Bioanalytical Method Validation- A Review

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Abstract-Bioanalytical method development is the process of preparing a procedure to know a compound of interest to be identified and quantified in a biological matrix. A compound can be examined by several methods and the choice of analytical method involves many considerations. Analysis of drugs and their metabolites in a biological matrix is carried out using different extraction techniques like liquid-liquid extraction, solid phase extraction (SPE) and protein precipitation. These methods and choice of analytical method describes the process of method development and includes sampling, sample preparation, separation, detection and evaluation of the results. The developed process is then validated. These bioanalytical validations play a pivotal role in evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic studies and different parameters like accuracy, precision, selectivity, sensitivity, reproducibility, and stability are performed.

Key words: Bioanalytical method validation, Biological matrix, Bioavailability, Bioequivalence

INTRODUCTION

Measurement of drug concentrations in biological matrix (such as serum, plasma, blood, urine, and saliva) is an essential aspect of medicinal product development. Such data may be required to support applications for new active substances and generics as well as variation to authorised drug products. The results of animal toxicokinetic studies and of clinical trials, including bioequivalence studies are used to make significant decisions supporting the safety and efficacy of a medicinal drug substance or product. 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. In this methods determine the drugs in biological fluid are becoming increasingly important for the study of bioavailability, bioequivalence (BE) Pharmacokinetics (PK) studies, quantitative evaluation of drugs, concentration and their metabolites, new drug development, research in basic biomedical and pharmaceutical sciences and therapeutic drug monitoring etc.

High pressure liquid chromatography (HPLC) most widely applied analytical technique because of its highly selective and high reliability, especially in the pharmaceutical, environmental, forensic, clinical, and food department. The rapid growth in the use of LC-MS/MS in recent years due to its advantages of high Sensitivity, Extreme Selectivity and increased rate of analysis. The other advantages of LC-MS/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. In modern bioanalysis a good sample preparation and a hyphenated instrumentation are required. In pharmaceutical research companies the development of comprehensive bioanalytical methods is very important during the process of drug discovery and development. In addition the method validation has an important role in regulatory bioanalysis to ensure the quality of the applied method. Bioanalytical method validation is very important for supporting of new drug applications or biologics license applications.

Bio-analytical method validation is a procedure employed to demonstrate that an analytical method used for quantification of analytes in a biological matrix is reliable and reproducible to achieve its purpose: to quantify the analyte with a degree of accuracy and precision. Both HPLC and LC-MS/MS can be used for the bioanalysis of drugs in plasma.

Biological matrices relevant in bioanalysis

In bioanalytical studies, various types of biological matrices (e.g., blood, plasma, serum, urine, hair, human breast milk, saliva, sweat, cerebrospinal fluid (CSF), and tissue) need to be investigated. In addition,

every matrix has unique challenges. For example, plasma contains more phospholipids, whereas urine contains a large amount of salt. Conventionally, biofluids (e.g., blood, serum, plasma, saliva, sweat, urine, and tissue) are used extensively in bioanalysis. Recently, hair, human breast milk, and feces have also been used as biological specimens. Hair is a stable and tough matrix that is easy to handle and hardly tampered with during collection, and it has a high degree of degradation in post-mortem studies. Human breast milk is an excellent marker of drugs and ecological pollutants. As has been known for a long time, drug and metabolite excretion in breast milk is a crucial issue for breastfeeding mothers. Similar to excretion in breast milk, some herbal medicines may be metabolized by intestinal microbiota and excreted in feces. Feces are non-digested, non-homogeneous, complex, and laden with macromolecules and particulates, which can present problems for analytical systems.

In bioanalysis, plasma is the most important and often utilised biological fluid. Compared to other biological matrices, plasma has various advantages. including the fact that it's easily accessible, inexpensive, and provides an accurate indicator of drug concentration in the blood. If the drug concentration in the blood is low, plasma is favored above any other matrix. Plasma has the highest rate of recovery of any matrix. One of the best matrices is plasma.to employ if the treatment has a strong protein binding capability

Why plasma?

In bioanalysis, plasma is the most important and often utilised biological fluid. Compared to other biological matrices, plasma has various advantages. including the fact that it's easily accessible, inexpensive, and provides an accurate indicator of drug concentration in the blood. If the drug concentration in the blood is low, plasma is favored above any other matrix. Plasma has the highest rate of recovery of any matrix. One of the best matrices is plasma.to employ if the treatment has a strong protein binding capability. Because the sample size in animal studies is so small, assessing plasma drug concentration is the preferred method. Plasma has a significant edge versus serum in this regard a sample is less likely to be lost in plasma. Blood is centrifuged with anticoagulant and the supernatant is collected to obtain plasma. When entire blood gets collected in anticoagulant-treated tubes, plasma is formed. [1] For estimating and measuring pharmaceutical substances in various biological matrices, a number of detection techniques have been published. Plasma, urine, serum, cerebrospinal fluid (CSF), tissue, and other biological matrices all have their own criticality when it comes to sample preparation in Bioanalysis.

There are several valid reasons for developing new methods of analysis.

There may not be a suitable method for a particular analyte in the specific sample matrix Existing method may be too error or contamination prone or they may be unreliable

Existing method may be too expensive,

Time consuming, or energy intensive, or they may not be easily automated

Existing method may not provide adequate sensitivity or analyte selectivity in samples of interest Newer instrumentation and techniques may have evolved that provide opportunities for improved methods, including improved analyte identification or detection limits, greater accuracy or precision, or better return on investment.

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

Steps involved in method development: Method development is a complex process that involves a number of steps, which are as follows:

Method selection and information of sample

Selection of initial method conditions

Checking the analytical method in aqueous standards

Development and optimization of sample processing method

Checking the analytical method in biological matrix

Pre-validation

Step 1: Method selection and information of sample: Literature survey shall be conducted to have first hand information on drug profile and its pharmacokinetic properties. Collection of physicochemical properties of the analytes and the related compounds are essential for the development of the analytical method. Based on the drug's physicochemical properties such as molecular size, shape, structure, functional groups, polarity, partition coefficient, solubility, dissociation constant etc., choose the internal standard having comparable molecular structure and physicochemical properties with respect to the analytes. Same molecule

with different isotopes like deuterium, C13and N15 will be a better alternative for internal standards.

Step 2: Selection of initial method conditions: Setting the initial method conditions include diluent selection based on the solubility of the drug, drug metabolites and internal standard and compatibility with analytical method. The lowest concentration to be quantified shall be assessed using aqueous solutions during this phase. Run time and resolution between the peaks should be taken care during this phase.

Step 3: Checking the analytical method in aqueous standards: Before going to analyze a method in biological matrix, first check the analytical method in aqueous standards. Prepare aqueous calibration curve standards, at least with four concentrations, including the highest and lowest. Concentration of the highest standard shall be based on Cmax and lowest standard shall be tentatively fixed based on the preliminary studies. Make injections of each calibration curve standard and find the correlation coefficient. Correlation co-efficient (r) should not be less than 0.99. If required, adjust the mobile phase, mass spectral parameters (if applicable) and chromatographic conditions such as mobile phase constituents, buffer strength, ratio, pH, flow rate, wavelength, column, column oven temperature etc., to get the clear resolution with required sensitivity.

Step 4: Development and optimization of sample processing method When the instrumental method is concluded with aqueous standards, prepare matrix sample. Based on the literature survey data on analyte and internal standard's physicochemical properties like structure, functional groups, pH, partition co efficient, dissociation constant, polarity and solubility, set and optimize the sample preparation technique like protein precipitation, liquid-liquid extraction and solid phase extraction.

Step 5: Checking the analytical method in biological matrix: When sensitivity of the drug is more, prefer protein precipitation and check for recovery, precision and interferences. When sensitivity of the drug is less, prefer liquid-liquid extraction and check for recovery, precision and interferences. When the recovery and reproducibility is less in liquid-liquid extraction, prefer solid phase extraction for better sensitivity, recovery, precision and low interferences. Checking the developed bioanalytical method with matrix samples for accuracy, precision and recovery is essential before finalizing the method for pre-validation. Minimum three aliquots each of Higher Quality Control (HQC) and Lower Quality Control (LQC) and Lower Limit of Quantification (LLOQ) matrix samples are analysed with one set of extracted calibration curve standards including matrix blank and zero standard (blank with only internal standard) and the results shall be compared for recovery with aqueous quality control samples of equivalent concentration. The method is accepted if it meets the criteria of accuracy, precision and recovery. If needed, the method shall be considered for modification.

Step 6: Pre-validation: When the method is evaluated to be reliable, prepare a brief procedure with the details of sample preparation, instrumental conditions and method conditions, to proceed for pre-validation. Selectivity, Accuracy, Precision, Recovery parameters should be evaluated in Pre-validation stage.

Extraction Techniques

A.Liquid-liquid extraction (LLE) is a common sample preparation choice in regulated bioanalysis. LLE is useful for separating analytes from interferences by partitioning the sample between two immiscible liquids or phases. One phase in LLE will usually be aqueous and the second phase an organic solvent. LLE can generate high analyte recoveries, clean extracts, and is perceived as low cost. Extraction solvents may need to be acidified, basified or low percentages of more polar solvents may be required to simultaneously achieve high recoveries for metabolites and related compounds, as well as the primary analyte. Protein precipitation extraction method is simplest ,cheap but very poor cleaned sample. Liquid-liquid extraction (LLE) is an very useful technique in sample preparation and it has been widely used for the preparation of aqueous and biological samples. Liquid-liquid extraction involves the extraction of a substance from one liquid phase to another liquid phase. Liquid-liquid extraction is useful for separating analytes from interferences by partitioning the analyte between two immiscible liquids or phases. One phase in LLE is aqueous and second one an organic solvent. More hydrophilic compounds prefer the polar aqueous phase; whereas 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 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 an appropriate solvent (generally mobile phase).

LLE was used for the extraction of basic and acidic drugs from biological samples with high extraction recovery. Quantitative recoveries (90%) of most drugs can be obtained through multiple continuous extractions. Now-a-days traditional LLE has been replaced with advanced and improved techniques like liquid phase microextraction (LPME), single drop-liquid phase micro extraction (DLPME) and supported membrane extraction (SME).

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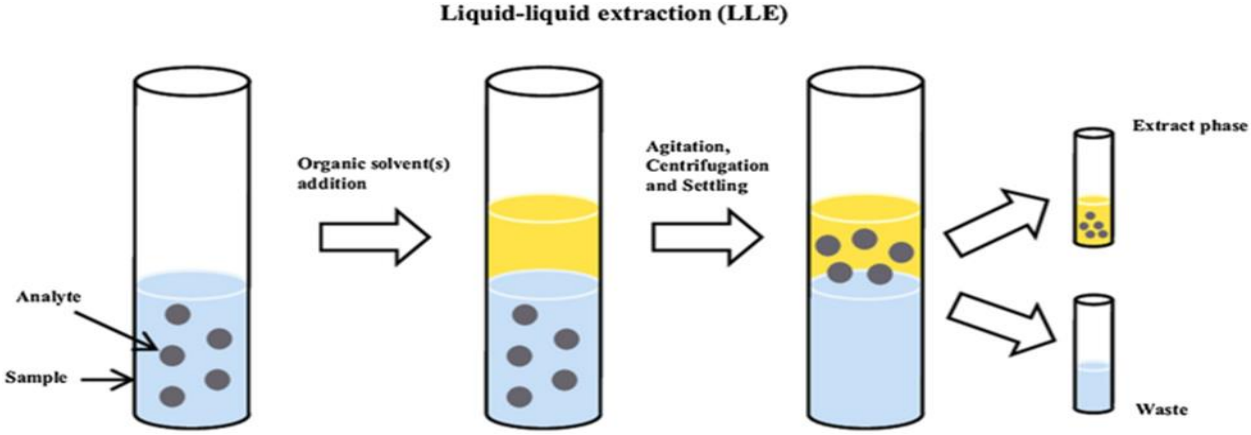

Fig.1: Liquid-liquid Extraction

B. Solid phase extraction (SPE) At the present time, SPE is the most well-known sample preparation method due to the high efficiency, cost-effective, high-reproducibility, relatively green and easy to operate and automate. SPE is beneficial such as separating and concentrating of trace analytes in biological samples. SPE is a straight forward method that uses a sorbent of 50–200 mg as cartridge to separate required analytes from a complex matrix. Recently, other formats of SPE have also been developed such as, flat disks and μ SPE. The nature and amount of the sorbent, loaded sample volume (with enough recovery), composition and volume of the washing and elution solutions (without loss of the analytes) are the effective parameters in SPE performance.Solid phase includes four steps; conditioning, sample loading, washing and elution are shown in Fig. 2. Conditioning The column is triggered with an organic solvent that acts as a wetting agent on the packing material and solvates the functional groups of the sorbent. Water or aqueous buffer is added to activate the column for proper adsorption mechanisms. Sample loading After adjustment of pH, the sample is entering on the column by gravity feed, pumping or aspirating by vacuum. III. Washing Interferences from the matrix are removed while retaining the analyte. IV. Elution Distribution of analyte - sorbent interactions by suitable solvent, removing as little of the remaining interferences as possible. Generally, sorbents used in SPE consists of 40 μm diameter silica gel with around 60 A^0pore diameters. To this silica gel, functional groups are chemically bonded. The most commonly used formatisa syringe barrel that contains a 20 μm frit at the bottom of the syringe with the sorbent material and another frit on top, referred to as packed columns. Extractions disks are placed in syringe barrels. These disks consist of 8-12 μm particles of packing material fixed into an inert matrix. Disks are conditioned and

used in a similar way as packed columns. The major advantage of disks compared to packed columns is that higher flow rates can be easily applied. Analytes can be classified into four categories; acid, basic, neutral, and amphoteric compounds. Amphoteric analytes have both acid and basic functional groups and can, therefore, functions as cations, anions or zwitterions, depending on pH, mainly the pH is 13.Solid phase extraction is the most important technique used in sample pretreatment for HPLC. 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 un-retained (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 v/s. LLE

Complete extraction of the analyte Effective separation of interferences from analyte Reduced organic solvent consumption Easier collection of the total analyte fraction Convenient manual procedures Removal of particulates More easily automated

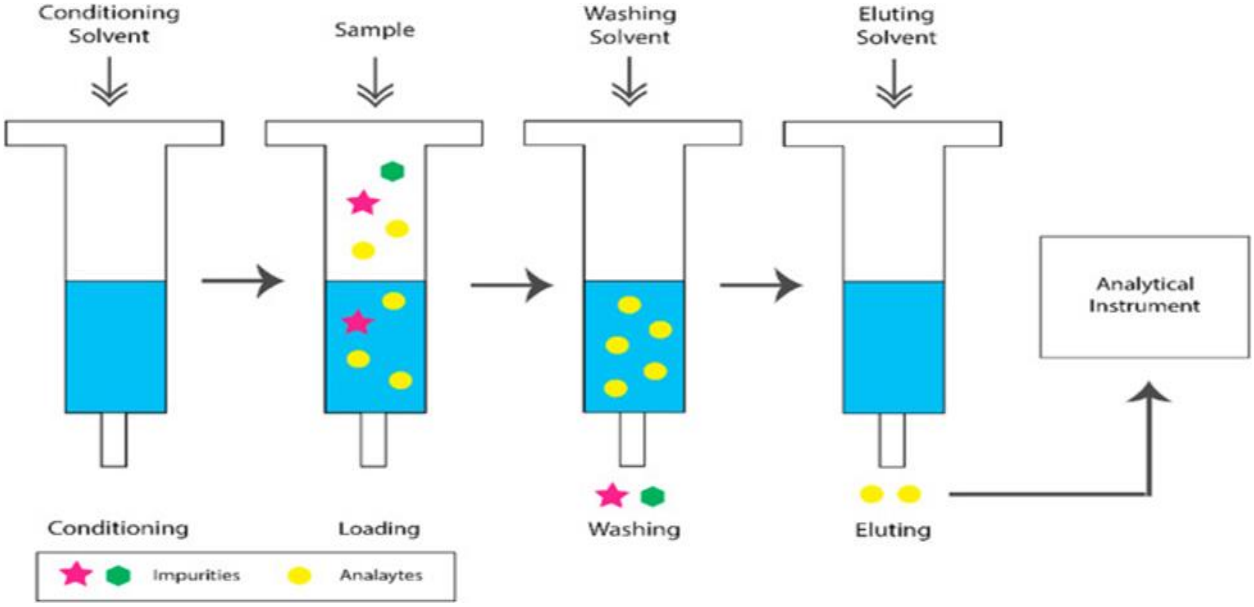

Fig. 2: Solid-Phase Extraction

C. Protein precipitation: Protein precipitation is the simple method of extraction as compared to the LLE and SPE 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. 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.

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. 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. Preliminary evaluation of lower limit of quantification to be done after fixing the sample processing technique. Using the biological matrix with lowest interference, prepare at least three aliquots at each concentration level, with the concentrations of 1/20 of Cmax, 1/30 of Cmax and 1/40 of Cmax of the analytes. The wash volume and washing pattern of auto injector needle to be evaluated to avoid carryover of previous injections to next injections

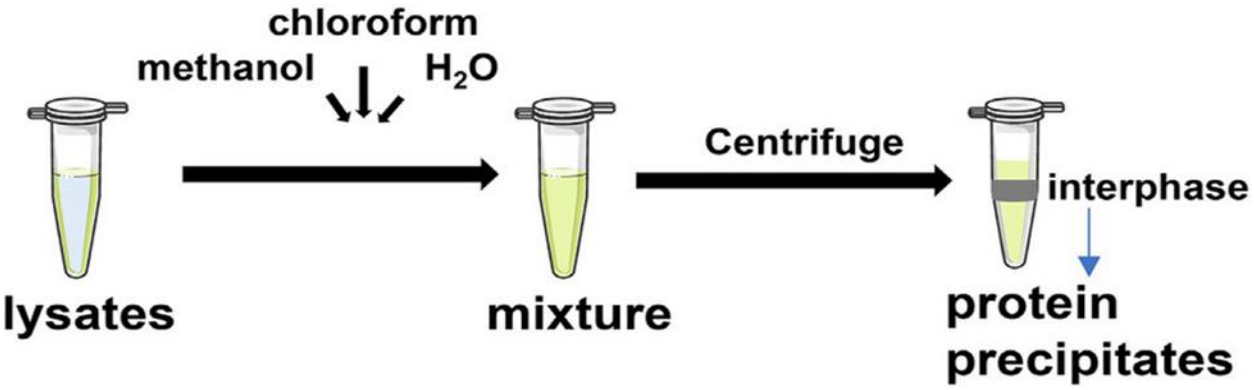

Fig.3: Precipitation of Proteins

Parameters to be optimized: The various parameters to be optimized during method development includes 1. Mode of separation 2. Selection of stationary phase 3. Selection of mobile phase

1. Mode of separation: In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the suitable mode is reverse phase. The nature of the analyte is the primary factory in the selection of mode of separation.

2. Selection of stationary phase/column: 10 Selection of the column is the first and the most important step in method development, because the column is the heart of separation process.

The appropriate choice of separation column includes different approaches Column dimensions, Nature of packing material, Shape of the particles, Size of the particles Surface area, Pore volume, End capping.

The optimum length of the column required for a particular separation is dictated by the number of theoretical plates needed to give the desired resolution. If the column is too short, then the clearly the column will not have enough ‘resolving power’ to achieve the separation and if it is too long, then analysis time is needlessely extended. The most common column lengths used in regular analytical HPLC are 10, 12.5, 15 and 25 cm, with 15 cm columns being perhaps the most popular. Most analytical HPLC columns have internal diameters (i.d.) of around 5 mm, majority being 4.6mm. It is generally considered that spherical forms give superior column packing properties to the non spherical forms. As the particle size of the column packing decreases, the superiority of spherical materials become more apparent and therefore spherical forms are often used for silica particles of 5µm diameter, 3µm diameter or less. Currently, most HPLC separation are carried out with 5µm diameter packing materials. Columns with 5µm particle size give the best compromise of efficiency, reproducibility and reliability. As the particle size decreases the surface area for coating increases. Generally high specific surface area will increase the retention of solutes by increasing the capacity factor.Reverse phase mode of chromatography facilitates a wide range of columns like dimethylsilane (C2), butylsilane (C4), octyl silane (C8), octadecyl silane (C18), cyanopropyl (CN), nitro, amino etc.

3.Selection of Mobile phase: The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degradants from each other and analyte peak. The following are the parameters to be considered during selection and optimization of

mobile phase. Buffer, pH of the buffer, Mobile phase composition.

Buffer and its role: Buffer and its strength play an important role in deciding the peak symmetries and separations. The retention time depends on molar strength of buffer. Molar strength is proportional to retention time. In order to achieve better separation the strength of the buffer can be increased. Commonly used buffers are Acetic buffers includes ammonium acetate, sodium acetate. Acetic acid buffers are prepared using acetic acid. Another important component is the influence of the pH since this can change the hydrophobicity of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate, to control the pH. The buffers serve multiple purposes: they control pH, neutralize the charge on any residual exposed silica on the stationary phase and act as ion pairing agents to neutralize charge on the analyte. Ammonium formate is commonly added in mass spectrometry to improve detection of certain analytes by the formation of ammonium adducts. A volatile organic acid such as acetic acid, or most commonly formic acid, is often added to the mobile phase if mass spectrometry is used to analyze the column eluent. Trifluoroacetic acid is used infrequently in mass spectrometry applications due to its persistence in the detector and solvent delivery system, but can be effective in improving retention of analytes such as carboxylic acids in applications utilizing other detectors, as it is one of the strongest organic acids. The effects of acids and buffers vary by application but generally improve the chromatography.

pH of buffer: pH plays an important role in achieving the chromatographic separation as it controls the elution properties by controlling the ionization characteristics. A different concentration of buffer was chosen to achieve required separations. It is important to maintain the pH of mobile phase in the range of 2.0 to 8.0 as most of the columns does not withstand out of this range . As Siloxane linkages are cleaved below pH 2 and at above pH 8 silica dissolves.

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

Types of Bioanalytical Method Validation

A. Full Validation: Full validation is important when developing and implementing a Bio analytical method for the first time. Full validation is important for a new drug entity. A full validation of the revised assay is important if metabolites are added to an existing assay for quantification.

B. Partial Validation: Partial validations are modifications of already validated bio analytical methods. Partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation. Typical bio analytical method changes that fall into this category include, but are not limited to: • Bio analytical method transfers between laboratories or analysts • Change in analytical methodology (e.g., change in detection systems) • Change in anticoagulant in harvesting biological fluid • 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 • Limited sample volume (e.g., pediatric study) • Rare matrices • Selectivity demonstration of an analyte in the presence of concomitant medications • 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 bio analytical methods are used to generate data within the same study or across different studies. An example of cross-validation would be a situation where an original validated bio analytical method serves as the reference and the revised bio analytical method is the comparator. The comparisons should be done both ways. When sample analyses within a single study are conducted at more than one site or more than one laboratory, cross-validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter laboratory reliability.

The Fundamental parameters involved in bio analytical validation are: 1.Selectivity 2. Sensitivity 3. Linearity 4. Accuracy 5. Precision 6. Recovery 7. Matrix effect 8. Dilution integrity 9. Stability

1.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.

2. 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 coefficient 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.

3. Accuracy: Accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. This is sometimes termed as trueness. The two most commonly used ways to determine the accuracy or method bias of an analytical method, are (i) analysing control samples spiked with analyte and (ii) by comparison of the analytical method with a reference method.

4. Precision: It is the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix. There are various parts to precision, such as repeatability, intermediate precision, and reproducibility ruggedness). Repeatability means how the method performs in one lab and on one instrument, within a given day. Intermediate precision refers to how the method performs, both qualitatively and quantitatively, within one lab, but now from instrument to instrument and day to-day. Finally, reproducibility refers to how that method performs from lab-to lab, from day-to-day, from analyst-to-analyst, and from instrument-to-instrument, again in both qualitative and quantitative terms. The duration of these time intervals are not defined. Within/intraday, assay, run and batch are commonly used to express the repeatability. Expressions for reproducibility of the analytical method are between/interday, assay, run and batch. The expressions intra/within-day and inter/between- day precision are not preferred, because a set of measurements could take longer than 24 hours or multiple sets could be analysed within the same day.

5. Linearity: According to the ICH-definition ‘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. The concentration range of the calibration curve should at least span those concentrations expected to be measured in the study samples. If the total range cannot be described by a single calibration curve, two calibration ranges can be validated. It should be kept in mind that the accuracy and precision of the method will be negatively affected at the extremes of the range by extensively expanding the range beyond necessity. Correlation coefficients were most widely used to test linearity. Although the correlation coefficient is of benefit for demonstrating a high degree of relationship between concentration-response data, it is of little value in establishing linearity. Therefore, by assessing an acceptable high correlation coefficient alone the linearity is not guaranteed and further tests on linearity are necessary, for example a lack of-fit test.

6. Recovery: It can be calculated by comparison of the analyte response after sample workup with the response of a solution containing the analyte at the theoretical maximum concentration. Therefore

absolute recoveries can usually not be determined if the sample workup includes a derivatisation step, as the derivatives are usually not available as reference substances. Nevertheless, the guidelines of the Journal of Chromatography require the determination of the recovery for analyte and internal standard at high and low concentrations. Recovery does not seem to be a big issue for forensic and clinical toxicologists as long as precision, accuracy (bias), LLOQ and especially LOD are satisfactory. However, during method development one should of course try to optimize recovery.

7. Matrix effect: Matrix effect is the effect on bio analytical 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. Matrix effect is done in LCMS-MS to find out if there is any ion suppression or enhancement effect by the matrix.

8. 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 in a biological fluid: Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The 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. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution.

Freeze and Thaw Stability: Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze-thaw cycle should be repeated two more times, then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -7^0C during the three freeze and thaw cycles.

Short-Term Temperature Stability: Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

Long-Term Stability: The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

Processed sample Stability: The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards. Although

the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits for evaluation of analyte stability in a biological matrix can be used.

Bench top Stability: The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

Specific Recommendations for Method Validation: 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. 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 coefficient of variation and/or appropriate confidence interval. The LLOQ should serve as the lowest concentration on the standard curve and should not be confused with the limit of detection and/or the low QC sample. The highest standard will define the upper limit of quantification (ULOQ) of an analytical method. For validation of the 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. Other methods of assessing accuracy and precision that meet these limits may be equally acceptable. The 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 concentrations, QC samples from an equivalent biological matrix. At a minimum, three concentrations representing the entire range of the standard curve should be studied: one within 3x the lower limit of quantification (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. The stability of the analyte in biological matrix at intended storage temperatures should be established. The influence of freeze-thaw cycles (a minimum of three cycles at two concentrations in triplicate) should be studied. The stability of the analyte in matrix at ambient temperature should be evaluated over a time period equal to the typical sample preparation, sample handling, and analytical run times. Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalyzed in the case of instrument failure. The specificity of the assay methodology should be established using a minimum of six independent sources of the same matrix. For hyphenated mass spectrometry-based methods, however, testing six independent matrices for interference may not be important. In the case of LC-MS and LC MS-MS-based procedures, matrix effects should be investigated to ensure that precision, selectivity, and sensitivity will not be compromised. Method selectivity should be evaluated during method development and throughout method validation and can continue throughout application of the method to actual study samples. Acceptance/rejection criteria for spiked, matrix-based calibration standards and validation QC samples should be based on the nominal (theoretical) concentration of analytes. Specific criteria can be set up in advance and achieved for accuracy and precision over the range of the standards,

CONCLUSION

The efficient development and validation of analytical methods are critical elements in the development of pharmaceuticals. In bioanalytical method

development should not be restricted to pure and neat analyte solutions. The objective of this paper is to review the sample preparation of drug in biological matrix and to provide practical approaches for determining selectivity, specificity, lower limit of quantitation, linearity, accuracy, precision, recovery, stability, ruggedness of chromatographic methods to support pharmacokinetic, toxicokinetic, bioavailability, and bioequivalence studies.

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