Bioanalytical Method Development and Validation by HPLC: A Review

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ABSTRACT
The development of selective, sensitive and reliable bioanalytical 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 bioanalytical 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 bioanalytical HPLC method for low dose drugs. This article reviews current progress in HPLC based bioanalytical method development and validation of different drugs. So, far drugs like Anti malarials, Omeprazole, Clofarabine, Palonosetron HCl, etc have been analyzed bioanalytically.

Key words: Bioanalytical method development, HPLC, validation, documentation and application.

INTRODUCTION:
Bioanalysis is the method used to determine the concentration of drugs, their metabolites and/or endogenous substances in the biological matrices such as blood, plasma, serum, cerebrospinal fluid, urine, and saliva [1-3]. The method includes collection, processing, storage and analysis of a biological matrix for a drug. Bioanalytical method validation involves documenting established and verified specific laboratory investigations for quantitative measurement of a drug substance in a given biological matrix. The basic parameters of validation comprises all criteria determining data quality such as selectivity, sensitivity, calibration model, accuracy, precision, stability, lower limit of quantification (LLOQ), recovery, linearity, limit of detection, reproducibility, and ruggedness [4-5]. Results from validated bioanalytical methods are used by quality control laboratories to confirm identity, purity, quality, potency and bioavailability of drug...
products [6]. When a study requires sample analysis in multiple laboratories, it is essential to validate the bioanalytical method(s) at each lab and provide suitable validation information for different labs to establish inter-laboratory reliability [7]. Existing bioanalytical methods and techniques are often modified suitably as per the requirements of an analytical procedure. Therefore, it is important to establish well defined and validated bioanalytical methods to get reliable results that can be adequately deduced [5]. Biological matrices contain high concentrations of various endogenous substances and drug concentrations are often low. Sometimes the endogenous compounds are structurally similar to the drug to be estimated. For analysis, the drug must be isolated in pure state using a suitable extraction technique. Invention of modern analytical instruments and extraction techniques resulted in advanced 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 [8]. The review focuses on bioanalytical method development using High performance liquid chromatography (HPLC) and validation including efficient sample preparation.

**Method Development:** 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 [9].

**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 preparation is to free analyte of interest from all possible unwanted substances without significant loss of analyte [10].

**Biological matrices used in bioanalysis:**

Determination of drug in urine is an indirect method to ascertain the bioavailability of a drug. Estimation of drug in feces may reflect drug that has not been absorbed after an oral dose or may reflect drug that has been expelled by biliary secretion after systemic absorption. Salivary drug levels indicate free drug rather than total plasma drug concentration as only free drug diffuses into the saliva. Therefore, the saliva/plasma drug concentration ratio is less than 1 for many occasions. Measurement of a drug concentration in the blood, serum or plasma is the most direct approach to assess the Pharmacokinetics of the drug in the body. Assuming that a drug in the plasma is in dynamic equilibrium with the tissues, and then changes in the drug concentration in plasma will reflect changes in tissue drug concentrations [11].

**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 [12].

**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 0.1M or 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 acetone in 1:2 ratio by centrifugation, and use the supernatant for the extraction.
- Treat the biological fluid with acids or inorganic salts, such as formic acid, perchloric acid, trichloroacetic acid, ammonium sulfate, sodium sulfate, or zinc sulfate to precipitate proteins. Adjust the pH of the resulting supernatant, sonicate for 15 minutes, dilute with water or buffer, centrifuge, and use the supernatant for the extraction [13].
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 fluid is usually done by using cooling centrifuge at 4°C to avoid decomposition of the analyte and the clear supernatant liquid is used for analysis. For samples containing insoluble 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. [14]

Phases of drug metabolism:
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 [15].

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 [16].

Phase I
Phase I metabolic processes include oxidation, reduction and hydrolysis reactions which typically provide functional groups capable of undergoing Phase 2 reactions. The enzymes which catalyze Phase 1 reactions are found in a number of subcellular 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 metabolite which enters phase II metabolic process to get converted to a polar metabolite. In phase II the drug undergoes conjugation and gets metabolized as polar metabolite 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 glucuronide acetics or sulfate esters 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 30 min at 90°C to 100°C using 2N to 5N hydrochloric acid 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 [14].

Separation of analyte

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) [19].

(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 [20].

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 a side 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 in to 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 thermolabile substances as high temperature is used during evaporation [1].

(b) Solid Phase Extraction (SPE)

SPE is a common and 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 0.5 g of sorbent which is commonly RP material (C18 or C8). 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 [20].

Steps in the extraction of the analyte from plasma by SPE

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 pores and 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 wet ness of the cartridge up to sample loading.

c) Loading the sample - Sample size must be scaled to suit the size of the cartridge bed. The capacity of a typical RP cartridge is up to 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 [21& 22].

(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 (trichloroacetic acid and perchloric acid)/ organic solvents (methanol, ethanol, aceton and acetonitrile) or salts (ammonium sulphate). After precipitation the sample is centrifuged, analyte gets into supernatant. Among the solvents methanol is preferred as it 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 Ammonium sulphate

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.

Solvent Precipitation

When large amounts of a water-miscible solvent such as ethanol or aceton are added to a protein solution, proteins precipitate. This is due to decrease in the dielectric constant, which would make interactions between charged groups on 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 at higher temperatures [23, 24].

Detection of analyte

HPLC Instrumentation:

High-performance liquid chromatography (HPLC) is used 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 [25]. The sample to be analyzed is introduced in small volume to the stream of
mobile phase. Detector shows the retention times of the molecules. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time. [26]

**Injection of the sample:** Sample solution can be injected by using septum injectors, when the mobile phase is flowing or it is stopped. A new advanced rotary valve and loop injector can be used to produce reproducible results [26]

**Steps involved in HPLC Method development [27]**

**Bioanalytical Method Validation:** 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 an analyte in a specific biological matrix [6].

**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 [3,4].

**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 a nearly full validation [4]. 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 [3,4]

**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 [3,4].

**Validation Parameters [28-30]**
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 [3-5 & 31].

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.[3]

- For selectivity, blank samples of the appropriate biological matrix should be analysed from at least six sources. Each blank should be tested for interference of other substances and selectivity should be ensured at LLOQ [4].

2. Accuracy
Accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte.
- Accuracy should be measured for a minimum of three concentrations in expected 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 [4].

3. Precision:
Precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots 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].

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 [4].

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 sample is not available, e.g. in case of cerebrospinal 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.
At least four out of six non zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration [4].

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 at least 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% [4].

8. 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, and analysis. 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 determined is largely dependent on the nature of the analyte, the biological matrix, and the anticipated time period of storage (before analysis) [4].

a) Freeze-thaw stability:

The influence of freeze/thaw cycles on analyte stability should be determined after at least 3 cycles at 2 concentrations in triplicate. At least three aliquots 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 freshly made stock 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°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:

At least 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 at least 6 hours. % 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.

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 [32].

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 the method development as problems that may occur during validation are often detected in advance [5].

Quality control of samples

QC samples in duplicates at three concentration levels (one near the 3 x LLOQ, one in mid range, one close to high end) should be incorporated at each assay run. Minimum number QCs should be at least 5% of total number of samples in a run or six total QCs whichever is greater [4].

Acceptance criteria

The results of the QC samples provide the basis for accepting or rejecting a run. At least 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 [4].

**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 [4].

**CONCLUSION:**

In recent times, 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 validation assist in performing quality control of pharmaceuticals as well as in obtaining 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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