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Bioanalytical Method Development –Determination of Drugs in Biological Fluids

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ABSTRACT

One of the major challenges facing the pharmaceutical industry today is finding new ways to increase productivity, decrease costs whilst still ultimately developing new therapies that enhance human health. One of the major challenges facing the pharmaceutical industry today is finding new ways to increase productivity,decrease costs whilst still ultimately developing new therapies that enhance human health.To help address these challenges the utilisation of analytical technologies and high-throughput automated platforms has been employed; in order to perform more experiments in a shorter time frame with increased data quality. During the last decade, quantification of low molecular weight molecules using liquid chromatography–tandem mass spectrometry in biological fluids has become a common procedure in many preclinical and clinical laboratories. This overview highlights a number of issues involving "small molecule drugs", bioanalytical liquid chromatography–tandem mass spectrometry, which are frequently encountered during assay development. Since plasma is one of the most widely adopted biological fluid in drug discovery and development, the focus of this discussion will be limited to plasma analysis. Bioanalytical method development largely depends on the experience and the preference of the developer.Mathematical models could help in selecting the proper conditions to develop a selective and robust method, using liquid chromatography, liquid–liquid extraction, solid phase extraction and protein precipitationSpecial attention has been paid to matrix effects, the most important issues in bioanalysis and possible solutions to handle these issues are discussed.By proper use of the proposed models a more structured method development is accomplished, resulting in a description of the method that could be used for future use to control the complete Bioanalytical method

Keywords: *LC–MS; Validation; Matrix effect; Biological fluids; Mass spectrometry; Extraction*

Introduction.

Bioanalytical methods are widely used to quantitate drugs and their metabolites in physiological matrices, and the methods could be applied to studies in areas of human clinical pharmacology and nonhuman pharmacolgy/toxicolgy. Bioanalytical method employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic (PK), and toxicokinetic studies.[1] The major bioanalytical services are method development, method validation and sample analysis (method application).

Whatever way the analysis is done it must be checked to see whether it does what it is purported to do; i.e it must be validated. Each step in the method must be investigated to determine the extent to which environment, matrix or procedural variables can effect the estimation of analyte in the matrix from the time of collection up to the time of analysis. Both HPLC and LCMS-MS can be used for the bioanalysis of drugs in plasma. Each of the instruments has its own merits and demerits. HPLC coupled with UV, PDA or fluorescence detector can be used for estimation of many compounds but it does not give the high sensitivity as required by some of the potent, low dose drugs and lacks selectivity. The main advantages of LCMS-MS include low detection limits, the ability to generate structural information, the requirement of minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities. Despite their high sensitivity and selectivity LC/MS/MS instruments are limited to some extent due to matrix-induced differences in ionization efficiencies and ion suppression/enhancement effects due to biological matrix. HPLC coupled with UV, PDA or fluorescence detector offers a cost effective bioanalytical method. Depending on the sensitivity, selectivity and cost effectiveness of the method a choice needs to be made between HPLC AND LCMS-MS.

ESTIMATION OF DRUGS IN BIOLOGICAL FLUIDS

The choice of sampling media is determined largely by the nature of the drug study. For example, drug levels in a clinical pharmacokinetic study demand the use of blood, urine, and possibly saliva. A bioavailability study may require drug level data in blood or urine. Steps involved in the estimation of drugs in biological fluid are collection of the sample, sample treatment and separation of the compound of interest from the matrix and analysis.

BASIC PRINCIPLES OF MASS SPECTROMETRY

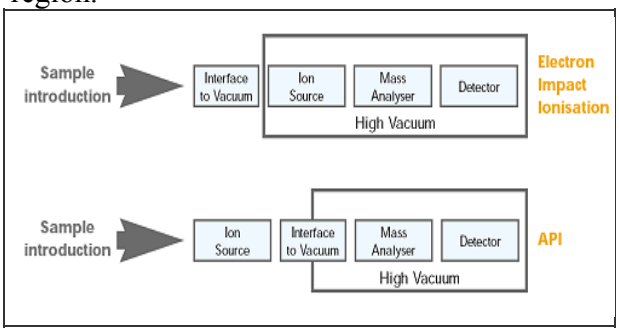
Mass spectrometry is based on slightly different principles to the other spectroscopic methods.

The physics behind mass spectrometry is that a charged particle passing through a magnetic field is deflected along a circular path on a radius that is proportional to the mass to charge ratio, m/e. In a mass spectrometer, a ***radical cation*** is formed by displacing an electron from the organic molecule known as the ***molecular ion*****.** If the molecular ion is too unstable then it can ***fragment*** to give other smaller ions. The collection of ions is then focused into a beam and accelerated into the magnetic field and deflected along circular paths according to the masses of the ions. By adjusting the magnetic field, the ions can be focused on the detector and recorded.There are a number of different mass spectrometers which are characteristic of the instrument manufacturer, ionisation technique or the instrument design (some instruments are designed so that they are optimised for particular applications). There are also technological differences in the mass analyser. For example, in sector instruments, ions are produced in the source of a mass spectrometer that is operating under vacuum. They are then accelerated by an electric field into a magnetic region.

Scanning the magnetic field of the electromagnet sequentially focuses ions of differing mass at the detector. In contrast, quadrupole mass spectrometers operate by filtering masses through a radio frequency voltage field. Whilst there are differences in the instruments, the scientific principles and overall components are the same for all mass spectrometers. In each case there is a need to introduce the analysed sample into the mass spectrometer; in practice this is the interface with the chromatographic separation technique (primarily LC). The analyte molecule is then converted into an ionic compound by the ionisation process; following ionisation the ions are progressed to the mass analyser and ion detector.[2]

PRINCIPLES OF LC/MS

LC/MS is a hyphenated technique, combining the separation power of HPLC, 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. 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. 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.[3]



Ionization modes

Electrospray Ionization(ESI)

The electrospray ionization mode transforms ions in solution into ions in gas. In ESI, ions are produced and analyzed as follows:

1. The sample solution enters the ESI needle, to which a high voltage is applied

2. The ESI needle sprays the sample solution into a fine mist of droplets that are electrically charged at their surface.
3. The electrical charge density at the surface of the droplets increases as solvent evaporates from the droplets.
4. The electrical charge density at the surface of the droplets increases to a critical point known as the Rayleigh stability limit. At this critical point, the droplets divide into smaller droplets because the electrostatic repulsion is greater than the surface tension. The process is repeated many times to form very small droplets.
5. From the very small, highly charged droplets, sample ions are ejected into the gas phase by electrostatic repulsion.
6. The sample ions enters the mass spectrometer and are analyzed.[4]

Atmospheric Pressure Chemical Ionization (APCI)

APCI is a soft ionization technique, but not as soft as ESI. APCI is used to analyze compounds of medium polarity that have some volatility.

In APCI, ions are produced and analyzed as follows:

1. The APCI nozzle sprays the sample solution into a fine mist of droplets.
2. The droplets are vaporized in a high temperature tube.
3. A high voltage is applied to a needle located near the exit end of the tube. The high voltage creates a corona discharge that forms reagent ions through a series of chemical reactions with solvent molecules and nitrogen sheath gas.
4. The reagent ions react with sample molecules to form sample ions.
5. The sample ions enter the mass spectrometer and are analyzed.

Matrix Assisted Laser Desorption/Ionization (MALDI)

MALDI permits the analysis of high molecular weight compounds with high sensitivity. It uses a solid matrix and the ionization beam is laser light. Ion formation in MALDI is accomplished by directing a pulsed laser beam onto a sample suspended or dissolved in a matrix.[6]

MASS ANALYZERS

These forms the heart of the LCMS-MS instruments. There are different types based on their mechanism some of them are as follows

Types of Analyzers	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)

SCAN TYPES

Full Scan

The full scan type provides a full mass spectrum of each analyte. Full scan experiments are used to determine or confirm the identity of unknown compounds or the identity of each component in a mixture of unknown compounds.

Selected Ion Monitoring(SIM)

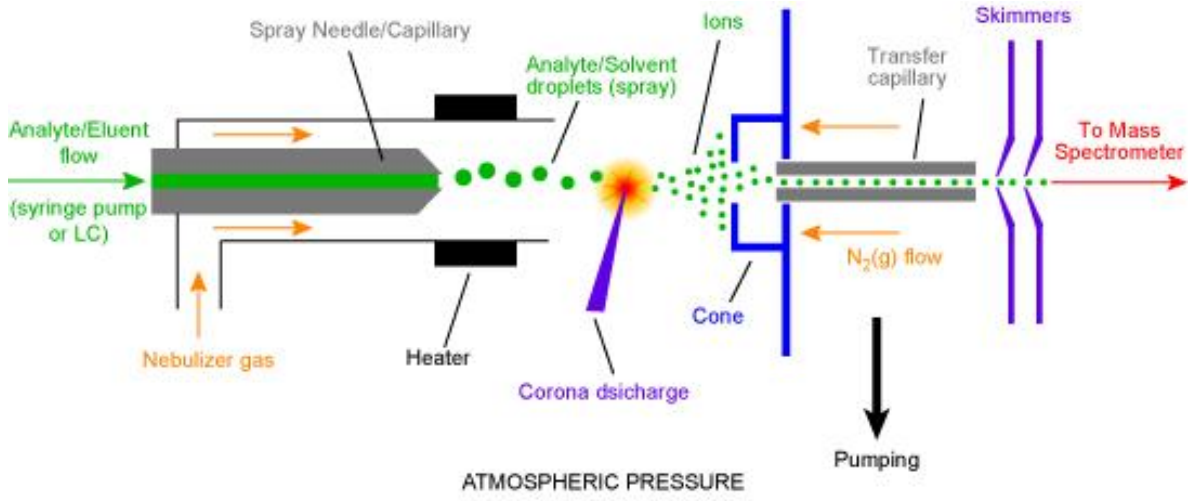
SIM is a technique in which a particular ion or set of ions is monitored. SIM experiments are useful in detecting small quantities of a target compound in complex mixture when the mass spectrum of target compound is known.

Selected Reaction Monitoring(SRM)

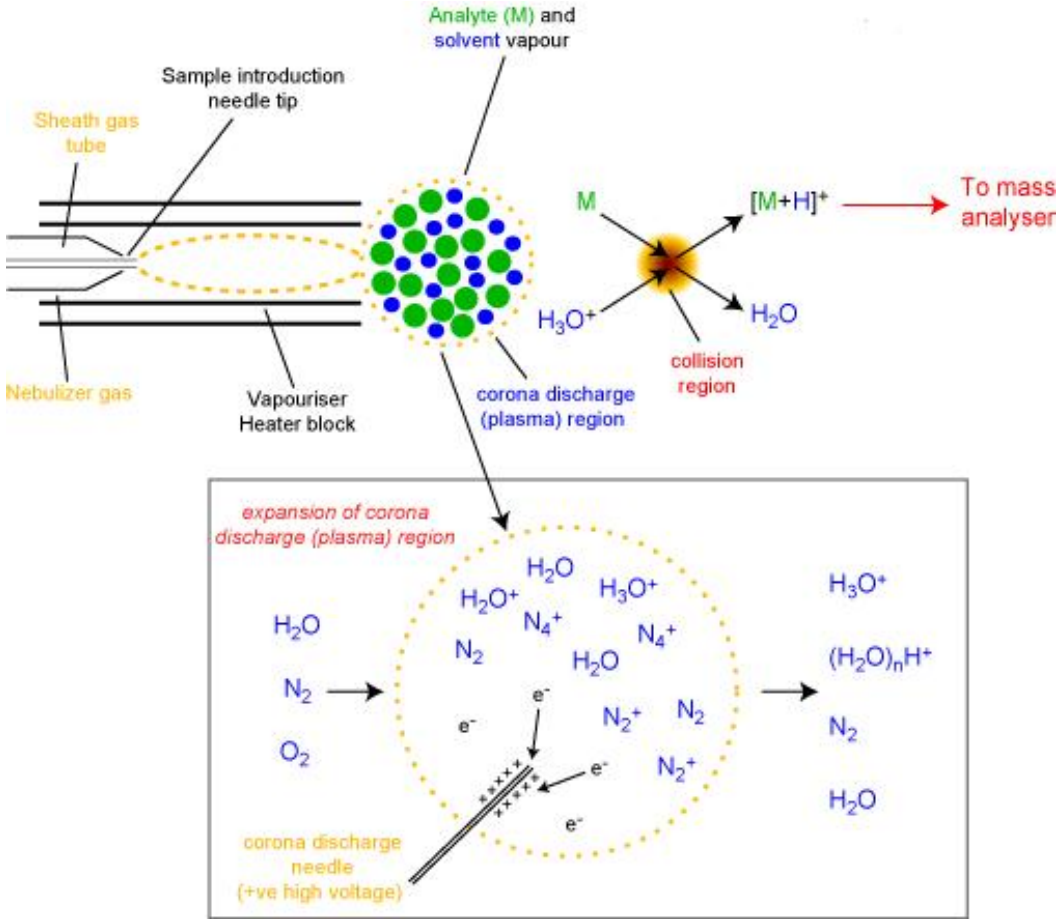
In SRM, a particular reaction or set of reactions, such as the fragmentation of an ion or the loss of a neutral moiety is monitored.[7]

DETECTORS

The detector is the device which detects the ions separated by the analyser. 3 different types of detector are used with the analysers i.e. Electron multipliers, dynolyte photomultiplier, microchannel plates , out of which Electron multiplier is most widely used .



A schematic of the components of an APCI source



A more detailed view of the mechanism of APCI [5]

EXTRACTION PROCEDURES FOR DRUGS AND METABOLITES FROM BIOLOGIC SAMPLES

Sample preparation prior to chromatographic separation there are three major objectives

1. The dissolution of analyte in suitable solvent
2. Removal of interfering compound as possible
3. Preconcentration of the analyte

Different types of extraction technique are:

a. Liquid-Liquid Extraction

Liquid-liquid extraction is useful for separating analytes from interferences by partitioning the sample between two immiscible liquids or phases. One phase in LLE often is aqueous and second phase an organic solvent. More hydrophilic compounds prefer the polar aqueous phase; where as more hydrophobic compounds will be found mainly in the organic solvents. Analyte extracted into the organic phase are easily recovered by evaporation of the solvent, while analytes extracted in to the aqueous phase can often be injected directly on to a reversed-phase column.

The technique is simple, rapid and has relatively small cost factor per sample when compared to others. The extraction containing drug can be evaporated to dryness and the residue reconstituted in a smaller volume of a appropriate solvent (preferably mobile phase). Near quantitative recoveries(90%) of most drugs can be obtained through multiple continuous extraction.

b. Solid phase extraction:

Solid phase extraction is the most important technique used in sample pretreatment for HPLC. SPE occur between a solid phase and a liquid phase. SPE is more efficient separation process than LLE. It is easier to obtain a higher recovery of analyte. SPE employs a small plastic disposable column or cartridge, often the barrel of a medical syringe packed with 0.1 to 0.5 g of sorbent. The sorbent is commonly reversed phase material (C18-silica), and a reversed phase SPE (RP-SPE) assembles both LLE and reversed phase HPLC in its separation characteristics. In SPE, a liquid sample is added to the cartridge and wash solvent is selected so that the analyte is either strongly retained ($k>>1$) or unretained ($K=0$). When the analyte is strongly retained, interferences are eluted or washed from the cartridge so as to minimize their presence in the final analyte fraction. The analyte is then eluted in a small volume with strong elution solvent, collected, and either (1) injected directly or (2) evaporated to dryness followed by dissolution in the HPLC mobile phase. In the opposite case, where analyte is weakly retained, interferences are strongly held on the cartridge and the analyte is collected for the further treatment.

Advantages of SPE vs. LLE

- More complete extraction of the analyte
- More efficient separation of interferences from analyte
- Reduced organic solvent consumption
- Easier collection of the total analyte fraction
- More convenient manual procedures
- Removal of particulates
- More easily automated

c. Precipitation method

Protein precipitation is the simple method of extraction as compared to the LLE and SPE. This can be carried out by using the suitable organic solvents which has good solubility of the analyte and protein precipitating properties. Acetonitrile is the first choice of solvent for protein precipitation due to its complete precipitation of proteins and methanol is the second choice of organic precipitant provided the solubility of the analyte in these solvents. After protein precipitation the supernatant obtained can be injected directly in to the HPLC or it can be evaporated and reconstituted with the mobile phase and further clean up of the sample can be carried out by using micro centrifuge at very high speed. [8]

METHOD DEVELOPMENT

Methods for analyzing drugs by HPLC and LCMS-MS can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character, pK_a values and the solubility parameter. Method development cannot be standardized across the board because method development is unique and specific for each drug candidate. It also depends on the nature of the sample, sensitivity required etc. While there are a number of HPLC methods available to the development chemist, perhaps the most commonly applied method is reversed phase and reverse phase coupled with ion-pairing probably account for more than 85% of the applications for a typical pharmaceutical compound. The typical pharmaceutical compounds is considered to be an active

pharmaceutical ingredient of less than 1,000 Daltons, either soluble in water or in an organic solvent

General conditions to initiate HPLC method development [9]

Method development starts with literature survey of the molecule in which we find the nature of the molecule its pKa, solubility, molecular weight etc.Either isocratic or gradient mode may be used to determine the initial conditions of the separation, following the suggested experimental conditions given in the Table#1

Depending on the number of active components to be resolved or separated, the mode of run can be determined. If the number of components is large or the pKa values of components are wide apart then gradient mode is preferred over isocratic mode.

In deciding whether a gradient would be required or whether isocratic mode would be adequate, an initial gradient run is performed and the ratio between the total gradient time and the difference in gradient time between the first and last component are calculated. When the calculated ratio is <0.25, isocratic is adequate; when the ratio is >0.25, gradient would be beneficial for the separation of complex mixture and when there are many compounds or degradation products, a long gradient run may be needed. In this case, two separation modes using an isocratic method for product release and gradient method for stability assessment.

In general, one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble. The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography.

Table # 1 General Experimental Conditions for an Initial HPLC Run

Initial parameters			
Chromatographic variables	**Neutral compounds**	**Ionic-acidic compounds (carboxylic acids)**	**Ionic-basic compounds (amines)**
Column			
Dimension (length, ID)	25cm x 0.46cm	25cm x 0.46cm	25cm x 0.46cm
Stationary phase	C18 or C8	C18 or C8	C18 or C8
Particle size	10µm or 5µm	10µm or 5µm	10µm or 5µm
Mobile phase			
Solvents A and B	Buffer-acetonitrile	Buffer-acetonitrile	Buffer-acetonitrile
%B (organic) isocratic	50%	50%	50%
%B (organic) gradient	20%/80%	20%/80%	20%/80%
Buffer			
Type	Phosphate	Phosphate	Phosphate
Concentration	50 mM	50 mM	50 mM
pH	3.0	3.0 &7.5(gradient)	3.0 &7.5(gradient)
Peak modifier	10mM triethylamine and 1% acetic acid	1% acetic acid	25 mM Triethylamine
Flow rate	1.5-2.0 mL/min	1.5-2.0 mL/min	1.5-2.0 mL/min
Temperature	Ambient to 35°C	Ambient to 35°C	Ambient to 35°C
Sample size			
Volume	10µL-25µL	10µL-25µL	10µL-25µL
Mass	< 100mcg	< 100mcg	< 100mcg

Gradient can be started with 5-10 % organic phase in the mobile phase and the organic phase concentration can be increased up to 100 % within 20-30 min. Separation can then be optimized by changing the initial mobile phase composition and the slope of gradient according to the chromatogram obtained from preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest is eluted, namely, at what mobile phase composition, retention time and the pKa of the component. Changing the polarity of the mobile phase will alter elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic samples (acidic or basic) can be separated, if they are present in undissociated form. Dissociation of ionic samples may be suppressed by proper selection of pH. The buffer selected for a particular separation should be used to control pH over the range of ≈ pKa ± 1.5. The buffer should transmit light at or below 220nm so as to allow low UV detection and pH of the buffer should be adjusted before adding organic.

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that a near symmetrical peaks detect all the compounds, a good separation and a reasonable run time.

The peak resolution can be increased by using a more efficient column (column with higher theoretical plate, N), which can be achieved by using a column of smaller particle size, or a longer column. These factors, however, will increase the analysis time. Flow rate does not influence resolution, but it has a strong effect on the analysis time.

The parameters that are affected by the changes in chromatographic conditions are,

- Capacity factor (k'),
- Selectivity (α),
- Column efficiency (N) and
- Peak asymmetry factor (As).

Selection of Mobile phase

If the sample contains ionic or ionizable compounds, then use of a buffered mobile phase to ensure reproducible results. Under unfavorable circumstances, pH changes as little as 0.1 pH units can have a significant effect on the separation. On the other hand properly used buffer allows controlling the pH easily. Buffer works best at the pKa of its acid. At this pH, the concentration of the acidic form and the basic form of the buffering species is equal, and the buffering capacity is maximum. Phosphate has two pKa values in the range of interest for silica- based- chromatography. One at pH-2 and the other at pH-7. The pKa of the acidic buffer is 4.75.Citrate has three-pKa value: 3.08, 4.77 and 6.40. Between citrate and phosphate buffers, the entire pH range useful for silica chromatography can be covered.

For LCMS-MS ammonium acetate and formate are suitable for buffer mobile phase as they are volatile in nature; usually 2-10mM concentration is adequate but concentration upto 50mM can be used.

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.

In many cases, silanophillic interactions cause tailing, mostly due to ion-exchange interaction. This can usually be reduced or suppressed by the use of amine-based buffers or by using acidic mobile phases, or a combination thereof.

Whenever buffers or other mobile phase activities are used, check the solubility in mobile phase. This is especially true for gradient applications. Acetonitrile is the preferred organic modifier in reverse-phase-chromatography. Acetonitrile based mobile phases can give an up 2-fold lower pressure drop than methanol based mobile phase at equal flow rate. This means that column efficiency is higher.

The elution strength increases in the order methanol, acetonitrile and tetrahydrofuran. The retention changes by roughly 10 % for every 1 % change in the concentration of organic modifier.

Role of pH

pH is another factor in the resolution that will affect the selectivity of the separation in

reversed-phase HPLC. Selecting the proper buffer pH is necessary to reproducible separation of ionizable compounds by Reversed-Phase HPLC. Selecting an improper pH for ionizables analyte often leads to asymmetric peaks that are broad, tail, split, or shoulder. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low Relative Standard Deviation (RSD) between injections, and reproducible retention times.

Sample retention increases when the analyte is more hydrophobic. Thus when an acid (HA) or base (B) is ionized (converted from the unionized free acid or base) it becomes more hydrophilic (less hydrophobic, more soluble in aqueous phase) and less interacting with column binding sites, as a result the ionized analyte is less retained on the column, so that the ḱ is reduced, some times dramatically. When the pH = pK_a for the analyte, it is half ionized, i.e. the concentrations of the ionized and unionized species are equal. As mostly all of the pH caused changes in the retention occur within ± 1.5 pH units of the pK_a value, it is best to adjust the mobile phase to pH values at least ± 1.5 pH units of above or below the pK_a to ensure practically 100% unionization of analyte for retention purposes. Generally at low pH peak tailing is minimized and method ruggedness is maximized. On the other hand, operating in the intermediate pH offers an advantage in increased analyte retention and selectivity

Role of Buffer

In Reverse-Phase liquid chromatography mobile phase pH values are usually between 2.0 and 7.5. Buffers are needed when an analyte is ionizable under Reverse-Phase conditions or the sample solution is outside this pH range. Analytes ionizable under Reverse-Phase conditions often have amine or acid functional groups with pK_a between 1.0 and 11.0. A correctly chosen buffer pH will ensure that the ionizable functional group is in a single form, whether ionic or neutral. If the sample solution is at pH damaging to the column, the buffer will quickly bring the pH of the injected solution to a less harmful pH.

If the analyte contain only amine functional groups Buffer selection is easier. Most amines will be in cationic form at pH value less than 9.0, so any buffer effective at pH 7.0 or lower will work. Buffer at pH 7.0 are used, even though pH of water is 7.0, because amine retention and peak shapes are pH dependent. As pH is lowered amine retention time shortens and peak shapes sharpens as the buffer protonates the acidic silanols on the silica surface. Any buffer with a pK_a less than 7.0 is suitable, but phosphate buffer of pH 3.0 is found to be best for amines.

Selection of Column:

The HPLC column is the heart of the method, critical performing the separation. The column must possess the selectivity, efficiency and reproducibility to provide good separation. Commonly used reversed phase columns are C_{18} (octadecyl silane,), C_8 (octyl silane,) phenyl and cyano. They are chemically different bonded phases and demonstrate significant changes in the selectivity using the same mobile phase

During method development selection of column can be streamlined by starting with shorter columns (150,100 or even 50mm long.). By selecting a shorter column with an appropriate phase run time can be minimized so that an elution order and an optimum mobile phase can be quickly determined. It is also advantageous to consider the column internal diameter, many laboratories use 4.6mm ID as standard, but it is worth considering use of 4mm ID column as an alternative. This requires only 75% of the solvent flow than that of 4.6mm column.

Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, a octyl phase (C_8) can provide time saving over a octadecyl (C_{18}) as it doesn't retain analytes as strongly as the C_{18} phase. For normal phase applications Cyano phases are the most versatile.

C_{18} (250*4.6mm) column are more often used in the laboratory. These columns are able to resolve a wide variety of compounds due to their selectivity and high plate counts.

Role of temperature

Temperature variations over the course of a day have quite significant effect on HPLC separations. This can even occur in air conditioned rooms. While temperature is a variable that can affect the selectivity, its effect is relatively small. Also retention time generally decreases with an increase in temperature for neutral compounds but less dramatically for partially ionized analytes. Snyder et al. (29) reported that an increase of 1°C will decrease the retention time by 1to 2%. Because of possible temperature fluctuations during method development and validation, it is recommended that the column be thermostated to control the temperature.

Role of Flow rate

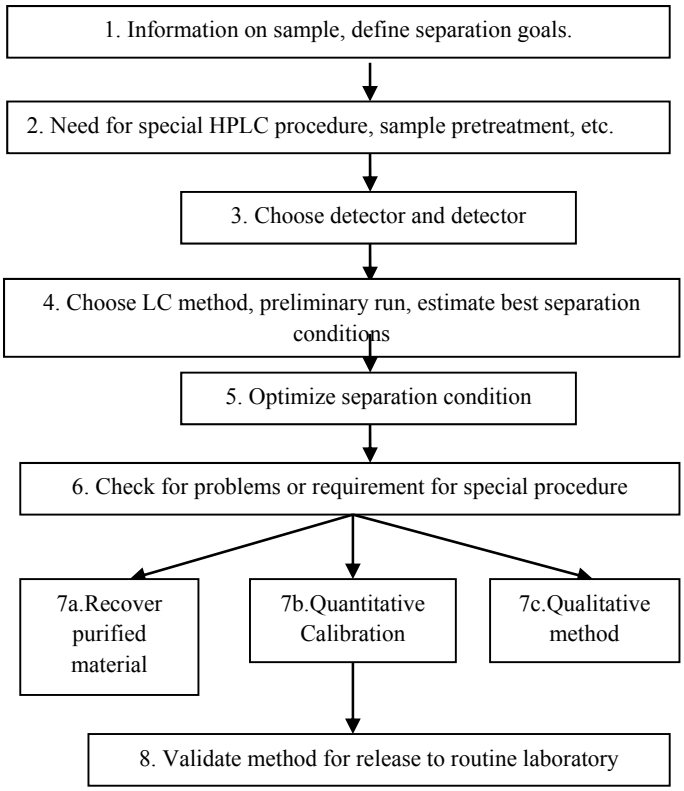
Flow rate, more for isocratic than gradient separation, can sometimes be useful and readily utilized to increase the resolution, although its effect is very modest. The slower flow rate will also decrease the column back pressure. The disadvantage is that when flow rate is decreased, to increase the resolution slightly, there is a corresponding increase in the run time.

Method development involves considerable trial and error procedures. Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that all the compounds are detected by more or less symmetrical peaks on the chromatogram.[10]

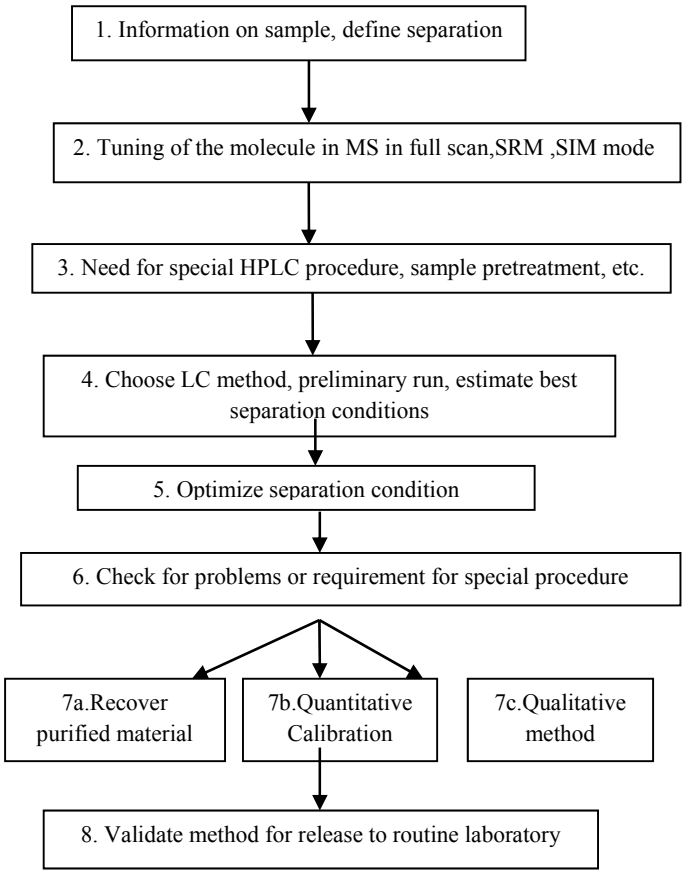
Selection of Internal Standard

A compound added to a sample in known concentration to facilitate the qualitative indentification and/or quantitative determination of the sample components. The best internal standard is an isotopically labeled version of the molecule you want to quantify. An isotopically labeled internal standard will have a similar extraction recovery, ionization response in ESI mass spectrometry, and a similar chromatographic retention time. Often it is difficult to procure an isotopically similar compound than a compound with similar characteristics to that of analyte is chosen.

Steps Involved in HPLC Method Development



Steps Involved in LCMS-MS Method Development



Method validation

Owing to the importance of method validation in the whole field of analytical chemistry, a number of guidance documents on this subject have been issued by various international organizations and conferences [11–17]. All of these documents are important and potentially helpful for any method validation.

However, only few specifically address analysis of drugs, poisons, and/or their metabolites in body fluids or tissues [11,15,17]. Of these, the most influential guidance documents are the reports on the conference on ‘‘Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies’’ held inWashington in 1990 (Conference Report) [11] and the follow-up conference in 2000 (Conference Report II) [15], in which experiences and progress since the first conference were discussed. Both of these reports were published by Shah et al. and had an enormous impact on validation of bioanalytical methods in the pharmaceutical industry. Because of the close relation to bioanalysis in the context of bioavailability, bioequivalence and pharmacokinetic studies, Conference Report II is probably also the most useful guidance paper for bioanalytical method validation in clinical and forensic toxicology. It was therefore also used as basis for the guidance document recently issued by the Germanspeaking Society of Toxicological and Forensic Chemistry (GTFCh) [17], and to the authors’ knowledge, the only available comprehensive guideline specifically addressing method validation in analytical toxicology. Besides these official guidance documents, a number of review articles have been published on the topic of analytical method validation [18–23]. Again, all of these papers are interesting and helpful for any method validation, while only part of them specifically address analysis of drugs, poisons, and/or their metabolites in body fluids or tissues [18,19,23]. This

includes the excellent review on validation of Bioanalytical chromatographic methods which includes detailed discussions of theoretical and practical aspects [8]. The other two deal with the implications of bioanalytical method validation in clinical and forensic toxicology [19] and with theoretical and practical aspects in method validation using LC–MS(/MS) [23]. The latter also describes a proposed experimental design for validation experiments, as well as statistical procedures for calculating validation parameters.

Selective and sensitive analytical methods for the quantitative evaluation of drug and their metabolites are critical for the successful conduct of preclinical, biopharmaceutical and clinical pharmacology studies.

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

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

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

Types of method validation

A. Full Validation

Establishment of all validation parameters to apply to sample analysis for the bioanalytical method for each analyte.

B. Partial Validation

It is modification of already existing validated bioanalytical method; it can range from as little as one intra-assay, accuracy and precision determination to nearly full validation.

Typical bioanalytical method changes that fall into this category include, but are not limited to

a) Change in analytical methodology
b) Change in matrix within species
c) Change in sample processing procedures
d) Changes in instruments/ or softwares platform

e) Limited sample volume
f) Selectivity demonstration of an analyte in the presence of specific metabolites

C. Cross Validation

Cross validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within same study or across different studies. An example of cross validation would be a situation where an original validated bioanalytical method serves as an reference and the revised bioanalytical method as the comparator.

2.1. Validation parameters

Analytical methods in clinical and forensic toxicology may either be used for screening and identification of drugs, poisons and/or their metabolites in biological fluids or tissues, for their quantification in these matrices, or for both. For quantitative bioanalytical procedures, there is a general agreement, that at least the following validation parameters should be evaluated: selectivity, calibration model (linearity), stability, accuracy (bias), precision (repeatability, intermediate precision) and the lower limit of quantification (LLOQ). Additional parameters which may be relevant include limit of detection (LOD), recovery, reproducibility, and ruggedness (robustness) [11,15,18,24]. For qualitative procedures, a general validation guideline is currently not available [22], but there seems to be agreement that at least selectivity and the LOD should be evaluated and that additional parameters like precision, recovery and ruggedness (robustness) might also be important [13,22,25,26]. For methods using LC–MS, experiments for assessment of possible matrix effects (ME), i.e. ion suppression
or ion enhancement, should always be part of the validation process, particularly if they employ electrospray ionization (ESI) [15,23,27–29].

Fundamental parameters

1. Selectivity
2. Sensitivity
3. Linearity
4. Accuracy
5. Precision
6. Recovery
7. Matrix effect
8. Dilution integrity
9. Stability

Selectivity

Selectivity is defined as, "the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample The definition of selectivity is quite similar to the definition of specificity: "the ability to assess unequivocally the analyte in the presence of components which might be expected to be present .

Selectivity is evaluated by injecting extracted blank plasma and comparing with the response of extracted LLOQ samples processed with internal standard.

There should be no endogenous peak present within 10% window of the retention time of analyte and an internal standard. If any peak is present at the retention time of analyte, its response should be ≤ 20% of response of an extracted Lower calibration standard i.e. LLOQ standard If any peak is present at the retention time of an internal standard, its response should be ≤ 5% of the response of an extracted internal standard at the concentration to be used in study.

Sensitivity

Sensitivity is measured using Lower Limit Of Quantification (LLOQ) is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. The LLOQ should be established using at least five samples independent of standards and determining the co-efficient of variation and appropriate confidence interval. The LLOQ should serve the lowest concentration on the standard curve and should not be confused with limit of detection and low QC sample. The highest standard will define the upper limit of quantification (ULOQ) of an analytical method.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

A calibration curve is the relationship between instrument response and known concentrations of analyte. The matrix based standard curve should consist of a minimum of six standard points, excluding blanks, using single or replicate samples. The standard curve should cover the entire range of expected concentrations. Standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for goodness of fit.

Accuracy and Precision

For validation of bioanalytical method, accuracy and precision should be determined using a minimum of five determinations per concentration level (excluding blank samples). The mean value should be within ± 15% of the theoretical value, except at LLOQ, where it should not deviate by more than ± 20%. The precision around the mean value should not exceed 15% of the CV except for LLOQ, where it should not exceed 20% of the CV.

The accuracy and precision with which known concentrations of analyte in the biologic matrix can be determined should be demonstrated. This can be accomplished by analysis of replicate sets of analyte samples of known concentrations QC samples from an equivalent biologic matrix. At a minimum three concentrations representing the entire range of the standard curve should be studied: one within 3x the LLOQ (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC).

Reported method validation data and the determination of accuracy and precision should include all outliers. However, calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported.

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

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

Recovery

An important parameter associated with Bioanalytical methods is recovery. The recovery of an analyte in an assay is defined as, "the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery reflects the degree of extraction; in general recovery is impacted by the interaction of the analyte with endogenous and/or exogenous components of the matrix. Analytes that interact strongly with the aforementioned components may not be completely extracted. An extraction method need not result in 100% recovery of the analyte; however, the degree of recovery must be consistent, precise and reproducible.

Knowledge of the physical and chemical characteristics of the analyte would provide a starting point. Depending on the extraction scheme, factors such as solvent composition (organic), buffer ionic strength and pH, acid or base concentration and temperature are just some of the parameters that could be investigated. It is not always possible to improve the degree of recovery. Consequently one should determine the amount of time to be spent on such investigations. If the recovery can't be improved, different type of the matrix should be extracted to ensure that the process is consistent, precise and reproducible.

% Recovery =100 x Mean response of extracted samples/Mean response of unextracted samples

Matrix effect (for LCMS-MS only)

Matrix effect is the effect on an bioanalytical method caused by all other components of the sample except the specific compound to be quantified. It happens due to ion suppression/enhancement by the others ions present in the biological matrix which might get ionized during detection and will give false results.

Matrix effect studied by comparing the response of extracted samples spiked before

extraction with response of the blank matrix sample to which analyte has been added at the same nominal concentration just before injection(Causon,1997).Matrix effect is done in LCMS-MS to find out if there is any ion suppression or enhancement effect by the matrix. Process and analyze calibration standards and three sets each of LQC and HQC samples using six different lot of matrix that were post spiked along with internal standard.

Dilution integrity

Dilution integrity is performed in order to check the validity of method incase the sample needs to be diluted during analysis.

It is done by spiking analyte working standard in drug free and interference free plasma to get concentration of 2xULOQ. Two and four fold dilution made of the original concentration using screened and pooled plasma and analysed against a fresh calibration curve. The concentration will be calculated using the dilution factor.

Stability

Stability is an extremely important parameter to consider during the Bioanalytical method validation process. Stability is defined as the, "chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

Should the analyte change in any respect, chromatographic behavior could be affected; in turn, this complicates the process of method development. To reduce the occurrence of changes in the analyte during the method development process, the following activities should be considered:

- determine the short term (up to 6 hrs) stability,
- determine the post preparative stability (auto sampler),
- use fresh samples for extraction and freshly prepared stock solutions for matrix spiking
- determine the short term (up to 6 hrs) stability of the spiked extract
- determine the post preparative stability (auto sampler) of the spiked extract

The parameters listed above can greatly aid implementation of the development process and validation process. If selectivity can't be demonstrated, the procedure is not suitable for its' intended purpose. If recovery is inconsistent and the analytes are fractionated after being altered, development of the method would be quite difficult. Under such conditions, characteristics such as accuracy, precision, range, etc. would be most likely be greatly affected.

Another issue often raised in the context of analytical method validation is measurement uncertainty. However, measurement uncertainty is not a validation parameter of itself. It can be obtained from validation data, most notably bias and precision, or from routine QC data obtained during application of the analytical method. For this reason, it is not covered in detail here. Interested readers are referred to the EURACHEM/CITAC guide to quantifying uncertainty in analytical measurement [30].

CONCLUSION

Although it is a good starting point, modelling analytical techniques in bioanalytical method development should not be restricted to pure and neat analyte solutions.Unknowncompounds originating from the biological matrix could interfere with the analyte response and should also be taken into account in the modeling process.Carefully built models provide a good description of the behaviour of the complete analytical system under different conditions. These results could be considered as a summary of the results of the performed experiments. Also, method development could be performed much more structured and the models could be helpful for future use for trouble shooting purposes.Care should be taken to include responses into the models that are not affected by unexpected effects. Besides matrix effects affecting the analytical response, other effects could also blur the results.Solvent evaporation of extractsmay lead to incomplete reconstitution due to the loss of analyte or adsorption effects and a decrease in the amount of thermal, oxidation sensitive or pH labile

compounds may be interpreted as a low recovery. Also, enzymatic activity in biological matrices may decrease the analyte concentration resultingin misinterpreted recovery values.Moreover, one has to be sure that a state of equilibrium has been reached and that reactions have been complete as in protein precipitation..A bioanalytical method which was set up using theoretical models and where matrix effects are taken into account could be considered as a controlled method since the effect of changes in analytical parameters can be predicted quantitatively. Validation of new methods in analytical toxicology is an integral part of quality assurance, accreditation, and publication.Methods intended for routine use or publication must be fully validated to objectively demonstrate their applicability for the intended use. For methods used for analysis of rare analytes or in single cases, the number of validation experiments and parameters to be evaluated may be reduced, but a minimum validation is necessary to ensure sufficient quality of the results.

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