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Review on Bioanalytical Method Development and Validation by Chromatographic Techniques

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Bhusnure O.G.*[1], Gandge N.V[1], Gholve S.B[1]. Sugave B.K.[1], Giram P.S[2].

1. Channabasweshwar Pharmacy College, Department of Quality Assurance, Latur (MS), India.

2. Channabasweshwar Pharmacy College, Department of Pharmacology, Latur (MS), India.

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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. To help address these challenges the utilization 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 is 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.[1]. 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 precipitation Special 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.[2,3].

INTRODUCTION

In analytical chemistry, the main goal is to determine the identity and/or concentration of one or more species in a sample. The samples analyzed are often naturally occurring samples in our environment or body fluids. The aim during method development is to achieve a robust and reproducible method, with high accuracy and precision, to determine analytes in a matrix. With accurate planning of all the steps in the analytical chain i.e. sampling, sample treatment, separation, detection and evaluation of the results, the analytical errors can be minimized. Reversed-phase (RP) is today the most commonly used technique in liquid chromatography (LC) methods, for separation and determination of chemical components in complex biological mixtures. Compounds that differ in their molecular properties, like hydrophobicity, polarity and ionic character can be separated and analysed with the large number of techniques that LC offers. Compounds to be analysed is generally contained in a complex biological matrix and can rarely be measured directly without sample pretreatment. Interfering compounds have to be eliminated. This becomes particularly important during trace analysis in the presence of abundant matrix components. Solid-phase extraction (SPE) is a commonly used technique for sample clean up. It is especially useful when high sensitivity is needed and it is easy to automate and can often achieve high sample throughput. SPE and LC are both based on differential migration processes in which compounds are adsorbed and eluted as they are traveling through a porous medium carried by a mobile-phase flow [4].

The time it takes depends on the compounds differential affinities between the sorbent material and the mobile phase. Finally, a selection of an accurate detection method has to be done based on the properties of the compounds to be analysed, of the required concentration level and the nature of the matrix. When a method has been developed, it is evaluated during method validation, based on international guidelines. This is a process to establish that the method developed is suitable for its intended purpose, that it provides reliable and valid data for a specific analyte in the selected matrix. Also to ensure that the method can be implemented for analysis of actual patient samples. Typical parameters to validate are; accuracy, precision, selectivity, sensitivity, linearity, recovery and stability. The present review focuses on analytical methods for measuring a variety of analytes in various biological matrices.

Needs of Bioanalytical method Development and Validation

1. It is essential to use well-characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactorily 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. Moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study. When sample analysis for a given study is conducted at more than one site, it is necessary to validate the bioanalytical method (s) at each site and provide appropriate validation information for different sites to establish inter-laboratory reliability.

Method development:

Analytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a matrix. A compound can often be measured by several methods and the choice of analytical method involves many considerations, such as chemical properties of the analyte, concentrations levels, sample matrix, cost of the analysis, speed of the analysis, quantitative or qualitative measurement, precision required and necessary equipment. The analytical chain describes the process of method development and includes sampling, sample preparation, separation, detection and evaluation of the results.

Sample collection

The first step in the analytical chain is to decide which matrix to utilize. Most often venous blood, withdrawn from the arm, or capillary blood, withdrawn from the fingertip, or urine is used. More rarely, saliva and cerebrospinal fluid are utilized. If the venous blood is withdrawn into tubes with an anticoagulant, plasma is obtained after centrifugation. Plasma is an accepted sampling method and it allows sample volumes in the range of ml.

Saliva was used since a less invasive method was required. The levels of melatonin in saliva reflect the level in blood daytime and 30% of it night time [5-6]. Saliva may be sampled with

saliva-collecting tubes, by spitting in a tube or by chewing on parafilm or gum to stimulate saliva production and then spit in a tube saliva samples were collected using Salivette sampling device (fig 1). The procedure is straightforward and involves chewing on a cotton roll that absorbs the saliva. The cotton roll is placed in the sampling device which is stored in refrigerator until brought to the laboratory for centrifugation and later analysis. The use of saliva for biochemical analysis has a number of advantages compared to blood. Collection is non-invasive, stress free, very convenient and cost effective. In addition, the patient can collect the samples either at home or at work, without the assistance or supervision of medical personnel since instructions are simple and contain few precautions. These advantages makes it possible to study/ reach larger group of patients than if a more invasive method would be used.

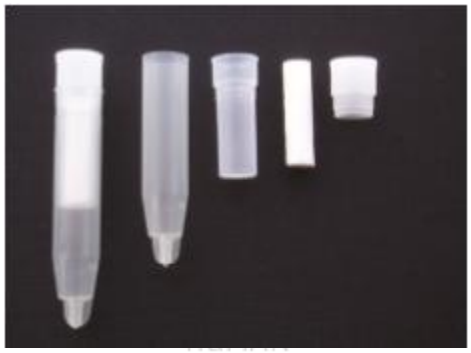

Figure 1. Salivette sampling device

When performing therapeutic drug monitoring (TDM) in rural areas, facilities may not be available (i.e. centrifuges or freezers) for storage of blood samples. This problem can be overcome by capillary blood sampling applied onto sampling paper i.e. dried blood spots, DBS. In DBS, blood is obtained by puncturing a fingertip (or heal/earlobe) with a lancet. The blood is collected with a capillary pipette of fixed size and the blood is applied on to sampling paper (fig. 2). The DBS is dried completely (at ambient temperature) before storing the samples in plastic bags, for transportation to the laboratory, since moisture may cause bacterial or fungus growth [7]. The DBS sampling technique, compared with vein-puncture, requires minimal training to perform, it is less invasive and the DBS represents also a low infectious risk from viruses such as HIV-1 and -2 and hepatitis C as they are dried on the sampling paper. The hepatitis B virus can remain infectious for at least seven days [8-9]. With DBS normally only 50 or 100 µl blood (sample) is collected, compared to 500-5000 µl

for whole blood/plasma, which is an advantage when collecting blood from children. The dried blood spot method was used.

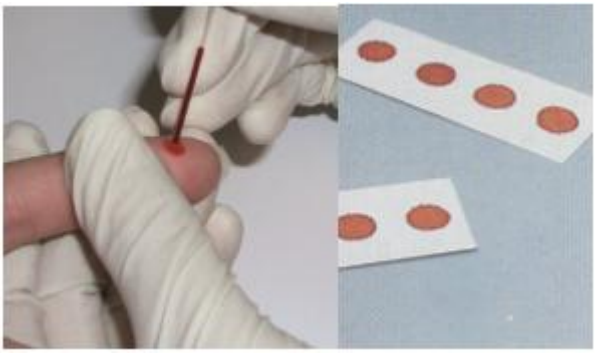

Figure 2. Capillary blood collection and dried blood spots

Sample preparation -

Good sample preparation is often the key to successful analytical results. It has a direct impact on accuracy, precision and quantification limits and is often the rate determining step for many analytical methods. The purpose of sample preparation is to clean up the sample before analysis and/or to concentrate the sample. Material in biological samples that can interfere with analysis, the chromatographic column or the detector includes proteins, salts, endogenous macromolecules, small molecules and metabolic byproducts [10-11]. Injection of matrix substances can also cover up and hide the drug or analyte being analyzed, making quantification difficult or even impossible. A goal with the sample preparation is also to exchange the analyte from the biological matrix into a solvent suitable for injection into the chromatographic system. General procedures for sample preparation are dilution followed by injection, filtration, liquid/liquid extraction, solid-phase extraction (SPE), protein precipitation or desalting.

1. Extraction from DBS

Over the past decades, many applications with the DBS method have been reported [12]. It is a sampling method that offers a number of advantages over conventional whole blood sample collection. Low sample volumes, non-invasive sampling compared to vein-puncture, no need for cooling (e.g. freezer) and easy to transport. There are also some disadvantages with the method. The low amount of blood in the dried spot gives a reduced sensitivity in detecting drugs at low concentration. There is no control over the volume and origin of the blood.

Interactions between the paper and the drug can give raise to low recovery and it is also an additional step in the analytical chain which can result in additional errors in precision and accuracy. There are a few parameters that have to be optimized when the DBS method should be used for a drug. Sampling paper should be selected with respect to extraction recovery and reproducibility of the drug and also availability and cost. The liquid used for the extraction of drug from the blood spot should be optimized and organic modifier, ionic strength and pH should be selected. Some drugs interact strongly with the sampling paper why it sometimes is necessary to modify the surface to get high and reproducible recovery. In some earlier reported methods the papers have been modified with a solution of plasma–protein, formic acid and ammonium acetate [13] or dimethyl ammonium bromide [14] to achieve enhanced recovery of the drugs the sampling paper used was 3MMChr and 3TC, AZT and NVP was extracted with zinc sulphate and methanol which gave rather clean extracts with a satisfying extraction recovery. Extraction of the drugs was achieved with a solution of acetonitrile and acetic acid. To improve the recovery of the drugs the sampling paper, 31ETChr, had to be modified with tartaric acid, which also enhances the stability of the analyte.

2. Solid-phase extraction

Over the past decade, SPE techniques have largely replaced liquid/liquid extraction methods as the preferred technique to extract drugs from biological fluids prior to detection. Advantage with SPE over liquid/liquid extraction is reduction in the consumption of organic solvents which leads to lesser disposal of large quantities of organic solvents. Other benefits with SPE include high enrichment factor, reduced laboratory work, purified extracts, ease of automation, compatibility with chromatographic analysis, and easily adaptable for very selective extraction. Another advantage compared to liquid/liquid extraction is the wide range of sorbents available which makes it possible to apply SPE to a variety of substances e.g. highly polar, ionic and nonpolar compounds [15]. In SPE a separation or extraction of one or more compounds from a mixture is achieved by selective distribution between a solid phase (sorbent) and a liquid phase (solvent). The extraction is performed by absorbing the analyte (s) from matrix onto a solid support (sorbent) by chemical attraction. Retention is achieved through strong, but reversible, interactions between the analyte and the sorbent. Typical interactions in SPE are hydrophobic (van der Waals forces), polar (hydrogen bonding and dipole-dipole forces) or ion exchange interactions [16]. All steps (fig. 11) in SPE are subjects to optimization during the method development.

Conditioning: The column is activated 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 loaded on the column by gravity feed, pumping or aspirating by vacuum.

Washing: Interferences from the matrix are removed while retaining the analyte.

Elution: Disruption of analyte-sorbent interaction by an appropriate solvent, removing as little of the remaining interferences as possible.

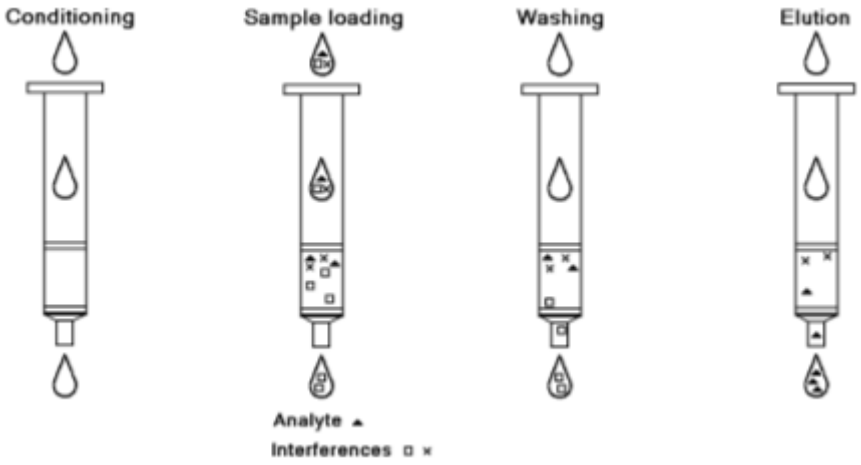

Figure 3. General solid-phase extraction procedure

3. Protein precipitation

Protein precipitation is often used in routine analysis to remove proteins. Precipitation can be induced by the addition of an organic modifier, a salt or by changing the pH which influences the solubility of the proteins [16]. 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. In 300 µl cold methanol was added to 75 µl plasma, the samples were

mixed and kept at 4ºC for 30–60 minutes, mixed again and centrifuged for 10 minutes at 12000 x g. The samples were then kept at -37ºC to freeze the precipitated proteins, transferred into new glass tubes and evaporated to dryness. The samples were dissolved in 75 µl deionised water and derivatizated before analysis.

4. Derivatization

Derivatization is a technique used to modify an analyte in order to change its properties like solubility, polarity or boiling point. Generally, a specific functional group of the drug participates in the derivatization reaction resulting in a derivative. There are a number of reagents on the market, targeting specific functional groups. An additional reason for derivatization is if the analyte is absence of chromophoric moiety i.e. is absent of UV- or fluorescence chromophores. The derivatization is then performed in order to be able to detect the analyte of interest with a suitable detector.

5. Separation

1 **Chromatography-** Chromatography is an analytical method that is widely used for the separation, identification and determination of chemical components in a complex biological mixture. In chromatography one phase is stationary while the other, the mobile phase, passes through the chromatographic bed. Molecules in a sample will have different interactions with the stationary support and the mobile phase, leading to separation of similar molecules. Molecules that display stronger interactions with the support will tend to move more slowly than those molecules with weaker interactions. In this way, different types of molecules can be separated from each other as they move over the support material (fig 4). The stationary phase may be a solid, porous, surface-active material in small-particle form or a thin film of liquid coated on a solid support or column wall. The mobile phase may be a liquid, a mixture of liquids, a gas or a supercritical fluid with the characteristics of gas and liquid, depending on the chromatographic conditions [17]. As in SPE, there are several types of forces that individually, or in combination, result in retention of the analyte by the stationary phase; non polar, Van der Waals forces, dipole-dipole interactions, hydrogen bonding, dielectric interactions and electrostatic attractions, i.e. ion-ion and ion-dipole [18].

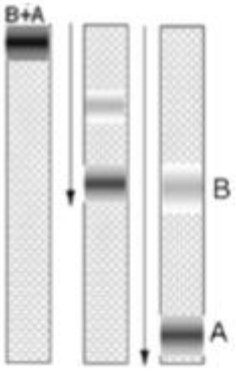

Figure 4. Illustration of chromatographic separation of a mixture of components A and B in a column

2 Liquid Chromatography

Liquid chromatography (LC) is today the main tool for analysis of various substances in different matrices in which the mobile phase is liquid. LC is suitable to separate compounds over a wide range of polarity, without previous derivatization. The analyte is forced through a column by a liquid (mobile phase) at high pressure, which decreases the time the separated components remain on the stationary phase and thus the time they have to diffuse within the column [17]. The interaction of the analyte with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases which result in a high degree of versatility compare to other chromatographic systems. A wide variety of chemical mixtures can easily be separated with LC. The basic LC system consists of six units; the mobile phase, the pump, the injector, the column, the detector and a data handling system. A block diagram is shown in figure 5.

Figure 5. Basic liquid chromatography system

In reversed-phase chromatography, a non polar stationary phase is used in conjunction with polar, aqueous mobile phases. Most stationary phases are silica-based bonded phases, but polymeric phases, phases based on inorganic substrates other than silica and graphitized carbon have found their place as well. There are also chiral stationary phases used for the separation of enantiomers [18]. In the selection of stationary phase for a particular application, two things have to be considered. The chemistry of the packing material in the

column which should ideally be the most appropriate for the separation in mind and the physical properties of the column, especially particle size and column dimensions since it influences the resolution, the speed of the analysis, the column back pressure, the detectability and the solvent consumption per analysis [19]. The LC column is typically 5, 10, 15 or 25 cm in length and the internal diameter of the column varies from 0.5 to 5 mm. For analytical purposes flow rates between 0.2–1.5 ml/min are normal [19]. To increase the lifetime of the column, a precolumn or guard column is often used. These columns are recommended for samples that contain material/substances that can strongly adsorb on the column, such as proteins. The guard column is normally 0.4 to 1 cm long and most often of the same stationary phase as the analytical column [20].

Detection

There are several different detectors suitable for detection of analytes after the chromatographic separation. Some detectors used in LC are ultraviolet (UV) detectors, fluorescence detectors, electrochemical detectors and mass spectrometry (MS) detectors. The choice of detector depends on the sample and the purpose of the analysis. The ideal detector has the following characteristics; good sensitivity, good stability, reproducibility, linear response over a few orders of magnitude, short response time and ease of operation [19].

1 Fluorescence detection

Many compounds have the ability to absorb UV-light of a given wavelength, followed by the emission of light at a longer wavelength. The excited electron will return to its ground state, with the emission of electromagnetic energy producing fluorescence. As some energy is always lost in the process the fluorescent light always has a longer wavelength than the excitation light [20]. Fluorescence has been shown to be extremely useful as detection process and detectors based on fluorescent measurement have provided some of the highest sensitivities available in LC. The sensitivity may be up to 1000 times greater than with UV-detection [20]. In addition, many substances can, by forming appropriate derivatives, get the ability to fluoresce.

2 Absorbance detection

Absorbance detection is based on the principle that functional groups, of a chemical compound, can absorb light at one or more wavelengths in the UV or the visible light range,

190–600 nm. The absorbance detector is one of the most commonly used detectors. It is relatively inexpensive, robust and easy to operate. A majority of organic compounds have some absorbance in the UV-visible light range and a high molar absorptivity (ε) of the compound allows low concentration levels to be measured.

Method validation

Validation parameters Method validation is a process used to verify/confirm that an analytic method developed is suitable for its intended purpose, that it provides reliable and valid data for a specific analyte. Typical parameters to validate are; accuracy, precision, selectivity, sensitivity, linearity, recovery and stability. General recommendation for analytical method validation, i.e. for pharmaceutical methods, can be found in The US Food and Drug Administration (FDA) guideline [21].

US FDA guidelines for bioanalytical method validation

Bioanalytical validation methods	**US FDA guidelines**
Selectivity (specificity)	Analyses of blank samples of the appropriate biological matrix (plasma, urine or other matrix) should be obtained from at least six sources. Each blank should be tested for interference and selectivity should be ensured at LLOQ.
Accuracy	Should be measured using a minimum of six determinations per concentration. Minimum of three concentrations in the range of expected concentrations is recommended for determination of accuracy. The mean should be ±15% of the actual value except at LLOQ, where it should not deviate by ±20%. This deviation of mean from the true values serves as the measure of accuracy.
Precision	Precision should be measured using a minimum of fi ve determinations per concentration. Minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the CV except for the LLOQ, where it should not exceed 20% of the CV.
Recovery	Recovery experiments should be performed at three concentrations (low, medium and high) with unextracted standards that represent 100% recovery
Calibration curve	Should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard) and six to eight non-zero samples covering the expected range, including LLOQ
LLOQ	Analyte response should be five times the response compared to blank

	response. Analyte peak should be identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80–120%.
Freeze-thaw stability	Analyte stability should be determined after three freeze–thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at intended storage temperature for 24 hours and thawed at room temperature. When completely thawed, refreeze again for 12–24 hours under same conditions. This cycle should be repeated two more times, then analyze on third cycle. Standard deviation of error should be <15%. If analyte is unstable, freeze at -70°C for three freeze–thaw cycles
Short-term stability	Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature for 4–24 hours and analyzed. Percent deviation should be <15%
Long-term stability	At least three aliquots of each of low and high concentrations at same conditions as study samples. Analyze on three separate occasions. Storage time should exceed the time between the date of first sample collection and the date of last sample analysis
Stock-solution stability	Stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. Percent deviation should be <15%
QC samples	QC samples in duplicates at three concentration levels (one near the 3× LLOQ, one in mid range, one close to high end) should be incorporated at each assay run. At least four out of every six should be within 15% of the respective nominal value. Two of the six may be outside of 15% but not both at the same concentration. Minimum number QCs should be at least 5% of total number of unknown samples or six total QCs, whichever is greater

Accuracy -

The degree of closeness of the observed concentration to the nominal or known true concentration [22-24]. It is typically measured as relative error (%RE) [27]. Accuracy is an absolute measurement and an accurate method depends on several factors such as specificity and precision [25, 26]. Accuracy is sometimes termed as trueness. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte (i.e., QCs). Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The mean value should be within 15% of the nominal value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the nominal value serves as the measure of accuracy. The two most commonly used ways to determine the accuracy or method bias of an analytical method are (I) analyzing control samples spiked

with analyte and (II) by comparison of the analytical method with a reference method [23, 28]. Accuracy is best reported as percentage bias which is calculated from the expression [27]

$$\text{Abso\% Bias} = \frac{\text{measured value} - \text{true value}}{\text{true value}} \times 100$$

Precision-

The precision of a bioanalytical method is a measure of the random error and is defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [24, 30]. Measurement of scatter for the concentrations obtained for replicate samplings of a homogeneous sample. It is typically measured as coefficient of variation (%CV) [27] or relative standard deviation (R.S.D.) of the replicate measurements [27, 28].

$$\% \text{ CV} = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% coefficient of variation (CV) except for the LOQ where it should not exceed 20% CV. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability-

Repeatability expresses the analytical variability under the same operating conditions over a short interval of time (within-assay, intra- assay). Repeatability means how the method performs in one lab and on one instrument, within a given day. Precision measured under the best condition possible (short period, one analyst etc.).

Intermediate precision-

It includes the influence of additional random effects within laboratories, according to the intended use of the procedure, for example, different days, analysts or equipment, etc. (between- assay, inter-assay). Intermediate precision refers to how the method performs, both qualitatively and quantitatively, within one lab, but now from instrument-to-instrument and

from day-to-day [22, 28]. Precision measurements of the within laboratory variation due to different days, analysts, equipments, etc.

Reproducibility-

Reproducibility is the precision between laboratories (collaborative or inter laboratory studies), is not required for submission, but can be taken into account for standardization of analytical procedures. Ability of the method to yield similar concentration for a sample when measured on different occasions [27]. 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 [28, 29].

Linearity-

The ability of the bioanalytical procedure to obtain test results that are directly proportional to the concentration of an analyte in the sample within the range of the standard curve [22,27, 30,31]. 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.

Selectivity and Specificity-

The ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. These could include metabolites, impurities, degradants, or matrix components [27]. Selectivity is the documented demonstration of the ability of the bioanalytical procedure to discriminate the analyte from interfering components [28, 29]. It is usually defined as "the ability of the bioanalytical method to measure unequivocally and to differentiate the analytes in the presence of components, which may be expected to be present" [30, 31]. Analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ) [32]. These interferences may arise from the constituent of the biological matrix under study. They may depend on characteristics

of the individual under study, be it an animal (age, sex, race, ethnicity, etc.) or a plant (development stage, variety, nature of the soil, etc.), or they could also depend on environmental exposure (climatic conditions such as UV-light, temperature and relative humidity) [28]. The actual FDA guidance for bioanalytical method validation requires the use of at least six independent sources of matrix to demonstrate methods selectivity.

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present [33, 34]. For example, in high-performance liquid chromatography with UV detection (HPLC-UV), a classic chromatographic method, the method is specific if the assigned peak at a given retention time belongs only to one chemical entity; in liquid chromatography with mass spectrometry detection (LC-MS) the detector could measure selective an analyte, even if this is not fully separated from endogenous compounds etc. Despite this controversy, there is a broad agreement that specificity/ selectivity is the critical basis of each analytical procedure.

Limit of Detection (LOD) -

The lowest amount of analyte that can be detected but not quantified [22]. The calculation of the LOD is open to misinterpretation as some bioanalytical laboratories just measure the lowest amount of a reference solution that can be detected and others the lowest concentration that can be detected in the biological sample [23]. There is an overall agreement that the LOD should represent the smallest detectable amount or concentration of the analyte of interest.

Limit of Quantitation -

The Quantitation limit of individual analytical procedures is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy [23].

Quantification Range -

The range of concentration, including the LLOQ and ULOQ that can be reliably and reproducibly quantified with suitable accuracy and precision through the use of a concentration response relationship [27, 34]. The FDA Bioanalytical Method Validation document defines the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) as following,

Lower limit of quantification (LLOQ) -

The lowest concentration of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy [27, 28, 30, 31].

Upper limit of quantification (ULOQ)

The highest amount of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy [27, 30]. Several approaches exist in order to estimate the lower limit of quantification (LLOQ). A first approach is based on the well-known signal-to-noise (S/N) ratio approach. A 10:1 S/N is considered to be sufficient to discriminate the analyte from the background noise [25]. The other approaches are based on the "Standard Deviation of the Response and the Slope".

The computation for LLOQ is: LLOQ = 10σ/S

Where σ is the standard deviation of the response and S = the slope of the calibration curve. Another approach to estimate the LLOQ is to plot the RSD versus concentrations close to the expected LLOQ.

Standard Curve (Calibration Curve)

The standard curve for a bioanalytical procedure is the existing relationship, within a specified range; between the response (signal, e.g., area under the curve, peak height, absorption) and the concentration (quantity) of the analyte in the sample i.e. Calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. It is also called as calibration curve. This standard or calibration curve should be described preferably by a simple monotonic (i.e. strictly increasing or decreasing) response function that gives reliable measurements, i.e. accurate results as discussed thereafter [28]. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ. The lowest standard on the calibration curve should be accepted as the limit of quantification if the analyte response is at least five times the response compared to the blank response and if the analyte response is

identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80 to 120% [23].

Recovery

The extraction efficiency of an analytical process reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method [27]. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery [29]. It also be given by absolute recovery,

$$\text{Absolute recovery} = \frac{\text{response of analyte spiked into matrix(processed)}}{\text{response of analyte of pure standard(unprocessed)}} \times 100$$

Stability

The chemical or physical stability of an analyte in a given matrix under specific conditions for given time intervals. The aim of a stability test is to detect any degradation of the analytes of interest during the entire period of sample collection, processing, storing, preparing, and analysis. The condition under which the stability is determined is largely dependent on the nature of the analyte, the biological matrix, and the anticipated time period of storage (before analysis). The FDA guidelines on bioanalytical method validation, as well as the recent AAPS/FDA white paper, require evaluating analyte stability at different stages. Stability should be confirmed for every step of sample preparation and analysis, as well as the conditions used for long-term storage. They also include the evaluation of the analyte stability in the biological matrix through several freeze–thaw cycles, bench-top stability (i.e. under the conditions of sample preparation), long term stability at for example -20°C or -70°C (i.e. during storage conditions of the samples) and stability of samples on the auto-sampler. Generally, stability should be evaluated at least at two concentration levels, using blank biological matrix matched samples spiked at a low and high concentration level. It should be assessed in each matrix and species in which the analyte will be quantified. Also, the stability of the analyte must be investigated under various conditions: in the standard solutions used to prepare calibration curves, in any biological matrix stored at -20°C and at

room temperature prior to analysis and also in the final extract awaiting analysis. There may also be the need to investigate the stability of the analyte between the sample being taken and stored: some compounds are metabolized by esterases in the blood and have very short half-lives, therefore to stabilize the compound an inhibitor should be added, the effectiveness of which will need to be assessed and validated [27]. Percent stability could be calculated as follows:

$$\% \text{ stability} = \frac{\text{Mean response of stability samples}}{\text{Mean response of comparision samples}} \times 100$$

Stability samples should be compared to freshly made calibrators and/or freshly made QCs. At least three replicates at each of the low and high concentrations should be assessed. Assessments of analyte stability should be conducted in the same matrix as that of the study samples. All stability determinations should use samples prepared from a freshly made stock solution. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis (e.g., short-term, long-term, bench top, and room temperature storage; and freeze-thaw cycles). If during sample analysis for a study, storage conditions changed and/ or exceed the sample storage conditions evaluated during method validation, stability should be established under the new conditions. Stock solution stability also should be assessed. Stability sample results should be within 15% of nominal concentrations [28].

Short-term stability

The stability of the analyte in biological matrix at ambient temperature should be evaluated. Three aliquots of low and high concentration should be kept for at least 24 hours and then analysed [30].

Long-term stability

The stability of the analyte in the matrix should equal or exceed the time period between the date of first sample collection and the date of last sample analysis [24].

Freeze and Thaw Stability

During freeze/thaw stability evaluations, the freezing and thawing of stability samples should mimic the intended sample handling conditions to be used during sample analysis. Stability should be assessed for a minimum of three freeze-thaw cycles [35].

Bench-Top stability

Bench top stability experiments should be designed and conducted to cover the laboratory handling conditions that are expected for study samples [35].

Stock solution stability

The stability of stock solutions of drug should be evaluated. When the stock solution exists in a different state (solutions vs. solid) or in a different buffer composition (generally the case for macromolecules) from the certified reference standard, the stability data on this stock solution should be generated to justify the duration of stock solution storage stability [35].

Processed Sample Stability

The stability of processed samples, including the time until completion of analysis, should be determined [35].

Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity [23]. The range of a bioanalytical assay is the concentration interval over which an analyte can be measured with acceptable precision and accuracy [25].

Robustness

According to ICH guidelines, the robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [24]. Robustness can be described as the ability to reproduce the (analytical) method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained

result(s), and a robustness test as an experimental set-up to evaluate the robustness of a method.

Ruggedness

This includes different analysts, laboratories, columns, instruments, sources of reagents, chemicals, solvents. Ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test condition. The ruggedness of the method was studied by changing the experimental condition such as,

a. Changing to another column of similar type

b. Different operation in the same laboratory

Specific Recommendation for Bioanalytical Method Validation

1. 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. Other methods of assessing accuracy and precision that meet these limits may be equally acceptable.
2. 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.
3. 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.
4. The stability of the analyte in biological matrix at intended storage temperatures should be established.
5. 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.
6. Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalyzed in the case of instrument failure.

7. The specificity of the assay methodology should be established using a minimum of six independent sources of the same matrix [30].

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