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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION BY LC-MS/MS: A REVIEW

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ABSTRACT

LC-MS/MS is commonly used in laboratories for the qualitative and quantitative analysis of drug substances, drug products and biological samples. LC-MS/MS has played a significant role in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. This article discuss about various extraction techniques like liquid–liquid extraction, solid phase extraction and protein precipitation which are important in the development of bioanalytical method. After developing a method with desired attributes, the method is validated to establish that it will continue to provide accurate, precise, and reproducible result during sample analysis. This review serves as a general guidance recommended for the validation of bioanalytical methods to ensure adequate reproducibility and reliability. The validation of bioanalytical methods and the analysis of study samples for clinical trials in humans should be performed following the principles of Good Clinical Practice (GCP). The stability of the method can be determined by several methods including freeze and thaw method, short term stability, bench-top stability, long term stability, stock solution stability and processed sample stability. The applications of LC-MS to the studies of in vitro and in vivo drug metabolism, analysis and identification of impurities and degradation products in pharmaceuticals and analysis of chiral impurities in pharmaceuticals are described in this article.

KEYWORDS: Bioanalytical Method Development, Validation, LC-MS/MS, Sample preparation, Extraction, Application.

INTRODUCTION

In the development of medicinal products, bioanalytical methods are used in clinical and non-clinical pharmacokinetic studies (including toxicokinetic studies) to evaluate the efficacy and safety of drugs and their metabolites. Drug concentrations determined in biological samples are used for the assessment of characteristics such as in vivo pharmacokinetics (adsorption, distribution, metabolism, and excretion), bioavailability, bioequivalence, and drug-drug interaction.[5]

Bioanalytical methods employed for the quantitative determination of drugs and their metabolites in biological matrix (plasma, urine, saliva, serum etc.) play a significant role in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. For development of bioanalytical method various methods such as Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), combined GC and LC mass spectrometric (MS) procedures, such as LC-MS, LC-MS-MS, GC-MS, and GC-MS-MS; and ligand binding assays (LBAs), and immunological and microbiological procedures that are performed for the quantitative determination of drugs and/or metabolites, and therapeutic proteins in biological matrices such as blood, plasma, urine, saliva, serum, tissue and skin.[5]
It is important that these bioanalytical methods are well characterized throughout the analytical procedures to establish their validity, reproducibility, and reliability. It also provides a framework for analyses of study samples by using validated methods to evaluate study results supporting applications for drug marketing authorization.

Validation of a bioanalytical method is the process by which it is established that the performance characteristics of the method meet the requirements for the intended bioanalytical application. The fundamental bioanalytical method characteristics are expressed in terms of bioanalytical method validation parameters. The fundamental bioanalytical method validation parameters include accuracy, precision, selectivity, sensitivity, reproducibility and stability. However, the stability of the method can be determined by several methods including freeze and thaw method, short term stability, bench-top stability, long term stability, stock solution stability and processed sample stability.[1]

The objective of validation of bioanalytical procedure is to demonstrate that it is suitable for its intended purpose. The most widely accepted guideline for method validation is the ICH guideline Q2 (R1), which is used both in pharmaceutical and medical science. This guideline provides assistance to sponsors of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs) and supplements in developing bioanalytical method validation information used in human clinical pharmacology, bioavailability (BA) and bioequivalence (BE) studies requiring pharmacokinetic (PK) evaluation.[6]

Liquid chromatography-mass spectrometry (LC-MS)

Liquid chromatography-mass spectrometry (LC-MS) is an analytical technique that couples high resolution chromatographic separation with sensitive and specific mass spectrometric detection. LC-MS is a powerful technique that has very high sensitivity and selectivity and so is useful in many applications.

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. MS works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios. In a typical MS procedure, a sample is loaded onto the MS instrument and undergoes vaporization. The components of the sample are ionized by one of a variety of methods (e.g., by impacting them with an electron beam), which results in the formation of charged particles (ions). The ions are separated according to their mass-to-charge ratio in an analyzer by electromagnetic fields. The ions are detected, usually by a quantitative method. The ion signal is processed into mass spectra.[7]

Additionally, MS instruments consist of three modules. An ion source, which can convert gas phase sample molecules into ions (or, in the case of electrospray ionization, move ions that exist in solution into the gas phase). A mass analyzer, which sorts the ions by their masses by applying electromagnetic fields. A detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present.

There are several discrete stages in LC-MS analysis, typically these include.

Separation of the sample components using an HPLC column where the analytes are differentially partition between the mobile phase (eluent) and the stationary phase (coated onto a support material and packed into the column). The mechanism of retention and separation will depend on the mode of chromatography but may include, Hydrophobic Interaction, Ion Exchange, Ion-Pair, Surface Localization, etc.

The separated sample species are then sprayed into an Atmospheric Pressure Ion Source (API) where they are converted to ions in the gas phase and the majority of the eluent is pumped to waste. The mass analyser is used to sort ions according to their mass to charge ratio. Most popular analyser types include Quadrupole, Time of Flight, Ion Trap and Magnetic Sector. The mass analyser may be used to isolate ions of specific mass to charge ratio or to 'scan' over all ion m/z values present.

The detector is used to 'count' the ions emergent from the mass analyser, and may also amplify the signal generated from each ion. Widely used detector types include: electron multiplier, dynode, photodiode and multi-channel plate.

All mass analysis and detection is carried out under high vacuum –established using a combination of foreline (roughing) and turbomolecular pumps.

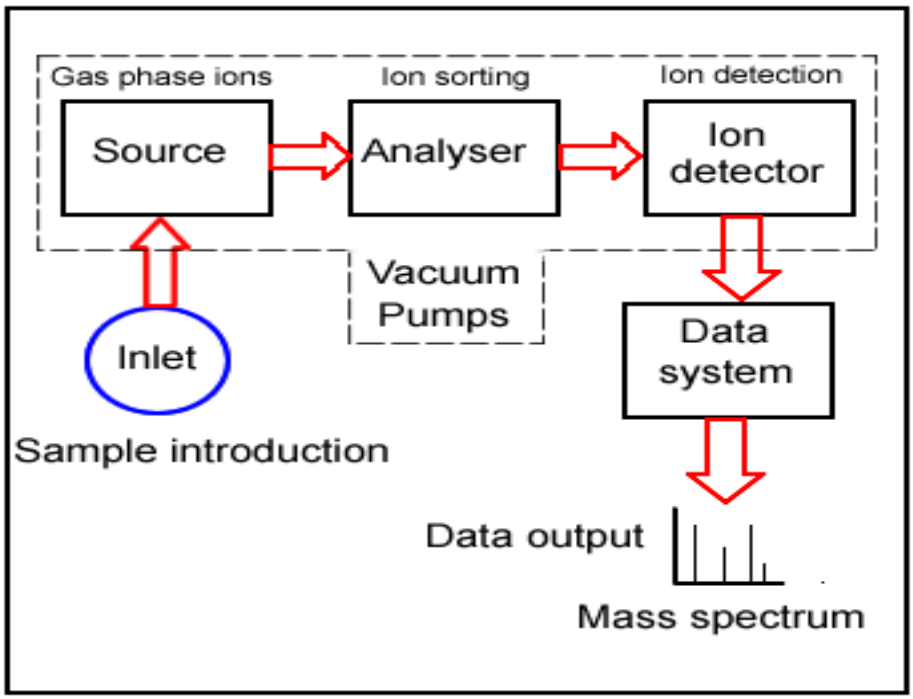

Fig. 1. Simplified diagram and function of instrumentation of typical mass spectrometer

Ionization method

Ionization method refers to the mechanism of ionization while the ion source is the mechanical device that allows ionization to occur. The different ionization methods work by either ionizing a neutral molecule through electron ejection, electron capture, protonation, cationization, or deprotonation, or by transferring a charged molecule from a condensed phase to the gas phase.[8]

The most common ionization methods in LC-MS include.

- Electrospray Ionization (ESI)
- Atmospheric Pressure Chemical ionization (APCI)
- Atmospheric Pressure Photo ionization (APPI)

a. Electrospray ionization

ESI is a "soft" ionization technique in that little fragmentation is produced, forming protonated or de-protonated ions respectively for positive and negative mode MS. Generally, negative mode is less sensitive, except where the nature of the analyte leads to the formation of stable anions, such as with carboxylic acids. An important feature of ESI is that multiply charged ions may be produced with large molecules, enabling their analysis in mass analyzers with limited mass range. Mass analyzers measure mass (m) to charge (z) ratio, m/z. In most ionization processes predominantly singly charged ions are produced where z=1 and m/z=mass, but z can be very large in ESI, especially for proteins. However, for molecules below about 1000 Da, then ESI produces predominantly singly charged ions usually. This would be the case for the vast majority of drug substances currently in use, but peptides and proteins are growing in importance in the pharmaceutical field. The development of ESI led to a great increase in the use of LC-MS, it proving to be an efficient ionization technique, especially for polar compounds and very compatible with solvents used for reversed-phase HPLC.[10]

In electrospray ionization there are three important processes that occur in order to transfer sample ions from the HPLC eluent into the gas phase within the mass spectrometer. These processes are.

• Production of charged droplets at the capillary tip • Desolvation of the droplets

• Production of gas phase ions from small / highly charged droplets

b. Atmospheri Pressure Chemical Ionization (APCI)

Atmospheric Pressure Chemical Ionization uses analyte desolvation and charge transfer

reactions in the vapour phase to produce vapour phase analyte ions. In APCI the eluent is introduced into the interface using a capillary of similar design to the ESI source. However, no potential is applied to the capillary but instead the liquid emerges from the capillary surrounded by a flow of inert, nebulising gas into a heated region. The combination of nebulising gas and heat forms an aerosol that begins to rapidly evaporate. A pin is placed within the heated region that has a high potential applied to it and produces an electrical discharge that ionizes eluent molecules, these ionized molecules impart charge to the analyte molecules via charge transfer reactions or molecular association.

Both ESI and APCI are termed "soft" ionisation methods. This means that in the process of producing ions there is negligible energy transferred to the ion. As a consequence the ion formed does not fragment to small mass ions. The resultant mass spectrum therefore consists predominantly of **pseudomolecular** ions, either $[M+H]^+$ or $[M-H]^-$ or adduct ions like $[M+Na]^+$.

The ionised form of the molecule: $M \cdot \rightarrow M^{+\cdot} + e^-$

The $M^{+\cdot}$ is known as the molecular ion. Note that molecular ions do not typically occur in LC/MS

Pseudomolecular ion formation

If the analyte (M) has a larger proton affinity than the solvent (S), then: $M + [S + H]^+ \rightarrow [M + H]^+ + S$

If the analyte (M) has a lower proton affinity than the solvent (S), then: $M + [S - H]^- \rightarrow [M - H]^- + S$

c. Atmospheric Pressure Photo Ionization (APPI)

APPI, is a complement to ESI and APCI and has been developed to broaden the range of ionizable analytes at atmospheric pressure. APPI is important in the analysis of certain compounds that are not easily ionizable by ESI or APCI like low- and non-polar compounds (APPI has been used in the analysis of polycyclic aromatic hydrocarbons). In APPI, the ionization process is accomplished by exposing an aerosol of droplets to photo irradiation. A molecular radical ion is formed when the molecule absorbs a photon. This process is possible only when the irradiating Photon of energy exceeds the ionization potential (IP) of the

molecule. APPI, allows the formation of charged species in positive or negative ion mode.

BIOANALYTICAL METHOD DEVELOPMENT

A specific, detailed, written description of the bioanalytical method should be established. This can be in the form of a protocol, study plan, report, and/or SOP. Each step in the method should be investigated to determine the extent to which environmental, matrix, or procedural variables could affect the estimation of analyte in the matrix from the time of collection of the samples to the time of analysis. Appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method, especially if the matrix used for production batches is different from the matrix used during method validation. Matrix effects on ion suppression or enhancement or on extraction efficiency should be addressed. A bioanalytical method should be validated for the intended use or application. All experiments used to make claims or draw conclusions about the validity of the method should be presented in a report (method validation report), including a description of validation runs that failed.[1]

Sample collection and sample preparation

The objectives of sample preparation are.

1. The dissolution of analyte in suitable solvent

2. Removal of as many interfering compounds as possible 3. Pre-concentration of the analyte

The biological media that contain the analyte are usually blood, plasma, urine, serum etc. Blood is usually collected from human subjects by vein puncture with a hypodermic syringe up to 5 to 7 ml (depending on the assay sensitivity and the total number of samples taken for a study being performed). The venous blood is withdrawn into tubes with an anticoagulant, e.g. EDTA, heparin etc. Plasma is obtained by centrifugation at 4000 rpm for 15 min. About 30 % to 50 % of the volume is collected.[2]

The purpose of sample preparation is to clean up the sample before analysis and to concentrate the sample. Material in biological samples that can interfere with analysis, the chromatographic column or the detector includes proteins, salts, endogenous macromolecules, small molecules and metabolic byproducts.[11] A goal with the sample preparation is also to exchange the analyte from the biological matrix into a solvent suitable for injection into the chromatographic system. General procedures for sample preparation are liquid/liquid extraction, solid-phase extraction (SPE) and protein precipitation.

a. Liquid – Liquid extraction

Liquid-liquid extraction is an important separation technology, with a wide range of applications in the modern process industry. The extraction process is based on different solubilities of components in two immiscible, or partially miscible, liquids. The components that need to be recovered are extracted from the feed stream with the help of an extractant (often called solvent). Both liquids have to be thoroughly contacted and subsequently separated from each other again. To achieve high purities and yields, it is necessary to operate with multiple stages and with the liquids flowing counter currently. For successful separation by extraction, all components must meet certain specifications. The main criteria for the extractant are a favorable partition coefficient, a high selectivity and an easy separation from the extracted product. The basic conditions for the pair of liquids are a low mutual solubility and a difference in density, which is the driving force for the motion of the droplets. In most multistage extractors, the liquids are transported counter currently. The viscosity and interfacial tension are additional important parameters. In nearly all liquid-liquid extraction processes one of the liquids is dispersed into the second liquid in the form of droplets. The key for a high process performance is an adapted droplet size and a uniform hold-up profile throughout the column. This requires specially adapted equipment and in-depth know-how about two phase liquid flows.

Disadvantages of LLE

1. Formation of emulsions which can be difficult to break even using centrifugation or ultrasonication and can cause loss of analyte by occlusion within the emulsion. The use of less rigorous mixing or larger volumes of extracting solvent can help reduce the problem with emulsions.
2. Not very readily automatable.
3. Time consuming as compared to other methods of sample preparation. 4. Contamination issues

b. Solid Phase Extraction[12]

Solid-phase extraction (SPE) is a separation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. Analytical laboratories use solid phase extraction to concentrate and purify samples for analysis. Solid phase extraction can be used to isolate analytes of interest from a wide variety of matrices, including urine, blood,

water, beverages, soil, and animal tissue. SPE uses the affinity of solutes dissolved or suspended in a liquid (known as the mobile phase) for a solid through which the sample is passed (known as the stationary phase) to separate a mixture into desired and undesired components. The result is that either the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase. The portion that passes through the stationary phase is collected or discarded, depending on whether it contains the desired analytes or undesired impurities. If the portion retained on the stationary phase includes the desired analytes, they can then be removed from the stationary phase for collection in an additional step, in which the stationary phase is rinsed with an appropriate eluent. The stationary phase comes in the form of a packed syringe-shaped cartridge, a 96 well plate or a 47- or 90-mm flat disk, each of which can be mounted on its specific type of extraction manifold. The manifold allows multiple samples to be processed by holding several SPE media in place and allowing for an equal number of samples to pass through them simultaneously. A typical cartridge SPE manifold can accommodate up to 24 cartridges, while a typical disk SPE manifold can accommodate 6 disks. Most SPE manifolds are equipped with a vacuum port. Application of vacuum speeds up the extraction process by pulling the liquid sample through the stationary phase.

The analytes are collected in sample tubes inside or below the manifold after they pass through the stationary phase. Solid phase extraction cartridges and disks are available with a variety of stationary phases, each of which can separate analytes according to different chemical properties. Most stationary phases are based on silica that has been bonded to a specific functional group. Some of these functional groups include hydrocarbon chains of variable length (for reversed phase SPE), quaternary ammonium or amino groups (for anion exchange), and sulfonic acid or carboxyl groups (for cation exchange).

SPE method development generally involves five steps[13]

1. Sample pretreatment
2. Conditioning/ equilibrating the packing. 3. Sample application.
4. Washing the packing (removal of interferences) 5. Recovery of the analyte

1. Sample pretreatment

Sample pre-treatment may simply be a dilution of the sample with an appropriate solvent in order to reduce viscosity, or it could involve addition of a buffer to control the pH of the

sample prior to retention by non-polar or ion exchange sorbents.

2. Conditioning/ equilibrating the packing

Conditioning wets or activates the bonded phases to ensure consistent interaction between the analyte and the sorbent functional groups. Reversed-phase sorbents are often conditioned with 1-2 tube volumes of a water miscible solvent such as methanol or acetonitrile. Equilibration introduces a solution similar to the sample load in terms of solvent strength and pH in order to maximize retention. 1-2 tube volumes of buffer (used in sample pre-treatment) or water are good choices for reversed-phase equilibration.

3. Sample application

Apply sample at a consistent and reduced flow rate of 1-2 drops/second to ensure optimal retention.

4. Washing the packing (removal of interferences)

Sample interferences are often co-retained with compounds of interest during sample load. A wash step is necessary to elute interferences without prematurely eluting compounds of interest. 5-20% methanol in water or sample pre-treatment buffer is typical for wash solvents.

5. Recovery of the analyte

A good elution solvent should elute the analyte in as low a volume as possible. Choose a solvent or solvent mixture in which the analyte is highly soluble. Elution solvents should overcome both primary and any secondary interactions by which the analyte is retained.

Disadvantages of SPE

1. SPE columns are more expensive than conventional solvent extraction columns. i.e. the use of expensive instrumentation to load the separation column with the slurry.
2. Application of SPE to particle-laden water samples can be a vexing problem because repeated extraction is required for extraction of strongly adsorbed substances, added to the fact that suspended particles may plug the SPE membrane and decreases the flow rate so much that it effectively stops, but it is not a serious problem in simple solvent extraction.
3. The set-up in SPE is more complicated.

c. Protein Precipitation

Protein precipitation is often used in routine analysis to remove proteins. Precipitation can be

induced by the addition of an organic modifier, a salt or by changing the pH which influence the solubility of the proteins.[14] The samples are centrifuged and the supernatant can be injected into the LC system or be evaporated to dryness and thereafter dissolved in a suitable solvent. A concentration of the sample is then achieved. There are some benefits with the precipitation method as clean-up technique compared to SPE. It is less time consuming, smaller amounts of organic modifier or other solvents are used. But there are also disadvantages. The samples often contain protein residues and it is a non-selective sample cleanup method, there is a risk that endogenous compounds or other drugs may interfere in the LC-system. However the protein precipitation technique is often combined with SPE to produce clean extract. 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.[15, 16]

BIOANALYTICAL METHOD VALIDATION

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of a method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method. For pivotal studies that require regulatory action for approval or labeling, such as BE or PK studies, the bioanalytical methods should be fully validated. For exploratory methods used for the sponsor's internal decision making, less validation may be sufficient. When changes are made to a previously validated method, additional validation may be needed. For example, published methods of analysis are often modified to suit the requirements of the laboratory performing the assay, and during the course of a typical drug development program, a defined bioanalytical method often undergoes many modifications. These modifications should be validated to ensure suitable performance of the analytical method. The evolutionary changes needed to support specific studies call for different levels of validation to demonstrate the validity of method performance.[1]

The following define and characterize the different types and levels of methods validation.

Full validation

A full validation should be performed when establishing a new bioanalytical method for

quantification of an analyte. A full validation should also be considered when a new analyte, such as a metabolite, is added to an existing, fully validated analytical method. A full validation is also required when using an analytical method that has been published in the literature.[5] The main objective of method validation is to demonstrate the reliability of a particular method for the determination of an analyte concentration in a specific biological matrix, such as blood, serum, plasma, urine, or saliva. Moreover, if an anticoagulant is used, validation should be performed using the same anticoagulant as for the study samples. Generally a full validation should be performed for each species and matrix concerned.[2]

Usually one analyte or drug has to be determined, but on occasions it may be appropriate to measure more than one analyte. This may involve two different drugs, but can also involve a parent drug with its metabolites, or the enantiomers or isomers of a drug. In these cases the principles of validation and analysis apply to all analytes of interest.

The matrix used in analytical validation should be as close as possible to the intended study samples, including anticoagulant and additives. When an analytical method is to be established for a matrix of limited availability (rare matrix, e.g., tissue, cerebrospinal fluid, bile), one may encounter a problematic situation where a sufficient amount of matrix cannot be obtained from an adequate number of sources (subjects or animals). In such a case, a surrogate matrix may be used to prepare calibration standards and QC samples. However, the use of a surrogate matrix should be rigorously justified in the course of establishing the analytical method.[5]

Partial Validation

Partial validation may be performed when minor changes are made to an analytical method that has already been fully validated. A set of parameters to be evaluated in a partial validation are determined according to the extent and nature of the changes made to the method.[5]

Typical bioanalytical method modifications or changes that fall into this category include but are not limited to[1]

- Bioanalytical method transfers between laboratories or analysts
- Change in analytical methodology (e.g., change in detection systems)
- Change in anticoagulant in harvesting biological fluid (e.g., heparin to EDTA)

- Change in matrix within species (e.g., human plasma to human urine)
- Change in sample processing procedures
- Change in species within matrix (e.g., rat plasma to mouse plasma)
- Change in relevant concentration range
- Changes in instruments and/or software platforms
- Modifications to accommodate limited sample volume (e.g., pediatric study)
- Selectivity demonstration of an analyte in the presence of concomitant medications Acceptance criteria used in partial validation should in principle be the same as those employed in the full validation.

Cross Validation

Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. In the cross validation conducted after full or partial validation in each laboratory or for each analytical method to be compared, the same set of QC samples spiked with the analyte or the same set of study samples is analyzed at both laboratories or by both analytical methods, and the mean accuracy at each concentration level or the assay variability is evaluated.[1] In the cross validation between different laboratories within the same study, the mean accuracy of QC samples (low-, mid-, and high-levels) at each level should be within ±20% of the theoretical concentration, considering the intermediate precision and reproducibility (inter-laboratories precision). When using a set of study samples, the assay variability should be within ±20% for at least two-thirds of the samples. In the cross validation between different analytical methods, both validation procedure and acceptance criteria (i.e., acceptable assay variability) should be separately defined based on scientific judgment by considering the nature of the analytical methods.[5]

Reference standard

Reference standard serves as the standard in quantifying an analyte, and is mainly used to prepare calibration standards and quality-control (QC) samples, which are samples spiked with a known concentration of the analyte of interest. The quality of the reference material is critical, as the quality affect measurement data. Therefore, a material of known chemical structure from an authenticated source should be used as a reference standard. When this is not possible, an established chemical form (free base or acid, salt or ester) of known purity

can be used.[5]

Three types of reference standard are usually used: (1) certified reference standards (e.g. USP compendial standards), (2) commercially-supplied reference standards obtained from a reputable commercial source, and/or (3) other materials of documented purity custom-synthesized by an analytical laboratory or other noncommercial establishment. The source and lot number, expiration date, certificates of analysis when available, and/or internally or externally generated evidence of identity and purity should be furnished for each reference and internal standard (IS) used. If the reference or internal standard expires, stock solutions made with this lot of standard should not be used unless purity is re-established.[1]

Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Selectivity is evaluated using blank samples (matrix samples processed without addition of an analyte or internal standard) obtained from at least individual sources. The absence of interference with each analyte and internal standard should be confirmed. In case the matrix is of limited availability, it may be acceptable to use matrix samples obtained from less than 6 sources. The evaluation should demonstrate that no response attributable to interfering components is observed with the blank samples or that a response attributable to interfering components is not more than 20% of the response in the lower limit of quantification (LLOQ) for the analyte and 5% of the internal standard.[5]

Lower limit of quantification

The lower limit of quantification (LLOQ) is the lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision. The analyte response at the LLOQ should be at least 5 times the response of a blank sample. Mean accuracy and precision at the LLOQ should be within ±20% of the nominal (theoretical) concentration and not more than 20%, respectively.[5]

Calibration curve

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. The relationship between response and concentration should be continuous and reproducible. A calibration curve should be generated for each

analyte in the sample.[1] The calibration curve should be prepared using the same matrix as the intended study samples, whenever possible, by spiking the blank matrix with known concentrations of the analyte. Ideally, before carrying out the validation of the analytical method it should be known what concentration range is expected. This range should be covered by the calibration curve range, defined by the LLOQ being the lowest calibration standard and the upper limit of quantification (ULOQ), being the highest calibration standard. The range should be established to allow adequate description of the pharmacokinetics of the analyte of interest. A calibration curve should be generated with a blank sample, a zero sample (blank sample spiked with internal standard)), and at least 6 concentration levels of calibration standards, including an LLOQ sample. In general, the simplest model that adequately describes the concentration-response relationship should be used for regression equation and weighting conditions of the calibration curve. A multiple regression equation may be used. Blank and zero samples should not be included in the determination of the regression equation for the calibration curve. The calibration curve parameters should be reported (slope and intercept in case of linear fit). In addition, the back calculated concentrations of the calibration standards should be presented together with the calculated mean accuracy values. All the available (or acceptable) curves obtained during validation, with a minimum of 3 should be reported.

The back calculated concentrations of the calibration standards should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%. At least 75% of the calibration standards, with a minimum of six calibration standard levels, must fulfil this criterion. In case replicates are used, the criteria (within ±15% or ±20% for LLOQ) should also be fulfilled for at least 50% of the calibration standards tested per concentration level. In case a calibration standard does not comply with these criteria, this calibration standard sample should be rejected, and the calibration curve without this calibration standard should be re-evaluated, including regression analysis. In case all replicates of the LLOQ or the ULOQ calibration standard are rejected then the batch should be rejected from the validation, the possible source of the failure be determined and the method revised (if necessary). If the next validation batch also fails, then the method should be revised before restarting validation.[2]

Accuracy

The accuracy of an analytical method describes the closeness of the determined value obtained

by the method to the nominal concentration of the analyte (expressed in percentage). Accuracy should be assessed on samples spiked with known amounts of the analyte, the quality control samples (QC samples). The QC samples should be spiked independently from the calibration standards, using separately prepared stock solutions, unless the nominal concentration(s) of the stock solutions have been established.

Within-run accuracy should be determined by analyzing in a single run a minimum of 5 samples per level at a minimum of 4 concentration levels which are covering the calibration curve range: the LLOQ, within three times the LLOQ (low QC), around 30 - 50% of the calibration curve range (medium QC), and at least at 75% of the upper calibration curve range (high QC). The mean concentration should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.[2]

For the validation of the between-run accuracy, LLOQ, low, medium and high QC samples from at least three runs analysed on at least two different days should be evaluated. The mean concentration should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.

Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision is expressed as the coefficient of variation (CV). Precision should be demonstrated for the LLOQ, low, medium and high QC samples, within a single run and between different runs, i.e. using the same runs and data as for the demonstration of accuracy.[1]

For the validation of the within-run precision, there should be a minimum of five samples per concentration level at LLOQ, low, medium and high QC samples in a single run. The within-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.[2]

For the validation of the between-run precision, LLOQ, low, medium and high QC samples from at least three runs analysed on at least two different days should be evaluated. The between-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

Recovery

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 analyte in solvent. 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 unextracted standards that represent 100% recovery.[1]

Matrix effect

The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample. Matrix effect should be assessed when using mass spectrometric methods. Matrix effect is evaluated by calculating the matrix factor (MF). The MF is determined by comparing the analyte response in the presence of matrix with that in the absence of matrix. MF should be calculated using matrix from at least 6 different sources. The MF may be normalized using an internal standard. The precision of the MF calculated should not exceed 15%. Matrix effect can also be evaluated by analyzing QC samples, each prepared using matrix from at least 6 different sources. The precision of determined concentrations should not be greater than 15%. In case the matrix is of limited availability, it may be acceptable to use matrix obtained from less than 6 sources.[5]

If a formulation for injection to be administered to the subjects or animals contains excipients known to be responsible for matrix effects, for instance polyethylene glycol or polysorbate, matrix effects should be studied with matrix containing these excipients, in addition to blank matrix. The matrix used for this evaluation should be obtained from subjects or animals administered the excipient, unless it has been demonstrated that the excipient is not metabolised or transformed *in-vivo*. The effect of the excipients can be studied by the determination of the MF or by a dilution study of a study sample with a high concentration with blank matrix not containing the excipient.

Dilution integrity

Dilution of samples should not affect the accuracy and precision. Dilution integrity should be evaluated by the replicate analysis of at least 5 times per dilution factor after diluting a sample with blank matrix to bring the analyte concentration within the calibration range. Mean accuracy and precision in the measurements of diluted samples must be within ±15% of the theoretical concentration and not more than 15%, respectively. Dilution integrity should cover the dilution applied to the study samples. Evaluation of dilution integrity may be covered by partial validation. Use of another matrix may be acceptable, as long as it has been demonstrated that this does not affect precision and accuracy.

Carry-over

Carry-over should be addressed and minimized during method development. Carry-over is an alteration of the measured concentration due to a leftover analyte in the analytical instrument used.

The carry-over should be evaluated by analyzing a blank sample following the highest concentration calibration standard. The response in the blank sample obtained after measurement of the highest concentration standard should not be greater than 20% of the analyte response at the LLOQ and 5% of the response of internal standard. If these criteria cannot be met, the extent of carry-over needs to be examined, and appropriate procedures should be taken to avoid any impact during the analysis of actual study samples.

Stability

Evaluation of stability should be carried out to ensure that every step taken during sample preparation and sample analysis, as well as the storage conditions used do not affect the concentration of the analyte. The stability of the samples should be assessed under conditions that is as close as possible to those under which the samples are actually stored or analyzed. Careful consideration should be given to the solvent or matrix type, container materials, and storage conditions used in the stability-determination process.[2]

Stability testing 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 freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. If, during sample analysis for a

study, storage conditions changed and/or exceeded the sample storage conditions evaluated during method validation, stability should be established under these new conditions.

Stability of the analyte in the studied matrix is evaluated using low and high QC samples (blank matrix spiked with analyte at a concentration of a maximum of 3 times the LLOQ and close to the ULOQ) which are analysed immediately after preparation and after the applied storage conditions that are to be evaluated. The QC samples are analysed against a calibration curve, obtained from freshly spiked calibration standards, and the obtained concentrations are compared to the nominal concentrations. The mean concentration at each level should be within ±15% of the nominal concentration.

Stability of the stock and working solutions should be tested with an appropriate dilution, taking into consideration the linearity and measuring range of the detector. Stability studies should investigate the different storage conditions over time periods that equal or exceed those applied to the actual study samples.

a. Freeze and Thaw Stability: During freeze/thaw stability evaluations, the freezing and thawing of stability samples should mimic the intended sample handling conditions to be used during sample analysis. Stability should be assessed for a minimum of three freeze-thaw cycles.

b. Bench-Top Stability: Bench top stability experiments should be designed and conducted to cover the laboratory handling conditions that are expected for study samples.

c. Long-Term Stability: The storage time in a long-term stability evaluation should equal or exceed the time between the date of first sample collection and the date of last sample analysis.

d. Stock Solution Stability: The stability of stock solutions of drug and internal standard should be evaluated. When the stock solution exists in a different state (solution vs. solid) or in a different buffer composition (generally the case for macromolecules) from the certified reference standard, the stability data on this stock solution should be generated to justify the duration of stock solution storage stability.

e. Processed Sample Stability: The stability of processed samples, including the resident time in the auto sampler, should be determined.[1]

APPLICATION OF LC-MS/MS FOR THE QUANTITATIVE BIOANALYSIS OF ANALYTES IN VARIOUS BIOLOGICAL SAMPLES[10]

The major applications of LC-MS in pharmaceutical analysis have been in drug metabolism studies, the analysis and identification of impurities and degradation products in pharmaceuticals and the isolation and characterization of potential drug substances from natural or synthetic sources.

a. LC-MS in Drug Metabolism Studies

The metabolism of a potential new pharmaceutical must be studied before it can be considered for further development into a therapeutic agent. A good drug should ideally be metabolically stable and show a good pharmacokinetic profile with high bioavailability and long half-life. Some metabolites may also be more pharmacologically active or more toxic than the parent drug. Characterization of the major and active metabolites helps in the discovery and design of new drug candidates with improved pharmacological activity, metabolic stability and toxicology profile. LC-MS is the method of choice for the study of drug metabolism because of its sensitivity and specificity. It also provides molecular weight information and fragmentation patterns for structure elucidation. Which ionization technique is used will depend on the structure and the acidity or basicity of the drug studied. In general, positive or negative ion ESI is preferred for strongly basic or acidic drugs respectively, while APCI is more suited for the less basic/acidic or neutral molecules.

b. Analysis and Identification of Impurities and Degradation Products in Pharmaceuticals

Drug Regulation Authorities require the purity of a pharmaceutical to be fully defined and the presence of impurities be fully tested and evaluated. This is important to ensure that the observed pharmacological and toxicological effects are truly those of the pharmaceutical and not due to the impurities. Analysis and monitoring of impurities and degradation products in formulated pharmaceuticals are essential for ensuring that no compounds with deleterious effects are generated during their shelf life. The identification of degradation products will aid in the understanding of potential side effects associated with degradation and in the design of a more favorable formulation and synthesis of new drugs with greater stability. Impurities present in pharmaceuticals are mainly formed during the synthetic process from raw chemicals, intermediates, solvents and by-products. Raw chemicals and

intermediates for drug manufacturing do not have the same purity requirements as the final pharmaceuticals. They are likely to contain components that affect the purity of the final manufactured pharmaceutical. Solvents used in the synthesis may also contain impurities that could react to generate pharmaceutical impurities. By-products are often generated during synthesis and are another source of pharmaceutical impurities. Monitoring and controlling the penultimate step of pharmaceutical synthesis is crucial for the production of a relatively pure drug and the identification of any potential impurities present. Impurities in pharmaceuticals may also arise from the ingredients used in dosage formulation and/or in the process of formulation where temperature, humidity and light may all play a part. Drug degradation is often complex and unpredictable. The most common degradation processes are oxidation, hydrolysis and dehydration; other processes include adduct formation with excipients, dimerization and rearrangement. The International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use has published guidelines for the reporting, identification and qualification of impurities in pharmaceuticals (ICH Q3A Impurities in new drug substances and ICH Q3B Impurities in new drug products).

c. Analysis of Chiral Impurities in Pharmaceuticals

The separation and detection of chiral impurities in pharmaceuticals are of great importance because the D-isomer of a drug can have very different pharmacological, metabolic and toxicological activity from the L-isomer. LC-MS has been used for the analysis of chiral amino acids in the identification of chiral impurities present in diastereomeric peptide drugs. The peptide is hydrolyzed and the amino acids released are derivatized with N^2-(5-fluoro-2,3-dinitrophenyl)-L-alanine amide (Marfey's reagent), which converts the amino acid enatiomers into the corresponding diastereoisomers for separation and detection by HPLC-MS. The specificity of the MS detection eliminated interference from other peaks encountered when an UV detector was used for detection. Diastereoisomeric derivatization followed by LC-MS separation and detection is also useful for the analysis of chiral acidic drugs *e.g.* 2-arylpropionic acids. Although chiral stationary phases can be used for the enantiomeric separation of these drugs, the sensitivity of detection by negative ion ESI-MS was poor. They have been derivatized with benzofuran fluorescent reagents for enatiomeric separation by HPLC with fluorometric detection. These benzofuran derivatives are also ideal for sensitive and specific detection by positive ion ESI-MS without interference. Diastereomeric derivatization with a suitable reagent that confers favourable MS properties to

the molecules is a technique worth exploring for chiral drug analysis.

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