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A REVIEW ON "BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION"

P. Sahithi, Ch. Prasad*, Y. Srinivasa Rao, K. Vara Prasada Rao, R. Deepthi, P. Srinu

Vignan Institute of Pharmaceutical Technology, Duvvada, 530049, Visakhapatnam, Andhra Pradesh, India

***Corresponding author E-mail:deepthi.pharma7@gmail.com**

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ABSTRACT

In this review article, bio analytical methods are generally used to quantitate drugs and their metabolites in the plasma matrices and the methods should be applied to studies in human clinical and non-human study. Bioanalytical development is the process to create a procedure to enable a compound of interest to be identified and quantified in a biological matrix. A compound can be measured by several methods. Analysis of drugs and their metabolites in a biological matrix is carried out by using different extraction techniques like liquid-liquid extraction, solid-phase extraction, and protein precipitation. From these extraction methods samples are spiked with calibration (reference) standards and using method describes the process of method development and including sampling, sample preparation, separation, detection and evaluation of the results. Then developed process is validated. Techniques such as high pressure liquid chromatography (HPLC) and liquid chromatography coupled with double mass spectrometry (LC-MS-Ms) can be used for the bioanalysis of drugs in body. Each of the instruments has its own merits and demerits. These bioanalytical validations play an important role in evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic and toxicokinetic studies. Linearity, accuracy, precession, selectivity, sensitivity, reproducibility and stability are some of the parameters regularly used. In this review article, we are proposed to add some points regarding bio analytical method development and validation parameters, which are beneficial to quality assurance to determine the drug concentration and its metabolite.

INTRODUCTION

Bioanalytical methods development and validation play an important role in the discovery, development and manufacture of pharmaceuticals [(1)]. A bio analytical method is a set of procedures involved in the collection, processing, storage, and analysis of a biological matrix for a chemical compound[(2)].Method development and validation has a great importance in the field of pharmacokinetics (PK), bioavailability (BA), and bioequivalence (BE) studies for new drugs approval and investigation by regulatory bodies [(3-5)]. High Pressure liquid chromatography (HPLC) most widely applied analytical techniques because of its highly selective and high reliability, especially in the pharmaceuticals, environmental, forensic, clinical and food departments[(6)]. Recent progress in methods development has been largely a result of improvement in analytical instrumentation. This is true especially for chromatographic and detectors. Isocratic and gradient high performance liquid chromatography (HPLC) have evolved as the primary techniques for the

analysis of non-volatile active pharmaceutical ingredients and impurities. The emphasis on the identification of analytes and impurities has led to the increased use of hyphenated technique such as liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-nuclear magnetic resonance spectroscopy (LC-NMR). Bioanalytical method validation is the process used to establish that a quantitative analytical method is suitable for biochemical applications. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum or urine is reliable and reproducible for the intended use.Various agencies namely US food and drug administration (USFDA), American association of pharmaceuticals scientist (AAPS), health protection branch (HPB), association of analytical chemists (ADAC), centre for veterinary medicine (CVM), U.S. Department of health and human services food and drug administration, centre for drug evaluation & research (CDER), European medicine agency (EMA), china food and drug administration (CFDA), European bio analytical forum (EBF) global CRO council (GCC), ANVISA(brazil) had done collective efforts at different timings to regulate and harmonize bioanalytical method development and validation. In May 2018, U.S. Department of health and human services, food and drug administration, centre for drug evaluation and research (CDER) and centre for veterinary medicine (CVM) were published guidance for industry regarding bioanalytical method development and validation.[7]

2. Need of bio analytical method development and validation

- Sponsors are applying for investigational new drug application (IND), new drug application (NDA), abbreviated new drug application (ANDA) to FDA. To fulfil the formalities, they have to submit human clinical pharmacological, bioavailability (BA), and bioequivalence (BE) studies, requiring pharmacokinetic (PK) evaluation including non-human pharmacology and toxicology studies and preclinical studies, for this purpose there is a need to develop and validate bioanalytical method. Generally, for industrial use, thebioanalytical methods are developed in biological matrices such as blood, serum, plasma, or urine. [8]
- It is essential to used well-characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactory interrupted.
- It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements; they are at the cutting edge of the technology.
- It is also important to emphasize that each bioanalyticaltechnique has its own characteristics, which will vary from analyte to analyte, specific validation criteria need to develop for each analyte.
- Moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study is conducted at more than one site, it is necessary to validate the bioanalytical methods at each site and provide appropriate validation information for different sites to establish inter-laboratory reliability. [9]

3. Instruments used for bio analytical method development and validation

Gas chromatography, high pressure liquid chromatography, LC and GC, combined with mass spectrometric (MS) procedures such as LC-MS, LC-MS-MS, GC-MS, and GC-MS-MS are used for quantitative analysis. For the quantification of conventional, low molecular weight drugs in biological fluids has shifted dramatically in favour of mass spectrometry – based methods, particularly LC-MS and LC-MS-MS. In the year of 90's there have been tremendous advancements in the field of mass spectrometry with the development of new interfaces, ionization and detection techniques. These advancements resulted in the rapid emergence and wide spread commercial use of hyphenated mass spectrometry-based assays, which have largely replaced conventional HPLC, GC and GC-MS assays.

4. Bio analytical Method development

Bioanalytical method development is the process of making a procedure to unknown

compound or novel compound to be identified and measured in a matrix. A compound can often be measured by several methods and the choice of analytical method involves, that is chemical properties of an analyte, concentration, samples matrix, cost of the analysis method and instruments, speed and time of analysis, quantitative or qualitative measurements, precision and accuracy. Method development includes sample preparation, sampling, separation, detection and evaluation of the results and finally conclusion. [10]In the development stage, decisions regarding choice of column, mobile phase, detectors and method of quantification must be considered. There are several valid reasons for developing new methods of analysis.

- There may not be a suitable method for analyte in the specific sample matrix.
- Presently existing method may have few errors or contamination prone.
- Presently existing method may be too expensive, time consuming.
- Presently existing method may not provide adequate sensitivity. [11]

Steps involved in method development:

Method development is complex process that involves a number of steps, which are as follows:

1. Method selection and information of sample.
2. Selection of initial method conditions.
3. Checking the analytical method in aqueous standards.
4. Development and optimization of sample processing method.
5. Checking the analytical method in biological matrix.
6. Pre-validation.

1. Method selection and information of sample:

Literature survey shall be conducted to have information on drug profile and its pharmacokinetic properties. For the development of analytical method collection of physico-chemical properties of the analytes and the related compounds are essential. Based on the Analyte physico-chemical properties such as molecular size, structure, functional groups, polarity, partition coefficient, solubility, dissociation constant etc.

2. Selection of initial method conditions:

The initial method conditions include diluent selection based on the solubility of the drug, drug metabolites and internal standard and compatibility with analytical method. During this phase lowest concentration to be quantified shall be assessed using aqueous solutions. Run time and resolution between the peaks should be taken care during this phase.

3. Checking the analytical method in aqueous standards:

Before going to analyse a method in biological matrix, first check the analytical method in aqueous standards. Aqueous calibration curve standards are prepared initially, at least with four concentrations, including the highest and lowest. Concentration of the highest standards shall be based on C_{max} and preliminary studies. Each concentration is injected for plotting calibration curve standards and the correlation coefficient should be plotted. Correlation co-efficient (r^2) should not be less than 0.999.

4. Development and optimization of sample processing method:

When the instrumental method is concluded with aqueous standards, same experiment is carried on matrix sample. Based on the literature survey data on analyte and internal standards physic-chemical properties like structure, functional groups, pH, partition coefficient, dissociation constant, polarity and solubility, set and optimize the sample preparation technique like protein precipitation, liquid-liquid extraction and solid phase extraction. [12]

A. Liquid-liquid extraction (LLE):

Liquid-liquid extraction is useful for separation of analytes from interferences by partitioning the sample between two immiscible liquids or phases. Liquid-liquid

extraction generally involves the extraction of a substance from one liquid phase to additional liquid phase. [13]

B. Solid phase extraction (SPE):

Solid phase extraction is selective method for sample preparation where the analyte is bound onto a solid support, interferences are washed off and the analyte is selectively eluted. Due to many different choices of sorbents, solid phase extraction is a very powerful technique. [14]Solid phase consists of four steps: Conditioning, Sample loading, Washing, and Elution. [15-17]In SPE, multiple sampling generally 12-24 with a lower quantity of solvent with automation are major contributing factors. In SPE the recovery of the sample is higher, small disposable column or cartridges is employed for partitioning. The SPE consists of the medical syringe which is packed with 0.1-0.5g of sorbent generally C18 silica. Liquid samples are added to the cartridges and wash solvent is selected to their strongly retain or unretain the analyte. To minimize the presence of interferences, this method is advantageous. Even though analyte get retained on the cartridge, the interferences can be eluted or washed, that results in the analyte-free from interferences. Then the analyte is eluted with elution solvent and their directly injected in or evaporated to dryness followed by dilution with the HPLC mobile phase. [18-19]

C. Protein precipitation:

Protein precipitation is the one method to avoid interferences. This can be done by denaturation and precipitation. Trichloroacetic acid and per chloric acid has a more choice as precipitating agent. Various organic solvents like methanol, acetonitrile, acetone, and ethanol are widely used for removing plasma proteins, one part of sample matrix is diluted with three-fourth parts of the precipitating agent then vortex is carried out. After centrifugation, filtration is done to remove protein mass. The supernant liquid or filtrate is obtained which is directly analysed. For quantitative analysis, the supernatant can be isolated, evaporated to dryness and then reconstituted with suitable solvent before analysis [20].

5. Checking the analytical method in biological matrix

When drug sensitivity is more, protein precipitation is used and recovery, precision and interferences are checked. When drug sensitivity is less, liquid-liquid extraction is used and recovery, precision and interferences are checked. When recovery and reproducibility is less in liquid-liquid extraction then solid phase extraction is done and the optimized conditions were applied to biological matrix and the system suitability parameters are observed.

6. Pre-validation

In pre-validation accuracy, precision, recovery, stability, sensitivity/ specificity, ruggedness, linearity parameters are performed. During method development various parameters are to be optimized.

a) Mode of separation.
b) Selection of stationary phase.
c) Selection of mobile phase.

a) Mode of separation:

The nature of the analyte is the primary factor for selection of mode of separation. In reverse phase mode, the mobile phase is more polar than stationary phase. For separation of polar or moderately polar compounds, reverse phase mode is used.

b) Selection of stationary phase/column:

Column selection is the first and the important step in the method development. Column is the heart for the separation process [21].

Selection of column includes different approaches:

- Column dimensions
- Nature of packing material.
- Shape of the particles.
- Size of the particles.
- Surface area.
- Pore volume.
- End capping.

c) Selection of Mobile phase:

To achieve optimum separation, selection of mobile phase is the primary objective. The following parameters are to be considered

during selection and optimization of mobile phase [22].

- Buffer.
- pHof the buffer.
- Mobile phase composition.

5. Bio analytical Method validation:

Method validation is the process which is used to confirm the optimized bio analytical procedure. Results from the method validation can be used to check the quality, reliability and consistency of the bio analytical results [23-28]. In case if a new drug, its metabolites or biomarkers or any revision to existing method are determined, then full validation is necessary[29].

Three types of validations are:

a) Full validation.

b) Partial validation.

c) Cross validation.

a) Full Validation:

As per US-FDA guidelines, after developing new method for new drug entire step should be validated. It is very important for new drug and its metabolites.

b) Partial Validation:

Partial validation means modification of validated bio analytical method.

- Change in analytical method
- Transfer Bio analytical method between analysts or laboratory.
- Changing anticoagulant in harvesting biological fluid.
- Change matrix within species
- Change processing procedure.
- Change species within matrix.
- Change in instrument.

c) Cross Validation:

In Cross Validation, comparison between two bio analytical method validation parameters within the same study or different studies. Forexample, data generated by using different analytical technique like LC-MS-MS vs. ELISA in different studies. [30-33]

Steps of Bioanalytical validation

1) In development manual documents about company policy and requirements for validation are available.
2) Selection of analytical method and instrument depends on the aim of analysis.
3) In installation qualification documents about the instrument which meets the requirement of the system are available.
4) In operational qualification verification of the installed system is suitable for intended purpose. [34-36]

Validation Parameters:

According to FDA guidance following are the validation parameters:

1. Accuracy
2. Precision
3. Recovery
4. Selectivity
5. Reproducibility
6. Linearity
7. Limit of detection(LOD)
8. Limit of quantification(LOQ)
9. Calibration curve
10. Ruggedness
11. Robustness
12. Stability of the analyte in matrix.

1. Accuracy: Degree of closeness of the observed concentration to the nominal or known true concentration. It is measured as coefficient of variation (% CV) [37-38].

2. Precision: Measurement of scattering for concentrations which obtained from the replicate sampling of a homogenous sample. It is measured by coefficient of variation (%CV).

3. Recovery: The extraction efficiency of an analytical process is reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.

4. Selectivity: To measure and differentiate the analyte in the presence of the components that may be expected to be present.

5. Reproducibility: The ability of a method to yield similar concentration for a sample when measured.

6. Linearity: Test results which are obtained are directly proportional to the concentration of an analyte in the sample within the range of the standard curve.

7. Limit of Detection: The lowest amount of the analyte that can be detected but not quantified. [39]

8. Limit of Quantitation: The lowest concentration of an analyte in the sample that can be quantified and determined within acceptable precision and accuracy.

9. Calibration curve: Calibration curve or standard curve means the relationship between experimental response value and the analytical concentration.

10. Robustness: Robustness which is defined as the measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and which provides indication of its reliability during normal usage. Or Robustness means the ability to reproduce the method in different laboratories or under different circumstances without the occurrence of unexpected in obtained results and robustness test is an experimental set up to evaluate the robustness of the method.

11. Ruggedness: This includes different analysts, laboratories, columns, instrument, source of reagents, chemicals, solvents. It is the degree of reproducibility of the test results obtained by the analysis of the same samples under variety of normal test conditions. The ruggedness is the method by changing the experimental conditions such as, changing to another column of similar type and different operations in the same laboratory.

12. Stability: The chemical or physical stability of an analyte in a given matrix under specific conditions for given time intervals. Various stability parameters can be explained as below:

Stock solution stability: The stability of the stock solution should be evaluated at room temperature for 6 hrs.

Short-term temperature stability: The stability of analyte in biological fluid at ambient temperature should be evaluated. Three aliquots of low and high concentration kept at least 24 hours and then analysed.

Long-term temperature stability: The stability of the analyte in the matrix should beyond the time from sample collection until the last day of analysis.

Freeze and thaw stability: The stability of the analyte should be determined, after three and thaw cycles.Three aliquots of low and high concentration should be stored at intended temperature and after 24 hours thawed at room temperature.

Post-preparative stability: The stability of the analyte during the stage of process of analysis should be evaluated. [39-41]

Whole blood stability: The immediate spinning down of aliquot of whole blood containing drug which is taken immediately following preparation zero time followed by spinning down of another aliquot following stability period. The whole blood stability should be performed during method validation.

Dilution effects: The integrity of the dilution should be monitored during validation by QC samples above the ULQC with like matrix to bring to within quantitation range, if the method measures the diluted samples. There should be a proper demonstration of accuracy and precision of these diluted QC's.

CONCLUSION:

Bioanalysis and the production of pharmacokinetic, toxicokinetic, and metabolic data play an important role in pharmaceutical research. The quantitative estimation of drug and its metabolites in the biological fluids can be performed.Now a day, LC-MS-MS plays an important role in developing bioanalytical method. While developing the bioanalytical

method there should be complete clarity about nature of the analyte, that whether it is micro molecule or macromolecule. The objective of this paper is to review the sample preparation of the drug in biological matrix and to provide practical approaches for determining selectivity, specificity, lower limit of quantification, linearity, accuracy, precision, recovery, stability, ruggedness of the methods to support bioavailability, toxicokinetic, pharmacokinetic and bioequivalence studies. The aim has been made to understand and explain the development of bioanalytical method and its validation from the basic of point view.

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