



## REVIEW ON DEVELOPMENT AND VALIDATION OF BIOANALYTICAL METHOD BASED ON LC-MS/MS TECHNIQUE

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### ABSTRACT

In this review article, the bioanalytical methods are broadly used to give quantity of drugs and its metabolites in the matrices of plasma and this methods should be applied to studies in areas of human clinical and non-human evaluation. Bioanalytical method employ for that quantitative assessment of drugs along with their biological media in metabolites and plays an important role in assessment and construal of bioequivalence, pharmacokinetic, and toxic kinetic study. The foremost bioanalytical role is method development, its method validation, and sample analysis. Each step in that process required to be properly investigated to fix on the level to which surroundings, matrix, or else procedural variables that can obstruct the evaluation of analyte in the matrix from the time of set up to the time of analysis. Techniques such as high pressure liquid chromatography (HPLC) and liquid chromatography coupled with double mass spectrometry (LCMS-MS) can be used for the bioanalysis of drugs in body. The instruments has its individual advantages and disadvantages. Chromatographic methods of HPLC and having a gas chromatography that are for the most part used for bioanalysis of small as well as large molecules, with LC/MS/MS. Linearity, accuracy, precision; selectivity, sensitivity, reproducibility, and stability are some of the commonly used parameters. In this bioanalytical method review article, we are anticipated to append a few points on the subject to its method development and validation parameters, of assistance to quality assurance to determine the drug, concentration and its metabolite.

**KEYWORDS:** Bioavailability, Bioequivalence, extraction technique, LC-MS/MS.

### INTRODUCTION

Bioavailability it is defined as, “the rate and extent to which the active constituent or active moiety from the drug is absorbed and having development onto the site of action”. Or “the rate and extent of absorption of unaffected drug from its dosage form”.

#### Objectives of Bioavailability Studies

1. Most important development in stages it is an appropriate new drug entity of dosage form.
2. New development of formulation of the existing drugs.
3. purpose of influence of patient correlated factors, excipients and possible interface with further drugs on the effectiveness of absorption absolute bioavailability.

#### Bioequivalence

“The lack of a considerable dissimilarity in the rate at which the active ingredient or else active moiety in pharmaceutical equivalent become existing at the site of drug action, when administered at the same molar dose under related conditions in an properly designed study.

### Bioanalysis

Bioanalysis is the term generally used to describe the quantitative measurement of a compound (drug) or their metabolites in biological fluids, primarily blood, plasma, serum, urine, faeces.

#### Need for Biopharmaceutical Analysis

Methods of measuring drugs in biological media are increasingly important related to following studies. These are highly dependent on biopharmaceutical analytical methodology.

- Bioavailability and Bioequivalence studies
- New drug development
- Clinical pharmacokinetics
- Research in basic biomedical and pharmaceutical sciences
- Therapeutic drug monitoring

#### A. Bioavailability and Bioequivalence studies

There are two different types of bioavailability studies. The first type involves an evaluation of the bioavailability of a new drug formulation. That is

pharmacokinetics consideration following special routes of administration of the innovative drug are obtained are utilized in mounting in optimum prescribed amount regimen. Finally, the new drug is formulated correctness for projected routes of administration and its bioavailability is assessed.

The second type of bioavailability study involves a comparison of a test formulation with that of reference standard dosage form that is proved to have therapeutic efficacy and safety. This type of study is known as bioequivalence studies.

### B. New drug development

A New Drug Application (NDA) is regulatory mechanism that is designed to give that the Food and Drug Administration (FDA) sufficient information to make a meaningful evaluation of a new drug.

### C. Clinical pharmacokinetics

Clinical pharmacokinetics attempts to afford both a more quantitative correlation involving dose and effect and the agenda with which to interpret capacity of concentrations of drugs in biological fluids. The importance of pharmacokinetics in patient care rests on the enhancement in efficacy that can be attained by attention to its principles when doses regimens are preferred and modified. The three most important parameters are clearance, volume of distribution, bioavailability.

### D. Research in basic biomedical and pharmaceutical sciences

It should be used in main stages of expansion of a suitable dosage forms for an innovative drug unit, development of new formulations of the offered drugs, determination of influence of excipients, patient related factors and probable communication with extra drugs on the competence of absorption.

### E. Therapeutic drug monitoring

There is at times marked variability between patients in their pharmacokinetics parameters and in drug responsiveness. This renders the concept of generally accepted 'therapeutic dose' rather inaccurate and invalid. An alternative would be to monitor and maintain drug concentration at level which is necessary to produce a therapeutic effect. Such a procedure is designed as therapeutic drug monitoring (TDM).

### Primary Treatment of biological samples

Biological matrices e.g. Plasma, Serum, Urine, Faeces, Saliva, Bile and sweat are associated with diverse physiological characteristics and texture. Each of these samples has its own set of factors that must be considered before an appropriate pretreatment method can be selected. Such factors as texture and chemical composition of the sample, degree of drug protein binding, chemical stability, types of interferences can affect the final measurement step. Primary treatment of biological samples is as follows:

### Hydrolysis of Conjugates

The drug metabolite presents in the form of conjugate such as sulphate, glucuronides. These conjugates cannot be ignored so therefore it may be important to isolate the actual conjugates. They are usually treated by:

- Enzymatic Hydrolysis
- Acid Hydrolysis

Nonspecific hydrolysis is done by heating a sample for 30 minutes at 100°C in 2 to 5 N HCl. Then cooled and metabolite removed by solvent extraction procedure. Stable conjugate requires hydrolysis in autoclave.

### Homogenization

The samples which include insoluble protein, such as muscle or other linked tissues, a homogenization/solubilizing step have need of by using 1N hydrochloric acid. Intended for gelatinous sample it can be completed by sonication. A solid sample such as faeces can be homogenized by using least amount of alcohol. It generally performed by a blade homogenizer.

### Protein Precipitation

Biological sample such as plasma contains significant amount of protein which can bind a drug. The drug may have to be free from protein before further manipulation. Protein precipitation is significant because the being there of lipid salts and also interfere the assay. In protein precipitation acid or water miscible organic solvent are used to remove protein by denaturation and precipitation. Generally used solvent for protein precipitation are methanol, acetonitrile, trichloro acetic acid, and perchloroacetic acid and ammonium sulphate.

### Challenges in Bioanalysis

- **Matrix-**Plasma, serum, urine and tissues are common matrices which have differing implication for cleanup and accounting for interferences in the sample.
- **Assay Specificity:** - Co-administered drug may influence the selectivity of assay.
- **Urgency-**To meet a required short turnaround time.
- **Number of analyte to be quantified-**If there are many analytes, this will determine the complication to the partition and the span of the method development process.
- **Required level of sensitivity-**The primary consideration should be that detection and quantification of lower level of analyte of possible.
- **Pharmacokinetic profile of the analyte-**An understanding pharmacokinetic profile will allow the analyst to determine the lower level of quantification required.

Apart from above mentioned points following aspects are also important in bioanalytical method development.

1. Bioavailability, half-life, metabolism and excretion of drug play a key role in measurement of drug from plasma or serum.

2. For the revival of drug from biological matrices, different organic solvents must be tried.
3. In order to minimize the matrix effect altered sample dealing out techniques should be useful like protein precipitation, solid phase extraction and liquid-liquid extraction.

### Extraction procedure for drugs and metabolite from biological samples

After pre-treating biological samples, the next step is usually the extraction of the drugs from the biological samples. For extraction of biological samples various methods used are as follows;

#### 1. Protein precipitation

Biological samples such as plasma contains significant amount of protein which can bind a drug. The drug may have to be freed from protein before further manipulation. Protein precipitation is significant because the protein, lipid, salts and additional endogenous objects can cause rapid deterioration of HPLC columns and also interfere the assay. In protein precipitation acid or water miscible organic solvents are used to take out protein by denaturation and precipitation.

Generally used solvents for protein precipitation are methanol, acetonitrile, trichloro acetic acid, perchloro acetic acid, ammonium sulphate.

#### 2. Liquid-Liquid Extraction

The basic principal of liquid-liquid extraction is that a sample is distributed or partitioned between two immiscible liquids or phases in which the compound and matrix have divers solubility's. Usually, one phase is aqueous and the further stage is an organic solvent. The basis of the extraction process is that the more polar hydrophilic compounds prefer the aqueous phase and the more non-polar hydrophobic compounds prefer the organic solvent.

Liquid-liquid extraction is possibly the most widely used technique because,

- The analyst can remove a drug or metabolite from larger attention of endogenous matter that might interfere with the final investigative determination.
- The technique is simple, rapid and has a relatively small cost factor per sample.
- The extraction solvent containing drug can be evaporated to drought and the residue can be re-dissolved in a slighter volume of a more appropriate solvent. In this comportment, the sample becomes well-suited with a particular analytical methodology in the measurement step, such as a mobile phase in HPLC determination.
- Close to quantitative recoveries (>90%) of most drug preserve to obtained throughout several or constant extraction.

LLE is the direct extraction of biological material with water immiscible solvents. The analyte is isolated by

partitioning between aqueous phase and organic phase. Partition or distribution of a drug between two immiscible liquid phases can be expressed in terms of a distribution coefficient.

### Factors affecting the partition coefficient

Factors that affect partition coefficient and recovery of drug in extract are choice of solvent, pH and ionic strength of the aqueous phase.

In almost all cases, one of the liquid phases is aqueous and the second is the organic. It is highly describing to select organic solvent which shows great affinity for the analyte to be analyzed, and leaves contaminants in the aqueous or biological phases. The solvent should be immiscible with the aqueous phase and should have less polarity than water. It should also have less viscosity.

The solvent should have low boiling point to facilitate the removal at the end of extraction, low viscosity to facilitate mixing with sample matrix. Other considerations are cost, toxicity, flammability and the nature of the solvent. It is accepted that Tertiary Butyl Methyl Ether (TBME), diethyl ether and chloroform are the solvent which is used for acidic & basic drug especially when sample is unknown. Proper pH adjustment of a sample permits quantitative conversion of the ionized drug to the unionized species, which is more soluble in a non polar solvent. The suitable pH for extraction can be considered from the Henderson-Hasselbalch equation with the pKa of the compound.

$$\text{pH} = \text{pK}_a + \log_{10} \left( \frac{[\text{conjugate base}]}{[\text{conjugate acid}]} \right) = \text{pK}_a + \log_{10} \left( \frac{[\text{proton acceptor}]}{[\text{proton donor}]} \right)$$

Third factor influencing extraction of drug is ionic strength. Addition of highly water soluble ionized salts such as NaCl, to an aqueous phase creates a high degree of interaction between the water molecule & the inorganic ion in solution. Fewer water molecules are free to interact with the unionized drug. Therefore, the solubility of the drug in the aqueous phase decreases and increases the partitioning in the non-polar or organic phase. This technique is commonly called as salting out. Emulsification is often experiential when organic solvents are used at basic pH whereas certain organic solvents such as n-hexane and diethyl ether are less emulsion-prone.

The most important factor in distribution in organic phase is relative lipophilicity or hydrophobicity of analyte must be soluble in which the analyte is soluble.

The extraction procedure can be controlled using pH ionized compounds are less efficiently partitioned in organic phase. Shifting the pH enables the processes to be inverted, the charged analyte re extracted into aqueous phase for advance purification. The use of pH control allows the fractionation of sample into acid, neutral acid and basic components.

### 1. Solid Phase Extraction (SPE)

In solid phase extraction the analyte is retained on solid phase sorbent which samples passed through it, followed by elution of analyte with suitable solvent. A usual SPE sorbent contain of 40-60 $\mu$ m silica particles having in which has a bonded hydrocarbon phase. This achieved bonding by response of chlorosilane through the silica gel hydroxyl group that results are silicon-oxygen-silicon relation.

- Solid phase taking out is typically proper for polar compounds that would or else tend to remain in aqueous phase. The method is also useful for amphoteric compounds that cannot be extracted easily from water.
- Factor leading the elution and adsorption of drug commencing the resin column incorporate with solvent polarity, flow rate of the solvent during the column and the degree of make contact with the solvent has with the resin beds.
- Samples of biological that can be prepared for cleanup by transient the sample during the resin bed anywhere drug component are adsorbed and to end with eluted with a suitable solvent.
- collective sorbent for acidic or basic or neutral compounds. It is completed from stability ratio of two monomers the hydrophilic nucleophilic vinyl pyrrolidine and lipophilic divinyl benzene.
- These sorbents are opposed to the pH extremities, show astonishing retention of polar compounds, three times relative retention ability as balance to conventional SPE silica based sorbent like C18.

#### Advantages

- 1) Effective in selective removal of interferences enabling selective, sensitive and robust.
- 2) SPE enable the enrichment of selected analytes without concentrating interferences.
- 3) SPE technology join with robotic mechanization create not single cost valuable but also time competent sample preparation system which

improves analytical system presentation by correlate with the analyte in a MS compatible solvents.

- 4) extend the systematic column life, compact system maintenance, minimize the ion restraint while civilizing signal response.

#### Introduction to Chromatographic method

Chromatography is separation technique that is based on differing affinities of a mixture of solutes between at least two phases. The result is a physical separation of the mixture interested in its various components. The affinities or communications can be classify in terms of a solute adhere to the surface of a polar solid (adsorption), a solute dissolve in a liquid (partition), and a solute passing through or impeded by a porous substance based on its molecular size.

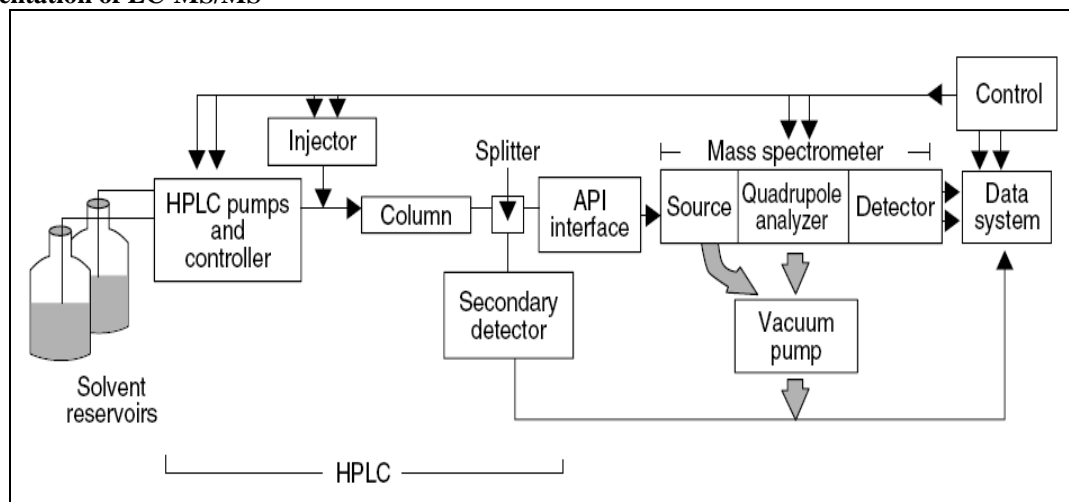
#### Liquid chromatography and mass spectrometer

It is the way of interfacing of high vacuum domain of mass spectrometer with the condensed phase domain of liquid chromatography.

#### Need of LC system interfacing with mass spectrometer

The separation is absolute prior to mass study for the reason that the mass spectrometer is not sufficiently expert to directly shaping every analyte in all type samples. The liquid chromatography can be regarded as part of preparative procedure required for sample cleanup up regarded as part of Preparative procedure required for sample cleanup which improves linearity, accuracy and better sensitivity. Spectrometer of mass provides larger selectivity and also sensitivity for the chromatographic development for the reason that endogenous matrix can co-elute among analyte up till now not interferes on condition that these constituent possess precursor masses. The most important intention of interface is to disperse the mobile phase and shift the analyte from the higher pressure/ atmospheric pressure at which chromatographic taking apart is achieved to the lower pressure essential for the mass analysis.

#### Instrumentation of LC-MS/MS





### 1. Sample inlet system

There are mainly two types of sample inlet system that are,

- The sample introduces as neutral group during a restricted vacuum leak followed by ionization in vacuum chamber.
- construct the ion at atmospheric pressure along with the introduced the ion in to the mass spectrometer during a controlled vacuum leak with aid of electrostatic field, this process called as API (atmospheric pressure ionization) it provides proper way, when a forceful coupling of liquid chromatography done.

API is largely responsible for dramatic growth of mass spectrometry in, so API-LC/MS –MS is now days commonly used.

### 1. Ionization Source

Ionization proceeds by two fundamental processes:

1. Loss/gain of electron
2. Loss/gain of charged particle

An odd electron ion is generated by the loss /gain of an electron. In vacuum generating method ionic species of identical nominal molecular weight differ only by the mass of an electron to the neutral species from which it was generated. An even electron is produced by gain or loss of even electron species from a molecule.

There are four common modes of ionization

- Atmospheric Pressure Ionization (API)
- Electron Ionization (EI)
- Chemical Ionization (CI)
- Matrix-Assisted Laser Desorption Ionization (MALDI)

In LC-MS/MS Interfacing Atmospheric Pressure Ionization (API) is the potential ionization technique because,

- It gives softer ionization.
- It provides convenient interface with liquid chromatography.
- The less fragmentation of the molecular ions and a convenient interface with liquid chromatography at ambient pressure to mass.
- Spectrometry at high vacuum.

### 3. Types of API Source

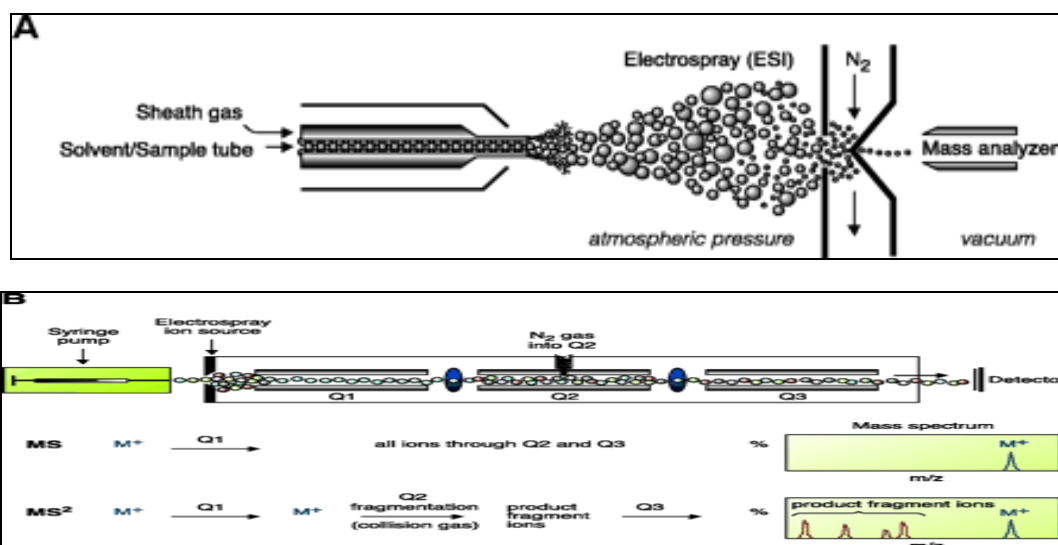
There are two types of API source

- Atmospheric Pressure Electro Spray Ionization (ESI)
- Atmospheric Pressure Chemical Ionization (APCI)

#### Atmospheric pressure Electro Spray Ionization (ESI)

It is an atmospheric ionization technique in which ions are generated in the solution phase by evaporation of carrier solvent and ion are produces in gas phase. An Appropriate solvent from Lc (liquid chromatography) system is passed through a metal capillary to which a static DC voltage is applied to create ionization of effluents. When the solvent got evaporate the charge density increases creating columbic repulsion and subsequent dissociation of droplet. Further evaporation of droplet creates an environment in which charge transfer takes place from the solvent to the analyte.

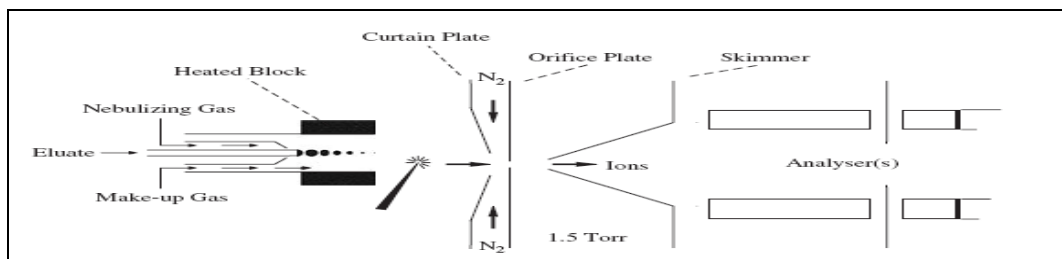
Typically, a voltage of 2.5 to 5 kV will be applied to generate an even electron ion in gas phase. This method is commonly used for high molecular compounds. Presently most of the designs on coaxial gas flow (nitrogen) which improves desolvation. These sources with coaxial gas flow are called nebulization assisted electro spray ionization. Low ionization is observed in this technique due to solvent elustering and analyte adduct formation, so this is most applicable for LC-MS/MS system.



### Atmospheric pressure chemical ionization

It is an ionization technique in which the ionization occurs not in vacuum but at atmospheric pressure. It is gas phase ionization process whereby gas molecules are

isolated from the carrier solvent before ionization. Generally, less polar compounds are ionized by this method.

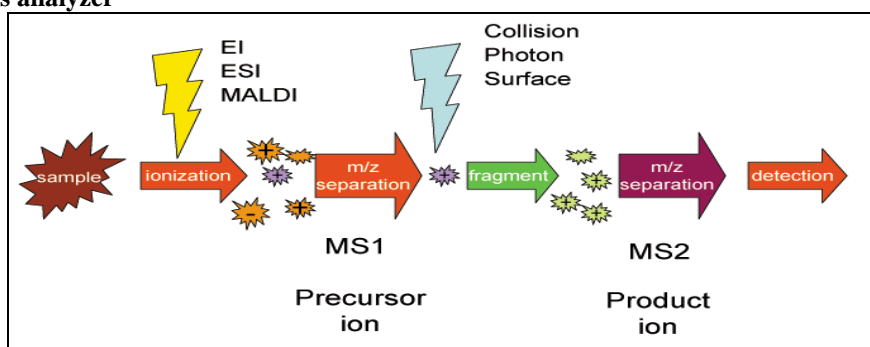


### Mass analyzer

Any mass analyzer contains electrical or magnetic field or combination of two, which is capable of manipulating the trajectory of ions in a vacuum chamber. For any mass

analyzer the analyte particle should be charged in order to separate the ion from the other ions. There are so many types of analyzers used in case of LC-MS/MS.

### Quadra pole mass analyzer



It consists of two pair of electricity conducting rods/ electrodes on to which a concurrent ration frequency (RF) and direct current (DC) voltage is applied. The rod is made of molybdenum or gold, having 1cm diameter and 30cm length. Opposite quadruples pairs are connected electrically such that they carry identical RF/DC ratio. For a given RF/DC voltage ratio the quadrupole analyzer filter only the ion within a selected mass/charge ratio has a trajectory as they pass from one quadrupole rod set to other. If RF/DC is held constant only a narrow  $m/z$  range will transmit the quadrupolein this centrifugation quadrupole mass analyzer act as a mass filter or quadrupole filter. In case of bioanalysis it is used as a tandem (MS/MS).

It consists of capillary interface for liquid introduction, a heated nebulization system and a high voltage corona needle, it requires high liquid flow rate. Nebulization of effluent is done by heated gas flow (nitrogen) generating gas phase molecule of both (effluent and analyte). A metal needle is placed between nebulization probe and first vacuum orifice.

A DC voltage is applied on needle which develops a charge from atmosphere and evaporated solvent, leads to production of ions in gas phase. The process of introducing the ions into the mass analyzer through differential pumping and their subsequent separation and

detection are identical. This method used to improve ionization efficiency. This method is particularly useful for non-polaranalyte or weakly polar analyte in normal phase. Tandem means when two or more analyzers either of different type (TOF/Q) or the same type (Q/Q) configured. The quadrupole mass analyzer configured in tandem commonly called Triple quadrupole mass analyzer.

In between the two quadrupole a collision cell is used as a false quadrupole for the fragmentation of precursor ions. This cell is either hexapole or octapole not true quadrupole so this configuration is called as triple quadrupole.

### Ionization polarity

In API method either positive or negative charge ion can be generated. The type of ions generated is dependent on the charge of adduct gain or loss during ionization. A positive DC voltage on the capillary will generate positive ion from the basic compound such as amine will favor positively charged ion (protonated). A negative DC voltage on the capillary will generate negative ion from the acidic compound such as carboxylic acid will favor negatively charged ion (deprotonated).

**Tandem mass spectrometry**

Tandem mass spectrometry is also called as mass spectrometry- mass spectrometry (MS/MS) because the instrument contains two mass analyzer in tandem. A collision cell is placed between the two analyzers.

The basic approach of MS/MS is the measurement of mass to charge ratio ions before and after the fragmentation of selected ions in collision cell with high pressure gas. This collision process is known as collision induced dissociation.

The tandem arrangement allows the  $m/z$  relating to be selected at the second quadrupole. This arrangement provides good selectivity and sensitivity. The first quadrupole is used to select the parent ion and fragmentation occurs in the collision cell. The fragmented ion goes to second quadrupole, which select the ions of the specific reaction product only. In this process the internal energy of analyte is increasing which induce the fragmentation this is achieved by collision activation. Collision with neutral gas molecules called Collision Induced Dissociation (CID).

***CID is a two-step process***

First collision occurs when an ion's translational energy is converted into internal energy to obtain an ion in excited state and a secondary slow unimolecular decomposition occurs which yields various ion products from a number of competitive reactions. As a result, there is increase in internal energy which leads to the fragmentation of ions. In case of CID, the fragmentation is induced in a collision cell with higher pressure. In principle two mass analyzers are required; one is for selecting the precursor ions and other for analyzing the product ions after the collision.

**Detector**

The detector either measure the ions current directly or with possible single amplification or using ion conversion followed by solid state amplification. The solid state detectors are Electron multiplier, multichannel plate detector, photomultiplier. These are the modern type of detector, photomultiplier. These are the modern type of detectors mostly used in MS system. It utilizes the dynode to convert ions signal into secondary particle before amplification. Signal response is a function of ions mass and kinetic energy. In case of LC/MS where quadrupole using as a mass analyzer, photomultiplier is used as a detector.

**Photomultiplier**

It provides signal detection through conversion and subsequent amplification. The conversion dynodes of a multiplier detector generate electron that impinge on a phosphorescent screen, which subsequently generate photon that are detected and amplified.

The mass spectrometer that utilizes the photomultiplier must be isolated from ambient light because it is sensitive to light background.

**Modes of LC-MS/MS monitoring**

Typically, the mass spectrometer is set to scan a specific mass range. This mass scan can be wide as in the full scan analysis or can be very narrow as in selected ion monitoring. A single mass scan can be acquired during an LC/MS analysis. LC/MS data is represented by adding up the ion current in the intensity point against time. The most common modes of acquiring LC/MS data are:

- Full scan acquisition resulting in the typical total ion current plot (TIC)
- Selected Ion Monitoring (SIM)
- Selected Reaction Monitoring (SRM)
- Multiple Reaction Monitoring (MRM)

**Full Scan Mode**

The MS total ion current plot similar to HPLC-UV traces apart from the fact that the mass spectrometer can analyzed many more components. The ion current is a plot of total ion existing in each MS check plotted as a strength point. As a small molecule or peptide elutes from the HPLC column the comparative intensity goes up and a peak appears in the total ion current scheme as the points (TIC of scan) are plotted adjacent to time.

**Selected Ion Monitoring (SIM)**

In selected ion monitoring in which the mass spectrometer is lay down on the search over a especially undersized mass range, usually only mass unit. The current of ion in a SIM plot that resulting from that is in a very small mass range. The reason is that the peaks that are showing in that SIM plot very small works in the TIC plot. In which the SIM plot is a further detailed plot than the complete scan TIC plot. The SIM experiment is more sensitive because the mass spectrometer can settle for a longer time over a less significant mass range.

**Selected Reaction Monitoring (SRM)**

A preferred reaction monitoring is the method used by the majority of scientists performing mass spectrometric Quantitation. SRM delivers a unique fragment ion that can be monitored and quantified in the midst of a very complicated matrix. SRM plots are very simple. This characteristic makes the SRM plot ideal for sensitive and specific quantification.

**The Multiple Reactions Monitoring (MRM)**

The Multiple Reaction Monitoring (MRM) experiment is accomplished by specifying the parent mass of the compound for MS/MS fragmentation and then specially monitoring for a signal fragment ion.

**Bioanalytical Method Development and Validation**

A bioanalytical method is a set of all of the procedures involved in the collection, processing, storing, and

analysis of a biological matrix for an analyte. Analytical methods employed for quantitative determination of drugs and their metabolite in biological fluids are the key determinants in generating reproducible and reliable data that in turn are used in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetics.

### Method Development for LC-MS/MS

Various stages involved in method development are,

- Literature survey
- Stock solution preparation and tuning of analyte
- Selection and tuning of Internal standard
- Selection and optimization of chromatographic conditions
- Selection and optimization of Extraction method

### Literature survey

In general, the process of method Development being with literature survey of the drug/metabolite with the help of available books, reference, published articles, internal search.

Literature survey should contain following information about the drug/metabolite:

- Molecular weight, molecular formula
- Physicochemical properties such as solubility, pKa, C<sub>max</sub>, LLOQ, protein binding, Strength and dosage.
- Pharmacology and pharmacokinetic properties.
- Analytical method if any, specifying the chromatographic condition such as mobile phase, temperature, flow rate, column, internal standard.

### Stock solution preparation and tuning of analyte

Prepare the stock solution and its dilution of suitable concentration by using appropriate solvent and diluents. These stock dilutions for ex. 500ng/mL to 1000ng/mL are used for setting tuning process. Select an ion source (ESI or APCI) and ion mode (positive or negative) based on chemical properties of the compound. Infuse suitable stock dilution in full scan mode. Select the m/z of the parent ion based on the molecular weight of the compound (If molecular weight of the compound is 200 then m/z of the parent ion in positive mode is 201 and 199 in negative mode). Select the m/z of parent ion. Then for fragmentation of parent ion, inject suitable stock dilution in product ion mode and check for m/z of various daughter ions obtained. Select the prominent and suitable daughter ions by altering the various parameters. For ex. Compound dependent parameter are DP, FP, EP, CE, CXP source dependent parameter – Nebulizer Gas, Curtain Gas, Temperature and ion spray voltage then inject suitable concentration using m/z of selected parent and daughter ions in multiple reaction monitoring (MRM) mode. {Shah, 1992 #4}.

### Selection and optimization of Chromatographic conditions

Column choosing is according to polarity of drug. For polar drugs non-polar stationary phase such as C-18, C-8, -CN, -NH<sub>2</sub> are used, while for nonpolar drugs polar stationary phases such as porous silica column is used.

Different size column should also be tried i.e. 50mm, 75mm, 100mm, 150mm. Internal diameter of the column is also being also important, mostly 3.5µm, 4.6µm are used. Select the best column based on the based on the retention time, peak shape and response.

Optimize the mobile phase composition by altering the followings

Buffer salt, Buffer concentration, Buffer pH, Solvent, Solvent proportion. Ionic samples (acidic or basic) can be separated only, if they are present in undissociated form. Dissociation of ionic samples can be suppressed by selection of proper pH.

### Selection and optimization of Extraction method

Process blank matrix samples along with spiked middle point of calibration curve range by following extraction techniques.

Choose a column and sample processing techniques which gives best possible retention time, peak shape, response, maximum recovery and no significant interference.

Process and inject blank matrix sample to check for any late eluting interference. Optimize the run time to avoid presence of late eluting interference in consecutive injections.

### Selection and tuning of internal standard (I.S)

Selection of the I.S based on; Detectable under chromatographic conditions and getting extracted in extraction procedure of the main compound. No significant interference at the retention time of internal standard in the processed blank matrix sample.

### Bioanalytical Method validation

Method validation can be defined (as per ICH Guideline) as “Establishing documented evidence, which provides a high degree of assurance that a specific method or activity will consistently produces a desired result or product meeting its predetermined specification quality characteristics”.

Selective and sensitive analytical method for the quantitative evaluation of drug and their metabolite (analyte) are critical for the successful conduct.

Bioanalytical method validation includes all of the procedure that demonstrate that a particular method used for quantitative measurement of analyte in a given biological matrix, such as blood, plasma, serum and urine is reliable and reproducible for the intended use.



The process by which a specific bioanalytical method is developed, validated and used in routine sample analysis can be divided into

1. Reference standard preparation
2. Bioanalytical method development establishment of assay procedure
3. Application of validated bioanalytical method to routine drug analysis and acceptance criteria for the analytical run or batch.

#### Types of method validation

- Full validation
- Partial validation
- Cross validation

#### Full validation

Full validation is essential when on the increase and implementing a bioanalytical method for the first time. Full validation is significant for a new drug creature. Full validation of the revised assay, if metabolites are other to the presented assay for quantification.

#### Partial validation

Partial validation is modification of earlier validated bioanalytical method. the range of Partial validation it is small like one intra assay, accuracy and precision purpose to a almost full validation.

A Bioanalytical method change includes:

- a) Changes in analytical methodology.
- b) Changes in matrix within species.
- c) Changes in sample processing procedure.
- d) Changes in sample instrument or software platform.
- e) Limited sample volume.

#### Cross validation

Cross validation is evaluation of validation parameters in the bioanalytical methods there is an two or more method that are used to produce data within the same or across diverse studies. Cross validation is to site where a unique validated bioanalytical method that gives the actual and the revised bioanalytical method is the comparator.

#### CONCLUSION

Bioanalysis and the production of pharmacokinetic, toxic kinetic and metabolic data plays a essential role in pharmaceutical research, progress involved in the drug discovery and development process. An attempt has been made to understand and explain the bioanalytical method development and validation from a quality assurance department point view. Some of the method and how is validation carried out were described in different situations encountered in the study sample analysis has been reported in this article. These various essential development and validation characteristics for bioanalytical methodology have been discussed with a view to improving the standard and acceptance in this area of research.

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