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BIO ANALYTICAL METHOD DEVELOPMENT AND VALIDATION USING HPLC: A REVIEW

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ABSTRACT

The development of selective, sensitive and reliable bio analytical methods for the quantitative evaluation of drugs and their metabolites in biological matrices is crucial for the successful drug development. The data obtained from these methods is required in the pharmacokinetic and toxicokinetic studies of investigational new drug applications (INDs), new drug applications (NDAs) and abbreviated new drug applications (ANDAs). The results of animal toxicokinetic studies and of human clinical trials, including bioavailability and bioequivalence studies requiring pharmacokinetic evaluation are used to make critical decisions supporting the safety and efficacy of a drug. Therefore, it is of paramount importance that the developed bio analytical methods are well designed, adequately validated and documented to a satisfactory standard to apply in drug analysis in order to obtain reliable results. High-pressure liquid chromatography is a versatile analytical tool useful in identification and quantitative estimation of low concentration of drugs and metabolites in biological matrices. So it is advantageous to develop and validate bio analytical HPLC method for low dose drugs. This article reviews current progression HPLC based bio analytical method development and validation of different drugs. So, far drugs like Antimalarials, Omeprazole, Clofarabine, Palonosetron HCl, etc have been analyzed bio analytically.

Keywords: Bio analytical method development, HPLC, validation, documentation and application.

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1. INTRODUCTION

The field of bioanalysis has matured significantly from early studies in drug metabolism using many simple and advanced techniques, and in today's Bioanalyst is well equipped to deal with the modern challenges. A bioanalytical method is a set of procedures involved in

the collection, processing, storage, and analysis of a biological matrix for a chemical compound. Bioanalytical method validation (BMV) is the process used to establish that a quantitative analytical method is suitable for biochemical applications. Bioanlysis covers the quantitative measurement of Xenobiotics of drugs such as their metabolites, and biological molecules in unnatural locations or concentrations and Biotics like macromolecules, proteins, DNA, large molecule drugs, metabolites in biological systems. Bioanalysis is a progressive discipline for which the future holds many exciting opportunities to further improve sensitivity, specificity, accuracy, efficiency, assay throughput, data quality, data handling and processing, analysis cost and environmental impact. The main impact of bioanalysis in the pharmaceutical industry is to obtain a quantitative measure of the drug or its metabolites for the study of pharmacokinetics, toxicokinetics, bioequivalence and exposure-response like pharmacokinetic/ pharmacodynamic studies. The focus of bioanalysis in the pharmaceutical industry is to provide a quantitative measure of the active drug and/or its metabolite(s) for the purpose of pharmacokinetics, toxicokinetics, bioequivalence and exposure–response (pharmacokinetics/ pharmacodynamics studies). The reliability of analytical findings is a matter of great importance in forensic and clinical toxicology, as it is of course a prerequisite for correct interpretation of toxicological findings. Unreliable results might not only be contested in court, but could also lead to unjustified legal consequences for the defendant or to wrong treatment of the patient. In the last decade, similar discussions have been going on in the closely related field of pharmacokinetic (PK) studies for registration of pharmaceuticals[1-5].

As per Bioanalytical Method Validation (BMV) guidelines for industry, these guidelines are applied to bioanalytical methods that are used for the quantitative determination of drugs and their metabolites in biological matrices such as plasma, urine and preclinical studies [1]. Bioanalytical method validation includes all of the procedures that

demonstrate that a particular method developed and used for quantitative measurement of analytes in a given biological matrix is reliable and reproducible [2]. Validation of a bioanalytical method is the process by which it is established that the performance characteristics of the method meet the requirements for the intended bioanalytical application. These performance characteristics are expressed in terms of bioanalytical method validation parameters [3,4]. The fundamental bioanalytical method validation parameters include precision and accuracy, sensitivity,

1. Chromatographic methods HPLC(high performance liquid chromatography)

1.1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High performance liquid chromatography is a form of column chromatography used frequently in bio chemistry and analytical chromatographic packing material (stationary phase),a pump that moves the mobile phase(s) through the column, and a detector that shows the retention time of the molecules, retention time varies depending on the interactions between the stationary phase, the molecules being analyzed and the solvent(s) used. Bionalytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a matrix. By using biological products can be measured by several methods and the choice of bioanalytical method involves several considerations of quantitative or qualitative measurement, and precision are required with necessary equipment. The bioanalytical chain describes the process of method development by biological samples includes sampling, sample preparation, separation, detection and evaluation of the results.[3]

2.1.1. Some General procedures for sample preparation are

Liquid/Liquid Extraction

Solid-Phase Extraction (SPE) and Protein Precipitation.

2.1.2. Liquid – Liquid extraction

It is based on the principles of differential solubility and partitioning equilibrium of analyte molecules between aqueous (the original sample) and the organic phases. Now a day's Liquid-Liquid extraction has been replaced with advanced and improved techniques like liquid phase micro extraction, single drop liquid phase micro extraction and supported membrane extraction. Liquid – Liquid extraction generally involves the extraction of a substance from one liquid phase to another liquid phase [4].

2.1.3. 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 different sorbents, solid phase extraction is a very powerful technique. Further Solid phase consists of four steps they are; conditioning, sample loading, washing and elution.

2.1.4. Protein Precipitation

Protein precipitation is often used in routine analysis to remove proteins. Precipitation can be induced by the addition of an organic modifier, a salt or by changing the Ph which influence the solubility of the proteins [15]. The samples are centrifuged and the supernatant can be injected into the HPLC system or be evaporated to dryness and thereafter dissolved in a suitable solvent. A concentration of the sample is then achieved. However, the protein precipitation technique is often combined with SPE to produce clean extract. Salts are other alternative to acid organic solvent precipitation. This technique is called as salt induced precipitation. As the salt concentration of a solution is increased, proteins aggregate and precipitate from the solution [6].

2.1.5.Conditioning

The column is activated with an organic solvent that acts as a wetting agent on the packing

material and solvates the functional groups of the sorbent. Water or aqueous buffer is added to activate the column for proper adsorption mechanisms.

2.1.6. Sample Loading and elution

Distribution of analyte–sorbent interactions by appropriate solvent, removing as little of the remaining interferences as possible**.** Typically, sorbents used in SPE consists of 40µm diameter silica gel with approximately 60 A0 pore diameters. The most commonly used format is a syringe barrel that contains a 20µm frit at the bottom of the syringe with the sorbent material and another frit on top, referred to as packed columns. Analytes can be classified into four categories; basic, acid, neutral and amphoteric compounds. Amphoteric analytes have both basic and acid functional groups and can therefore functions as cations, anions or zwitterions, depending on pH [7].

Figure.1-HPLC Instrument [8]

Bioanalytical methods development and validation. High performance liquid chromatography (HPLC) analysis is useful in identification and quantitative determination of drugs and metabolites in biological fluids, particularly plasma, serum or urine. HPLC requires good selections of detectors, good stationary phase, eluents and adequate program during separation. UV/VIS detector is the most versatile detector used in HPLC. UV detection is preferred as it provides excellent linearity and rapid quantitative analysis against a single standard of the drug being determined. The review focuses on bioanalytical method

development using High performance liquid chromatography (HPLC) and validation including efficient sample preparation.

2.1.7. Method Development[9-10]:

A well organized method development is important in drug development. Method development comprises of three essential inter-related components: sample preparation, separation of analyte and detection of analyte.

2.1.8. Sample preparation:

Sample preparation is important step for analysis of drugs and metabolites in bioanalytical study. Biological samples contain proteins, various endogenous and exogenous substances that may interfere with analyte. The objective of sample preparations to free analyte of interest from all possible unwanted substances without significant loss of analyte concentrations.

2.1.9. Preservation of Biological Samples:

Biological fluids are highly susceptible to physicochemical changes as they contain different substances. Processing or purifying biological samples is often time consuming therefore optimal storage conditions must be established for biological samples. Samples sensitive to oxidation can be protected by using air tight containers. Dehydration of moisture sensitive drugs could be achieved largely by freeze-drying or lyophilisation.

2.1.10. Sample Pretreatment:

Pretreatment of serum and plasma samples is not necessary if the analyte is protein-bound. In such cases, one of the following methods can be followed.

- Using 0.1Mor greater concentration of acids or bases make the Ph of the sample to pH<3 or pH>9. Separate the resulting supernatant and use it as the sample for extraction.

- Precipitate the proteins from biological fluid with a polar solvent such as acetonitrile, methanol,or acetonein 1:2 ratio by centrifugation, and use the supernatant for the extraction.
- Treat the biological fluid with acids or inorganic salts, such as formicacid, perchloricacid, trichloroaceticacid, ammonium sulfate, sodiumsulfate, orzincsulfate toprecipitate proteins. Adjust the pH of the resulting supernatant, sonicate for 15 minutes, dilute with water or buffer, centrifuge, and use the supernatant for extraction.

If the analyte is not protein bound, the pre- treatment methods include centrifugation, homogenization and hydrolysis of conjugates. Centrifugation is the process of separating cells from serum and plasma. Centrifugation of biological fluids usually done by using cooling centrifuge at $4^{O}C$ to avoid decomposition of the analyte and the clear supernatant liquid is used for analysis. For samples containing in soluble proteins, such as muscle or other related tissues, a homogenization or solubilizing step, using 1N hydrochloric acid, may be required before treating the sample further. A solid sample such as faeces can be homogenized with a minimum amount of methanol with a blade homogenizer or tissue homogenizer.

Phases of drug metabolism [6-8]:

Metabolism requires the interaction of drug (substrate) with an enzyme.

Enzyme + drug→ enzyme-drug complex → enzyme + metabolite

The major enzymatic biotransformation reactions of drug metabolism are oxidation, reduction, hydrolysis and conjugation. Conjugation is a synthesis reaction. A drug may have a chemical structure suitable for biotransformation by one or more of these reactions. Each enzymatic reaction results in a metabolite that itself may be further metabolized.

Phase 0

Phase 0 has been described as the transport of drug from the blood into the hepatocytes in the liver, the basolateral (sinusoidal) uptake processes.

Phase I

Phase1 metabolic processes include oxidation, reduction and hydrolysis reactions which typically provide functional groups capable of undergoing Phase2 reactions. The enzymes which catalyze Phase1 reactions are found in a number of sub cellular components including cytoplasm, mitochondria and endoplasmic reticulum.

Phase II

Phase II reactions include Conjugation reactions which involve the addition of molecules naturally present in the body to the drug molecule [17-18]. A lipid soluble drug may enter phase I or Phase II metabolic process. In phase I the drug after biotransformation may form a water soluble metabolite which can be excreted from urine or it may form a non-polar rmetabolite which enters phase II metabolic process to get converted to a polar rmetabolite. In phase II the drug undergoes conjugation and gets metabolized as polar rmetabolite which can be easily excreted from urine. The effect of a drug depends largely on the biotransformation reactions. Therefore, it may be important to isolate the actual conjugates. Samples containing either glucuronideacetals or sulfateesters are usually pre-treated using enzymatic or acid hydrolysis. The unconjugated metabolites that result from the hydrolysis procedure are less hydrophilic than their conjugates and usually can be extracted from the biological matrix. Heating a biological sample for 30min at 90^{O}C to 100^{O}C using 2N to 5N hydrochloricacid results in non-specific acid hydrolysis of the drug. After cooling , the pH of the sample can be adjusted to the desired level and the metabolite can be separated by solvent extraction.

Separation of analyte [13-15]

3.0. Extraction procedures for drugs and metabolites from biological samples

Extraction of analyte from biological matrix is traditionally carried out by (a) liquid-liquid extraction (LLE), (b) solid-phase extraction (SPE) and (c) precipitation of plasma proteins (PP).

(a) Liquid-Liquid Extraction(LLE)

LLE is a method used for the separation of compounds in a mixture using water and an immiscible organic solvent. Separation of analyte occurs based on its partition coefficient between two immiscible liquids and extraction can be done by using a suitable solvent. LLE method is simple, rapid, and relatively cost effective compared to other techniques. Most of the drugs can be recovered to the extent of 90% by multiple continuous extraction technique.

First, dissolve the component mixture in a suitable solvent and then add an immiscible solvent with the first solvent. Mix the contents thoroughly and set aside to separate the two immiscible solvents into layers. The components of the mixture will be distributed amongst the two immiscible solvents based on their partition coefficients. Separate the two immiscible solvent layers, transfer and isolate the component from each solvent. After extraction hydrophilic compounds get in the aqueous phase and hydrophobic compounds are found in the organic solvents. Non polar analytes extracted into the organic phase can be easily recovered by evaporation of the solvent, the residue reconstituted with a small volume of an appropriate solvent preferably mobile phase. Polar analytes extracted into the aqueous phase can be directly injected into a reverse phase (RP) column [10&20]. Sometimes the method requires pH control of samples for extraction. The method is not suitable for thermo labile substances as high temperature isused during evaporation.

(b) Solid Phase Extraction (SPE)

SPE is a commonand effective technique for isolation and concentration of analyte in trace amounts in a variety of sample matrices. With SPE the level of interferences can be reduced and final sample volume is minimized to maximize analyte sensitivity. Higher recovery of analyte can be obtained by using a small plastic disposable column or cartridge packed with 0.1 to 0.5g of sorbent which is commonly RP material (C18orC8). The components of interest may either preferentially adsorbed to the solid, or they may remain in the liquid phase. If the desired analyte is adsorbed on the solid phase, it can be selectively desorbed by washing with an appropriate solvent. If the component of interest remains in a liquid phase, it can be recovered through concentration, evaporation and or recrystallization. Extraction by SPE is more time taking and extraction of high density materials is difficult.

Steps in the extraction of the analyte from plasma by SPE [16]

a) Pretreatment of sample- which includes dilution of sample or pH adjustment, filtration to avoid the blocking of the SPE cartridge and for better adsorption.

b) Conditioning of the cartridge- It is the main step in case of reverse phase SPE cartridges. Preconditioning of the cartridge is necessary to obtain reproducible results and is done by solvents such as methanol, acetonitrile, isopropyl alcohol or tetrahydrofuran. Otherwise, a highly aqueous solvent cannot penetrate the pore sand wet the surface. As a result only limited fraction of the surface is available for interaction with the analyte. So it is important to maintain wetness of the cartridge up to sample loading.

c) Loading the sample-Sample size must be scaled to suit the size of the cartridgebed. The capacity of atypical RP cartridge is upto 100 mg of very strongly retained substances.

d) Washing the SPE bed- A suitable solvent or water mixture is passed through SPE bed to remove the contaminants.

e) Elution of fraction- A suitable solvent or buffer is used to elute the analyte from the SPE bed for analysis.

(c) Protein Precipitation (PP)

Protein precipitation is a very simple technique for extraction of the analyte from blood or plasma. The main requirement for this technique is that the analyte should be freely soluble into reconstituting solvent. In this technique, the sample is prepared by protein precipitation by using acids (trichloroaceticacid and perchloricacid) organic solvents (methanol, ethanol, acetone and acetonitrile) or salts (ammonium sulphate). After precipitation the sample is centrifuged, analyte gets into supernatant. Among the solvents methanol is preferred a sit produces clear supernatant which is suitable for direct injection. PP can be employed for extraction of hydrophilic and hydrophobic substances. The limitation is PP may clog the column.

Salting out with Ammoniumsulphate

Ammonium sulphate is used for salting out, because of its high solubility and high ionic strength. Its solubility changes little with temperature and is cheap. The density of a concentrated solution is less than that of protein. So that protein can be easily centrifuged from the concentrated solutions.

(d) Solvent Precipitation

When large amounts of a water-miscible solvent such as ethanol or acetone are added to a protein solution, proteins precipitate. This is due to decrease in the dielectric constant, which would make interactions between charged group son the surface of proteins stronger.

Solvent precipitation for the protein is performed at 0°C and the solvent colder, 20°C in an ice-salt bath, because proteins tend to denature higher temperatures.

4.0. HPLC Instrumentation[4-9]:

High-performance liquid chromatography (HPLC) isused in biochemistry and analysis to separate, identify and quantify the active compounds. HPLC instrumentation consists of a pump, injector, column, detector, integrator and a display system. The heart of the system is the column where separation occurs. Stationary phase is composed of micron sized porous particles, hence a high pressure pump is required to move the mobile phase through the column. The sample to be analyzed is introduced in small volume to the stream of mobile phase. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time.

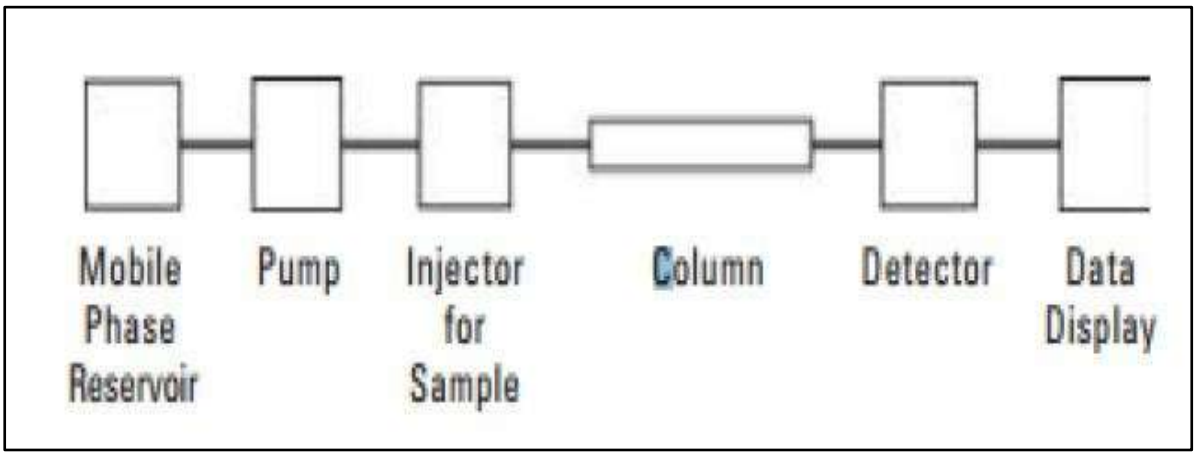

Figure: Schematic diagram of HPLC [8]

4.1. Injection of the sample: Sample solution can be injected by using septum injectors, when thermolabile phase is flowing or it is stopped. A new advance drotary valve and loop injector can be used to produce reproducible results

4.2. Steps involved in HPLC Method development

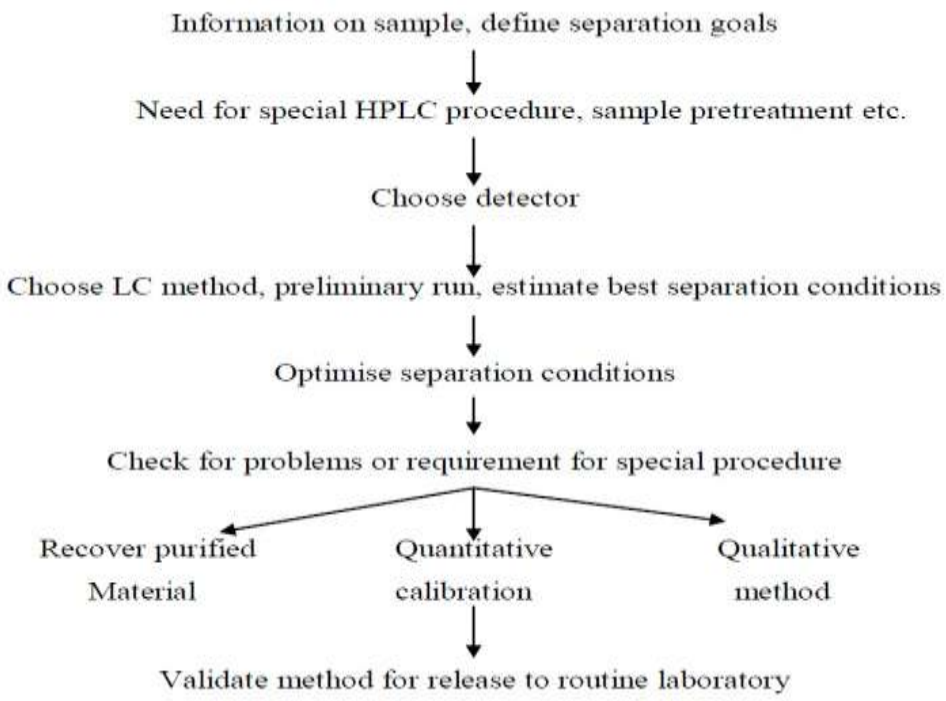

Figure2: HPLC Method Development [8]

4.3. Bioanalytical Method Validation [2-6]:

Validation is mandatory by the regulatory agencies. The main objective of method validation is to demonstrate the reliability of a particular method developed for the quantitative determination of ananalyte in a specific biological matrix.

4.4. Full Validation

Full validation is necessary when developing and implementing an analytical method for analysis of a new drug entity, when developing and implementing a bioanalytical method for the first time and when an existing assay method is modified-metabolites are added to an existing assay for quantification of a drug.

4.5. Partial Validation

Partial validations are modifications of existing validated bioanalytical methods. Partial validation can range from as little as one intra-assay accuracy and precision

determination to an early full validation. Bioanalytical method changes that require partial validation are:

- Method transfers between laboratories or analysts
- Modification of analytical methodology (e.g., change in detection systems)
- Addition of different anticoagulant in harvesting biological fluid
- Changes in matrix within the same species (e.g., human plasma to human urine)
- Alteration of sample processing procedures
- Changes in species within matrix (e.g., rat plasma to mouse plasma)
- Variation in relevant concentration range
- Changes in instruments and/or software platforms
- Limited sample volume (e.g., pediatric study)
- Rare matrices
- Selectivity demonstration of an analyte in the presence of concomitant medications
- Selectivity demonstration of an analyte in the presence of specific metabolites

4.6. Cross-Validation

In cross-validation two bioanalytical methods for the same drug are compared. Original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator. Cross validation is required when two or more bioanalytical methods are used to generate data within the same study. When sample analyses within a single study are conducted at more than one site or more than one laboratory, cross-validation with spiked matrix standards and subject samples should be conducted to establish inter-laboratory reliability. Cross-validation should also be considered when data generated using different analytical techniques in different studies**.**

4.7. Validation Parameters[5-13]

The basic parameters for the validation of a chemical assay comprises of all criteria determining data quality such as selectivity, accuracy, precision, recovery, linearity, calibration model, limit of detection (LOD), lower limit of quantification (LLOQ), stability, reproducibility, and ruggedness.

1. Selectivity (specificity)

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics For selectivity, blank samples of the appropriate biological matrix should be analysed from atleast six sources. Each blank should be tested for interference of other substances and selectivity should be ensured at LLOQ.

2. Accuracy

Accuracy of an analytical method describes the closeness of mean test results obtained by theme to the true value (concentration) of the analyte. Accuracy should be measured for a minimum of three concentrations inexpected range of concentrations using a minimum of 6 determinations per concentration. The mean should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. This deviation of mean from the true value serves as the measure of accuracy.

3. Precision:

Precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple a liquots of a single homogeneous volume of biological matrix. Intraday precision is precision during single

analytical run. Interday precision measures with time, and may involve different analysts, equipment, reagents and laboratories.

Precision should be measured using a minimum of three concentrations in the expected range of concentrations with five determinations per concentration. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

4. Recovery

Recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery experiments should be performed at three concentrations (low, medium and high) with un-extracted standards that represent 100% recovery. Recovery of the analyte need not be 100% but the extent of recovery of an analyte and an internal standard should be consistent, precise and reproducible.

5. Linearity:

Linearity is the relationship between instrument response to known concentrations of the analyte. Linearity assesses the ability of the method to obtain test results that are directly proportional to the concentration of the analyte in the sample.

6. Calibration curve:

It is the relationship between experimental response value and known concentrations of the analyte. A calibration curve should be designed by using the same biological matrix in which the intended study is to be done by spiking the matrix with known concentrations of the analyte. If enough blank samples are not available, e.g. in case of cerebro spinal fluid,

0.9% NaCl can be used as calibration matrix and response from both matrices should be compared. Concentration of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard) and 5-8 non zero samples covering the expected range of analyte including LLOQ. Calibration curve should be designed by applying simplest model that satisfactorily describes the concentration response relationship using suitable weighting and statistical tests for goodness-of-fit. The following conditions should be met when developing a calibration curve:

- 15% deviation of standards other than LLOQ from true concentration and 20% deviation of the LLOQ from authentic concentrations.
- Atleast four out of six non zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration.

7. Limit of Detection (LOD)

It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions.

8. Lower Limit of Quantification (LLOQ)

It is the lowest amount of analyte in a sample

That can be detected but necessarily quantitated under the stated experimental conditions with acceptable accuracy and precision. The lowest standard on the calibration curve should be accepted as LLOQ if the following criteria are met:

- The analyte response should be atleast five times the response compared to blank response. Analyte peak should be identifiable, discrete and reproducible with a precision of maximum 20% and accuracy of 80-120%.

9. Stability

It is the chemical stability of an analyte in a given matrix under specific conditions for given time intervals. The aim of a stability test is to detect any degradation of the analyte(s) of interest, during the entire period of sample collection, processing, storage, preparation, andanalysis. All but long term stability studies can be performed during the validation of the analytical method. Long term stability studies might not be complete for several years after clinical trials begin. The condition under which the stability is determine dislargely dependent on the nature of the analyte, the biological matrix, and the anticipated time period of storage (beforeanalysis).

a) Freeze-thaw stability:

The influence of freeze/thaw cycles on analyte stability should be determined after at least 3cycles at 2 concentrations in triplicate. At least three a liquots at each of the low and high concentrations should be stored at intended storage temperature for 24 hours and thawed at room temperature. When completely thawed, refreeze again for 12-24 hours under the same conditions. This cycle should be repeated two more times, then analyse on 3rd cycle. All stability determinations should use a set of samples prepared from a fresh lymadestock solution of the analyte in the appropriate blank, interference-free biological matrix. Standard deviation of error should be <15%. If analyte unstable freeze at -70^{O}C for three freeze-thaw cycles.

b) Short-term stability:

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature for 4-24 hours and analyse. % Deviation should be <15%.

c) Long-term stability:

Atleast three aliquots of each of low and high concentrations should be thawed at room temperature and kept at this temperature for 4- 24 hours and analyse. Analyse on three separate occasions. Storage time should exceed the time between the date of first sample collection and the date of last sample analysis.

d) Stock-solution stability:

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for atleast 6hours. % Deviation should be <15%. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

e) Post-Preparative Stability:

The stability of processed samples, including the resident time in the auto sampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.

f) Reproducibility:

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology). Reproducibility only has to be studied, if a method is supposed to be used in different laboratories.

10. Ruggedness: (Robustness)

Ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis due to pH, mobile phase composition, temperature, etc. Ruggedness should be tested if a method is supposed to be transferred to another laboratory. Ruggedness is not mandatory under full validation, but it would be helpful during themethod development as problems that may occur during validation are often detected in advance.

5.0. Quality control of samples [16-18]

QC samples in duplicates at three concentration levels (one near the 3xLLOQ, one in mid range, one close to high end) should be incorporated at each assay run. Minimum number QC should be atleast 5% of total number of samples in a run or six total QCs whichever is greater.

a. **Acceptance criteria**

The results of the QC samples provide the basis for accepting or rejecting a run. Atleast four out of every six should be within 15% of respective nominal values. Two of the six may be outside of 15% but not both at the same concentration.

b. **Repeat analysis**

The guidelines for repeat analysis should be established. The rationale for the repeat analysis should be clearly documented such as sample processing errors, equipment failure, poor separation and resolution of analyte.

6.0. CONCLUSION

This review is aimed in focusing the role of bioanalytical techniques in pharmaceuticals and gives a thorough literature survey of the bioanalytical methods and instruments in drug analysis. Modification of existing bioanalytical method development and validation has become common with the availability of modern instruments and novel

purification methods of biological matrix. The data generated from a well developed, validated and documented bioanalytical method is helpful in improving existing bioanalytical methods. Development of better bioanalytical methods using HPLC and preclinical analysis, pharmacokinetic and toxicokinetic data of drugs in lesser time. Bioanalytical method development is useful in identity, purity, potency and bioavailability of drugs. Therefore, bioanalytical HPLC method development and validation is essential for the determination of a drug concentration in bulk and in pharmaceutical dosage forms and to monitor and control impurities in drugs

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