography (HPLC) and liquid chromatography coupled with double mass spectrometry (LCMS-MS) can be used for the bioanalysis of drugs in body. Each of the instruments has its own merits and demerits. Chromatographic methods are HPLC and gas chromatography have been mainly used for the bioanalysis of small/ large molecules, with LC/MS/MS. Linearity, accuracy, precision, selectivity, sensitivity, reproducibility, and stability are some of the regularly used parameters. In this review article, we are proposed to add some points regarding bioanalytical method development and validation parameter, beneficial to quality assurance to determine the drug, concentration and its metabolite.

INTRODUCTION

For the study of bioavailability, bioequivalence (BE), pharmacokinetics (PK), quantitative evaluation of drugs, concentration and their metabolites, new drug development, basic biomedical and pharmaceutical sciences research, therapeutic drug monitoring, etc.,

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methods to determine the drugs in biological fluid are becoming more and more crucial [1,2]. Due to its excellent selectivity and dependability, high pressure liquid chromatography (HPLC) is one of the most often used analytical methods, particularly in the pharmaceutical, environmental, forensic, therapeutic, and food sectors [3]. Guidelines for the validation of bioanalytical methods were generally proposed by the USFDA in 2001 and more recently by the EMEA. By using specialized laboratory investigations, validation entails documenting that a method's performance characteristics are appropriate and trustworthy for the intended bioanalytical applications. The validity of the procedure is related to how well the analytical data are accepted. The bioanalytical techniques might be thoroughly verified for significant investigations that call for regulatory action for approval, such as BE or PK studies. Less validation may be necessary for advance techniques used for the sponsor's internal decision-making, whereas more validation may be required when improvements are made to a previously validated method [4,5]. Many changes are frequently made; these changes should be evaluated to ensure the analytical technique is performing as intended. Evolutionary shifts need to back certain research for the various stages of validation to show the method's viability. Validation of bioanalytical techniques was done:

- During development and implementation of a novel bioanalytical method.
- For analysis of a new drug entity.
- For revisions to an existing method that add metabolite quantification [6].
- Bioanalytical method transfers between laboratories or analysts.
- Change in analytical methodology.
- Change in matrix within species (e.g., human plasma to human urine).
- Change in sample processing procedures [7].

METHOD DEVELOPMENT

The process of creating a procedure to identify and quantify a novel or unknown component in a matrix is known as developing a bioanalytical method. The chemical characteristics of the analyte, concentrations, sample matrix, cost of the analysis method and tools, speed and time of the analysis, quantitative or qualitative measurement, precision, and required equipment all play a role in the choice of analytical method when determining how to measure a compound. Sample preparation, sampling, separation, detection, assessment of the findings, and ultimately conclusion are all included in method development [8].

Sample collection and preparation

The living media that contain the analyte are usually blood, plasma, urine, serum, etc. Blood is usually collected from human volunteers/ subjects by vein puncture with a hypodermic syringe up to 5-7 ml. The venous blood is withdrawn into tubes with an anticoagulant, generally ethylenediaminetetraacetic acid, heparin is used. Plasma is obtained by centrifugation at 4000 rpm for 15 minutes. Around 30-50% of the volume is collected. The aim of sample preparation is to clean up the sample before analysis. Material in biological samples that can affect with analysis, the chromatographic column or the detector includes endogenous macromolecules, proteins, salts, small molecules, and metabolic by products. The sample preparation is also to conversation the analyte from the biological matrix into a solvent suitable for instillation into the chromatographic system. General methods for sample preparation such as liquid/liquid extraction, solid-phase extraction (SPE) and protein precipitation, chromatography, and ligand binding assay (LBA) [9,10].

BIOANALYTICAL METHOD

Some of the following bioanalytical method:

- Extraction method
- Protein precipitation
- Chromatography method



- Ligand binding assay (LBA).

Extraction method

Liquid-liquid extraction It is founded on the theories of differential solubility and analyte molecule partitioning equilibrium between aqueous (the sample) and organic phases. The process of extracting a material from one liquid phase into another liquid phase is known as liquid-liquid extraction [11]. Nowadays, enhanced and more modern technologies such as single drop liquid phase micro extraction, supported membrane extraction, and liquid phase micro extraction are used instead of liquid extraction [12].

SPE SPE is a selective sample preparation technique that involves binding the analyte to a solid support, washing off the interferences, and then selectively eluting the analyte. Despite the variety of sorbent options, SPE is a very effective method. In the solid phase, there are four steps: conditioning, sample loading, washing, and elution.

- I. **Conditioning:** An organic solvent that also serves as a wetting agent for the packing material and solvates the functional groups of the sorbent is used to activate the column. In order to properly activate the column for the adsorption processes, water or aqueous buffer is introduced.
- II. **Sample loading:** The sample is fed into the column by gravity, pumping, or vacuum aspiration following pH correction.
- III. **Washing:** Interferences from the matrix are removed while retaining the analyte.
- IV. **Elution:** Distribution of analyte-sorbent interactions using an appropriate solvent, removing the fewest probable interferences. Typically, silica gel with a pore size of 60 Å is employed as the sorbent in SPE processes. Functional groups are chemically linked to this silica gel. The most popular type is a syringe

barrel, also known as a packed column, that has a 20 m frit at the bottom of the syringe with the sorbent material and another frit on top. Disks for extraction are put in syringe barrels. These disks are made up of 8–12 m packing material particles that have been bonded to an inert matrix. Similar to packed columns, disks are utilized and condition in the same manner. When opposed to packed columns, disks have the significant benefit of simply implementing larger flow rates. Analytes may be divided into four groups: chemicals that are acidic, basic, neutral, and amphoteric. Amphoteric analytes can act as cations, anions, or zwitterions depending on pH, namely the pH range of 13 to 15 [13-15]. They have both acidic and basic functional groups.

Protein precipitation

In routine analysis, protein precipitation is frequently used to eliminate proteins. A salt, an organic modernizer, or a change in pH can all cause precipitation by affecting the solubility of the proteins. After centrifuging the samples, the supernatant can either be added to the HPLC system or dried off and then dissolved in a suitable solvent. After then, the sample is concentrated. Precipitation method cleaning techniques have several advantages than SPE [16]. Little organic modifier or other solvent is employed, and it takes less time. However, there are drawbacks as well. Since it is a non-selective sample cleansing technique and samples frequently contain protein particles, there is a chance that endogenous substances or other medications may inhibit the reversed phase HPLC system. To generate clean extract, however, the protein precipitation method is frequently used with SPE. Among the organic solvents, methanol is typically used since it may yield a clear supernatant that is suitable for immediate addition to HPLC. Another option to



acid organic solvent precipitation is salts. Precipitation caused by salt is the name of this method. Proteins clump together and precipitate out of a solution when the salt concentration rises [17,18].

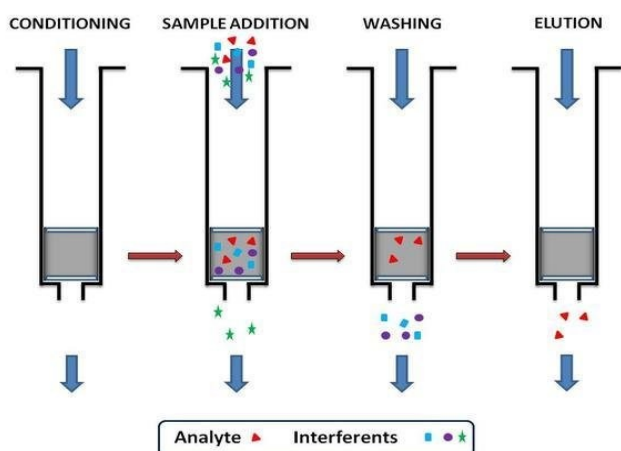


Fig. 1: Steps in solid phase extraction

Chromatographic method

Reference standards

Utilizing calibration standards and quality control samples (QCs) spiked with reference standards, medicines and their metabolites are analyzed in biological fluids. Study results may be impacted by the reference standard's purity, which was utilized to create spiked samples. For this reason, solutions with known concentrations must be prepared using authenticated analytical reference standards with recognized identities and levels of purity. The reference standard should, wherever feasible, match the analyte exactly. In the event that this is not feasible, a predictable chemical form (free base or acid, salt, or ester) with known purity may be utilized [12].

Three types of reference standards are usually used:

- Certified reference standards (e.g., USP compendial standards).
- Commercially supplied reference standards obtained from a well know and reputed material source.

- Other materials of documented purity custom-synthesized by a chemical laboratory or other non-commercial firms.

For every reference and internal standard (IS) used, the source, expiry date, lot number, documentations of analysis, and/or externally or internally created evidence of identity and purity should be disclosed. Stock solutions created with this amount of standard should not be utilized if the reference or IS has expired until purity has been restored [19,20].

LBA

Numerous of the ideas and metrics stated above for bioanalytical validation also apply to microbiological and LBA. These assays come in a range of design configurations and have certain distinctive characteristics that should be considered during technique validation.

Key reagents

Important reagents such reference standards, antibodies, tracers, and matrices need to be properly described and kept in predetermined environments. When critical reagents change, the assay may need to be reoptimized or validated.

For instance: Analytes (tracers) with labels: Binding has to be re-optimized, and performance needs to be tested using QCs and standard curves. Antibodies: It is important to look for cross-reactivates. Repeated the above tracer experiments.

Matrices: Repeat the above tracer experiments [18].

BIOANALYTICAL METHOD VALIDATION

Need of bioanalytical method validation

- To provide accurate data that can be satisfactorily understood, it is crucial to adopt bioanalytical procedures that have been well described and verified.
- It is acknowledged that bioanalytical methods and procedures are at the leading edge of technology and are continually undergoing modifications and advancements.

- It's also crucial to stress that each bioanalytical method has unique qualities that depend on the analyte being used, thus each analyte-specific validation standard must be created.
- In addition, the study's final goal may have an impact on whether the approach is acceptable. In order to achieve inter-laboratory reliability when samples analysis for a specific research is carried out at many locations, it is required to evaluate the bioanalytical procedures at each location and supply the relevant validation information for various locations [21].

Linearity and range

The correlation between the response and the analyte's known concentration is called a calibration curve. Every analyte should have its own calibration curve, which should be created in the same biological matrix as the samples. The concentration range for which the technique has been verified in terms of accuracy, precision, and linearity is known as the range. The most basic model that accurately captures the concentration-response relationship should be utilized as the calibration curve. The variation from the nominal concentration of the lower limit of quantification (LLOQ) should not be more than 20%, and it shouldn't be more than 15% from the other standards in the curve.

Accuracy

An analytical method's accuracy refers to how closely test results produced using the method match the analyte's actual value. Replicate analyses of samples having known levels of the analyte are used to assess accuracy. For each concentration, accuracy should be evaluated using a minimum of five determinations. At least three concentrations within the anticipated range. Except at LLOQ, when it should not differ by more than 20%, the mean value should be within 15% of

the actual value. The accuracy is measured by the mean's departure from the real value. [22,23].

Bias

ISO defines bias as the discrepancy between the expected outcome of a test and a generally recognized reference value. It could include many systematic error components. The percentage departure from the recognized reference value can be used to quantify bias. The word "trueness" describes how much a broad series of measurements' mean values deviate from the established reference value. It can be characterized as prejudice. Trueness is typically not assessed during method validation but rather from the outcomes of a large number of QCs during ordinary application due to the high burden of evaluating such huge series. [24].

Precision

When the technique is conducted repeatedly to several aliquots of a single homogenous volume of biological matrix, the precision of an analytical method refers to the accuracy of individual measurements of an analyte. For each concentration, a minimum of five determinations should be used to quantify precision. It is necessary to have a minimum of three concentrations within the predicted concentration range. Except for the LLOQ, where it should not exceed 20% of the CV, the precision calculated at each concentration level should not be more precise than 15% of the coefficient of variation (CV). The measurement of repeatability, which assesses precision across time and may involve several analysts, equipment, reagents, and laboratories, further divides precision into three categories: interday, intraday, and distinct analyst. [25].

Intermediate precision

Intermediate precision describes variability within laboratories, such as various days, analysts, equipment, etc. [17] The M-factor, which represents the amount of variables (operator,

equipment, or time) that change between subsequent determinations, is used in the ISO definition's phrase "M-factor varied intermediate precision." Between-run, between-day, or inter-assay precision are other names for intermediate precision [8].

Selectivity

To determine the bioanalytical method's capacity to separate and quantify the analyte in the presence of other ingredients in the sample, a selectivity exercise is carried out. For selectivity, it is recommended to perform studies on blank samples of the relevant biological matrix (plasma, urine, or another matrix) that were obtained from at least six different sources. Selectivity should be guaranteed at the lower LOQ (LLOQ), and each blank sample should be checked for interference [7].

Limit of detection (LOD)

The LOD is a property that only applies to limit tests. Under the specified experimental circumstances, it is the smallest quantity of analyte in a sample that can be detected but not always measured. Common ways to express the detection are as a percentage, parts per million, or parts per billion.

LOQ

The LLOQ is the minimal quantity of analyte that can be quantitated with acceptable accuracy and precision. The most practical technique, which determines LLOQ based on accuracy and precision, defines LLOQ as the lowest concentration of the sample that can still be measured with acceptable accuracy and precision. If baseline noise is present, as in chromatographic procedures, LLOQ based on the signal to noise ratio can only be used [26].

Recovery

The difference between the detector response received from the real concentration of the pure genuine standard and the detector response obtained from the quantity of the analyte introduced to and extracted from the biological

fluids is known as the recovery of an analyte assay. Although a recovery of an analyte and of the internal standard should be exact, constant, and repeatable, a recovery of an analyte is not required to be 100%. Recovery tests should be done by contrasting the results for extracted samples at the three lowest concentrations (low, medium, and high) with unextracted standards, which reflect 100% recovery [2].

Robustness

The robustness of an analytical technique, as defined by the ICH standards, is a measure of its ability to be unaffected by little but intentional changes in method parameters and offers a clue as to its dependability under typical conditions. The capacity to replicate an analytical or bioanalytical procedure in diverse labs or environments without the development of unanticipated changes in the acquired result is referred to as robustness.

Ruggedness

Ruggedness is a metric measuring how sensitive a technique is to subtle changes that might happen during normal analysis, such as tiny variations in pH values, the make-up of the mobile phase, temperature, etc. Ruggedness testing may be highly beneficial during the method development/prevalidation phase, as issues that may arise during validation are frequently identified in advance, although it is not required for full validation. If a method is going to be moved to another lab, its toughness has to be assessed [27,28].

Stability

During the method validation process, it is also important to look at the analyte's stability under varied circumstances. Stability tests should be conducted in circumstances that are similar to those that would really be present while handling and analysing actual samples. FDA lists the following stability conditions as ones that should be looked into [29];

stable stock solutions



It is necessary to assess the stock solution's stability throughout the course of six hours at room temperature.

temperature stability immediately

It is important to assess the analyte's stability in biological fluids at room temperature. Three aliquots of each concentration, low and high, were maintained for at least 24 hours before being examined.

stable temperature throughout time

The analyte in the matrix should remain stable from the moment of sample collection until the last day of analysis.

thaw-freeze stability

Three freeze-thaw cycles should be completed before determining the stability of the analyte. Three aliquots of each concentration, low and high, should be kept frozen for 24 hours before being defrosted at room temperature.

Afterwards, stability

It is important to assess the analyte's stability throughout the analytical procedure' phases [30,31].

Application of validated method for the drug analysis

When stability data are available, all the samples of an analyte in a biological fluid should be tested within specified time frame. If the assay technique has adequate acceptable variability as given by validation data, biological samples may often be evaluated with a single result without duplicate or replication analysis [32]. This is valid for processes where precision, accuracy, and variances routinely fall within acceptable bounds. Duplicate or even triple analyses may be carried out for a more accurate estimation of the analyte in a challenging operation involving a labile analyte where high precision and accuracy standards may be challenging to meet.

The following recommendations should be noted in performing a bioanalytical method to routine drug analysis.

- A minimum of six to nine standard points, excluding blanks (either single or duplicate), encompassing the whole range, should be included in a matrix-based standard curve.
- Response function: Typically, the standard curve throughout the research would employ the same curve fitting, weighting, and goodness of fit established during pre-study validation. The proper statistical tests are used to define the response function based on the actual standard points throughout each run of the validation. Numerous issues are indicated by changes in the connection between the response function and pre-study validation and regular run validation [33].
- The run must be accepted or rejected using the QC samples. These QC samples have analyte matrix spikes [24].
- System suitability: Based on the analyte and technique, a specific standard operating procedure (or sample) must be identified to ensure optimum operation of the system used.
- Any required sample dilutions should use like matrix (e.g., human to human) obviating the need to incorporate actual within-study dilution matrix in Quality control samples.
- Repeat analysis: It's hard to create acceptance criteria and a standard operating procedure (SOP) for repetitive analysis. The justifications for repeating sample analysis are explained in this SOP or guideline. Repeat analyses of clinical or preclinical samples for regulatory purposes may be necessary for a variety of reasons, such as inconsistent replication analyses, samples that fall beyond the assay limit, sample processing mistakes, equipment malfunctions, subpar chromatography, and conflicting PK data. If the sample volume permits, the retest must be carried out in triplicate. It is important to have a precise



record of the repeat analysis's foundation and its reporting.

Reintegrating sample data: A standard operating procedure (SOP) or set of guidelines should be created. This SOP or guideline should outline the rationale for and specifics of the reintegration process. The justification for the reintegration should be spelled out in detail and supported by evidence. It is important to record both original and reintegration data [34].

CONCLUSION

Drug discovery and development processes in the pharmaceutical industry depend heavily on bioanalysis and the generation of pharmacokinetic, toxicokinetic, and metabolic data. From the perspective of the quality assurance department, an effort has been made to comprehend and explain the development and validation of bioanalytical methods. This article reports on some of the methods and how validation is carried out in various scenarios found in the analysis of the research sample. In order to raise the bar and increase acceptability in this field of study, the different crucial development and validation features for bioanalytical methods have been explored.

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