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Bioanalytical Method Development and Validation: Determination of Drugs in Biological Fluids

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ABSTRACT

In this review article, a brief discussion of bioanalytical method development and validation. Bioanalytical method employed for the quantitative estimation of drugs and their metabolites in biological media and plays an important role in estimation and interpretation of bioequivalence, pharmacokinetic, and toxicokinetic studies. Analysis of drugs and their metabolites in a biological matrix is carried out using different extraction techniques like liquid-liquid extraction, solid-phase extraction, and protein precipitation. Validation of analytical method gives information about various stages and parameters like accuracy, precision, linearity, limit of detection, limit of quantitation, specificity, range and robustness.

INTRODUCTION

Bioanalytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a biological matrix. A compound can often be measured by several methods and the choice of analytical method involves many considerations.[1] The process by which a specific bioanalytical method is developed, validated, and used in routine sample analysis can be divided into

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

Reasons for developing new methods of analysis

1. There may not be a suitable method for a particular analyte in the specific sample matrix.

2. Existing method may be having too many errors or is contamination prone or they may be unreliable.

3. Existing method may be too expensive, time consuming, or energy intensive, or they may not be easily automated.

4. Existing method may not provide adequate sensitivity or analyte selectivity in samples of interest.

5. Newer instrumentation and techniques may have evolved that provide opportunities for improved methods, including improved analyte identification or detection limits, greater accuracy and precision, or better return on investment.

6. There may be need for an alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.[1]

Bioanalytical Method Development

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, and toxicokinetic. The major bioanalytical services are method development, method validation and method application. 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. Depending on the sensitivity, selectivity and cost-effectiveness of the method a choice needs to be made between HPLC and LC-MS.[6]

Steps in method development

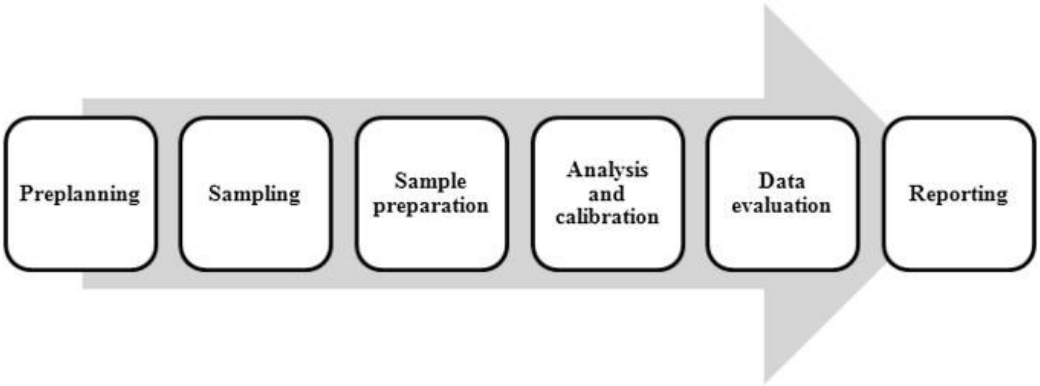

Fig 1: Steps in method development

1. Literature search for drugs
2. Identification of analytical techniques and optimization
3. Reference standard preparation
4. Selection of internal standard
5. Sample pre-treatment
6. Sample storage[7]

Sample collection and preparation

Biological media that contain analyte are blood, plasma, urine, serum. Blood is collected from human volunteers by vein puncture with a hypodermic syringe up to 5-7 ml. Venous blood is withdrawn into tubes containing an anticoagulant such as ethylene diamine tetra acetic acid or heparin. On centrifugation of blood sample at 4000 rpm for 15 mins, plasma sample is obtained. About 30-50 % of volume is collected. Aim of sample preparation is to clean up sample before analysis. Presence of macromolecules, proteins, salts, small molecules, metabolic by-products in blood sample can cause interference during analysis. Sample preparation involves conversion of analyte from biological matrix into a solvent suitable for analysis. Sample preparation methods are:[8]

- Liquid-liquid extraction
- Solid-phase extraction
- Protein precipitation
- Microextraction techniques
- Matrix solid phase dispersion

Liquid-liquid extraction

It is based on principle of differential solubility and partition equilibrium of analyte molecules between aqueous and organic phases. Liquid-liquid extraction involves extraction of a substance from one liquid phase to additional liquid phase. Recently advanced and improved methods like liquid phase micro extraction, supported membrane extraction, single drop liquid phase microextraction have been developed.[3]

It is useful for separating analytes from interferences by partitioning the sample between two immiscible liquids or phases. One phase is aqueous and second phase is organic solvent. More hydrophilic compounds prefer polar aqueous phase, whereas more hydrophobic compounds prefer organic solvent. Analytes extracted into aqueous phase can be injected directly onto a reverse-phase column, while analytes extracted into organic phase are recovered by evaporation of solvent. Organic solvents used are hexane, diethyl ether, tert-butyl methyl ether, ethyl acetate. This technique is simple, rapid, and has relatively small cost factor per sample. Extraction containing drug can be evaporated to dryness and residue reconstituted by using a small amount of appropriate solvent. Near quantitative recoveries of most drugs can be obtained through multiple continuous extractions.[1]

It is a smart technique in sample preparation in sample preparation. It has been extensively used for preparation of aqueous and biological samples. In liquid-liquid an aqueous sample and an immiscible organic solvent are mixed to eliminate analyte into organic phase for injection into an analytical system. This method can provide good recovery and clean sample. This method was used for extraction of basic and acidic drug from biological samples with high extraction recovery.[9]

Solid Phase Extraction

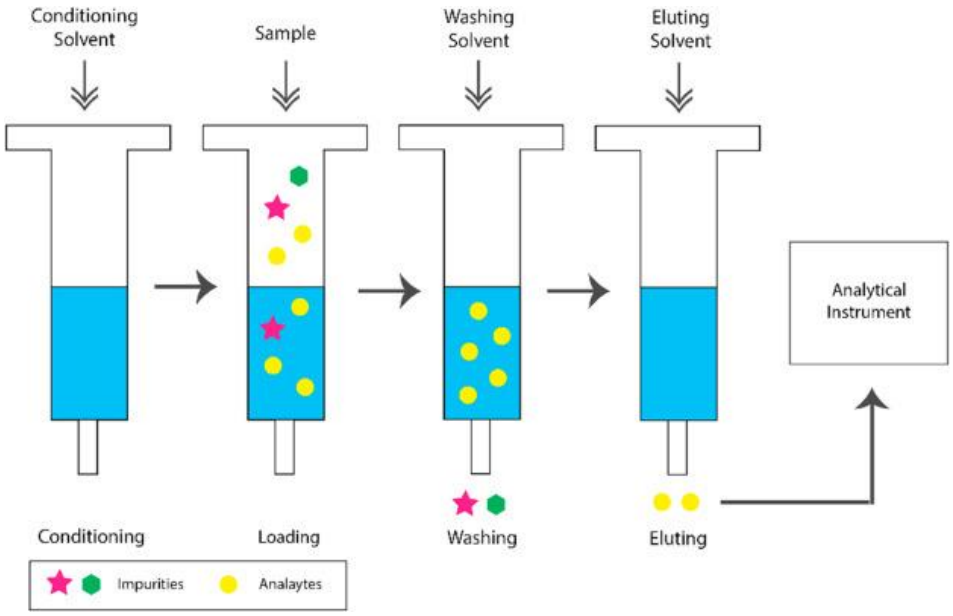

Fig 2: Solid Phase Extraction

It is a choosy method for sample preparation where analyte is bound onto solid support, impurities are washed off and analyte is selectively eluted. A number of solvent choices are available. It is a very powerful technique. It includes 4 steps-

1. Conditioning – Column is activated with organic solvent that acts as a wetting agent on packing material and solvates functional group of sorbents. Water or aqueous buffer is added to activate column for proper adsorption mechanisms.

2. Sample loading – After adjustment of pH, sample enters column by gravity feed, pumping or aspirating by vacuum.

3. Washing – Impurities from matrix are removed by retaining analyte.

4. Elution – Distribution of solvent by sorbent interaction by suitable solvent, removing as little of remaining impurities as possible. Analytes are classified into 4 categories – acid, base, neutral and amphoteric compounds. Amphoteric compounds have both acid and basic functional groups and can function as cations, anions, or zwitter ions depending on pH.[3]

It is the most important technique used in sample preparation for HPLC. It occurs between solid and liquid phase. It is more efficient separation process than liquid-liquid extraction. It is easier to obtain high recovery of analyte. Sorbent is commonly reversed phase material and reversed phase solid phase extraction combines both liquid-liquid extraction and reversed

phase HPLC in its separation characteristics. In solid phase extraction, liquid sample is added to cartridge and wash solvent is selected so that analyte is either strongly retained or unretained. When analyte is strongly retained, impurities are eluted or washed from cartridge so as to minimize their presence in final analyte fraction. Analyte is then eluted in a small volume with strong elution solvent, collected, and either injected directly or evaporated to dryness followed by dissolution in HPLC mobile phase. In opposite case, where analyte is weakly retained, impurities are strongly held on cartridge and analyte is collected for further treatment.[10]

It is the most well-known sample preparation method due to high efficiency, cost-effective, high reproducibility, comparatively green, easy to operate and automate. It is beneficial in separating and concentrating trace analytes from biological samples. Recently, other formats have also been developed such as flat disks and micro solid-phase extraction. Effective parameters in its performance are nature and amount of sorbent, loaded sample volume, composition and volume of washing and elution solutions.[9]

Advantages of SPE vs LLE

- More complete extraction of 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[1]

Types of Solid-Phase Extraction

A. Reversed phase

B. Normal phase

C. Ion exchange

A. Reversed phase

Sorbents used are bonded silica and polymer sorbent as polystyrene. Analytes are intermediate polar to non-polar and hydrophobic interactions between analytes and non-polar sorbent material occurred. This type is less selective compared to normal phase or ion exchange. Elution solutions like methanol, acetonitrile or mixed buffer can be used. Applications are drugs in biological fluids and environmental pollutants in water.[9]

B. Normal phase

Sorbents used are silica with polar functional groups. Retention mechanism is based on hydrogen bonding between analytes and solvent.[9]

C. Ion exchange

It is used for charged analytes in sample solution. Anionic analytes can be isolated with quaternary amine bonded silica as anion exchange. For basic drugs, strong cationic exchange, propyl sulfonic acid bonded with Na^+ counter ions and weak cationic exchange, carboxy propyl phase bonded with Na^+ counter ions can be used for isolating cationic analytes. It is the most selective method compared to normal phase and reversed phase.[9]

Protein Precipitation

It is a simple method used for extraction of analyte from plasma and blood sample. It is based on solubility of analyte in organic solvent which has good precipitation property. First preference solvent is acetonitrile and second preference is to methanol because of its complete protein precipitation property and solubility of analyte. Next step is to collect supernatant liquid and inject it directly into HPLC or it can be evaporated and reconstituted with mobile phase and further clean-up of sample can be carried out by using micro centrifuge at very high speed.[11]

Microextraction techniques

Use of microextraction techniques in bioanalysis has been gained attraction due to achieve high throughput sample preparation, to handle small sample volumes and to decrease solvents consuming. New microextraction techniques like solid-phase micro-extraction, stir bar sorptive extraction, micro-extraction by packed sorbent have been developed recently.

1. Solid phase microextraction

It is a powerful solvent free extraction technique especially for gas chromatography. It has been accepted as a straight forward and automated method in environmental analysis as compared to bioanalysis. A fused silica fibre is coated with a stationary phase. Equilibrium is established by exposing fibre with analyte with matrix. It is of two types – direct extraction of analytes with concentration of fibre into sample, or utilizing headspace to extract volatile compounds from fibre which are portioned between gaseous and liquid phases. To have an efficient process some parameters such as temperature, pH, salt concentration, stirring rate, equilibrium constant and equilibrium time need to be optimized. It is a practical technique but still suffering from some drawbacks such as short fibre lifetime and low sensitivity of analytes in complex matrices.[9]

2. Stir bar sorptive extraction

In this technique, a magnetic stirring bar of quartz coated with polydimethylsiloxane is used. Compared to solid phase micro-extraction, stir bar sorptive extraction has more coated polymer and so higher extraction efficiency is obtained. It is a cost-effective technique and it is commonly used in environmental applications but it was also applied in bioanalysis for extraction of some drugs and metabolites from biological fluids.[9]

3. Microextraction by packed sorbent

It is a novel, simple, fast, cost-effective, readily automated and green samples preparation method. It can be connected online to gas chromatography or liquid chromatography systems. In this, the sorbent is fitted in a liquid handling micro syringe as a plug with low void volume. When sample is drawn through syringe, analytes are adsorbed onto solid phase. Then sorbent is washed with water and finally analytes were absorbed by suitable solvent. Different sorts of sorbents can be used such as C18, C8, C2, C8/SCX, strong cation exchange, strong anion exchange, imprinted polymers and silica. It has been applied for determination of different drugs and metabolites in different types of biological fluids.[9]

Matrix solid phase dispersion

In this technique solid matrices are used for sample preparation. Sample required in a very low quantity with a solvent therefore it is termed as a microscale extraction technique. Near about 98% of solvent use is reduced and giving 90% sample turnaround time. In conventional

extraction of an organic analyte from tissue homogenization of sample analyte from tissue with bulk bonded silica-based sorbent has to perform, this can be achieved in mortar and pestle. Structure of tissue is getting disturbed due to mechanical shearing. Sample gets dispersed on surface of support sorbent, for this, hydrophilic and hydrophobic interaction plays a role which causes mixture to become semi dry and free flowing homogenous blend of sample. Interferences and analyte are eluted by transferring into a prefilled solid phase extraction cartridge. This technique has been applied, by using acid alumina, to extract organic analyte.[5]

Supercritical fluid extraction

This method is generally used for removing non polar to moderately polar analyte from matrix. Density of super critical fluid is like liquid while its viscosity and diffusivity is in gas and liquid values. By reducing pressure and by evaporation, recovery of super critical solvent can be obtained. Carbon dioxide dissolves many volatile polar compounds, acting as a good super critical solvent. This can be achieved in presence of trace amounts of polar cosolvents like water and short chain alcohols. Super critical fluids can be used to extract analytes from solvent.[5]

Chromatographic method

Depending on number of active components to be resolved or separated, mode of run can be determined. If number of components is large or the pKa values of components are wide apart then gradient mode is preferred over isocratic mode.[1]

In deciding whether a gradient would be required or an isocratic mode would be adequate, an initial gradient run is performed and ratio between total gradient time and difference in gradient time between first and last component are calculated. When calculated ratio is <0.25, isocratic is adequate, when ratio is >0.25, gradient would be beneficial for 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.[6]

In general, one begins with reversed phase chromatography, when compounds are hydrophilic in nature with many polar groups and are water soluble. Organic phase concentration required for mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, best way to start is with gradient reversed phase chromatography.[6]

Gradient can be started with 5-10% organic phase in mobile phase and organic phase concentration can be increased up to 100% within 20-30 min. Separation can then be optimized by changing initial mobile phase composition and slope of gradient according to chromatogram obtained from preliminary run. Initial mobile phase composition can be estimated on basis of where compounds of interest is eluted, namely, at what mobile phase composition, retention time and pKa of component. Changing polarity of mobile phase will alter elution of drug molecules. Elution strength of mobile phase depends upon its polarity, stronger polarity higher elution. Ionic samples can be separated, if they are present in undissociated form. Dissociation of ionic samples may be suppressed by proper selection of pH. Buffer should transmit light at or below 220 nm so as to allow low UV detection and pH of buffer should be adjusted before adding organic solvent.[6]

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that a near symmetrical peaks detect all compounds, a good separation and a reasonable run time.[6]

Peak resolution can be increased by using a more efficient column, which can be achieved by using a column of smaller particle size, or a longer column. These factors will increase analysis time. Flow rate does not influence resolution, but it has a strong effect on analysis time.[6]

Selection of mobile phase

If sample contains ionic or ionizable compounds, then use a buffer mobile phase to ensure reproducible results. Under unfavourable circumstances, pH changes as little as 0.1 pH units can have significant effect on separation. Properly used buffer allows controlling pH easily. Buffer works best at pKa of its acid. At this pH, concentration of acidic form and basic form of buffering species is equal buffering capacity is maximum. Phosphate has two pKa values in range of interest for silica-based chromatography. One at pH 2 and other at pH 7. pKa of acidic buffer is 4.75. Citrate has three pKa values – 3.08, 4.77 and 6.4. Between citrate and phosphate buffers, entire pH range useful for silica chromatography can be covered.[1]

Silanophilic interactions cause tailing due to ion exchange interactions. This can be reduced by 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 solubility in mobile phase. This is true for gradient applications. Acetonitrile is preferred ionic modifier in reverse phase

chromatography. Acetonitrile based mobile phase can give an up two-fold lower pressure drop than methanol based mobile phase at equal flow rate. This means that column efficiency is higher.[6]

Elution strength increases in order methanol, acetonitrile and tetrahydrofuran. Retention changes by roughly 10% for every 1% change in concentration of organic modifier.[6]

Role of pH

pH is another factor in resolution that will affect selectivity of separation in reverse phase HPLC. Selecting proper buffer is necessary to reproducible separation of ionizable compounds by reverse phase HPLC. Selecting an improper pH for ionizable analyte 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 between injections, and reproducible retention times.[1]

Sample retention increase when analyte is more hydrophobic. When an acid or base is ionized it becomes more hydrophilic and less interacting with column binding sites, as a result ionized analyte is less retained on column, so that the capacity factor is reduced dramatically. When pH = pKa for analyte, it is half ionized i.e., concentration of ionized and unionized species is equal. At low pH peak tailing is minimized and method ruggedness is maximized. Operating in intermediate pH offers an advantage in increased analyte retention and selectivity.[6]

Role of buffer

In reverse phase liquid chromatography mobile phase pH values are between 2 and 7.5. Buffers are needed when an analyte is ionizable under reverse phase conditions or sample solution is outside this pH range. Analytes ionizable under reverse phase conditions have amine or acid functional groups with pKa between 1 and 11. A correctly chosen buffer will ensure that ionizable functional group is in a single form, whether ionic or neutral. If sample solution is at pH damaging to column, buffer will bring pH of injected solution to a less harmful pH.[1]

If analyte contain only one amine functional group buffer selection is easier. Amines will be in cationic form with pH less than 9, so any buffer effective at pH 7 or lower will work. Buffer at pH 7 are used, even though pH of water is 7 because amine retention and peak

shapes are pH dependant. As pH is lowered amine retention time shortens and peak shapes sharpens as buffer protonates acidic silanols on silica surface. Any buffer with a pKa less than 7 is suitable, but phosphate buffer of pH 3 is found to be best for amines.[6]

Selection of column

HPLC column is heart of method, critical performing separation. Column must possess selectivity, efficiency and reproducibility to provide good separation. Commonly used reverse 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 selectivity using same mobile phase.[1]

During method development selection of column can be streamlined by staring with shorter columns. 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 determined. It is also advantageous to consider column internal diameter, many laboratories use 4.6 mm ID as standard, but it is worth considering use of 4 mm ID column as an alternative. This requires only 75% of solvent flow than that of 4.6 mm column.[6]

Selecting an appropriate stationary phase can also help improve efficiency of method development. For example, an octyl (C_8) phase can provide time saving over an octadecyl (C_{18}) it doesn't retain analytes as strongly as C_{18} phase. For normal phase applications cyano phases are most versatile.[6]

C_{18} columns are used in laboratory. These columns are able to resolve a wide variety of compounds due to their selectivity and high plate counts.[6]

Role of temperature

Temperature variations over course of a day have a significant effect on HPLC separations. This can occur in air-conditioned rooms. Temperature is a variable that can affect selectivity, its effect is relatively small. Retention time decreases with an increase in temperature for neutral compounds but less dramatically for partially ionized analytes. An increase of 1^0C in temperature will decrease retention time by 1 to 2%. Because of possible temperature fluctuations during method development and validation, it is recommended that column be thermostated to control temperature.[6]

Role of flow rate

Flow rate, more for isocratic than gradient separation, can sometimes be useful and readily utilized to increase resolution, but its effect is very modest. Slow flow rate will decrease column back pressure. Disadvantage is that when flow rate is decreased, to increase resolution slightly, there is a corresponding increase in run time.[6]

Selection of internal standard

An internal standard is a compound added to a sample in known concentration to facilitate qualitative identification and quantitative determination of sample components. Best internal standard is an isotopically labelled version of molecule you want to quantify. An isotopically labelled internal standard will have a similar extraction recovery, ionization response and retention time. It is difficult to procure an isotopically similar compound than a compound with similar characteristics to that of analyte chosen.[6]

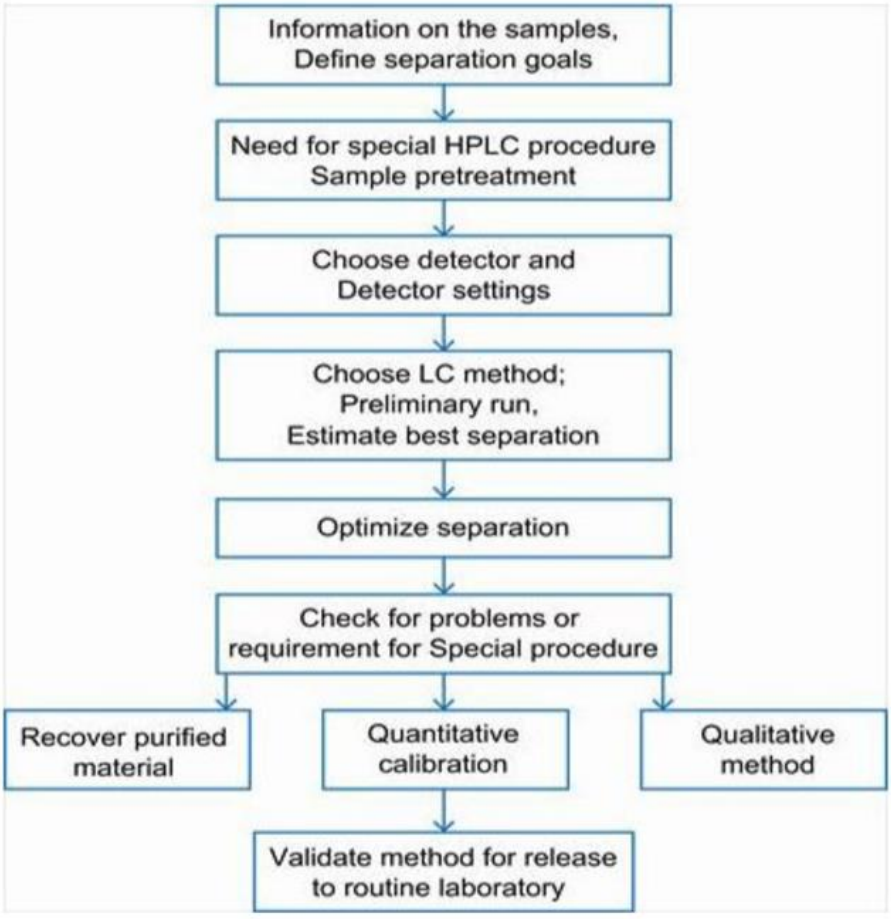

Fig 3: Chromatographic Methods

Bioanalytical method validation

Method validation is a process used to confirm that analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge quality, reliability and consistency of analytical results. It is an integral part of any good analytical practice.[1]

Bioanalytical method validation is performed when

- During development and implementation of a novel bioanalytical method
- For analysis of a new drug entity
- For revisions to an existing method that add metabolite quantification
- Bioanalytical method transfers between laboratories or analysts
- Change in analytical methodology
- Change in matrix within species
- Change in sample processing procedures[3]

Need for bioanalytical method validation

1. It is essential use well characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactory interpreted.

2. It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements; they are at the cutting edge of the technology.

3. It is also important to emphasize that each bioanalytical technique has its own characteristics, which will vary from analyte to analyte, specific validation criteria may need to be developed for each analyte.[4]

4. The bioanalytical technique has its own characteristics, which will vary from analyte to analyte, specific validation criteria may need to be developed for each analyst.

5. The bioanalytical methods to yields to reliable and satisfactorily results interpreted.

6. Validation involves documenting, through the utilization of specific laboratory investigations, that the performance characteristics of the technique that are suitable and reliable for the intended analytical applications.[5]

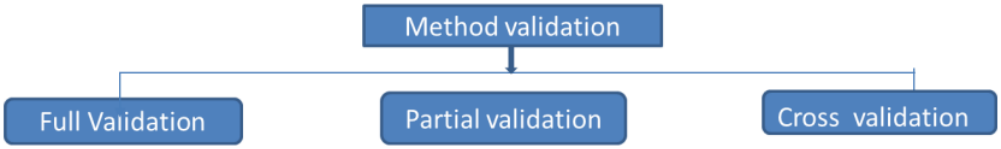

Fig 4: Types of Method Validation

Full validation

Full validation is necessary when developing and implementing a bioanalytical method for first time for a new drug entity. If metabolites are added to an existing assay for quantification, then full validation of revised assay is necessary for all analytes measured.[2]

Partial validation

Partial validations are modifications of validated bioanalytical methods that do not necessarily require full revalidations. Partial validation can range from one assay accuracy and precision determination to an almost full validation. Bioanalytical method changes that fall into this category include bioanalytical method transfers between laboratories or analysts, instrument or software platform changes, change in species within matrix, changes in matrix within a species, change in analytical methodology and change in sample processing procedures.[2]

Cross validation

Cross validation is a comparison of two bioanalytical methods. Cross validation is necessary when two or more bioanalytical methods are used to generate data within same study. For example, an original validated bioanalytical method serves as reference and revised bioanalytical method is comparator. Comparison should be done both ways. Cross validation with spiked matrix and subject samples should be conducted at each site or laboratory to establish interlaboratory reliability when sample analysis within a single study is conducted at more than one site, or more than one laboratory, and should be considered when data generated using different analytical techniques in different studies are included in a regulatory submission.[2]

Bioanalytical method validation involves the following parameters:

- Accuracy

- Precision
- Matrix effect
- Repeatability
- Reproducibility
- Linearity
- Selectivity and specificity
- Limit of detection
- Limit of quantitation
- Range
- Recovery
- Robustness
- Ruggedness

Accuracy

Degree of closeness of observed concentration to nominal or known true concentration. It is measured as relative error. Accuracy is an absolute measurement. An accurate method depends on specificity and precision. Accuracy is termed as trueness. Accuracy is determined by replicate analysis of samples containing known amounts of analyte.[4]

Accuracy should be measured using a minimum of five determinations per concentrations. A minimum of three concentrations in range of expected study sample concentrations is recommended. Mean value should be within 15% of nominal value except at lower limit of quantitation, where it should not deviate by more than 20%. Deviation of mean from nominal value serves as measure of accuracy. Two ways used to determine accuracy or method bias of an analytical method are (I) analysing control samples spiked with analyte and (II) by comparison of analytical method with a reference method.[7]

Precision

Precision of a bioanalytical method is a measure is random error and is defined as closeness of agreement between a series of measurement obtained from multiple sampling of some homogenous sample under prescribed conditions. Measurement of scatter for concentrations obtained for replicate samplings of a homogenous sample. It is measured as coefficient of variation or relative standard deviation of replicate measurement.[4]

Matrix effect

Matrix coextracted with analytes can alter signal response, causing either suppression or enhancement resulting in poor analytical accuracy, linearity and reproducibility.[8]

Matrix factor equal to 1 indicates no matrix effect, matrix factor less than 1 indicates suppression, and greater than 1 indicates enhancement. Variability in matrix factor is measured by coefficient of variation which should be less than 15%.[8]

Repeatability

Repeatability expresses analytical variability under same operating over a short interval of time. Repeatability means how method performs in one lab and one instrument, within a given day.[4]

Reproducibility

Reproducibility is precision between laboratories is not required for submission, but can be taken into account for standardization of analytical procedures. Ability of method to yield similar concentrations for a sample when measured on different occasions. Reproducibility refers to how that method performs from lab to lab, from day to day, from analyst to analyst, and from instrument to instrument, in qualitative and quantitative terms.[4]

Linearity

Ability of bioanalytical procedure to obtain test results that are directly proportional to concentration of analyte in sample within range of standard curve. Concentration range of calibration curve should at least span those concentrations expected to be measured in study samples. If total range cannot be described by a single calibration curve, two calibration ranges can be validated. Accuracy and precision of method will be negatively affected at

extremes of range by extensively expanding range beyond necessity. Correlation coefficients were used to test linearity.[4]

Selectivity and specificity

Ability of bioanalytical method to measure and differentiate analytes in presence of components that may be expected to be present. These could include metabolites, impurities, degradants or matrix components. Selectivity is documented demonstrations of ability of bioanalytical procedure to discriminate analyte from interfering components. It is defined as ability of bioanalytical method to measure unequivocally and to differentiate analytes in presence of components, which may be expected to be present. Analysis of blank samples of appropriated biological matrix should be obtained from at least six sources. Each blank sample should be tested for interference and selectivity should be ensured at lower limit of quantitation.[4]

Specificity is ability to assess unequivocally analyte in presence of components that may be expected to be present. For example, RP-HPLC-UV method is specific if assigned peak at a given retention time belongs only to one chemical entity. Specificity is critical basis of each analytical procedure.[7]

Limit of detection

Lowest amount of analyte that can be detected but not quantified. Calculation of LOD is open to misinterpretation as some bioanalytical laboratories measure lowest amount of a reference solution that can be detected and others lowest concentration that can be detected in biological sample. There is an overall agreement that LOD should represent smallest detectable amount or concentration of analyte of interest.[4]

Limit of quantitation

Quantitation limit of individual analytical procedure is lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. LLOQ is lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. Determining LLOQ on basis of precision and accuracy is a most practical approach and defines LLOQ as lowest concentration of sample that can still be quantified with acceptable precision and accuracy. LLOQ based on signal to noise ratio can only be applied when there is baseline noise, for example to chromatographic methods. A 10:1 S/N is

considered to be sufficient to discriminate analyte from background noise. Upper limit of quantitation is maximum analyte concentration of a sample that can be quantified, with acceptable precision and accuracy. ULOQ is identical with concentration of highest calibration standards.[4]

Range

Range of concentration, including LLOQ and ULOQ that can be reliably and reproducibly quantified with suitable accuracy and precision through use of a concentration response relationship.[4]

Range of analytical procedures is interval between upper and lower concentrations of analyte in sample for which it has been demonstrated that analytical procedures have a suitable level of precision, accuracy, and linearity. Range of bioanalytical assay is concentration interval over which an analyte can be measured with acceptable precision and accuracy.[7]

Recovery

Extraction efficiency of an analytical process, reported as percentage of known amount of an analyte carried through sample extraction and processing steps of method. Recovery pertains to extraction efficiency of an analytical method within limits of variability. Recovery of analyte need not be 100% but extent of recovery of an analyte and of internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing analytical results of extracted samples at three concentrations with unextracted standards that represent 100% recovery.[4]

Robustness

According to ICH guidelines, robustness of an analytical procedure is measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provide an indication of its reliability during normal usage. Robustness can be described as ability to reproduce method in different laboratories or under different circumstances without occurrence of unexpected differences in obtained results. A robustness test as an experimental set up to evaluate strength of a method.[4]

Ruggedness

This includes different analysts, laboratories, columns, instruments, sources of reagents, chemicals, solvents. Ruggedness of an analytical method is degree of reproducibility of test results obtained by analysis of same samples under a variety of normal test condition. Ruggedness of method was studied by changing experimental condition such as, changing to another column of similar type and different operations in same laboratory.[4]

Stability studies

Stability study are studies carried out during method development to determine analyte chemical stability in given matrix sample, during their sample collection, sample handling and storage. Stability studies should be performed in same matrix to that of subject sample. This include autosampler stability study, benchtop stability, processed and extracted sample stability, freeze thaw stability, stock solution stability, long term stability studies. For fixed combination drugs stability study should be performed for specific drug in presence of another drug. Validation of drug stability in a biological fluid is a function of storage conditions, physicochemical properties of drug, matrix, and container system. Stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. If storage condition is changed or validation studies should be carried outside of validated storage condition, stability study should be newly studied under same condition.[11]

1. Stock solution stability study

Stock solution stability of analyte and their internal standard for subject sample analysis should be evaluated at room temperature for at least 6 hours. Stability of stock solution is in refrigerator or deep freezer then this solution is stored in refrigerator or deep freezer respectively over specific period of time and evaluated stored stock solution with that of freshly prepared stock solution.[11]

2. Autosampler stability study (extracted sample stability study)

Autosampler stability study determine stability of extract of analyte when autosampler storage condition and extract processed sample storage condition are different. In these autosampler stability sample should be evaluated for 48-96 hrs to cover anticipated run time

for analytical batch. Then extracted QC samples are stored at autosampler temperature for specific time and analysed with fresh standard QC samples.[11]

3. Benchtop stability

Benchtop stability of samples is determined stability of samples under laboratory handling condition that are expected for study samples. Analyte samples in matrix are analysed after keeping them at ambient temperature for 4-24 hrs to cover at least time to extract samples. Observed samples concentrations are compared with their nominal values. Experiment is combinedly determine overall stability of sample during lab processing conditions.[11]

4. Freeze thaw stability

Freeze thaw stability is determined stability of samples after minimum freeze thaw cycles. In these samples are freeze and thaw and analysed according to standard procedure used for subject study samples. Minimum 2 concentrations are frozen overnight, at normal storage temperature and thawed these samples unassisted at room temperature. When samples are thawed, samples are again frozen at same temperature for 12-24 hrs and thawed. This freeze thaw cycle is repeated 2 more times, after 3rd cycle samples are analysed. The observed concentration is observed with their nominal values. If any degradation is observed in this concentration, then cycle 1st and 2nd is repeat to determine at which cycle instability occur. If needed number of freeze thaw cycles can be extended.[11]

5. Freezer storage stability

During validation, at nominal freezer storage stability temperature should be determine to extent possible. Long term stability should be determined and appropriately documented.[11]

6. Long term stability

This study is determined long term stability of sample over specific period of time. Time of long-term stability should be equal or more than period of sample collection and last sample analysis. Sample storage condition is same as that of study sample storage condition. After completion of validation, stability of analyte in matrix should be determine by storing sufficient number of QC samples at required long term storage temperature and analysing them in at least triplicate at minimum of two QC samples. Long term stability period should be depended on length of stability required. It can be determined at several period like 1, 3, 6,

9, 12, 18 months. In these long-term stability QC should be compared to freshly prepared calibration standard and QC samples.[11]

7. Matrix stability

At low temperature, if there may be denaturation of matrix protein. Therefore, matrix stability should be validated. For that purpose, additional stability should be carried out at lower temperature for sample matrix.[11]

8. Whole blood stability evaluation

Immediate spinning down of aliquot of whole blood containing drug taken immediately following preparation followed by spinning down of another aliquot following stability period. Whole blood stability should be performed during method validation. There are various approaches for determination of whole blood stability. It is not applicable for larger molecules.[11]

Specific recommendation for bioanalytical method validation

- Matrix based standard curve should consist of a minimum of six standard points, excluding blanks, using single or replicate samples. Standard curve should cover entire range of expected concentrations. Standard curve fitting is determined by applying simplest model that adequately describes concentration response relationship using appropriate weighting and statistical test for goodness of fit.

- LLOQ is lowest concentration of standard curve that can be measured with acceptable accuracy and precision. LLOQ should be established using at least five samples independent of standards and determining coefficient of variation or appropriate confidence interval. LLOQ should serve as lowest concentration on standard curve and should not be confused with LOD and low QC sample. Highest standard will define ULOQ of an analytical method.

- For validation of bioanalytical method, accuracy and precision should be determined using a minimum of five determinations per concentration level. Mean values should be within 15% of theoretical value, except at LLOQ, where it should not deviate by more than 20%. Precision around mean value should not exceed 15% of CV, except for LLOQ, where it should not exceed 20% of CV. Other methods of assessing accuracy and precision that meet these limits may be equally acceptable.

- Accuracy and precision with which known concentrations of analyte in biological matrix can be determined should be demonstrated. This can be accomplished by analysis of replicate sets of analyte samples of known concentration QC samples from an equivalent biological matrix. At a minimum, three concentrations representing entire range of standard curve should be studied: one within 3x LLOQ, one near centre, and one near upper boundary of standard curve.

- Reported method validation data and determination of accuracy and precision should include all outliners; calculations of accuracy and precision excluding values that are statistically determined as outliners can also be reported.

- Stability of analyte in biological matrix at intended storage temperatures should be established. Influence of freeze thaw cycles should be studied.

- Stability of analyte in matrix at ambient temperature should be evaluated over time period equal to sample preparation, sample handling, and analytical run times.

- Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalysed in case of instrument failure.

- Acceptance criteria for spiked, matrix-based calibration standards and validation QC samples should be based on nominal concentration of analytes. Specific criteria can be set up in advance and achieved for accuracy and precision over range of standards, if so desired.[2]

CONCLUSION

Bioanalysis and production of pharmacokinetic, toxicokinetic and metabolic data plays a fundamental role in pharmaceutical research and development involved in drug discovery and development process. Data should be produced to acceptable scientific standards and specifications laid by different regulatory agencies across globe. Bioanalytical methods should be validated to objectively demonstrate fitness for their intended use.[8]

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