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Review Article

ANALYSIS OF BIOLOGICAL SAMPLE BY LC-MS/MS: A COMPLETE REVIEW

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ABSTRACT

Biological sample analysis is an important aspect of medicinal product development for drug containing new active substances as well as generic products to get complete pharmacokinetic and toxicological profile which supporting the safety and efficacy of the drug substance or product. This study is mandatory, because it support new applications for drug approval by various respective regulatory authorities. As the sample concentration in biological matrix is very low, Hence LC-MS/MS is chosen as main instrument in this article, which support the analysis for quantitative application. This article provide complete review on the analysis of sample by proper selective biological matrix, sensitive analytical methods , recent advances in sample preparation and basic idea to select suitable technique for preparation of sample. It also provides a fundamental idea for assessing the reliability and overall performance of a bioanalytical method by validating the LC-MS/MS method based on various guidelines. Further various regulatory aspect of validation is also discussed in this paper.

KEY WORDS: *Biological sample, Bioanalytical method development, Validation, LC-MS/MS, Extraction.*

INTRODUCTION

Analysis of Biological sample is the measurement of drug concentration in various biological fluids. The main phases that comprise the analysis of biological sample are Method development, Method validation and Unknown Sample analysis (method application).

Bioanalytical methods is the process for quantitative determination of drugs and their metabolites in biological matrix (plasma, urine, saliva, serum etc) ,

It plays an innumerous role to generate the pharmacokinetic data by measuring bioavailability and hence bioequivalence.[1] The development of bioanalytical method is of paramount importance during the process of drug discovery and development, culminating in a marketing approval.

The reliability of bioanalytical methods is a matter of great importance in clinical and toxicological field because it is the prerequisite for correct interpretation of toxicological findings. Unreliable results might not only be contested in court, but could also lead to wrong treatment of the patient. Hence whatever way the analysis is done it must be checked to see whether it does what it was intended to do. Each step in the method must be investigated for the extent to which environment, matrix, or procedural variables can affect on the estimation of analyte in the biological media from the time of collection up to the time of analysis [2], Due to these facts importance of validation, at least of routine analytical methods, can therefore hardly be overestimated.

Moreover due to increased interdependence among countries in recent times it has become necessary for results of many methods to be accepted internationally. Consequently, to assure common level of quality, the need for and use of validated methods has increased [3]. Bio-analytical method is used to estimation of drugs in biological fluids for , Identification , Isolation and Quantification of different drugs and their metabolites from biological fluids .The ultimate is of detection techniques that should be highly sensitive and specific for the quantification of drugs. Both HPLC and LC-MS/MS are used for detection. HPLC itself coupled with UV, PDA or fluorescence detector is generally used but it does not give the high sensitivity as required by some of the potent, low dose drugs. The main advantages of LCMS-MS include low detection limits upto picogram, the ability to generate structural information, the requirement of minimal sample treatment and the possibility to cover a wide range of analyte differing in their polarities.[4]

Liquid chromatography linked to tandem mass spectrometry has played an important role in pharmacokinetics and metabolism studies at various drug development stages hence it is introduced to the pharmaceutical industry. Newly introduced techniques such as “ultra performance liquid chromatography” with small particles (sub-2µm) and monolithic chromatography offer improvements in speed, resolution and sensitivity compared

to conventional chromatographic techniques.[5]

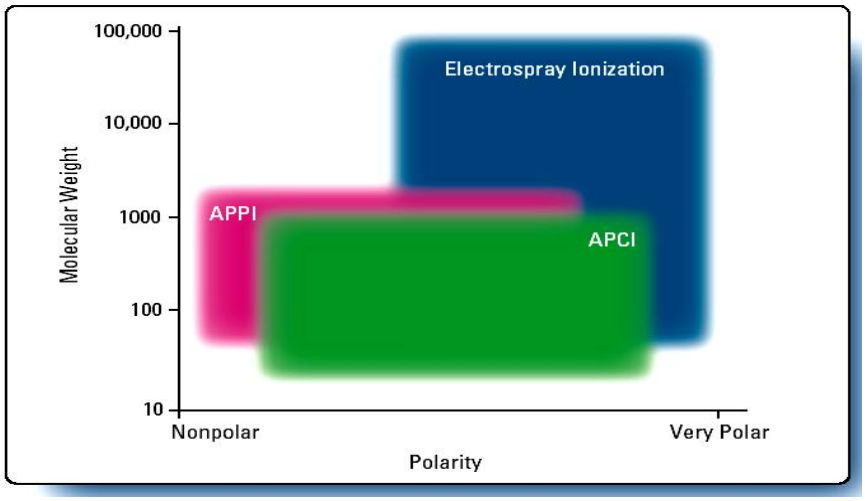
LIQUID CHROMATOGRAPHY MASS SPECTROMETRY[6, 7]

LC/MS is a powerful technique, combining the separation power of HPLC, or new modification of HPLC called UPLC with the detection power of mass spectrometry. The coupling of liquid chromatography is more delicate because gas-phase ions must be produced for mass spectrometry. For that reason an interface has been used to convert the liquid sample to gas phase ion. Liquid chromatography is not only used for compounds that are non volatile but also some thermoliable sample which are not suitable for gas chromatography[8]

Mass spectrometers can develop three dimensional data; they generate mass spectral data that can provide valuable information about the molecular weight, identity, quantity, and structure of a sample in addition to signal strength. For most compounds, a mass spectrometer is more sensitive and far more specific than all other LC detectors. It can analyze compounds that lack a suitable chromophore which is mandatory for UV detection. It reduced the need for perfect chromatography because it can identify components in unresolved chromatographic peaks.

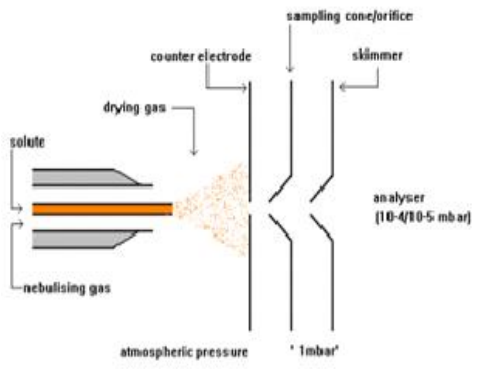
IONIZATION TECHNIQUES [8,9]

Different types of ionization techniques ESI, APCI, APPI are most commonly used . The mass spectrometric ionization techniques of electron ionization (EI) [11] and chemical ionization (CI) [12] required the analyte molecules to be present in the gaseous form. Hence there is the need for an interface that will eliminate the solvent and generate gas phase ions. Moreover the interface is the one which connect the LC to MS. Following diagram provides an idea in selection of proper ionization source.

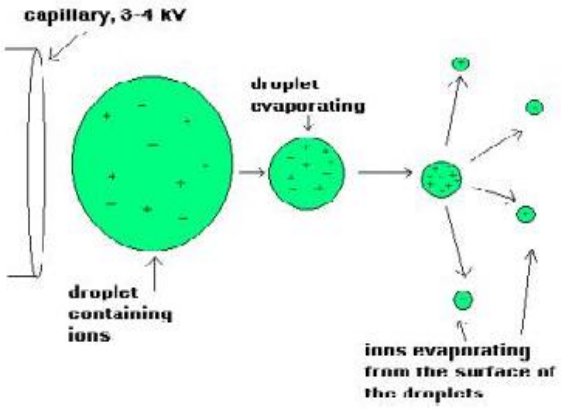


Electrospray Ionization [8.9]

Electrospray generates analyte ions from solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed (nebulized) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas. The electrostatic field causes further dissociation of the analyte molecules. The heated drying gas converted the solvent in to droplets to evaporate. As the droplets shrink, the charge concentration in the droplets increased. Eventually, the similar charge ion repulsive force exceeds the cohesive forces and ions are desorbed into the gas phase. These ions are attracted to and pass through a capillary sampling orifice into the mass analyzer



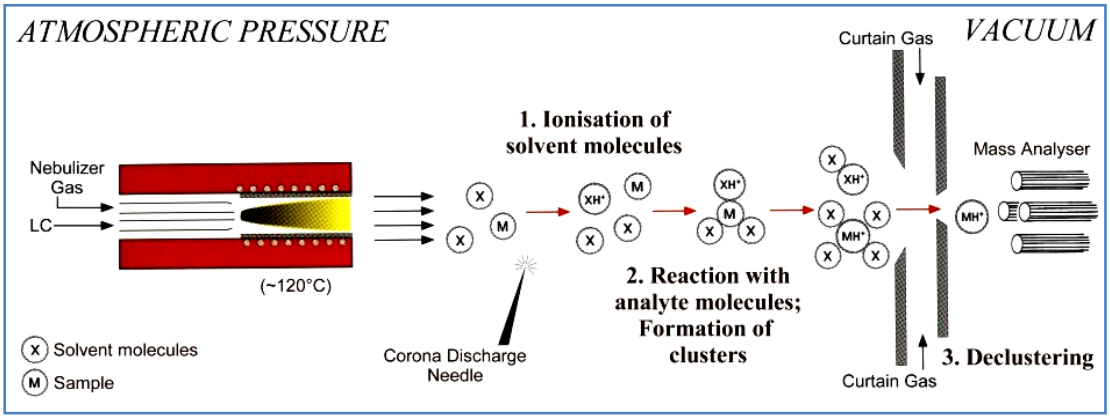
Standard electrospray ionisation source



The electrospray ionisation process

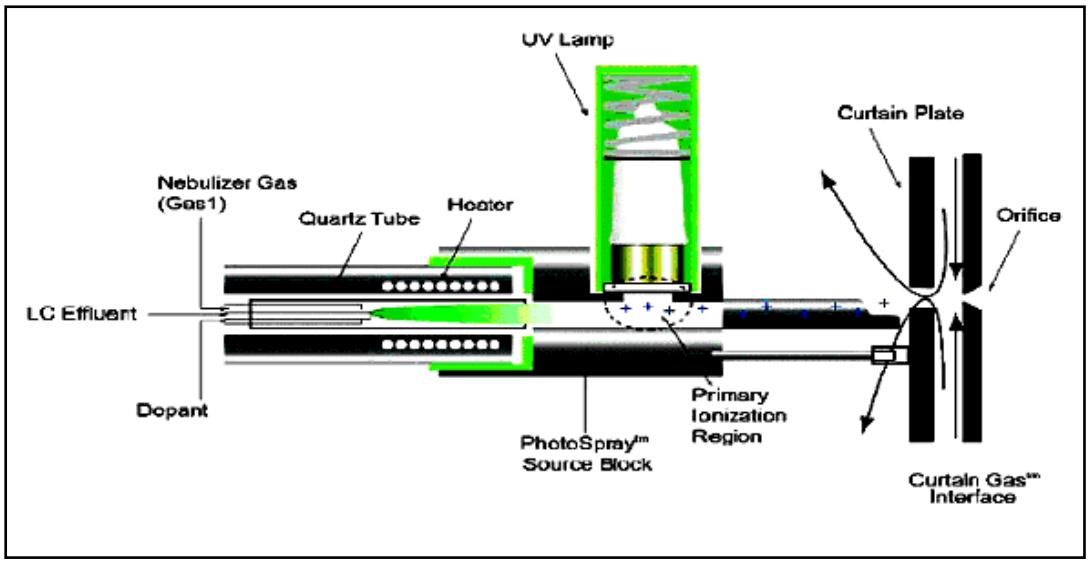
Atmospheric Pressure Chemical ionization[8, 9]

In APCI, the LC eluent is sprayed at atmospheric pressure through a heated (typically 250°C – 400°C) vaporizer. The heat vaporizes the liquid. As a result vaporized liquid converted to gas molecules which are further ionized by electrons discharged from a corona needle. The solvent ions then transfer charge to the analyte molecules through chemical reactions (chemical ionization).Then resultant analyte ions pass through a capillary sampling orifice into the mass analyzer. APCI is applicable to a wide range of polar and non polar molecules. It rarely results in multiple charging so it is typically used for molecules less than 1,500µ. Due to this, and because it involves high temperatures, APCI is less well-suited than electrospray for analysis of large biomolecules that may be thermally unstable. APCI is used with normal-phase chromatography more often than electrospray is because the analyte are usually nonpolar.



Atmospheric Pressure Photo ionization[8,9]

Atmospheric pressure photo ionization (APPI) is a relatively new technique. In APPI also a vaporizer converts the LC eluent to the gas phase. The only difference, a discharge lamp generates photons in a narrow range of ionization energies. The range of energies is carefully chosen to ionize as many analyte molecules as possible while minimizing the ionization of solvent molecules. The resulting ions pass through a capillary sampling orifice into the mass analyzer.



MASS ANALYZER (Quadrople) [8, 9]

A quadrople mass analyzer consists of four parallel rods arranged in a square. The analyte ions are directed down the center of the square. Voltages applied to the rods generate electromagnetic fields. These fields determine which mass-to-charge ratio of ions can pass through the filter at a given time. Quadroples tend to be the simplest and least expensive mass analyzers. In LC-MS/MS triple quadrople are used denoted as Q1, Q2,Q3. Among this three first will selectively scan parent ion where as 2nd causes the collusion deactivation of unwanted fragment .At last Q3 determine the most intense daughter ion.

Quadrople mass analyzers can operate in various modes:

- **Scanning (scan) mode**: The mass analyzer monitors a range of mass-to-charge ratios
- **Selected ion monitoring (SIM) mode**: SIM mode is significantly more sensitive than scan mode but provides information about fewer ions because the mass analyzer monitors only a few mass to-charge ratios
- **Selected/multiple reaction monitoring, MRM Mode**: This mode is applicable when there is more than one quadrople. Both of the analyzers are static in this case as user-selected specific ions are transmitted through the first analyzer and user-selected specific fragments arising from these ions are measured by the second analyzer. The compound under scrutiny must be known and have been well-characterized previously before this type of experiment is undertaken

METHOD DEVELOPMENT[6, 9,10, 13]

The method development of any biological assay needs to get through knowledge of the physiochemical and pharmacokinetic property of drug. Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification. Based on the information from the survey, the following can be done.

- Choice of instrument that is suitable for the analysis of analyte of interest.
- Choice of the column associated with instrument of choice, the detector and the mobile phase.
- Choice of internal standard, (It must have similar chromatographic properties of analyte.)
- Choice of extraction procedure, (which is time economical, gives the highest possible recovery without interference and has acceptable accuracy and precision.)
-

SELECTION OF BIOLOGICAL MEDIA

The most common samples obtained for biopharmaceutical analysis are blood, plasma and urine and Faeces. The choice of sampling media is determined largely by the nature of the drug study. A bioavailability study may require drug level data in blood or urine whereas a drug identification or drug abuse problem may be solved with any one type of biological sample. Faeces are utilized, especially if the drug or metabolite is poorly absorbed or extensively excreted in the bile.

Plasma is most favorable media for bioanalysis because drugs are more available in blood than any other media after absorption, as in blood clean up and extraction of drug is little difficult and the ultimate sensitivity and selectivity of the assay method may be limited by the efficiency of the cleanup methodology, plasma is more preferred. Blood, serum or plasma samples can be utilized for drug studies and may require protein denaturation steps before further manipulation.

Some drug like various immune suppressants are separated in RBC, in such cases blood is preferred.

Urine is easiest to obtain from the patient and also permits collection of large and frequently more concentrated samples. It can also identify components in unresolved chromatographic peaks. Because urine samples are readily obtained

and often provide the greatest source of metabolites, they are frequently analyzed in drug metabolism studies.

Saliva and biological media obtained from humans when constant ratio between plasma and salivary levels of certain drugs exists via non invasive sampling techniques. Saliva is advantageous in drug studies done with children. Although the concentrations of drugs in saliva are rarely equal to those in plasma, a constant ratio (over an effective therapeutic range) permits calculation of plasma levels based on salivary analysis.

ASSAY OF DRUGS AND THEIR METABOLITES[2]

Bio analytical assay is differ from normal analytical method because it needs through knowledge of the pharmacokinetics of the drug to distinguish drug and its metabolites..The method should have no of allusions that distinguish drugs from their metabolites. Drug metabolism reactions generally divided into phase I and phase II categories. Phase I typically involves oxidation, reduction, and hydrolysis reactions. In contrast, phase II transformations entail coupling or condensation of drugs. This involves glucoronidation, sulfation, amino-acid conjugation, acetylation, and methylation. Except for reduction processes, most phase I and phase II reactions yield metabolites that are more polar and hence more water soluble than the parent drug. Assays must distinguish between drug and its metabolites.

SELECTION OF INTERNAL STANDARD

An important issue in method development stage is the choice of internal versus external standardization. Internal standardization is common in LC-MS/MS methods .Especially with chromatographic procedures. For internal standard, a structural or isotopic analogue of the analyte was selected to the sample prior to sample pre-treatment. Further the ratio of the response of the analyte to that of the internal standard is plotted against the concentration.

SELECTION OF COLUMN[14]

The column must possess the selectivity, efficiency and reproducibility to provide good separation. During method development selection of column can be streamlined by starting with shorter columns (150,100 or even 50mm long.). By selecting a shorter column appropriate phase run time can be minimized. The primary basis of selection is the size of molecules. Small molecule needs column having small range of porosity For

example, a octyl phase (C8) can provide time saving separation over a octadecyl (C18) as it doesn't retain analytes as strongly as the C18 phase. The nature of the molecule also effect the selection, drug having higher pKa value needs basic column like beta basic column . Selecting an appropriate stationary phase can also help to improve the efficiency of method development. Stationary phases based on ultra pure silica gel is stable at high PH and it is preferred mostly on 6-9 pH range. If it is necessary to work at pH values above 8 with small bases as modifier (like Ammonia) must be there in column as base modifier.

OPTIMIZATION OF CHROMATOGRAM [14]

When reasonable chromatogram has been obtained Optimization can be started .The peak optimization can be done by using a column with higher theoretical plate, N which can be achieved by using a column of smaller particle size, or a longer column. These factors, however, will increase the analysis time. By changing flow rate the analysis time can changed.

SELECTION & OPTIMIZATION OF MOBILE PHASE

The main point in selection and optimization of mobile phase is to get optimum separation of all the individual impurities and degradrants from each other and from the analyte peak. These depend on elution strength of the mobile phase which is controlled by changing ionic strength, pH or type of anions. The mobile phases used in LC are typically aqueous salt solutions. The following condition should be taken in mind to get optimum separation

Compatibility with the detection mode - Suppressed or Non-suppressed: The selection should be done in such a way that the detector signal obtained by the background, i.e., the mobile phase itself, must not be too high.

Mobile phase's pH

The Mobile phase's pH is a key parameter in determination of its characteristics, as it influences the charges on both the mobile phase's ions and the solute ions. The effect of pH is most important in the separations of anions, ionizations most effected part. With pH the charge on the acid anion increases, so the eluting power of weak acid eluents increases with pH until the acid is completely dissociated. The reverse is for weak bases in the mobile phase. A higher degree of protonation occurs with

decreasing pH and the mobile phase becomes a stronger eluent. hence increase their retention. Examples of solutes showing these effects are F-, CO32-, PO43-, SiO32-, CN- and amines. When these ions are present in mixtures with other ions that show no pH dependency, the control of mobile phase's pH becomes an important variable to be manipulated in the optimization of the separation.

Buffering capacity of mobile phase [15]

Since both mobile phase's and solutes' can be effected by the pH, the buffering capacity of the mobile phase is very important, and should be maintained high. Polyprotic solute ions' retention can be significantly changed with pH, as their charge can increase from singly to doubly and triply charged. In such cases it is very important to make sure that the mobile phase pH is kept constant, using high capacity buffers. Some of the most, commonly employed buffers are: a) phosphate buffers prepared using salts like potassium dihydrogen phosphate, sodium dihydrogen phosphate etc. b) phosphoric acid buffers which are prepared using ortho-phosphoric acid (are not used in mass spectrometry since they have the tendency to get ionized on ion source) and c) acetate buffers (ammonium acetate, sodium acetate, etc) and acetic acid buffers prepared using acetic acid.[16]

EXTRACTION OF DRUG FROM ITH MATRIX

Liquid liquid extraction

Liquid-liquid extraction is the most widely used technique where it is possible to remove a drug or metabolite from larger concentrations of endogenous materials that might interfere with the final analytical determination. In this process the analye of interest is collected in non polar organic solvent after extraction. The selection of proper solvent is most important to get sufficient recovery. The solvent are selected on the basis of the log P value of the drug and solvent. The most used solvent are TBME, dichloromethane, ethyl acetate, N hexane etc. The technique is simple, rapid, and has a relatively small cost factor per sample. The extract containing the drug is then evaporated to dryness, and the residue can be reconstituted in a smaller volume of a more appropriate solvent particularly polar solvent as the most pharmaceutical drug separation is done by reverse phase chromatography. In this manner, the sample becomes more compatible with a particular analytical methodology in the measurement step, such as a mobile phase in LCMS/MS determinations. The

extracted material can be reconstituted in small volumes (e.g., 100 to 500 µl of solvent), thereby extending the sensitivity limits of an assay. It is possible to extract more than one sample concurrently. Quantitative recoveries (90% or better) of most drugs can be obtained through multiple or continuous extractions.

Protein precipitation method:

It is most widely used and simple method particularly used when the matrix are blood, plasma, serum. But the method is restricted to only those molecules which have very less plasma protein binding affinity. The protein precipitation provides the denaturation of protein causes the easy separation of drug from endogenous material of blood or serum. Precipitation of proteins can be done by changing the pH of sample by using inorganic reagents such as. Perchloric acid, trichloro-acetic acid etc. which causes the PH to reach the iso-electric point .At iso-electric pH, proteins have no net charge, results precipitation. Addition of organic solvent Methanol, Acetonitrile also induce precipitation by decreasing the dielectric constant of the medium, leads to insolubility thus cause precipitation. By using salt one can induce precipitation. Salts used for precipitation of proteins are citrates, phosphates, acetates, etc. At high concentration of salts solubility of proteins drop sharply, thus precipitates[17].After protein precipitation the supernatant obtained can be injected directly or it can be evaporated and reconstituted with the mobile phase and further clean up of the samples [18].

Solid phase extraction:

It is the most important technique used in sample preparation of bioanalysis. SPE occur between a solid phase and a liquid phase. SPE is most important because it provide higher recovery of analyte. Basic mechanism is selective adsorption. If the targeted analyte are adsorbed on the solid stationary phase, they can selectively be eluted by using an appropriate elution solvent. There are various types of commercially available solid phase called cartages. These are short inert plastic tube packed with an adsorbent, generally a reversed phase or an ion exchange resin[19].

Conditioning is the first step of SPE where all tubes are conditioned with appropriate solvents prior to sample application. Condition causes the activation of the sites of stationary phase bed. MeOH, small volumes of DCM, TBME or any other organic solvent, water, buffer are used for condition based on the need and mode of retention. Followed by

addition of suitable buffering reagent to the sample, with proper mixing. This step makes ready the sample to apply and called Sample Pre-treatment. Then Sample Application. The sample is applied from the top of the cartridge at a slow flow rate without any brake, which is necessary to allow analyte to interact with adsorbent thus to achieve the retention of analytes. Care must be taken to assure that no sample drop should remain on the inner wall of the cartridges.

Rinsing or Washing is the main step because by this step only removal of matrix components or other interferences is possible. Hence it is considered as proper clean up step

The cartridge with relative weak dilute solvent or solvent mixtures or buffer and interferences that are weakly retained than the analytes are drained out from the cartridge. Rinsing or washing solvents are water, buffers of different pH. Then the drying, which is intended for the removal of excess washing solvents/buffers Drying can be done by applying appropriate vacuum for recommended time period with the help of vacuum pump, recommended drying time is 2-3 minutes. Drying is done for following reason.

1. Avoid any interaction/precipitation possibility
2. Avoid any blockage of cartridges due to air bubble formation during elution

The ultimate step is the removal of analyte called elution which is done by passing of strong solvent through cartridge at a slow flow rate thus allowing more soak time on the packing of reach maximum extraction efficiency, solvents used are MeOH , ACN, small volumes of DCM, MTBE or any other organic solvent in combination with MeOH or ACN. It is used for cleavage of weak bonds formed between analyte and sorbent, usage of multiple small volumes is recommended to improve elution. Ultimately this final solution can be injected.

TUNING OF LC-MS/MS FOR DETECTION OF DRUG AND INTERNAL STANDARD.

As mass spectra are used as detection parameter hence, it must be set up correctly. Tuning the mass spectrometer involves optimizing voltages, currents, flows, and the ion source parameters to achieve the maximum mass spectral sensitivity and proper resolution. Generally tuning can be carried out by preparing nanogram concentration of the analyte of interest and then this concentration is infused in full scan mode.

From the result, m/z of parent ion was selected. This is called as Q1. Both negative and positive mode is there but most preferred mode is positive, which will generate M^+ parent ion and its positive fragment. Then for fragmentation of parent ion, infused the stock dilution in product ion mode and checked. m/z of various daughter ions obtained. Prominent and suitable daughter ion selected based on the response, this is generally called as Q3 ion. Finally both Q1and Q3 is optimized by changing various parameter of detector ,ion sources. E.g.:- Source dependent parameters:- Nebulizer gas, curtain Gas-2, Temperature and Ion spray voltage for APCI. Re-optimize the parameters and finally the parameter selected when the selected daughter ion shows maximum stable response. Then the m/z of selected parent and daughter ionis kept in Multiple Reaction Monitoring (MRM) mode . The similar is for the internal standard. Which will together use for detection when the drug is injected in mobile phase.

METHOD VALIDATION[3,10,21]

The search for the reliable range of a method and continuous application of this knowledge is called validation. It can also be defined as the process of documenting that the method under consideration is suitable for its intended purpose.

Method validation involves all the procedures required to demonstrate that a particular method for quantitative determination of the concentration of an analyte (or a series of analytes) in a particular biological matrix is reliable for the intended application. Validation is also a proof of the repeatability, specificity and suitability of the method. Bioanalytical methods must be validated if the results are used to support the registration of a new drug or a new formulation of an existing one. Validation is required to demonstrate the performance of the method and reliability of results. If a bioanalytical method is claimed to be for quantitative biomedical application, then it is important to ensure that a minimum package of validation experiments has been conducted and yields satisfactory results.

THE REGULATORY PROSPECTIVE OF VALIDATION

Method validation has received considerable attention in the literature and from industrial committees and regulatory agencies. The U.S. FDA CGMP[22] request in section 21 CFR 320.29 methods to be validated: The accuracy, sensitivity, specificity, and reproducibility of test methods employed by the firm shall be established and documented. Such validation and documentation may be

accomplished in accordance with Sec. 211.194 (a). These requirements include a statement of each method used in testing the sample to meet proper standards of accuracy and reliability, as applied to the tested product. The U.S. FDA has also proposed industry guidance for bioanalytical procedures and methods validation[23].A general guidance on analytical method validation was given at the 1990 AAPS/FDAworkshop [24] In 2000, new workshops addressed the validation of bioanalytical methods as well as the special case of assays dedicated to macromolecules[25] .ISO/IEC 17025 includes a chapter on the validation of methods[26] with a list of nine validation parameters. The International Conference on Harmonization (ICH)[27] has developed a consensus text on the validation of any analytical procedures. The document includes definitions for eight validation characteristics. ICH also developed guidance with detailed methodology[28].The United States Environmental Protection Agency (USEPA) prepared guidance for method's development and validation for the Resource Conservation and Recovery Act (RCRA) [29]. The AOAC, EPA and other scientific organizations provided methods validated through multi-laboratory studies. The implications of this bio analytical validation requirements in clinical and forensic toxicology is also addressed in various paper [30].

Validations are subdivided into the following three categories[3]:

Full Validation

Full validation should be performed to support pharmacokinetic, bioequivalence, and drug interaction studies in a new drug application (NDA).It is the validation performed when a bioanalytical method is implemented for the first time.

Partial Validation

Partial validations are performed when modifications is necessary of already validated bioanalytical methods. Partial validation includes as little as one intra-assay and precision determination to a nearly full validation depending on the modification. For example method transfers between laboratories, change of matrix within species or between the species and change in methodology. Which parameters to be revalidated are decided based on the logical consideration of the specific validation parameters which may affect due to change made to the bioanalytical method.

Cross Validation

Cross validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies.

VALIDATION PARAMETER

Pre method validation:

Before starting the validation by LC-MS/MS instrument one must check the System sensitivity, Auto sampler performance, and Calibration curve acceptance by a trial precision and accuracy batch and recovery after extraction. These all are come under validation parameter but first these parameter must be checked.

CALIBRATION STANDARD, QUALITY CONTROL SAMPLE AND LINEARITY:

Curve fitting procedures for bioanalytical assays are based on classical linear least squares (LSE) theory[31]. The calibration curve provides the best relationship between the detector response and concentration. Acceptable back calculated concentrations for the standards should be used to fit the calibration curve (C.C). If a weighting factor is used, it should be defined during validation. Generally in bio analytical technique the selection of linear calibration curve range is done based on the expected maximum drug concentration of that particular drug in that particular dose which needs to assayed [32-35] . Along with the CC concentration Quality control standard should be prepared from same stock where CC is prepared. The quality control sample acts as reference of calibration curve standard. In validation minimum six Calibrations standard are used but for better performance upto nine calibration standard can be used .Quality control sample are generally taken from high mid and low region of Calibration range and generally designated as HQC, MQC, LQC, LLOW. LLOQ is the lower limit of quantification. In most cases Calibration standards are calculated in such a way that highest point is double or triple of Cmax (maximum drug concentration) and LLOQ is 10% of Cmax.

Linearity is the phenomenon which represent that all Calibration standard must gives response where the response is directly proportional to the concentration range. Linearity is to be cheeked in both directly before extraction to see the accuracy of stock preparation, and after extraction to see the efficacy of method and determination of unknown concentration.

SYSTEM SUITABILITY

This test is performed in order to check suitability of system with optimized final condition and to maintain performance of changing environment.

The percent coefficient of variation for peak area ratio of analyte and internal standard and for retention time should be under the acceptance criteria i.e. %CV must be less than 5% for LCMS/MS procedures.

AUTO SAMPLER CARRIES OVER TEST (ASCOT)

In LCMS/MS instrument the sample is injected by the auto-sampler which itself automatically takes injection one after another in pre determined manner. The performance of auto sampler must be checked because if there is any interference of previous injection then the batch will failed .The ASCOT is performed by injecting an aqueous blank(without spiked drug) then highest aqueous concentration and then again blank then lowest aqueous concentration. No blank should show any interference response. The similar process is done on extracted sample where the blank is the matrix without drug[3].

PRECISION [3, 33]

The precision of a bioanalytical method is the measure of random error which is defined as the agreement between replicate measurements of the same sample. It is expressed as the percentage coefficient of variance (% CV) or relative standard deviation (R.S.D.) of the replicate measurements.

$$\% CV = \frac{\text{Standard deviation}}{\text{Mean}}$$

Within the batch Precision

This is also known as repeatability i.e. the ability to repeat the same procedure 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.

Between the batch Precision

It is the ability of a method to reproduce the same under different conditions, e.g. change of analyst, 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. The reproducibility of a method is generally got prime importance to the analyst since this will give a better representation of the precision during routine use as it includes the variability from a greater number of sources.

A minimum of four calibration concentrations in the range of expected concentrations should be match. The %CV determined at each concentration level, should be ±15 % except for the LLOQ, where it is ±20% as per US FDA guideline.

ACCURACY

The accuracy of a bioanalytical method is a measure of the systematic error or bias and is defined as the agreement between the measured value and the true value. Accuracy is best reported as percentage bias that is calculated from the formula[37]:

$$\% \text{ Bias} = \frac{\text{Measured value} - \text{True value}}{\text{True Value}} \times 100$$

Some of the possible error sources causing biased measurement are generally error in sampling, sample preparation, preparation of calibration line and sample analysis. The method accuracy can be studied by comparing the results of a method with results obtained, by analysis of certified reference material (CRM) or standard reference material (SRM).

Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended.

The mean value should be ± 15 % of the actual value except at LLOQ, where it should be ± 20 %.

PRECISION AND ACCURACY BATCH

As per various guideline minimum three Precision and accuracy batch must be performed. Each batch must run according to the following order- A calibration curve consisting a blank sample (matrix sample processed without the IS), a zero standard (matrix sample processed with internal standard), and 6 to 8 non zero calibration standard and followed by at least 3 level of six replicate of each quality control standard.

RECOVERY

The guidelines of the Journal of Chromatography B require the determination of the recovery for analyte and internal standard at high and low concentrations[38]. Recovery of a bioanalytical method means the measured response of a processed spiked matrix standard expressed as a percentage of the response of a pure standard, which has not been subjected to extraction. Good precision and accuracy can be obtained

from methods with moderate recoveries. It may be desirable to intentionally sacrifice high recovery in order to achieve better selectivity with some sample extraction procedure.

$$\% \text{ Recovery} = \frac{\text{Extracted sample Response}}{\text{Un-extracted sample response.}} \times 100$$

SELECTIVITY

Method selectivity is the ability of a method to produce a response for the target analyte distinguishing it from all other interferences. Interferences in biological samples arise from a number of endogenous (analyte metabolite, degradation products, co-administered drugs and chemicals normally accruing in biological fluids) and exogenous sources (impurities in reagents and dirty lab-ware). Zero level interference of the analyte is desired, but it is hardly ever the case. The main thing one must take care of is that, the response of the blank biological matrix standards should not be greater than the response from the LLOQ(Lower Limit of Quantification) by a defined factor. If all the efforts to get rid of interferences in the chromatographic process fail, changing to a more selective detector such as Mass Spectrometry (MS) or MS-MS may give a better result.

The following practical approach may be used during method development to investigate the selectivity of methods. Processing blank samples from different sources will help to demonstrate lack of interference from substances native to the biological sample but not from the analyte metabolite. Processing of reagent blank in the absence of biological matrix is normally adequate to demonstrate selectivity with regard to exogenous interferences mentioned above.

Although it would be preferable that all tested blanks, if obtained under controlled conditions, be free from interferences, factors like food and beverage intake and cigarette smoking can affect selectivity. Evaluate a minimum of six matrix sources to approve the selectivity of the method. The blank matrix should not give more than 1/5 of LLOQ response. As it checked the matrix interferences hence it is also called Screening of biological matrix.

MATRIX EFFECT

Matrix effect is carried out particularly for LC-MS/MS instrument. MS detector measure the response by the principle ionization. In addition of desired ions generated by tuning the sample others ions present in the biological matrix may also ionized in source at the same tuning

parameter and can cause ion suppression/enhancement. As a result false response may come. In matrix effect 6 different lots of blank plasma were processed along in duplicate and reconstituted with aqueous LQC and aqueous HQC of each set to determine the effect of matrix with drug and internal standard and it is calculated by comparing the response of extracted samples spiked before extraction with response of the blank matrix.

SESITIVITY

Sensitivity of the method is defined as the lowest concentration that can be measured with an acceptable limit of accuracy and precision. Although on the basis of Cmax the lowest concentration is calculated, but that concentration must be validated to check the instrument is capable to detect that concentration or not. The accuracy and precision at the lower limit of quantitation (LLOQ) is determined by analyzing at least 5 replicates of the sample at the LLOQ concentration. The accuracy as determined by the relative error (RE%) at this concentration should be within ± 20% and the CV should be less than 20%.

DILUTION INTEGRETY

The basic goal of bio analytical method development and validation is to determine the unknown concentration of the drug in the selective biological media chosen for selective purpose. But it is very difficult to predict whether all samples will fall within the calculated linear range. For this reason the integrity of dilution must be checked. It is performed by processing one or more additional QC (quality control) samples at concentrations two to four times higher than the upper limit of the calibration curve, covering the maximum expected dilution. These QC samples are diluted with blank matrix to bring the concentration to within the calibration range and then analyzed. The precision and accuracy of diluted QC sample within 15% of the calculated concentration.

LIPIDEMIC AND HEMOLYSED MATRIX EFFECT

This parameter is most useful when the sample matrix is plasma. This experiment determines the effect of hemolysed matrix on the analyte response. It is evaluated by processing at least six replicate HQC and LLOQ concentration by adding a 2% hemolysed matrix along with by a normal plasma and difference of response is calculated.

STABILITY

The stability of the analyte is often critical in biological samples even over a

short period of time. Degradation is usual even when all precautions are taken to avoid specifically known stability problems of the analyte (e.g. light sensitivity). It is therefore important to verify that there is not sample degradation between the time of collection of the sample and their analysis that would compromise the result of the study. Stability evaluation is done to show that the concentration of analyte at the time of analysis corresponds to the concentration of the analyte at the time of sampling.

An essential aspect of method validation is to demonstrate that analyte is stable in the biological matrix and in all solvents encountered during the sample work-up process, under the conditions to which study samples will be subjected.

According to the recommendations on the Washington conference report by Shah et al.(1992), the stability of the analyte in matrix at ambient temperature should be evaluated over a time that encompasses the duration of typical sample preparation, sample handling and run time. Similarly Dagar & Brunett (1995) gave the following details to be investigated.

LONG TERM STABILITY

This is done to assess whether the analyte is stable in the plasma matrix under the sample storage conditions for the time period required for the samples generated in a clinical study to be analyzed.

BENCH TOP STABILITY

Six replicates of low (LQC) and high (HQC) quality control samples were left at room temperature for 12 hours (stability samples). A calibration curve and 6 replicates of low and high quality control samples (comparison samples) were freshly processed along with the stability samples and analyzed in a single run.

AUTO SAMPLER STABILITY

Samples prepared at low (LQC) and high (HQC) quality control levels were extracted as per the procedure and kept in the auto sampler (stability samples). A calibration curve were freshly processed and analyzed with 6 replicates of stability samples in a run. Concentrations were calculated to determine % nominal over time.

FREEZE -THAW STABILITY

This stability test is done to ensure that the sample remains stable after it is subjected to multiple freeze-thaw cycles in the process of the study. This can be done by thawing samples at high and low concentrations unassisted and allowing them to freeze again for at least 12-24 hrs.

The cycle is repeated twice and the sample is processed at the end of the four cycles and its result is compared with freshly prepared sample.

CONCLUTION

The biological samples analysis is not restricted to the pure and neat analyte instead it needs through knowledge of the behavior of the drug sample in the biological matrix. Proper selection of cleaning of sample from biological matrix is most important in addition to the detection technique. Reliable method gives reliable result hence selection of method needs through knowledge on regarding the method selection and analyte response. Care should be taken to include responses into the models that are not affected by unexpected effects. This article provides some fundamental idea to the analyst to select proper method and sample preparation technique along with validation parameter.

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