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Original Article

Bioanalytical Method Development and Validation by Hyphenated Technique (LC-MS/MS)

Farah Iram*, Huma Iram*, Mohd Arif*, A.A. Siddiqui*, Asif Husain*

Abstract

Bioanalytical method development plays importance role in the preclinical and clinical studies. Pharmacokinetics of any drug and/or its metabolite can be recognised by bioanalytical studies. The quantitative analysis of drugs and their metabolites in the biological media is done by bioanalytical studies. Physico-chemical and biological techniques are used for these studies. Each bioanalytical method should be selective, sensitive and reliable for the quantitative evaluation in drug discovery process. Bioanalytical method development consists of sample preparation, chromatographic separation and detection by using proper analytical method. Each developed method should be validated as per the regulatory authorities, so as to give reliable and reproducible method for the intended use. Many analytical techniques can be use for bioanlysis, LCMS/MS is one of them. In Liquid chromatography-mass spectrometry (LC-MS/MS) the separation of analyte is done by LC and detection is carried out by MS. LC-MS/MS prominently used in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. This review also focused on various validation parameters such as: accuracy, precision, sensitivity, selectivity, standard curve, limits of quantification, range, recovery stability, etc.

Keywords: Bioanalytical; Liquid Chromatography; Spectrometry; Bioanalysis; Validation.

Author Affilation: *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Jamia Hamdard University, New Delhi-110 062, India.

Reprint Request: Asif Husain, Sr. Asst. Professor, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Hamdard University, New Delhi-110 062, India.
E-mail: drasifhusain@yahoo.com, ahusain@jamiahamdard.ac.in

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Introduction

Bioanalytical Methods are widely engaged for the quantitative analysis of the drugs. Bioanalysis is the method to investigate 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]. It also plays an important role in the evaluation of bioavailability, bioequivalence, pharmacokinetics studies [4-7]. The reason behind for new method of analysis is:

- Unavailability of suitable method for a particular analyte in the specific matrix. Already available method may have too pitfalls and poor in accuracy or precision.
- Present methods may be costly, laborious, and tedious.
- Poor in sensitivity and selectivity in samples of interest.
- If new instrumentation or techniques is incorporate in for developed method.
- There is a need for alternative methods to confirm, for legal or scientific reasons. [8-12].

Chromatographic techniques like, HPLC, Gas chromatography, LC-MS, GC-MS, Ligand binding assay, immunological and microbiological procedures are used for the bioanalysis purpose. The

method includes collection of sample, processing, storage in suitable conditions and finally analysis of a biological matrix for a drug. Method development consists of three essential parts sample preparation, chromatographic separation and detection by using proper analytical method. The documentation and verification of specific laboratory investigations, quantitatively of a drug substance in a given biological matrix is done by bioanlytical method validation. The basic parameters of validation comprises of various parameters such as selectivity, sensitivity, calibration curve, accuracy, precision, stability, lower limit of quantification (LLOQ), recovery, linearity, limit of detection, reproducibility, and ruggedness [13-17] .

This U.S. FDA guidance supports the sponsor of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs) and supplements in developing bioanalytical method validation. It also provides the assistance in the information used in human clinical pharmacology, bioavailability (BA) and bioequivalence (BE) studies requiring pharmacokinetic (PK) evaluation. The guideline also supports the bioanalytical methods used for non-human pharmacology/toxicology studies and preclinical studies [18].

The bioanalysis of drugs in plasma can be done by both HPLC and LCMS-MS method. Each analytical instruments has its own pros and cons. HPLC coupled with detector (UV, PDA or fluorescence) can evaluate many compounds and it offers a cost effective bioanalytical method. The demerit is poor sensitivity and selectivity for some of the potent compounds. Whereas low detection limits, good ability to generate structural information, minimal sample requirement and wider coverage of range of analytes differing in their polarities can be obtained by using LC-MS/MS. But some extent LC/MS/MS instruments are limited due to matrix-induced effect in ionization efficiencies and ion suppression or enhancement (due to presence of biological matrix). The integral use of LC-MS/MS can be seen from last few decade, as it provides high sensitivity, amazing selectivity, and rapid rate of analysis [19,20]. The review focuses on bioanalytical method development and validation using LC-MS/MS technology.

Bioavailability and Bioequivalence

The pharmaceutically equivalency between the test product and a reference product bioavailability/bioequivalence studies are required to done by regulatory bodies. Both objectives is on the release of drug substance from its dosage form and successive absorption into the systemic circulation. The equivalence can be assess by: comparative bioavailability/bioequivalence studies, comparative pharmacodynamic studies in humans, comparative clinical trials and In-vitro dissolution tests. For evaluation of two medicinal products containing the same active substance bioequivalence studies are done. The therapeutic equivalency should be present for two products marketed by different licensees, containing same active ingredient, in order to be considered interchangeable. Bioequivalence studies are a pivotal part of registering dossiers. Bioequivalence data is preliminary requirement for ANDA submission. The pharmacokinetic parameters such as area under the curve (AUC), peak concentration (Cmax), time to peak concentration (Tmax) can be evaluate by the plasma concentration data [20-22].

Method Development

A well organized method development is important in drug development. Analytical method development can be defined as the process of identifying the procedure to facilitate the identification and quantification of compound of interest in a matrix. Several methods can be used in identification of compound, analytical method involves in identification and characterisation depends on: chemical properties of the analyte, concentrations levels, sample matrix, cost of the analysis, and speed of the analysis, quantitative or qualitative measurement, precision required, the necessary equipment and on many other factors [4,9,23-30].

A Method development comprises of three components: sample preparation, separation of analyte and detection of analyte [31-33].

Collection of Sample and Its Preparation

The analyte are usually present in biological matrix that is blood, plasma, urine, serum etc. Hypodermic syringe (5 to 7 ml) is used to puncture the vein for blood collection. Further the venous blood is withdrawn into tubes, using an anticoagulant, e.g. EDTA, heparin etc is used. Finally centrifugation at 4000 rpm for 15 min is carried out by which plasma is separated out [34-36]. Sample preparation carried out to clean up the sample before analysis and to concentrate the sample. Much interference may occur

during analysis which may be due to proteins, salts, endogenous macromolecules, small molecules and metabolic by products. The sample preparations also allow the exchange of analyte from the biological matrix into a solvent suitable for injection into the chromatographic system. Sample preparation can be done by solid-phase extraction (SPE), liquid/liquid extraction and protein precipitation [37-40].

Biological Samples Preservation

Biological fluids are highly susceptible to physicochemical changes. 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. Moisture sensitive drugs dehydration could be achieved largely by freeze-drying or lyophilisation [41,42].

Sample Pretreatment [43-44]

If the analyte is protein-bound. In such cases, one of the following pre-treatment 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.
- Precipitate the proteins from biological fluid with a polar solvent such as acetonitrile, methanol, or acetone in 1:2 ratios.
- The biological fluid is then treated by acids or inorganic salts, such as formic acid, perchloric acid, trichloroacetic acid, ammonium sulphate etc.

If the analyte is not protein bound, the pre-treatment is done by centrifugation, homogenization and hydrolysis of conjugates.

Separation of Analyte

Extraction Procedures for Drugs from Biological Samples

Extraction of analyte from biological matrix is mainly carried out by three processes:

(i) liquid-liquid extraction (LLE),

(ii) solid-phase extraction (SPE) and

(iii) precipitation of plasma proteins (PP)

Liquid-Liquid Extraction (LLE)

It is based on the principle of differential solubility and partitioning equilibrium of the analytes between the aqueous and organic phases. It generally involves the extraction of analyte from one phase into another phase and the distribution of the analyte molecules between two immiscible phases. In LLE compounds separation is carried out in a mixture using water and an immiscible organic solvent. LLE method is simple, rapid, and relatively cost effective compared to other techniques. 90% of the drug can be recovered by multiple continuous extraction technique [9,38,45].

In LLE dissolve the component mixture in a suitable solvent and then add an immiscible solvent with the first solvent. Completely mix the content and allow the separation of two immiscible solvents into layers. Based on the partition coefficients of the solvents the components of the mixture will be scattered amongst the two immiscible solvents. Separate the two immiscible solvent layers, transfer and isolate the component from each solvent. After extraction the aqueous phase has hydrophilic compounds and hydrophobic compounds are found in the organic solvents. By evaporation the non polar analytes in organic solvents are recovered. Further the residue reconstituted with a small volume of an appropriate solvent preferably mobile phase. Whereas the analytes which are polar in nature can be extracted in to the aqueous phase and can directly inject into a reverse phase (RP) column [9,39,46]. Traditional LLE can be replaced with advanced and improved techniques like liquid phase micro extraction (LPME), single drop liquid phase micro extraction (DLPME) and supported membrane extraction (SME) [2,4].

Protein Precipitation (PP)

Protein precipitation is another important technique for extraction of the analyte from matrix. The principle behind PP is the precipitation (denaturation) of the proteins by using a range of reagents like acid (trichloroacetic acid and perchloric acid), organic solvents (methanol, acetone and acetonitrile) or by salts (ammonium sulphate). After denaturation the sample is centrifuged, that gives analyte into supernatant form. PP is less time consuming, smaller amount solvents are used. The samples often contain protein residues and it is a non-selective sample cleanup method. The limitation of PP is that it may clog the LC column. Of recently PP technique is combined with SPE to give clean extract. Methanol is generally preferred as solvent and can produce the appropriate for direct injection into LC-MS/MS [4,9,47,48].

Solid Phase Extraction (SPE)

SPE is frequent and effective technique for isolation of analyte in trace amounts in sample matrices. With SPE the level of interferences can be reduced. The final sample volume is minimized to maximize analyte sensitivity. SPE provide higher recovery of analyte. In SPE small plastic disposable column or cartridge packed with 0.1 to 0.5 g of sorbent which is commonly RP material (C18 or C8) is used. The analyte may either preferentially adsorbed to the solid, or they may remain in the liquid phase. The analyte can desorb by washing with an appropriate solvent, if the analyte is adsorbed. If the component of interest remains in a liquid phase, it can be recovered through concentration, evaporation and or recrystallization [4,9,46,49-51].

Solid phase consists of four steps: conditioning, sample loading, washing and elution.

- *Conditioning*:Conditioning is basically activation of the column. Organic solvent that acts as a wetting agent on the packing material are used for conditioning of the column. The solvents solvates the functional groups of the sorbent. For proper adsorption, water or aqueous buffer is added to activate the column.
- *Sample Loading:* The sample is loaded on the column, after the adjustment of pH
- *Washing*: Washing is done in which interferences from the matrix are removed and the analyte will retain.
- *Elution:* For elution a suitable solvent or buffer is used, which elutes the analyte from the SPE bed for analysis [52,53].

Types of Solid Phase Extraction Cartridges: [54-57]

1. *HLB Cartridge:* HLB is Hydrophilic-Lipophilic Balanced water-wettable reversed phase sorbent. Two monomers hydrophilic *N*-vinylpyrrolidone and lipophilic divinylbezene are present in specific ratio in HLB cartridg . It is available in various particle sizes such as (60ìm, 30ìm, 15ìm etc.)
2. *MCX Cartridge:* It is a mixed mode cation exchange, water-wettable, polymeric sorbent. It is a water-wettable, mixed-mode polymeric sorbent, to achieve higher selectivity and sensitivity for extracting basic compounds with cation-exchange groups.
3. *MAX Cartridge:* MAX (mixed-mode anion-exchange) is usually intended to overcome the drawback of silica-based mixed-mode SPE sorbents. This cartridge has a mixed-mode anion-exchange, water-wettable, polymeric sorbent which is stable from pH 0 to 14.
4. *WCX Cartridge:* WCX (mixed-mode weak cation-exchange) is usually intended to provide sample preparation for strong bases and quaternary amines. It has a water-wettable polymeric sorbent.
5. *WAX Cartridge:* WAX cartridge is for strong acids. WAX is mixed-mode weak anion-exchange reversed phase sorbent.
6. *Bond Elute Plexa:* It has non polar retention mechanism. It gives clean extracts which minimizes matrix interference

Strategy of LC-MS/MS Method Development

In many diverse ways method of analysis are being routinely developed, improved and validated. Type of sample will decide the required chromatographic conditions. So the knowledge of sample and chromatographic procedure is a must for systematic approach to LC-MS/MS method development [55,57-59].

- Physicochemical properties of drug molecules from literature
- Determine solubility profile
- MS scanning and optimization
- Mobile phase selection
- Selection of extraction method and optimization
- Selection of chromatographic method (based on solubility study, retention of compound)

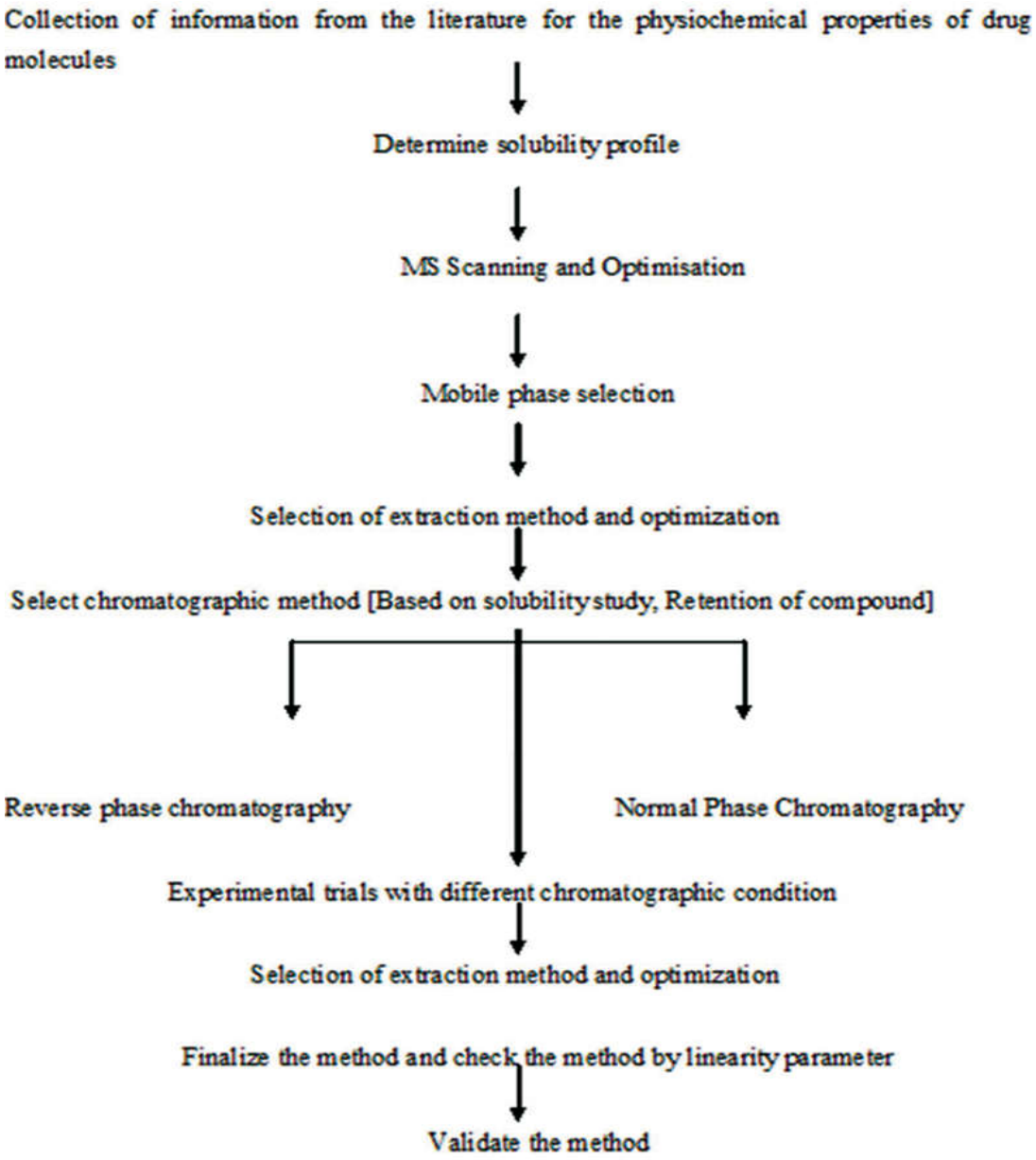
Flow chart for Method Development

During method optimization, the initial problems that have arise from the first stages of development are enhanced in terms of resolution and peak shape, retention time, limit of quantitation, and overall ability to quantify the specific analyte of interest. In method development various parameters need to be optimized: [4,9,60].

- Separation mode
- Stationary phase selection
- Mobile phase selection
- Selection of detector

Mode of Separation Technique

Most of the pharmaceutical compounds are polar



in nature so reverse phase chromatography is preferred first in which a non polar stationary phase and non polar mobile phase is used. The mobile phase comprises of water or buffer and organic phase (acetonitrile/methanol). Hence polar compounds get eluted first and non-polar compounds are retained for a longer time. The stationary phases used in reverse phase chromatography are n-octadecyl (RP-18), n-octyl (RP-8), ethyl (RP-2), phenyl, cyano, diol and hydrophobic polymers. It is the first choice for most samples; especially neutral or un-ionized compounds that dissolve in water-organic mixtures. Normal phase is try if reverse phase fails where the sample may be strongly retained with 100% acetonitrile as mobile phase. In reversed-phase chromatography the retention mechanism is between the column's stationary phase and sample analytes [61].

Selection of Stationary Phase

Selection of stationary phase is based on following parameter.

Column

The column is considered as the heart of separation process. The development of an accurate, precise, rugged and reproducible method is possible with the availability of a stable, high performance column. Commercial columns can differ widely. These differences can have a serious impact on method

development. The columns differ usually in plate number, band symmetry, retention, band spacing and lifetime.

Column Selection

Column selection should be based both upon knowledge of the sample and goals of the separation. Including sample knowledge and the goals of separation the following factors also considered for column selection:

Column Internal Diameter

Generally wider diameter columns are chosen for greater sample loading, whereas narrow columns are preferred for more sensitivity.

Particle Size

For complex mixture with similar components smaller particle (3-4 μm) are considered. Whereas bigger particle (5-10 μm) for sample with structurally different compounds [62,63].

Selection of Mobile Phase

Mobile phase composition plays significant role in improving peak resolution and peak sensitivity. By choosing the appropriate match between the stationary phase and mobile phase composition, the developed method simplifies the procedure and significantly decrease total analysis time as well as increase peak height.

In case of reverse phase chromatography, mobile phase with polar characteristic is used while as for normal phase a non polar mobile phase is used. More polar solvents cause increased retention in RPC or reduce retention in NPC. If the buffer pH is close to the pKa of the analytes, then selectivity altered. Subsequently, the mobile phase is modified by decreasing the proportion of water, and by increasing the addition of an organic solvent such as MeOH or acetonitrile, which causes the retained analyte to elute off the stationary phase. Changing the mobile phase composition in this way is the most efficient way of achieving chromatographic resolution.

Following parameters shall be taken into consideration while selecting and optimizing the mobile phase [60,64].

Buffer

Buffer and its strength play an essential role in deciding the peak symmetries and separations. The retention times also depend on the molar strengths of the buffer. Molar strength is proportional to retention times.

pH of the Buffer

pH plays an essential role for good chromatographic separations by controlling the ionization characteristics. It is essential in maintaining the pH of the mobile phase in the range of 2.0 to 8.0, columns does not withstand to the pH which are outside this range. The reason behind this is that the siloxane linkage area cleaved below pH 2.0, while pH valued above 8.0 silica may dissolve.

Mobile Phase Composition

Selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions in mobile phase. Methanol and acetonitrile are widely used solvents in reverse phase chromatography [24,47,65].

Estimation of Drugs by LC-MS/MS

LC/MS is a hyphenated technique, combining the HPLC separation ability and detection ability of mass spectrometry. The charged particle in LC-MS/MS passes through a magnetic field, which further deflected along a circular path on a radius that is proportional to the mass to charge ratio, m/e. In a mass spectrometer, an electron is displaced from the organic molecule. Too unstable molecular ions get fragmented. The ions is then focused into a beam and accelerated into the magnetic field. According to the masses of the ions, they get deflected along circular paths. The ions are further focused on the detector and finally recorded [47,66-68].

A MS detector consists of three main parts: the ***interface*** where the ions are generated, the ***mass analyser*** (separation) and ***the electron multiplier*** (detector).

Ionization Modes

Electrospray Ionization (ESI): From ESI needle the sample solution enters, the needle was at relatively higher voltage which sprays the sample solution into a fine mist of droplets that are electrically charged at their surface. At the surface of the droplets the electrical charge density increases as solvent evaporates from the droplets. From the very small, highly charged droplets, sample ions are ejected into the gas phase by electrostatic repulsion. The sample ions enter the mass spectrometer and finally analyzed [69].

Atmospheric Pressure Chemical Ionization (APCI)

In APCI, ions are produced when the sample solution in the form of fine mist of droplets enters to the APCI nozzle sprays. In high temperature tube droplets are vaporized. A high voltage is applied to a needle located near the exit end of the tube. This voltage creates a corona discharge that forms reagent ions through a series of chemical reactions with solvent molecules and nitrogen sheath gas. Further the reagent ions react with sample molecules and the sample ions enter the mass spectrometer and further they are analyzed [70].

Matrix Assisted Laser Desorption/Ionization (MALDI)

High molecular weight compounds with high sensitivity are ionised by MALDI. The ionization beam is laser light and matrix is solid. The pulsed laser beam is directed on a sample which is suspended or dissolved in matrix ions [71].

Mass Analyzers

Mass analyzer is the most important part of the LCMS-MS. There are different types of analyser based on their mechanism e.g. Electric sector (Kinetic Energy) Magnetic sector, Quadrople/Ion trap, Time of flight Flight time, FT-ion cyclotron resonance [47,72].

Scan Types

Full Scan: Each analyte is provided with full mass spectrum. Full scan is done to conclude or confirm the identity of unknown compounds or for the identification of each component in a mixture of unknown compounds.

Selected Ion Monitoring (SIM): In SIM a particular ion or set of ions is monitored. SIM experiments are applied when the mass spectrum of target compound is known in detection of small quantities of a target compound in complex mixture.

Selected Reaction Monitoring (SRM): A particular reaction or set of reactions, such as the fragmentation of an ion or the loss of a neutral moiety is monitored is determined in SRM [20,73]

Detectors

The ions from the analyser enter to the detector, where they get detected separately. Electron multipliers, dynolyte photomultiplier and microchannel plates are the different type of detectors used in LC-MS/MS [28,74].

Method Validation

Regulatory agencies have mandated the method validation. Selective and sensitive bioanalytical methods for the quantitative evaluation of drugs and their metabolites are critical for the successful biopharmaceutics and clinical pharmacology studies [75]. Bioanalytical method validation includes all procedures that display that a particular developed method for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for the intended use [76]. Bioanalytical method undergoes many modifications during a drug development process. Each modification should be validated to ensure suitable performance of the bioanalytical method. The objective of method validation is to exhibit the reliability of a particular method developed for the quantitative determination of an analyte in a specific biological matrix [30,77-81]

Goals

1. Well distinguish and completely validated bioanalytical methods should be used to yield reliable results that can be adequately interpreted.
2. To recognized the changes in the bioanalytical methods.
3. To highlight that each bioanalytical technique has its own features and characters, which will vary from analyte to analyte, specific validation criteria may need to be developed for each analyte
4. When sample analysis for a given study is carried out at more than one site, it is necessary to validate the bioanalytical method(s) at each site and provide suitable validation information for different sites to establish inter-laboratory reliability [82-85].

Types of Bioanalytical Method Validation

Full Validation: Full validation is done when developing a bio-analytical method for the first time. It is important for a new drug entity and if metabolites are new to an existing assay for quantification [86-90].

Partial Validation: Partial validations are modifications of previously validated bioanalytical methods. Partial validation can range from one intra-assay accuracy and precision determination to a nearly full validation. Bioanalytical method changes that fall into this category include [91-93]:

- Bioanalytical method transfers between laboratories or analysts
- Change in bioanalysis methodology (e.g., change in detection systems)
- Anticoagulant changes
- Within species if matrix changes (e.g., human plasma to human urine)
- If Sample processing procedures change
- Change in species within matrix (e.g., rat plasma to mouse plasma)

Cross Validation

Cross-validation is a evaluation and comparison of two bioanalytical methods; where an original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator [91,94-97].

Validation Parameters

System Suitability Test [97- 99]

System suitability test was carried out to verify that the analytical system is working appropriately and give accurate and precise result .It is perform prior to initiation of each analytical run instead of at the start of project. Circumstances where system suitability test is needed are as follows:-

1. After completion of repair of malfunction of chromatographic system in middle of project
2. On change of column in middle of project.
3. Interchange of system component in middle of project.

Method

System suitability test of the LC-MS system, to be used for validation is done by giving

a. One injection of reconstitution solution.

b. After that 6six injection of drug dilution.

c. Finally one injection of reconstitution solution was given.

Acceptance Criteria

%CV for peak area response ratio should be within 4%

%CV for retention should be within 5%

Selectivity [93,98,100]

Selectivity is the capability of an analytical method to discriminate and measure the analyte in the presence of other components in the sample. In selectivity, analyses of blank samples of the suitable biological matrix (plasma, urine, or other matrix) should be acquired from at least six sources. Each blank sample must be tested for obstruction, and selectivity should be ensured at the lower limit of quantification (LLOQ). Potential interfering substances in a biological matrix consist of endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is planned to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

Method

- Six lot of blank plasma were processed and run without addition of internal standard.
- Blank matrix with no or minimum peak area response at RT of all peaks of interest were selected.
- Analyte was spiked in selected blank matrix at LOQ concentration
- Six aliquot of spiked LOQ were processed. Response of interesting peak at retension time of the drug and internal standard in blank matrix was calculated.

Acceptance Criteria

» Response of interfering peak at the RT of analyte in blank matrix must be ≤ 20% of mean peak area response of analyte in LOQQC.

» Response of interfering peak at the RT of internal standard in blank matrix must be ≤ 5% of mean peak area response of internal standard in LOQQC.

» Atleast 80% screened matrix batches should pass.

» %CV should be ≤ 20% for both analyte and internal standard.

Sensitivity [101-103]

Sensitivity can be articulated as the slope of linear regression in the calibration curve, and it is calculated at the time in the linearity test. A technique is said to be sensitive if small changes in concentration cause large change in the response

function. The sensitivity achievable with an analytical method depends on the nature of the analyte and the revealing technique employed. The sensitivity required for a detailed response depends on the concentration to be calculated in the biological specimens generated in the specific study.

The lowest standard should be accepted as LOQ of the method if:-

1. Between batches precision (%CV) at LOQQC is ≤ 20%.
2. Between batches accuracy (% nominal) at LOQQC is 80-120%.
3. Analyte response at LOQQC is at least 5 times the response compared to blank matrix response.
4. S/N ratio of LOQQC sample should be at least 5 times of mean S/N ratio of black matrix samples.

S/N ratio was calculated by taking 4 replicate of spiked LOQQC samples and 4 replicate of pooled blank matrix samples. It was found greater than 5%.

Method

1. Process and analyze four replicate of spiked LOQQC samples and four replicates of pooled blank matrix sample.
2. Report S/N ratio for all and calculate mean of S/N ratio for pooled blank matrix sample
3. Compare the S/N ratio of each LOQQC with mean S/N ratio of blank matrix, it should be ≥ 5 for all LOQQC samples.

Precision and Accuracy [104-107]

Precision

The precision of an analytical method describe the closeness of individual measures of an analyte when the method is applied repetitively to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be considered using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended.

% CV = (Standard deviation/Mean value) x 100

Two Types of Precision

Inter-Day Precision

The ability to repeat the similar method under altered conditions, e.g. change of analyst, reagent, or equipment; or on subsequent occasions, e.g. over several weeks or months, is covered by the between batch precision or reproducibility, also known as inter-assay precision.

Intra-Day Precision

This is also known as repeatability i.e. the ability to repeat the same method with the same analyst, using the same reagent and equipment in a short interval of time, e.g. within a day and obtaining similar results.

Acceptance Criteria: The precision resolute at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LOQQC, where it should not exceed 20% of the CV.

Accuracy

The accuracy express as the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. By replicate analysis of samples containing known amounts of the analyte accuracy can be calculated. Accuracy should be calculated using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended.

% Nominal = (Mean concentration/Nominal concentration) x 100

Acceptance Criteria

The mean value must be within 15% of the actual value except at LOQQC, where it should not diverge by more than 20%. The difference of the mean from the true value serves as the measure of accuracy.

Method

Precision and accuracy of the method was evaluated by running three analytical batches. Determine within batch, between batch, inter-batch accuracy and precision. Each batch contained the following samples

- Reference standard solution (one sample, mixture with internal standard)
- Blank Matrix
- Blank Matrix with internal standard
- Spiked calibration standards (1 set of 8 non-zero concentration)
- LOQQC (6 samples)
- LQC (6 samples)

- ·MQC (6 samples)
- HQC (6samples)

Recovery [81,100,108]

The recovery can be define as 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 reliable standard. Recovery pertains to extraction efficiency of an analytical method. Recovery of the analyte need not to be 100% but extent of recovery of an analyte and of the internal standard is supposed to be consistent, precise and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, high) with unextracted standards that represent 100% recovery. The percentage recoveries for the drugs and the internal standard were determined by comparing the peak areas of the response of drug extracted with that of the peak areas of unextracted aqueous standard samples containing the same concentration of the drug and the internal standard. The percent recoveries were calculated at each QC concentration by the following equation:

% Recovery = Mean peak response of non-extracted samples/ Mean peak response of extracted samples X 100

Acceptance Criteria

The recovery is acceptable if CV is ≤ 20% for % mean recovery between low, middle & high QC concentrations.

Calibration/Standard Curve (Linearity) [89,102,109]

A calibration curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve is generated for each analyte in the sample. Adequate number of standards is used to adequately define the relationship between concentration and response. The biological matrix is same as the samples in the intended study by spiking the matrix with known concentrations of the analyte for calibration curve. The predictable range of analytical values and the nature of the analyte/ response relationship was the function of calibration curve.. A calibration curve be supposed to consist of a blank sample which has matrix sample processed without internal standard, a zero sample which has matrix sample processed with internal standard, and six to eight non-zero samples covering the expected range, including LLOQ.

The simplest model that sufficiently describes the concentration-response correlation should be used. Selection of weighting and use of a complex regression equation should be acceptable. The following conditions should be met in developing a calibration curve for particular analyte:

- ±20% deviation of the LLOQ from nominal concentration.
- ±15% deviation of standards other than LLOQ from nominal concentration.

At least four out of six non-zero standards must meet the above criteria, including the LLOQ and the calibration standard at the highest concentration.

Lower Limit of Quantification (LLOQ) [90,102,110]

The lowest standard on the calibration curve be supposed to be established as the limit of quantification. The analyte response at the LLOQ should be at least 5 times the response compared to blank response. Analyte peak (response) must be identifiable, distinct, and reproducible with a precision of 20% and accuracy of 80-120%.

Matrix Effect [94,105,111]

Direct or indirect alteration or intervention in response due to the existence of unintended analytes or other interfering material in sample is called matrix effect .Matrix effect calculated by comparing the response of extracted samples spiked before extraction with the response of the extracted blank matrix to which analyte has been added at the same supposed concentration just previous to injection. Matrix effect evaluated for six lots of plasma with aqueous dilution of LQC, MQC and HQC along with internal standard.

Method

Spike low and high QC samples into minimum six different batches of accepted blank matrix. Take 2 aliquot of LQC and HQC from each batch of blank matrix, add IS and process as per method SOP. Also prepare and process freshly spiked calibration standards .Inject CC standards and QC samples. The assessment of QC samples is back calculated against a calibration curve.

Acceptance Criteria

Mean concentration is inside ± 15% of nominal concentration at LQC and HQC level.

%CV should be ≤ 15% for LQC and HQC level

Matrix factor [103,108,112]

A quantitative evaluation of the matrix effect due to suppression or enhancement of ionization in a mass spectrometric detector is called as matrix factor.

Method

Prepared reference combination of analyte and internal standard at conc. representing 100% extraction of analyte and internal standard at LQC, MQC and HQC conc., used as reference samples. 12 aliquots of pooled plasma were taken and processed as per manner SOP without IS. For method not involving terminal drying step ,pipette out 12 processes pooled plasma samples and spike four aliquot each with IS and Analyte dilution to get concentration demonstrating 100% extraction of IS and Analyte at low, middle and high QC concentration. These samples serve as matrix samples reconstituted with reference sample. Inject these 12 samples along with 4 replicate of each reference mixture of IS and analyte at low, middle and high QC concentration .Tabulate peak area ratio of each .Calculate Matrix factor at each LQC, MQC, HQC concentration the following formula:

M.F = Mean peak area ratio of matrix samples reconstituted with reference samples/ Mean peak area ratio of reference samples X 100

Acceptance Criteria

% CV of matrix factor among LQC, MQC, and HQC level should be within ±15

Ruggedness [89,106,113]

It can be define as the degree of reproducibility of test results achieve by the analysis of the same samples under a range of background, which may be different laboratories, analysts, instruments, reagent lots.

Method

The ruggedness of the extraction process and the chromatographic process was calculated by analysis of a batch of six sets of quality control samples (including LOQQC) and a set of calibration standards using a new column by a changed analyst.

Acceptance Criteria

Same as precision accuracy batch

Stability [92,108,113-115]

Drug stability is a function of the storage situation, the chemical properties of the drug, the matrix and the container system. The stability of an analyte in a definite matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term and short-term storage and after going through freeze and thaw cycles and the analytical procedure. Setting used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The method should also include an assessment of analyte stability in stock solution.

The stability purpose must use a set of sample organized from a freshly made stock solution of the analyte in the suitable analyte free, interference free biological matrix .Stock solution of the analyte for stability estimate must be prepared in an suitable solvent at identified concentrations

Stock Solution Stability

Stock solution stability is the capability of a product maintain its composition and reliability after an intentional period of time

Method

Prepare analyte and IS solution and maintain aliquots of the same at refrigerated temperature. These shall serve as stability stock solutions. Following a particular storage period, prepare fresh stock solution of the analyte(s) and IS, these shall serve as evaluation stock solution. Inject 6 replicate from the vials of the stability stock dilutions and replicate from the comparison stock dilution. Tabulate the peak area response obtained from the stability and comparison stock dilution .Calculate the mean response, SD, %CV and %Stability.

% Stability = Mean peak area response of stability samples/ Mean peak area response of comparison samples X 100 X C.F

C.F = Concentration of stability samples/ Concentration of comparison samples

Acceptance Criteria

%Stability should be within the range of 85-115%

Freeze Thaw Stability

It is the ability of a product to uphold its

composition and reliability after repetitive cycles between freezing and ambient temperature levels. Even minor temperature variation can cause minor thawing of liquid within a product. Those ice crystals freeze at a big size, causing the breakdown of a products structure. Analyte stability should be resolute after three freeze and thaw cycles.

Method

Minimum of four aliquot at each of the low and high concentrations must be stored at the intended storage temperature for 24hrs and thawed unassisted at room temperature. When totally thawed, the sample should be refrozen for 12 to 24 hrs under the identical conditions. The freeze-thaw cycle should be repeated two or more times and the stability should be then analyzed on the third cycle .The QC concentrations are tabulated and the mean concentrations, SD, %CV and %nominal values are determined at low and high QC levels. If an analyte is not stable at the planned storage temperature, the stability sample should be frozen at -70^{0}C through the three freeze and thaw cycles

%Stability= Mean concentration of stability sample/ Mean concentration of comparison sample x C.F X 100

$$C.F.= \frac{\text{Stability Samples concentration}}{\text{Comparison Samples concentration}}$$

Acceptance Criteria: %Stability should be within the range of 85-115%

In-Injector Stability

The in injector stability duration is calculated as the time of injection of last QC sample less the time of their placement in auto injector.

Method

At least four aliquot at every low and high concentrations must be processed. The processed QC samples are placed in auto injector. The time of residency of QC samples must be recorded. Following the stability period four replicate of recently spiked LQO and HQC must be processed along with recently spiked calibration standard. Analyze the contrast QC, freshly spiked calibration standard along with stability QC samples. The in-injector stability was determined at LQC and HQC samples by analyzing 4 replicates at each level. The stability of the drug was resolute by back calculating the concentration of the stability samples against freshly processed calibration curve standards.

Acceptance Criteria: %Stability should be within the range of 85-115%

Bench Stop Stability

Stability of the drug in plasma at room temperature was examined by observance 4 sets LQC and HQC samples at room temperature for 4-24hrs. The concentration of the stability samples were considered and stability was evaluated by using a freshly prepare calibration curve and also by comparing against the set of QC samples at LQC and HQC level.

AcceptanceCcriteria: %Stability should be within the range of 85-115%

Re-Injector Stability

Any one of the three PA batch analyzed and meeting the acceptance criteria can be used for establishing reinjection reproducibility .Re-inject all LQC, MQC and HQC samples of the selected batch. Establish in injector stability duration for the intended period of time for which samples remained in auto injector. Calculate the re-injected QC concentration and determine the mean concentration, SD, %CV and %nominal values are determined at low and high QC concentration

Calculate % difference for each QC concentration.

$$\%\ \text{Difference} = \frac{\text{Absolute (original value} - \text{re-injected value)}}{\text{Original value}} \times 100$$

Acceptance Criteria: All the re-injected QC samples must meet the acceptance criteria of PA batch .The Reinjection Reproducibility is satisfactory if % difference of 80% of all QCs re-injected is within ±15%.

Conclusion

This review describes various aspects of the hyphenated technique; LC MS/MS, used for the bioanalysis. Bioanalytical method development and validation are required for the information of bioavailability and bioequivalence. These studies provide pharmacokinetic, toxicokinetic and metabolic data of drugs. Bioanalytical method development consists of sample preparation, chromatographic separation and detection by using LC MS/MS. Validation of a bioanalytical method comprises of various validation parameters which includes accuracy, precision, sensitivity, selectivity, standard curve, limits of quantification, range, recovery stability, etc. These studies can be carried

out by many analytical techniques including HPLC, UPLC, GC and LC MS/MS. LC MS/MS is most widely used analytical technique for bioanalytical method development and validation. Bioanalysis by LC MS/MS provides low detection limits, good ability to generate structural information, minimal sample requirement and wider coverage of range of analytes differing in their polarities.

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