IJARSCT
ISSN (Online) 2581-9429
International Journal of Advanced Research in Science, Communication and Technology (IJARSCT)
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
Impact Factor: 7.301
Volume 3, Issue 3, January 2023

A Review of the Development and Validation of Bioanalytical Methods

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Abstract: *This review article often uses bioanalytical techniques to assess pharmaceuticals and their metabolites in plasma matrices; these approaches must be used in clinical research including both humans and nonhuman subjects. An essential stage in the estimate and interpretation of bioequivalence, pharmacokinetic, and toxicokinetic studies is the use of the bioanalytical technique for the quantitative evaluation of medicines and their metabolites in biological medium. The three main bioanalytic tasks are method development, method validation, and sample analysis. To determine how much the environment, the matrix, or procedural factors might affect the estimate of analyte in the matrix from the time of set up to the time of analysis, each step of the process must be examined. Techniques for bioanalyzing pharmaceuticals in the body include high pressure liquid chromatography (HPLC) and liquid chromatography combined with double mass spectrometry (LCMS-MS). Each instrument has certain advantages and disadvantages. For the bioanalysis of both small and large compounds, gas chromatography and LC/MS/MS have been the most used chromatographic techniques. A few often used metrics include linearity, accuracy, precision, selectivity, sensitivity, repeatability, and stability. It is suggested that we include some information on the creation and verification of bioanalytical procedures in this review research. These details will help with quality control since they allow us to identify the medicine, its concentration, and its metabolite.*

Keywords: Clinical and nonclinical study, Method development, Validation parameter.

I. INTRODUCTION

It is becoming more important to use technology to evaluate medications in biological fluid for the research of bioavailability and bioequivalence. Basic biomedical and pharmaceutical science research, pharmacokinetics studies, quantitative drug evaluation, pharmaceutical concentration and metabolite analysis, development of new drugs, therapeutic drug monitoring, etc. One of the most widely used analytical techniques is high pressure liquid chromatography, especially in the pharmaceutical, environmental, forensic, medicinal, and food industries due to its exceptional selectivity and outstanding reliability.

In 2001, the USFDA and, more recently, the EMEA, developed generic guidelines for the validation of bioanalytical procedures. Validation comprises proving that a method's performance characteristics are suitable and reliable for the planned bioanalytical applications via specialized laboratory research. To what extent the analytical data are accepted determines the legitimacy of the technique. For important investigations, such BE or PK studies, that need regulatory action for approval, the bioanalytical methodologies may be fully evaluated. It could be necessary to conduct fresh validation when a previously validated technique is altered. Less validation, meanwhile, can be sufficient for advanced procedures utilized in the sponsor's internal decision-making. It is important to assess the many modifications that are often made to make sure the analytical method is operating as planned. In order to demonstrate the method's feasibility at different levels of validation, evolutionary changes must support specific study. Techniques for bioanalytical validation were carried out:

- During the development and use of a new bioanalytical technique.
- To analyze a brand-new drug entity.
- To add metabolite quantification to an existing technique via changes.
- Transfers of bioanalytical techniques between labs or analysts.

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• Modification of the analytical process.
• Matrix change within a species.
• Modifications to sample processing techniques.

II. METHOD DEVELOPMENT

The process of creating a technique to identify and quantify a new 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 eventually conclusion are all included in the creation of a method.

Sample collection and preparation

The analyte is often present in living media such as blood, plasma, urine, serum, etc. Human volunteers or subjects are typically bled by having a vein punctured and having a hypodermic syringe filled with 5-7 ml of blood. The venous blood is collected into tubes containing an anticoagulant; often, heparin is used together with ethylenediaminetetraacetic acid. Centrifugation at 4000 rpm for 15 minutes is used to produce plasma. 30 to 50 percent of the volume is gathered. Cleaning up the sample before analysis is the goal of sample preparation. Endogenous macromolecules, proteins, salts, tiny molecules, and metabolic byproducts are among the substances in biological samples that might have an impact on analysis, the chromatographic column, or the detector. As part of the sample preparation, the analyte from the biological matrix is also transferred into a solvent that may be injected into the chromatographic apparatus. Typical techniques for sample preparation include chromatography, protein precipitation, solid-phase extraction, liquid/liquid extraction, and ligand binding test.

III. 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 based on the concepts of differential solubility and analyte molecule partitioning equilibrium between aqueous and organic phases. The process of extracting a chemical from one liquid phase into another liquid phase is known as liquid-liquid extraction. Modern technologies such as liquid phase micro extraction, supported membrane extraction, and single drop liquid phase micro extraction have supplanted traditional methods of liquid extraction.

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 highly 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. To activate the column for appropriate adsorption processes, water or aqueous buffer is supplied.

II. Sample loading

Following pH correction, the sample is fed into the column by gravity, pump, or vacuum aspiration.

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III. Washing

The analyte is kept while matrix interferences are eliminated.

IV. Elution

Distribution of analyte - sorbent interactions with an appropriate solvent, eliminating as few interferences as feasible. Typically, silica gel with a 40 m diameter and 60 A0pore sizes is employed as the sorbent in SPE. 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. Since amphoteric analytes include both acidic and basic functional groups, they may act as cations, anions, or zwitterions depending on the pH, which is typically 13.

Protein precipitation

In routine analysis, protein precipitation is often used to eliminate proteins. A salt, an organic modernizer, or a change in pH may all cause precipitation by affecting the solubility of the proteins.

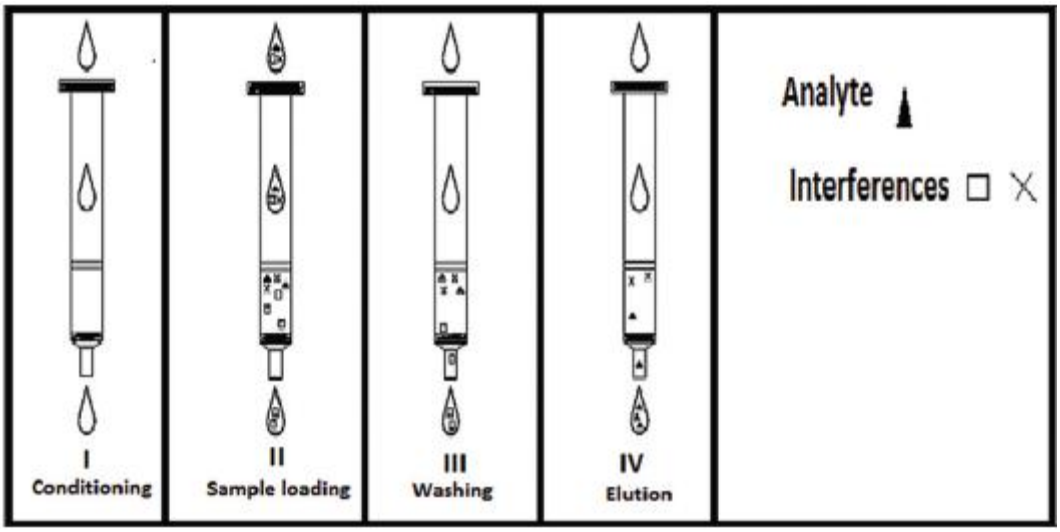

Fig. 1: Steps in solid phase extraction

After centrifuging the samples, the supernatant may either be added to the HPLC system or dried off and then dissolved in a suitable solvent. After then, the sample is concentrated. Precipitation approach as a clean-up technique has several advantages over SPE. 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 often include 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 often used with SPE. Among the organic solvents, methanol is often used since it may provide 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 gather and precipitate from a solution when the amount of salt in the solution rises.

Chromatographic method

Reference standards

Utilizing calibration standards and quality control samples laced with reference standards, medicines and their metabolites are analyzed in bodily 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 with known purity may be utilized.

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Commonly used reference standards include three categories:

• Certified benchmarks for comparison.

• Reference standards provided by industry, received from a reliable industry source.

• Other materials with proven purity that have been specially created by an analytical laboratory or other nonprofit organization.

For each reference and internal standard (IS) utilized, the source, expiry date, lot number, documentations of analyses, and/or internally or externally created proof of identity and purity should be disclosed. Stock solutions prepared with this lot of standard should not be used if the reference or IS has expired unless purity has been restored.

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 with particular characteristics that should be taken into account 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 example:

Labeled analytes (tracers): Binding has to be re-optimized, and performance needs to be tested using QCs and standard curves.

Antibodies: Checking key cross-reactivates is necessary. Repeated the aforementioned tracer experiments.

Matrices: Repeated the aforementioned tracer experiments.

IV. 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 crucial to note that each bioanalytical approach has unique properties that depend on the analyte being used. Accordingly, unique validation standards may need to be created for each analyte.

• In addition, the study's final goal may have an impact on whether the approach is acceptable. It is vital to verify the bioanalytical procedures at each site and provide the proper validation information for several locations when samples analysis for a specific research is carried out at more than one location in order to establish inter-laboratory reliability.

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 shouldn't be more than 20% from the lower limit of quantification's nominal concentration and shouldn't be more than 15% from the curve's other standards.

Accuracy

An analytical method's accuracy refers to how closely test results produced using the technique 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. Accuracy is determined by how much the mean deviates from the real value.

Bias

ISO defines bias as the discrepancy between the expected outcome of a test and a generally recognized reference value. It could have many systematic error components. The percentage departure from the recognized reference value may be used to quantify bias. The word "trueness" describes how much a broad series of measurements' mean values deviate

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from the established reference value. It may be characterized as prejudice. Trueness is often 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 big series.

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. The additional division of precision is into intraday, between days, and different analyst or repeatability. This repeatability measure is carried out, which assesses accuracy across time and may include several analysts, equipment, reagents, and labs.

Intermediate precision

Intermediate precision expresses within-laboratories variations:

The phrase "M-factor different intermediate precision," which refers to the number of factors that vary between subsequent determinations, is used in the ISO standard. Between-run, between-day, and inter-assay precision are other names for intermediate precision.

Selectivity

To determine the bioanalytical method's capacity to distinguish and quantify the analyte in the presence of other components in the sample, a selectivity exercise is performed. For selectivity, it is recommended to conduct studies on blank samples of the relevant biological matrix (plasma, urine, or another matrix) that were collected from at least six different sources. Selectivity at the lower LOQ should be guaranteed, and each blank sample should be checked for interference.

Limit of detection

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. Typically, the detection is given as a percentage, a part in a million, or a part in a 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. Only baseline noise, such as that found in chromatographic procedures, allows for the use of LLOQ based on the signal to noise ratio.

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 IS should be exact, constant, and repeatable, a recovery of an analyte is not required to be 100%. Recovery tests should be carried out by comparing the outcomes for the three lowest concentrations of extracted samples (low, medium, and high) with unextracted standards that reflect 100% recovery.

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, since issues that may arise during

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validation are often identified in advance, although it is not required for full validation. If a technique is intended to be transferred to another laboratory, ruggedness should be assessed.

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 analyzing actual samples. FDA lists the following stability conditions as ones that should be looked at.

Stock solution stability

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

Short-term temperature stability

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.

Long-term temperature stability

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

Freeze and thaw 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.

Post-preparative stability

It is important to assess the analyte's stability across the various phases of the analytical procedure.

Application of validated method for routine drug analysis

When stability data are available, all samples of an analyte in a biological matrix should be tested within that time frame. In general, if the assay technique has adequate acceptable variability as indicated by validation data, biological samples may be evaluated with a single result without duplicate or replication analysis. This is valid for processes where precision, accuracy, and variances frequently fall within acceptable bounds. Duplicate or even triple analyses may be carried out for a better estimate of analyte in a challenging method with a labile analyte where high precision and accuracy criteria may be challenging to attain.

When using a bioanalytical approach for standard drug analysis, keep in mind the following advice.

• A minimum of six to nine standard points, excluding blanks (either single or double), 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 response function connection between pre-study validation and regular run validation.

• The run must be accepted or rejected using the QC samples. These QC samples have analyte matrix spikes.

• **System suitability:** To achieve the best performance of the used system, a particular standard operating procedure must be defined based on the analyte and technology.

• Any necessary sample dilutions should be performed using a similar matrix (e.g., human to human), eliminating the requirement for QC samples to include real within-study dilution matrix.

• **Repeat analysis:** It's crucial to create acceptance criteria and a standard operating procedure (SOP) for repeat 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.

• **Sample data reintegration:** A standard operating procedure 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. Reporting of both original and reintegration data is required.

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V. 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. These numerous crucial aspects of bioanalytical technique creation and validation have been explored in order to raise the bar and increase acceptability of this field of study.

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