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A REVIEW ON LC-MS/MS IN BIOANALYTICAL STUDIES

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ABSTRACT

Liquid chromatography-mass spectrometry (LC-MS) is now became a routine technique with the development of electrospray ionisation (ESI) providing a simple and robust interface. It is applicable to a wide range of biological molecules and the use of tandem MS and stable isotope internal standards allows highly sensitive and accurate methods to be developed through some method optimisation to minimise ion suppression effects. The Method development of sound bioanalytical method(s) and its Method validation is of important during the process of drug discovery and development. The objective of this paper is to review the sample preparation of drug in biological matrix and to provide practical approaches for determining selectivity, specificity, limit of detection, lower limit of quantitation, linearity, range, accuracy, precision, recovery, stability, ruggedness, and robustness of liquid chromatographic methods to support pharmacokinetic (PK), toxicokinetic, bioavailability, and bioequivalence studies. Bioanalysis, employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, PK, and toxicokinetic studies. Selective and sensitive analytical methods for quantitative evaluation of drugs and their metabolites are critical for the successful conduct of pre-clinical and/or biopharmaceutics and clinical pharmacology studies.

Keywords: Bionalytical method development, validation parameters, LC-MS/MS.

INTRODUCTION [1-7]

Bioanalysis is a sub-discipline of analytical chemistry covering the quantitative measurement of xenobiotics (drugs and their metabolites, and biological molecules in unnatural locations or concentrations) and biotics (macromolecules, proteins, DNA, large molecule drugs, metabolites) in biological systems. Many scientific endeavours are dependent upon accurate

quantification of drugs and endogenous substances in biological samples; the focus of bio-analysis in the pharmaceutical industry is to provide a quantitative measurement of active drug and/or its metabolite(s) for the purpose of pharmacokinetics, toxicokinetics, bioequivalence and exposure–response (pharmacokinetics/pharmacodynamics studies). The 1930s also saw the rise of pharmacokinetics, and as such the desire for more specific assays. Over the last twenty five years, pharmacokinetics has emerged as an integral part of drug development, especially when identifying a drugs biological property. The most important property of any non-intravenous dosage form, intended to treat a systemic condition, is the ability to deliver the active ingredient to the blood stream in an amount sufficient to cause the desired response. This property of a dosage form has been identified as *physiologic availability, biologic availability or bioavailability*. Modern drugs are more potent, which has required more sensitive bioanalytical assays for accurate and reliable determination of these drugs at lower concentrations. This has driven the improvements in technology and analytical methods.

The blank matrix should be obtained from a reputable source and must have proper identification of species and matrix type, lot number, storage information and expiration dating. The matrix physiological properties (lipemic, haemolysed, etc.) can also be important to note. If an anticoagulant is used, it should be selected based on the known properties of the analyte, should not impart undesirable properties such as significant changes in pH, instability, precipitation or gel formation, and should be consistent for the entire toxicology or clinical program. If a stabilizer or enzyme inhibitor is required, it should not interfere with quantitation of the analyte. A priori acceptance criteria must be documented and are often driven by SOPs, but sound scientific judgment, based upon empirically derived method development data should dictate when modifications are warranted prior to finalization of the study protocol/study plan and execution of validation experiments.

The concentration range over which the analyte will be determined must be defined in the bioanalytical method, based on the evaluation of actual standard samples over the range, including their statistical variation. This defines the *standard curve*. It is necessary to use a sufficient number of standards to define adequately the relationship between concentration and response. The relationship between response and concentration must be demonstrated to be continuous and reproducible. The number of standards to be used will be a function of the dynamic range and nature of the concentration-response relationship. In many cases, five to

eight concentrations (excluding blank values) may define the standard curve. More standard concentrations may be necessary for nonlinear than for linear relationships. The ability, in terms of accuracy and precision, to dilute samples originally above the upper limit of the standard curve should be demonstrated in the validation. The accuracy and precision with which known concentrations of an analyte in biological matrix can be determined, must be demonstrated. This can be accomplished by analysis of replicate sets of analyte samples of known concentrations −quality control (QC) samples—from an equivalent biological matrix. At a minimum, three concentrations representing the entire range of the standard curve should be studied: one within 3× of the LLOQ (low QC sample), one near the centre (middle QC), and one near the upper boundary of the standard curve (high QC).

A *"bioanalytical method"* is a set of procedures involved in the collection, processing, storage, and analysis of a biological matrix for a chemical compound. The availability of selective and sensitive bioanalytical methods is a prerequisite for the generation of reliable data on pharmacokinetics, bioavailability, and bioequivalence of drugs. These methods should allow the quantification of drugs and their metabolites in biological matrices, e.g. plasma, urine, and cerebrospinal fluid, and must be validated with respect to their reliability for the intended use. Bioanalytical method validation comprises all criteria determining data quality, such as selectivity, accuracy, precision, recovery, sensitivity, and stability. This study is applicable for the analysis of preclinical as well as clinical samples. A specific, unique chemical moiety that would be found in a biological matrix is referred to as analyte. Some Bioanalytical Techniques commonly used in bioanalytical studies include: Hyphenated techniques, Chromatographic methods, Electrophoresis, Ligand binding assays, Mass spectrometry, Nuclear magnetic resonance. The bioanalytical sample analysis is categorized into two major processes: 1) Method Development 2) Method Validation

INTRODUCTION TO BIOANALYTICAL METHOD DEVELOPMENT

Quantitative determination of drugs and their metabolites in biological fluids is crucial during drug discovery and development. LC-MS is the preferred methodology for that purpose. The LC-MS method development is carried out by the following steps:

1. Considering the physicochemical properties of the analyte, such as chemical structure, functional group(s), molecular weight, purity, solubility, stability
2. Determination of the solubility in the required solutions

3. MS or MS/MS scanning and optimization, e.g. Electron Spray Ionization (ESI) or Atmospheric Pressure Chemical Ionization (APCI)
4. Development and optimization of the LC method

1. Selection of the best chromatography method (RP- (reversed phase) or NP- (normal phase) chromatography), selecting of a suitable column, temperature
2. Mobile phase selection and optimization (choosing best buffers, pH, flow rate)
3. Selection of the best internal standard, which possesses similar ionization response and similar chromatographic retention time as the requested substance
5. Development of sample preparation method - Selection of extraction method and optimization: The most critical step in the development of LC-MS methods is the sample preparation to obtain homogenous solutions suitable for injection onto column, as well as low ion suppression for reliable MS detection. The approaches of sample preparation in bioanalytical processes are: Solid-phase extraction (SPE), liquid-liquid extraction (LLE), protein precipitation techniques (PPT), filtration etc.

OPTIMIZATION OF LC-MS METHOD

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts asymmetry, capacity factor, elution time, detection limits, limit of quantification and overall ability to quantify the specific analyte of interest.

The various parameters to be optimized during method development are:

A) Selection of mode of separation B) Selection of column C) Selection of mobile phase

Selection of Mode of Separation

Modes of acquiring and visualizing LC/MS data: 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 take anywhere from 10 ms to 1 s depending on the type of scan. Many scans are acquired during an LC/MS analysis. LC/MS data is represented by adding up the ion current in the individual mass scans and plotting that "total" ion current as an intensity point against time.

The most common modes of acquiring LC/MS data are:

(1) Full scan acquisition resulting in the typical total ion current plot (TIC)

(2) Selected Ion Monitoring (SIM)

(3) Selected Reaction Monitoring (SRM) or multiple reaction monitoring (MRM).

The nature of the analyte is the primary factor in the selection of the mode of separation. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. The redesigned innovative turbo ion source includes the unique ability to switch ionization modes. The capability to rapidly switch between ionization modes is expected to increase system productivity and flexibility. Detection was performed on electro spray ionization (ESI), triple quadrupole mass spectrometer equipped with an ESI interface operated in positive and negative ionization mode. The selective reaction monitoring mode (SRM/MRM) is used to provide MS/MS detection. The selection of ion source is based on the chemical properties of the compound. If the drug is acid then negative mode is selected. If the drug is a basic compound, then positive ion mode is selected. Best suited is negative ion mode due to acidic nature of the compound.

Table No:1 The Following Table Shows The Process Of MS/MS Scan Modes In SRM/MRM

Precursor Ion Scan	**Neutral Loss Scan**	**Selected Reaction Monitoring (SRM)**
The ions formed in the ion source are introduced into Q1	The ions formed in the ion source are introduced into Q1	The ions formed in the ion source are introduced into Q1
Q1 scans a wide mass range	Q1 scans a wide mass range	Q1 selects the specific parent mass
Ions are fragmented into product ions in Q2	Ions are fragmented into product ions in Q2	Ions are fragmented into product ions in Q2
Q3 only transmits the mass of the selected fragment	Q3 nearly scans the same mass range as Q1; the mass range is lowered by the mass of a neutral fragment	Q3 selects the specific fragment mass
Spectrum illustrates all parent ions that produce a selected charged fragment	Spectrum illustrates all parent ions that loose a selected neutral fragment	
Mass Chromatogram shows the masses of precursors	Mass Chromatogram shows the masses of precursors	Mass Chromatogram shows the intensity of product ions
It is used for detection of structural homologues that have a common fragment ion	It is used for screening of several compounds for common function (e.g. glucuronides, sulphates, GSH adducts)	It is used for quantitative analysis

SELECTION OF COLUMN

The heart of the chromatographic system is the column. In order to achieve high efficiency of separation, the column material (micro-particles, 5-10 µm size) packed in such a way that highest numbers of theoretical plates are possible. Silica (SiO2, H2O) is the most widely used substance for the manufacture of packing materials. It consists of a network of siloxane linkages (Si-O-Si) in a rigid three dimensional structure containing inter connecting pores. Thus a wide range of commercial products is available with surface areas ranging from 100 to 800 m2/g. and particle sizes from 3 to 50 µm. The silanol groups on the surface of silica give it a polar character, which is exploited in adsorption chromatography using non-polar organic eluents. The attachment of hydrocarbon chains to silica produces a non-polar surface suitable for reversed phase chromatography where mixtures of water and organic solvents are used as eluents. The most popular material is octa decyl-silica (ODS-Silica), which contains C18, chains, but materials with C4 and C8 chains are also available.

In LC, generally two types of columns are used, normal phase columns and reversed phase columns. Using normal phase chromatography, particularly of nonpolar and moderately polar drugs can make excellent separation. Reversed phase chromatography is carried out using a polar mobile phase such as methanol, Acetonitrile, water, buffers etc., over a nonpolar stationary phase. Ranges of stationary phases (C18, C8, -NH2, -CN, -phenyl etc.) are available and very selective separations can be achieved.

SELECTION OF MOBILE PHASE

In LC, selectivity is generally altered by changing mobile phase composition or stationary phase functionality. The most successful and convenient option in the past, particularly for analysis of ionizable compounds, has been to optimize the mobile phase composition by changing solvent type and strength, buffer type and concentration, and pH.

Solvents

The most common solvents used in LC/ESI-MS are water, Methanol, Acetonitrile and mixtures of the above. The solvent composition (aqueous-to-organic ratio) is particularly important in ionization process. The effectiveness of the spraying process depends on the conductivity and surface tension of the liquid being vaporized. If more aqueous solvent is used, it is difficult to produce stable spray because conductivity and/or the surface area are too high, vaporization of droplet formed by the action of high voltage and nebulizing gas is also difficult.

Buffers and Additives

Volatile buffers are required for faster evaporation.

1. To act as a buffer for the chromatographic process in the traditional way, i.e. control and maintain the pH of the mobile phase in order to keep the ionization state of analyte constant.

2. To adjust the pH of the carrier solvent (mobile phase) in such a way as to present the analytes to the MS already in ionic form.

The most compatible buffer mobile phase are ammonium formate, ammonium acetate, and ammonium hydroxide and formate at concentrations of 10 to 20 mM in ESI and up to 50 mM in APCI, . Ammonium adducts can be frequently seen in positive ion mode and formate or acetate ions adduct in negative ion mode. Basic compound will usually show enhanced signal by lowering pH of mobile phase in LC/MS/MS. Other additives occasionally used include Trifluoroaceticacid (TFA), Triethylamine (TEA) and Diethyl amine (DEA) but these need to be used at low concentrations (< 0.1% v/v) since they may cause ionization suppression. Non-volatile buffers such as Phosphates, citrates, borate buffers and ion pairing agents, inorganic acids are generally avoided (may block the orifice and get deposited in ion source, analysers and detectors) in LC-MS method development.

LC-MS/MS (Tandem MS-MS):

The ***principle*** of LC-MS/MS is based on the fragmentation of charged ions and the detection of the resulting fragments. Thus it has a much higher selectivity and sensitivity than LC-MS and makes it possible to elucidate e.g. metabolite structures. LC/MS is a hyphenated technique, combining the physico-chemical separation power of liquid chromatography HPLC, and the mass analysis with the detection power of mass spectrometry. Even with a very sophisticated MS instrument, HPLC is still useful to remove the interferences from the sample that would impact the ionization. In this case, there is the need for an interface that will eliminate the solvent and generate gas phase ions, and then transferred to the optics of the mass spectrometer. The major advantage of LC-MS is its very high sensitivity and selectivity. Generally it is used for the detection and identification of chemicals in the presence of other chemicals (in a complex mixture or matrix).

Instrumentation

Mass Spectrometer contains the following components

a) Ion source/Ionization sources

The sample is introduced to the ionisation source either by manual direct infusion (DI) or via

HPLC, following which sample is delivered to the probe via a capillary, the probe functions to translocate the liquid sample from the capillary to the needle of the ion source.

A variety of ionization techniques are used for mass spectrometry. Most ionization techniques excite the neutral analyte molecule which then ejects an electron to form a radical cation. Other ionization techniques involve ion molecule reactions that produce adduct ions. The most important considerations are physical state of the analyte and the ionization energy. Most instruments now atmospheric pressure ionization (API) technique where solvent elimination and ionization steps are combined in the source and take place at atmospheric pressure. When electron impact ionization (EI) is the choice, the solvent elimination and ionization steps are separate. The interface is a particle beam type, which separates the sample from the solvent, and allows the introduction of the sample in the form of dry particles into the high vacuum region. Electron impact is of interest for molecules which do not ionize with API technique, or when an electron impact spectrum is necessary, since it provides spectral information independent of the sample introduction technique (GC or LC, or direct introduction) and instrument supplier.

Electron ionization (EI) and chemical ionization (CI) are only suitable for gas phase ionization. Fast atom bombardment (FAB), secondary ion mass spectrometry, electro spray (ESI), and matrix assisted laser desorption (MALDI) are used to ionize condensed phase samples. The ionization energy is significant because it controls the amount of fragmentation observed in the mass spectrum. Although this fragmentation complicates the mass spectrum, it provides structural information for the identification of unknown compounds. Some ionization techniques are very soft and only produce molecular ions; other techniques are very energetic and cause ions to undergo extensive fragmentation. Although this fragmentation complicates the mass spectrum, it provides structural information for the identification of unknown compounds.

Table No: 2 Summary of event according to ionization sources[10]

Ionization Source	Event
Electrospray ionization(ESI)	Evaporation of charged droplets
Nanoelectrospray ionization(nanoESI)	Evaporation of charged droplets
Atmospheric pressure chemical ionization (APCI)	Corona discharge and proton transfer

matrix-assisted laser desorption ionization (MALDI)	Photon absorption/proton transfer
Desorption/ionization on silicon(DIOS)	Photon absorption/proton transfer
Fast atom/ion bombardment(FAB)	Ion desorption/proton transfer
Electron ionization(EI)	Electron beam/electron transfer
Chemical Ionization(CI)	Proton transfer

Table No: 3 COMPARISION OF IONISATION METHODS

Ionisation method	Type of ion formed	Analytes	Sample Intro	Mass limits	Method type
EI	M+,M-	Small volatiles	GC, liquid or solid prob	10^3	Hard method structural info
CI	[M+H]+,[M+X]+	Small volatiles	GC, liquid or solid prob	10^3	Soft method
APCI	[M+H]+,[M+X]+, [M-H]-	Small volatiles(less polar species)	LC or syringe	$2x10^3$	Soft method
FI/FD	[M+H]+, [M+X]+	FI: Volatiles FD: Nonvolatiles	GC, liquid or solid probe	$2x10^3$	Soft method
ES	[M+ nH]n+, [M- nX]n-	Peptides, proteins nonvolatile	LC or syringe	$2x10^5$	Soft method multiply charged ions
FAB	[M+H]+, [M-H]-	Carbohydrates Organometalics Peptides, nonvolatiles	in viscous matrix	$6x10^3$	Soft but harder than ESI or MALDI
MALDI	[M+H]+, [M+X]+	Peptides, proteins, nucleotides	In solid matrix	$5x10^3$	soft

B) MASS ANALYSERS

The *m/z* analyzer (mass analyzer) is used to separate the ions according to their *m/z* ratio based on their characteristic behaviour in electric and/or magnetic fields. With the advent of ionization sources that can vaporize and ionize bio molecules, it has become necessary to improve mass analyzer performance with respect to speed, accuracy, and resolution. In the most general terms, a mass analyzer measures gas phase ions with respect to their *m/z*, where the charge is produced by the addition or loss of a proton(s), cation(s), anion(s) or electron(s). The addition of charge allows the molecule to be affected by electric fields thus allowing its

mass measurement. This is important aspect to remember about mass analyzers that they measure the *m/z* ratio, not the mass itself. The performance of a mass analyzers can be typically be defined by the following characteristics such as accuracy, resolution, mass range, tandem analysis capabilities, and scan speed.

Table No: 4

Types of Analysers	Basis of Separation
Electric sector	Kinetic energy
Magnetic sector	Momentum
Quadrople/Ion trap	m/z
Time of flight	Flight time
FT-ion cyclotron resonance	m/z (resonance activities)

SAMPLE PREPARATION METHODS[14,15]

Sample preparation is very important step for analysis of drugs and metabolites in bioanalytical study. The “unwanted substances” can interfere with small concentrations of the drug during analysis. The main objective of sample preparation is to eliminate all possible undesirable substances without significant loss of analyte of interest. Currently several techniques are available for extraction of analyte from the biological matrices like; solid phase extraction (SPE), liquid-liquid extraction (LLE) and protein precipitation (PP).

Protein Precipitation (PP)

Protein precipitation is often used as the initial sample preparation scheme in the analysis of a drug substance since it does not require any method development. It can be applied to extraction of plasma and blood samples. Principle of PP is based on precipitation (denaturation) of the proteins by using various reagents like acid (trichloroacetic acid and perchloric acid), organic solvent precipitants lowers the dielectric constant of the plasma protein solution and facilitate the precipitation(methanol, acetone and acetonitrile) or by salts (ammonium sulphate, ammonium chloride). The binding of positively charged metal ions reduces protein solubility and facilitate precipitation (zinc sulphate). After denaturation the sample is centrifuged, which results in extraction of analyte in the precipitating solvent. Methanol is generally preferred solvent amongst the organic solvent as it can produce clear

supernatant which is appropriate for direct injection into LC-MS/MS. Salts are other alternatives to acid and organic solvent precipitation. This technique is called as salt-induced precipitation. As the salt concentration of a solution is increased, proteins aggregate and precipitate from the solution It is simple, universal, inexpensive popular procedure. This method can be applied for extraction of both hydrophobic and hydrophilic substances. Matrix components are not efficiently removed and may be present in supernatant which may reduce the efficiency of ionisation process, loss in response and this phenomenon is referred as ionization suppression. Protein precipitation may clog the column.

Liquid-Liquid Extraction (LLE)

Liquid-Liquid Extraction, also called solvent extraction, is a technique used to separate analytes from interferences in the sample matrix by partitioning the analytes between two immiscible liquids or phases (aqueous and organic). Extraction of analyte occurs from aqueous phase into organic phase (when analytes are unionized and solubility in that organic solvent). Analytes get extracted into the organic phase (organic solvents eg., hexane, diethyl ether, methyl tert-butyl ether MBTH, ethyl acetate) are easily recoverd by evaporation of solvent in presence of nitrogen gas so as to get the dry form of sample and reconstituted (mobile phase) prior to chromatographic analysis, while analytes extracted into the aqueous phase can often be injected directly onto a reverse-phase HPLC column. Now-a-days traditional LLE has been replaced with advanced and improved techniques like liquid phase micro extraction (LPME), single drop-liquid phase micro extraction (DLPME) and supported membrane extraction (SME). Clean extracts can be obtained. Technique is simple, rapid and has relatively less cost per sample. pH control of the sample necessary for extraction. During evaporation, since the temperature is increased. Method cannot be used for thermo labile substances.

Solid Phase Extraction (SPE)

The principle of SPE is based on the partitioning of the analytes between two phases. The analyte of interest must have higher affinity to solid phase than the matrix components. Now-a-days, SPE employs a small plastic disposable column or cartridge (syringe-barrel format with 0.1 to 0.5 g of sorbent). Sorbent is commonly a reversed phase material (e.g., C18-silica) pack is held in syringe barrel by two fritted disks in the cartridge made up of polypropylene, PTFE and fiber glass based is for SPE disks. Silica-base with chemically bonded functional groups or highly cross-linked polymers such as styrene divinylbenzene and polymethacrylate

are generally used materials for preparation of SPE cartridges. These cartridges can be utilized only once so as to avoid any interference and mostly to eliminate carry-over effects. Different types of SPE sorbents used are non-polar, polar and ion exchange sorbents. For reverse- phase SPE, the stationary phase is usually silica bonded with alkyl and/aryl functional groups. C18 and C8 are the most common sorbents used. The packing materials required for SPE and liquid chromatography are comparable; only particle size is more in SPE as compared to that of liquid chromatography. SPE technique is modified by solid phase micro extraction. SPE method development involves generally four main steps includes conditioning the packing, sample application (loading), washing the packing (removal of interferences), recovery of analyte. No need of phase separation (as in LLE), total analyte fraction is easily collected, more recovery of analyte. Low concentration of drug can be detected. Effective in selective removal of interferences, different types of adsorbents can be used, extending the analytical column life, reduced system maintenance, minimizing ion suppression. Extraction is difficult for high-density materials, extraction processes a number of steps are to be carried out making it a time consuming process, variability of SPE catridges, irreversible adsorption of some analytes on catridges.

3 METHOD VALIDATION[9,11,13]

Bioanalytical method validation includes all of the procedures required to demonstrate that a particular bioanalytical method for the quantitative determination of the concentration of an analyte (or series of analytes) in a particular biological matrix is reliable for the intended application. Method validation is done by the use of specific validation parameters which explains that the performance characteristics of the method are appropriate and reliable for the proposed analytical applications. The suitability of analytical data relates directly to the criteria used to validate the method.

The guidelines for bioanalytical method validation are published by the United States Food and Drug Administration (USFDA) in May 2001. These guidelines are standard for validation parameters' evaluation and requirement. Bioanalytical method validation is the approach employed to indicate that the analytical method used to assess an analyte in biological matrix is reliable and also reproducible. There are three types of method validations, including "Full Validation, Partial Validation, and Cross Validation". These different types of bioanalytical method validations are defined and characterized as follows:

Full Validation

Full Validation should be performed to support pharmacokinetic, bioavailability, bioequivalence and drug interaction studies in a new drug application (NDA) or an abbreviated new drug application (ANDA).Full validation is necessary when developing and implementing a bioanalytical method for the first time and mandatory for any new drug entity.

Partial Validation

Partial validations are usually modifications of validated bioanalytical methods which do not essentially require complete revalidations. In partial validation either one intra-assay of precision and accuracy is carried out or "approximately" full validation is done. Partial validation can also be carried out when there is alteration in species within matrix (e.g. rat plasma to mouse plasma), changes in matrix within a species (e.g., human plasma to human urine), change in analytical methodology (e.g., change in detection systems), change in sample processing procedure(s), change in anticoagulant in harvesting biological fluid.

Cross Validation

Cross validation is comparison of two bioanalytical methods. Cross validations are essential when two or more bioanalytical methods are applied to generate information within same study. The evaluations should be done by considering an innovative validated bioanalytical method as the reference and the repeated bioanalytical method as the comparator and vice-versa. Cross validation with spiked matrix and subject samples should be carried out at each site or laboratory to establish inter-laboratory reliability when sample analyses within a single study conducted more than one site, or more than one laboratory. This should be taken into consideration when data is generated by using various analytical techniques (e.g. LC-MS-MS *vs* ELISA) in different studies are included in a regulatory submission.

System suitability

System suitability experiment was performed by injecting six consecutive injections at least once in a day with using aqueous MQC1 and MQC2 solutions. System performance experiment was performed by injecting sequence of injections at the beginning of analytical batch or before any re-injection. The Signal to Noise ratio should be more than or equal to **5** for LLOQ QC sample.

Carry Over Effect

Carryover can adversely affect accuracy and precision. During validation carryover is evaluated by injecting blank extracts after the highest calibration standard containing IS or QC high sample and LLOQ sample in duplicate with internal standard from biological matrix and extracted blank samples from the same matrix lot are used. If the peak response in the carryover blank exceeds 20% of the LLOQ response, carryover is considered significant. When carryover is inevitable, it needs to be noted in the method. The method should also contain recommendations for minimizing the impact of the carryover during analysis of study samples, such as placing extra blanks throughout the sequence or injecting the toxicology samples in a specific order. Unexpected carryover must be investigated. Major sources of carryover include inappropriate sample diluent, inappropriate mobile phases and adsorption of the analyte to instrument components. Carry over effect in the assay is also used to study the rinsing cycles or wash program of auto sampler required to wash the injection needle properly so as not to get any interference from the previous injection.

Selectivity

Selectivity is an important component of method validation. “The method must be able to quantify the analyte in the presence of endogenous compounds, degradation products, other medicines likely to be present in study samples, and metabolites of the medicine(s) under study There are several items to be considered when evaluating the selectivity of the assay. Evaluation of a minimum of 6 different lots or sources of matrix must be performed as matrix blanks (containing no analyte or IS). Additional lots beyond the six required lots should be added when needed in order to test each of the expected selectivity scenarios. For nonclinical studies with large animals, six individual lots are recommended, while studies with small animals (i.e., rat or mouse) may use pooled lots. For clinical assays, lots from individual donors are suggested. Choice of the matrix lots should be based on the expected composition of study samples. For example, if specific constraints are placed on the study samples, then the choice of selectivity lots should reflect these constraints. Some analytes generate dissimilar results in different genetic populations, different genders, or different age groups. For clinical studies in which there are fasted and fed components, the choice of selectivity lots during validation should include a choice of lots from fasted subjects and fed subjects in order to evaluate the potential impact of fed/fasted state on the matrix. In addition, hemolytic and lipemic plasma should be evaluated to determine the impact on quantitation. For evaluation of hemolytic and lipemic lots, it is suggested that QC samples be prepared at high

and low levels of hemolysis/lipemia and prepared per the analytical method. Acceptance criteria for these samples should mimic that of the assay (relative error ±15%, relative standard deviation ≤15%).

Sensitivity

Sensitivity is measured in terms of LLOQ (lower limit of quantification). For sensitivity six matrix spiked LLOQ samples using the same spiking dilutions should be used and the same for the preparation of LLOQ standard calibration curve. The lowest standard on the calibration curve is used to determine the limit of quantitation (LLOQ). The LLOQ is the lowest assessed concentration which can reproducibly give an analyte response that is both accurate (100±20% recovery) and precise (≤20% RSD). The Bioanalytical Guidance states that the LLOQ should be at least 5 times the response compared to the blank response. The EMA Guidance states that "the LLOQ should be adapted to expected concentrations and to the aim of the study". Typically ng/mL levels are utilized for pre-clinical bioanalysis; for example, 1-1000 ng/mL range. Clinical bioanalysis methods often require lower LLOQ, down to pg/ mL level, which may limit the choice of sample preparation techniques and instrument platforms.

Matrix effects (Ion suppression, ion enhancement)

"Matrix effect is investigated to ensure that precision, selectivity, and sensitivity will not be compromised within the matrix". "Matrix effect is the suppression or enhancement of ionization of analytes by the presence of matrix components in the biological samples. Quantitative measurement of matrix effect provides useful information in validation of MS-based bioanalytical methods. The quantitative measure of matrix effect can be termed as Matrix Factor (MF) and defined as a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions". Stable isotope labelled IS can compensate for matrix effects on quantification of the target analyte.

Three blank samples from each of at least six batches of matrix under screening are extracted. For matrix effect LQC (lower quality control), MQC (middle quality control) and HQC (higher quality control) spiking dilutions and internal standard dilution are spiked in the above extracted blank samples. Recovery comparison sample at LQC, MQC and HQC concentration level along with internal standard are prepared and screened.

Matrix factors

The matrix factor is defined as the ratio of analysis of the analytical response obtained from analysis of extracted blank matrix samples spiked after extraction with the analyte (fenofibric acid), at four concentrations (low, middle 1&2 and high) and Internal Standards (at the working concentrations) relative to the analytical response obtained from reference solutions. Six batches of human plasma (including hemolytic and lipidemic lot) were processed and after extraction of the blank plasma samples, was spiked with fenofibric acid at concentration equivalent to those in the low, middle 1&2 and high Quality control extracted samples and internal standard at its working concentration. At least 67% (2 out of 3) of total QCs injected at each level must be within ±15% of the respective nominal value. The matrix effect is nullified if the mean % Nominal is within ± 15 % and CV % ≤ 15 % at low and high QC .

Calibration of standard curve (Linearity and range)

Fresh calibration curves should be used for method validation. Frozen calibration curves may be used only after stability has been determined. Each run should contain two curves, one at the beginning and one at the end of the injection sequence. Calibration curves are prepared in the same biological matrix for the intended study samples; however, they may be prepared in a surrogate matrix if the study matrix is rare. As per FDA guidance, the simplest regression model that adequately describes the concentration-response relationship should be used. A linear regression with 1/x or $1/x^2$ weighting is typically sufficient. The use of a quadratic fit is allowed but should be limited, and the cause for non-linearity must be evaluated. The selection of final regression model and weighting factor should be based on all of the validation data, and evaluation should be documented.

The linearity of the method was determined by using standard plots associated with 8 point standard curve including LLOQ and ULOQ. Concentration of calibration curve standards was calculated against the calibration curve and the linearity of the method was evaluated by ensuring the acceptance of precision and accuracy of calibration curve standards. Two consecutive calibration curve standards should not be beyond the acceptance criteria. The lower limit of quantification (LLOQ) was the lowest concentration at which the precision expressed by relative standard deviations (RSD, CV %) is better than 20% and the accuracy (bias) expressed by relative difference of the measured and true value was also lower than 20%.

Accuracy & Precision

The purpose of validation is to ensure that the methods developed are sufficiently accurate and precise to quantify the actual concentrations of analyte which will be present in the study samples. The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. The QC samples are prepared by spiking analyte into matrix at Low (three times the LLOQ), Middle (around 50% of the logarithmic curve range) and High (at about 75% of the upper limit of quantitation (ULOQ)) concentrations. Dilution QC samples are prepared at 5 to 100 times ULOQ for assessment of ability of the method to accurately quantitative study samples which are initially above the limit of quantitation (ALQ) during sample analysis (this is also known as dilution integrity). QC samples are also prepared at the LLOQ to validate the sensitivity of the assay. Samples with concentration below the LLOQ are below the limit of quantitation (BLQ). "The QC samples are analyzed against the calibration curve, and the obtained concentrations are compared with the nominal value". An accuracy value of not more than 100±15% should be attained for Low, Middle, and High QC samples, and not more than 100±20% for QC samples at the LLOQ with at least 50% at each level meeting acceptance.

The ***precision*** of an analytical method describes the closeness of individual measure of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. Precision is the degree of reproducibility and is usually reported in terms of %CV or %RSD over the range of quantification for a single experiment. A value of not more than 15% for Low, Middle, and High QC samples and not more than 20% for the QC samples at the LLOQ is acceptable. Accuracy and precision should both be compared within independent runs (i.e., intra-run), and between at least two different runs (i.e., inter-run). It is highly recommended that at least one of the runs contains the same number of samples as will be expected in the longest anticipated sample run. Tracking the IS response variation is also critical to consistent assay performance. There is an assumption that the IS will correct for variability and compensate for differences between different matrix sources. The minimum IS response and the maximum IS response should be monitored during a run sequence.

The within-run and between-run percentage mean of precision and accuracy of the drug were measured by the percent coefficient by using 6 replicate samples of variation over the concentration range of LLOQ, low, middle 1&2 and high quality control samples for the three precision and accuracy batches to their nominal values. The acceptable % coefficient of precision and accuracy should be less than 15%. The between and within batch % mean precision and accuracy for LQC, MQC1, MQC2 and HQC samples were within the range of 85.00-115.00% and for the LLOQ QC within the range of 80.00-120.00% respectively.

The mean concentration, coefficient of variance (CV %) and accuracy (% Nominal) for all CC standards and at each QC concentration level are calculated using formula.

Within Batch Precision and Accuracy

$$\text{Accuracy (\% Nominal)} = \frac{\text{Mean of each QC concentration level within a batch}}{\text{Nominal Value}} \times 100$$

$$\text{Precision (CV \%)} = \frac{\text{STDEV of each QC concentration level within a batch}}{\text{AVERAGE of each QC concentration level within a batch}} \times 100$$

Between Batch / Inter Day Precision and Accuracy

$$\text{Accuracy (\% Nominal)} = \frac{\text{Mean of each QC conc. level of all batches run on different days}}{\text{Nominal Value}} \times 100$$

$$\text{Precision (CV \%)} = \frac{\text{STDEV of each QC concentration level of all batches}}{\text{AVERAGE of each QC concentration level of all batches}} \times 100$$

Ruggedness

One precision and accuracy batch will be processed and analysed with different analyst and another precision and accuracy batch will be processed and analysed using different column or with different sets of reagents. It should meet the acceptance criteria of precision and accuracy batch.

Recovery

"The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method." The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability.

Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible." Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with un-extracted standards that represent 100% recovery. Six sets each of quality control samples (LQC, MQC2 and HQC) will be withdrawn from deep freezer and thawed. These quality control samples (extracted samples) will be processed and injected.

Twenty four blank matrix samples will be processed and spiked with six sets of each QC dilutions at low, middle and high concentration without internal standard into the processed blank matrix samples which represents 100 % extraction of analyte(s) (non-extracted samples). Six blank matrix samples will be processed and spiked with working concentration of internal standard dilution into the six processed blank matrix samples which represents 100% extraction of internal standard (non-extracted samples).

The % mean drug recoveries was determined by comparing the mean peak area of the 6 replicates of extracted plasma quality control samples at high, middle 1&2 and low concentrations against respective mean peak area of the 6 replicates of un-extracted quality control samples at high, middle 1&2 and low concentrations. All replicates of non-extracted samples will be injected. Tabulate the peak area responses of extracted and non-extracted samples at each low, medium and high QC levels. The CV % of the mean analyte(s) and internal standard recoveries must be ≤ 15 % for each quality control concentration levels. The difference of % Recovery between the lowest % recovery and highest % recovery should not be more than 2

Mean Absolute % recovery of drug =

$$\frac{\text{Mean Peak Area Response of extracted samples at LQC, MQC2 and HQC}}{\text{Mean Peak Area Response of non-extracted samples at LQC, MQC2 and HQC}} \times 100$$

Mean absolute % recovery of internal standard =

$$\frac{\text{Mean Peak Area Response of extracted samples at medium QC level}}{\text{Mean Peak Area Response of non-extracted samples}} \times 100$$

Finally, calculate the overall recovery, S.D. and CV % for analyte only

Global % Recovery = Average of mean of absolute % recovery at LQC, MQC2 and HQC

Dilution Integrity

This exercise is carried out to assess the ability of the method to quantify the analyte and yield accurate and precise results after the sample dilution. Dilution integrity test can be used when the subject sample concentrations are above the validated calibration curve range. For dilution integrity six sets of quality control samples are prepared by diluting them twice and four times prior to extraction by addition of screened interference free blank matrix.

The dilution integrity of the method was evaluated by diluting the stock solution. Twenty-four sets of QCs will be prepared as spiked quality control sample at concentration 1.5-2 times above the concentration of the highest standard (ULOQ) in the calibration curve in the screened biological matrix. Conduct dilution integrity experiment by using six replicates each of diluted quality control (1/2) and diluted quality control (1/10) samples. Process and analyze the diluted quality control samples along with freshly spiked calibration curve standards and at least two sets of batch qualifying quality controls (at lower and higher).Within batch precision of QC having same dilution factors must be ≤ 15 %. Within batch accuracy of QC having same dilution factors must be ± 15 of the nominal value. At least 67% (4 out of 6) of total QCs injected at each level must be within ±15% of the respective nominal value.

Stability

Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process (incurred sample reanalysis). Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution. All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte free and interference free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

Standard Stock Solution Stability

Aliquots of Standard stock solutions of analyte and internal standard were prepared and stored in the refrigerator at temperature 2 - 8 °C for its stability and labelled with unique stock I.D. Room temperature stock solution stability will be carried out using remaining volume of the solution to assess stability of solution left at bench top conditions. Six replicates of stock dilutions of analyte and internal standard at 0 and 6 hours were prepared and injected. The concentration of the prepared dilutions will be equivalent to middle quality control (MQC2) sample concentration and working concentration, for analyte and internal standard respectively. Percent comparison response must be between 90 – 110 %

$$\text{Percent Comparison Response} = \frac{\text{Mean of response at 6 hours}}{\text{Mean of response at 0 hours}} \times 100$$

Room Temperature Spiking Solution Stability

Room temperature spiking solution stability of drug and internal standard are carried out using spiking solutions of drug at MQC2 level and internal standard at working concentration. Spiking solutions of drug and internal standard were prepared and left at room temperature for minimum of 6 hours. Final dilutions of drug and internal standard are prepared at 6 hours by spiking the drug and internal standard into the reconstitution solution and injected. The 6 hours samples are compared with 0 hour standard stock solution samples. Percent comparison response must be between 90 % and 110 %.

$$\text{Percent Comparison Response} = \frac{\text{Mean of response at 6 hours}}{\text{Mean of response at 0 hours}} \times 100$$

Refrigerator Stock Solution Stability

Refrigerated stock solution stability was carried out to assess the stability of stored stock over a period of time (e.g. 7, 14 and 21 days), during which it can be used. Fresh standard stock solutions of analyte(s) and internal standard are prepared. Prepared stock dilutions of both refrigerated stock (stability samples) and fresh stock solutions (comparison samples) equivalent to middle (MQC2) quality control sample concentration and working concentration of analyte(s) and internal standard, respectively. Six replicates, of prepared comparison and stability samples will be injected immediately after preparation. The response of comparison samples are corrected by multiplying with correction factor. Percent comparison response must be between 90 -110 %

Correction factor = $\frac{\text{Corrected concentration of stability standard stock solution}}{\text{Corrected concentration of comparison standard stock solution}}$

Percent Comparison Response = $\frac{\text{Mean response of stability samples}}{\text{Mean corrected response of comparison samples}} \times 100$

Working solution stability

Short term stability (at least 6 hours at ambient temperature) and long term stability (at least 4 days at 2-8°C) for working solutions of drug (stock solution ULOQ and LLOQ) and Internal Standard were performed by using six consecutive injections of equivalent aqueous standards prepared from fresh and stored solutions. Short term stability and long term stability of working solution were evaluated by comparing the mean response of stability samples against mean response of comparison samples.

Stability of Drug in Biological Matrix

Perform the matrix stability experiment by using freshly prepared calibration curve standard and two replicates of freshly prepared batch qualifying quality control samples at HQC and LQC levels. Stability studies in biological matrix were conducted in the various conditions at LQC and HQC levels as described below:

Freeze thaw stability

Freeze thaw stability will be carried out to assess the stability of the analyte in biological fluids during repeated freezing and thawing cycles. Freeze thaw stability of the spiked quality control samples were determined after 1st and 5th freeze thaw cycles stored at -20±5°C. Six replicates of each HQC and LQC samples were used for assessing each freeze thaw experiment (for first and fifth cycle at both the freezing temperatures). The first freeze-thaw cycle was of at least 24 hours followed by minimum of 12 hours for subsequent cycles. Process and analyze freeze thaw stability samples along with freshly spiked calibration curve and comparison samples (6 replicates of each LQC and HQC) in screened biological matrix. Evaluate the freeze thaw stability on the basis of % change of LQC and HQC samples. The % Change and % CV of LQC and HQC should be within ± 15.00 and □ 15.00 respectively. The quality control concentrations will be back calculated using the freshly prepared calibration curve data. Mean % Nominal concentration at each quality control sample (Stability samples) level must be between 85 % and 115 % and the precision must be ≤ 15 % of the CV %.

Mean % Nominal Concentration of Quality Control Samples (Stability samples) =

$$\frac{\text{Mean concentration of LQC and HQC having undergone 4 freeze thaw cycles}}{\text{Respective nominal concentration of LQC and HQC}} \times 100$$

Bench top stability

Spiked quality controlled samples (6 replicates of each LQC and HQC) were stored in deep freezer at temperature -20±5°C, which was retrieved after minimum 12 hours of freezing and was kept at ambient temperature on working bench for recommended period of at least 06 hours. Six replicates of each HQC and LQC samples were used for assessing the bench top stability experiment. Upon the completion of recommended period, process and analyze bench top stability samples along with freshly spiked calibration curve and comparison samples (6 replicates of each LQC and HQC) in screened biological matrix. Evaluate the bench top stability on the basis of % change of LQC and HQC samples. The quality control concentrations will be back calculated using the freshly prepared calibration curve data. Mean % Nominal concentration at each quality control sample (Stability samples) level must be between 85 % and 115 % and the precision must be ≤ 15 % of the CV %.

Mean % Nominal Concentration of Quality Control Samples (Stability Samples) =

$$\frac{\text{Mean concentration of LQC and HQC (stability samples)}}{\text{Respective nominal concentration of LQC and HQC}} \times 100$$

Bench top Stability will be carried out to assess the stability of the analyte in biological fluids over a period of time during which the samples are expected to be kept on the bench. Six sets each of quality control samples (LQC and HQC) will be withdrawn from the deep freezer and left at room temperature for at least 4 hours (stability samples). The stability samples will be processed and analysed along with freshly prepared calibration curve standards and Quality control samples.

Autosampler re-injection reproducibility

Autosampler re-injection reproducibility was evaluated by re-injecting accepted precision & accuracy batch, which were stored preferably in either autosampler or in refrigerator for at least 24 hours or as per requirement. Mean % Nominal concentration at each quality control

sample level must be between 85 % and 115 % and the precision must be ≤ 15 % of the CV %.

Mean % Nominal Concentration of Quality Control Samples (Stability samples) =

$$\frac{\text{Mean concentration of Stability LQC and HQC samples x 100}}{\text{Respective nominal concentration of LQC and HQC}}$$

Long Term Stability of Analyte and Internal Standard in Biological Matrix

The long-term stability of analyte stability samples of LQC, MQC1, MQC2 and HQC and internal standard samples were kept frozen in vials at -20 ± 5 0C for 1, 2 and 4 months (expected duration of the sample storage and analysis) were assessed along with freshly processed calibration and comparison samples (six samples each of LQC, MQC1, MQC2, HQC and internal standard). The initial analyte concentration freshly after sample treatment preparation was assumed to be 100%. The selection of stability duration is based on characteristic of the analyte(s). Compare the concentration of the stability samples with the mean of the back-calculated values for the QC samples (considered as nominal concentration) at appropriate concentration from the first day of long-term stability. Mean % Nominal concentration at each quality control sample (Stability samples) level must be between 85 % and 115 % and the precision must be ≤ 15 % of the CV %.

$$\text{Mean \% Nominal Concentration} = \frac{\text{Mean concentration of stability LQC and HQC samples}}{\text{Mean concentration of LQC and HQC samples of first day of stability testing}} \times 100$$

Short Term Stability at –20°C:

Stability in human plasma at –20°C will be conducted to assess the stability of drug in case of temporary storage of plasma samples in –20°C deep freezer. A minimum six sets of quality control samples (LQC and HQC) will be stored in –20°C deep freezer after bulk spiking. Six sets of quality control samples (LQC and HQC) after minimum of three days of storage will be withdrawn and processed along with freshly prepared calibration curve standards and QC samples. Back calculate the quality control concentrations using the freshly prepared calibration curve data. Compare the concentration of the stability samples with the mean of the back-calculated values for the QC samples (considered as nominal concentration) at appropriate concentration from the first day of stability. Mean % Nominal concentration at

each quality control sample (Stability samples) level must be between 85 % and 115 % and the precision must be ≤ 15 % of the CV%

Mean % Nominal Concentration =

$$\frac{\text{Mean concentration of stability LQC and HQC samples}}{\text{Mean concentration of LQC and HQC samples of first day of stability testing}} \times 100$$

Re-injection Stability

Re-injection stability will be carried out to assess the integrity of analyte samples re injected over a period of time after processing. One precision and accuracy batch will be processed and analysed. Back calculate the QC concentration using calibration curve data. The LQC and HQC samples over a period of time after 1st injection of QC sample (Maximum after 24 hours) will be re-injected. Back calculate the concentrations of re-injected QCs using the initial calibration curve data. Mean % Nominal concentration at each quality control sample level must be between 85 % and 115 % and the precision must be ≤ 15 % of the CV %.

Mean % Nominal Concentration =

$$\frac{\text{Mean concentration of QC reinjected over a period of time}}{\text{Mean concentration of QCs when injected for the 1st time}} \times 100$$

Wet Extraction Stability at Room Temperature

Wet extraction stability of the analyte and internal standard will be carried out at room temperature for the period of time. At least 12 sets (in multiple of six) of low and high QC samples will be processed to facilitate injection at proposed stability periods. The processed samples will be kept at room temperature for specified time interval. The QC samples will be injected along with freshly prepared calibration standards and QC samples. Data for stability period with minimum time interval of approximately 24 hours will be generated. Calculate the wet extraction stability duration as the time of injection of first QC, less the time of their placement at room temperature. Mean % Nominal concentration at each quality control sample level must be between 85 % and 115 % and the precision must be ≤ 15 % of the CV %.

Mean % Nominal Concentration of Quality Control Samples =

$$\frac{\text{Mean concentration of Stability LQC and HQC samples.}}{\text{Respective nominal concentration of LQC and HQC}} \times 100$$

Note: For all validation parameters, the batch should pass at least 67% (4 out of 6) of total QCs inject at each level must be within ±15% of the respective nominal value

CONCLUSION

The application of LC-MS, in combination with MS-MS, makes it a very valuable analytical tool LC–MS has become the standard method of quantization in bio-analysis and the bioanalyst should also be aware of recent developments in methodology that can improve data quality. MS ionisation methods can be applied to wider range of biological molecules Analytes can be determined with high through-put when specifically tandem MS is used for detection. LC-MS can be used to develop highly accurate and reproducible assays. Modern mass spectrometers are highly sensitive and LC-MS assays are now viable replacements for many immunoassays. LC-MS/MS techniques are most probably beneficial in the clinical laboratories when used for multiplexed and screening type assays. This review has focused on several areas of awareness that can enhance the quality of a bio-analytical method. It should be concluded from this review that method development, quality sample preparations, method validation, and understanding of the LC–MS/MS instrumentation all contribute to a quality of analytical method.

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