Bioanalytical Method Development and Validation at a glance: An Updated and concise Review

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Abstract : Bioanalytical Method Development and Validation is a process i,e intended to enable a compound of interest to be identified and quantified in a given Biological matrix. Bioanalysis is an essential part in the development and discovery of drugs. Bioanalysis involves analysis of analytes i.e. drugs, metabolites, biomarkers in biological matrix and it include several steps from collection of sample to sample analysis, data compilation, calculation and reporting. First step is sample collection from clinical studies, then sending sample for analysis. Second step is preparation of sample and it is most important step in bioanalysis so as to obtain reliable results. Last step is sample analysis, detection, data compilation and evaluation of results. For separation and detection LC-MS/MS is the most suitable method of choice due to its high selectivity and sensitivity. Bioanalysis intended for estimstion of drugs and their metabolites in biological matrix plays an important role in evaluation of Bioavailability, Bioequivalanece, Pharmacokinetic and Toxicokinetic studies. In which different parameters like Sensitivity, Selectivity, Precision, Matrix effect, etc. are performed. This Review provides an updated overview of Bioanalytical Method Developmnet and Validation.

Keywords : Biological matrix, Quality control samples, Bioanalysis, Bioanalytical Method development, Method Validation, Sample preparation, Chromatography.

1. Introduction

Bioanalytical method development and validation plays a significant roles in the discovery, development and manufacture of pharmaceuticals. Today bioanalysis is essential in pharmacokinetic and pharmacodynamics studies during drug development. The development of sound Bioanalytical method is of great importance in the process of drug discovery and development, culminating entry of generic drug into the market, hence we can say that Bioanalytical method development is one of the bottle neck for the drug development. Method Validation is important part in regulated bioanalysis. Method validation is necessary to demonstrate that bioanlytical procedure is suitable for its intended use. Isocratic and gradient HPLC is the primary technique for the analysis of non-volatile API and impurities. The emphasis on the identification and the quantification of analytes and impurities leds to increased use of advance hyphenated techniques such as LC-MS and LC-NMR. The development of appropriate Bioanalytical method is critical for the successful conduct of preclinical and clinical pharmacology studies. So, during the development stage, decision regarding the choice of column, selection and mobile phase and its composition, detectors, sample preparation method and method of quantification must be addressed.

Need of Bioanalytical Method Development and Validation

It is important to use well characterized and fully validated bioanlytical method to give reliable results that can be easily interpreted. Each bioanlytical method has its own characteristics, which will vary from analyte to analyte.

There are several valid reasons for developing new Bioanalytical method :

1. There may not be a suitable method for a particular analyte.

2. Existing method may be unreliable.

3. Existing method may be too expensive or may not be easily automated, etc.

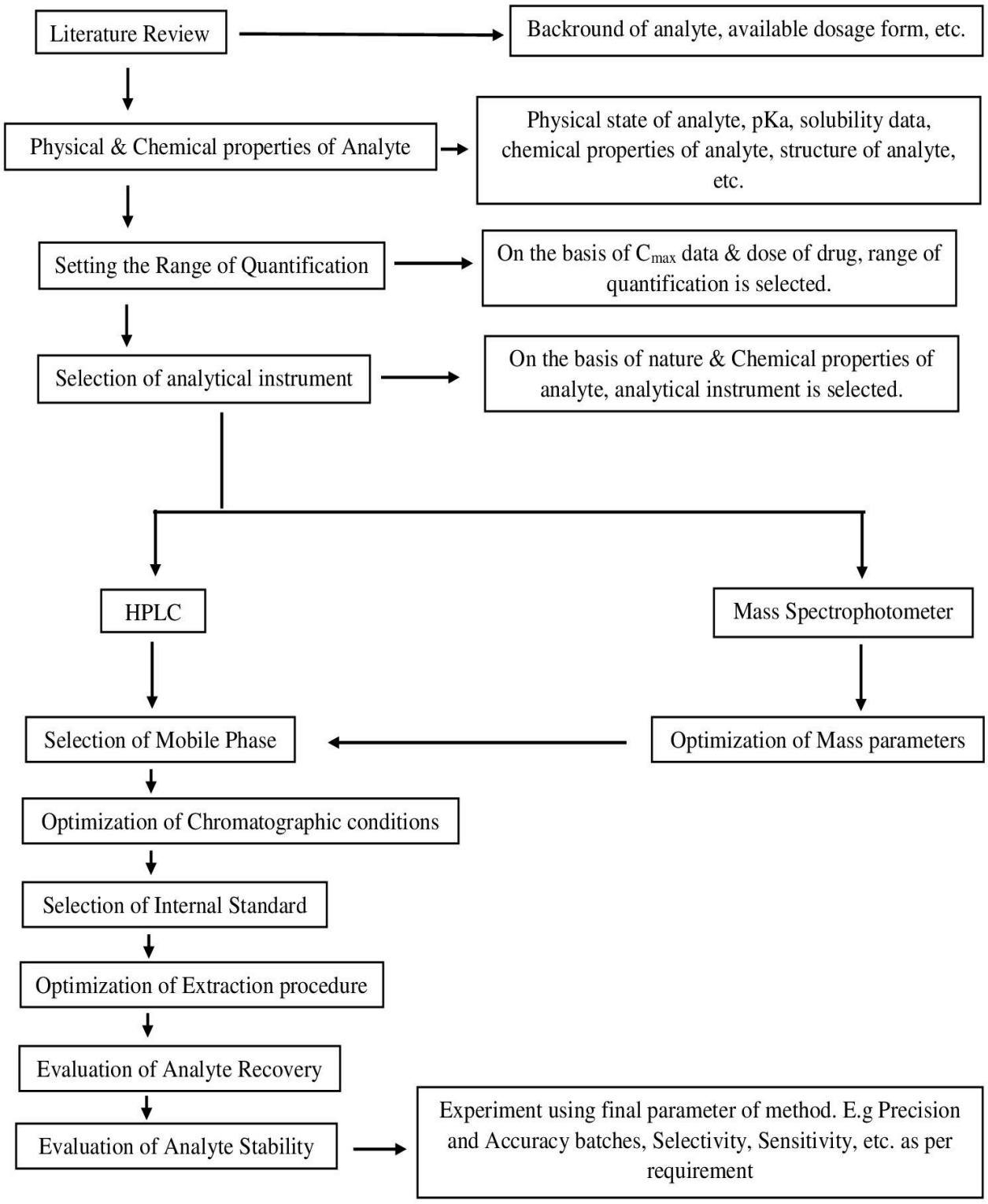

fig. schematic diagram of bioanalytical workflow

2. Method Development

Bioanalytical Method Development is the challenging process due to the complexity of sample matrix.

Steps involved in the Method Development :

1) Method selection and information of Sample

Before start of Bioanalytical method development, literature survey shall be conducted for information on drug profile and its pharmacokinetic as well as physicochemical properties. On the basis of physicochemical properties of drugs such as chemical structure, functional groups, solubility, partition coefficient, polarity, etc. the internal standard shall be selected which having comparable physicochemical properties and structure with respect to analyte. Deuterated form of analyte will be a better alternative for selection of Internal standard.

2) Initial method conditions

Selection of initial method conditions include following:

Selection of diluent on the basis of solubility of drug, Analyte and Internal Standard compatibility with analytical method, Selection of mobile phase and its composition, Selection of Column, Run time and resolution between the peaks should be taken care during this phase.

3) Processing the analytical method in aqueous standards

Before analysing developed method in biological matrix, first check the analytical method in aqueous standards. Aqueous calibration curve should be prepared with at least four concentration including the highest and lowest. Concentration of highest should be based on C_{max} of analyte and lowest shall be based on preliminary studies. Make the injections of each calibration curve standards and prepare the calibration curve and find out the correlation coefficient. It should not be less than 0.99. If so then adjust the chromatographic conditions so as to get clear resolution with the required sensitivity.

4) Selection of sample processing method

When the instrumental method is well suited with aqueous standards, prepare the matric sample. Based on literature survey data on physicochemical properties of analyte and internal standard, select and optimize suitable sample preparation technique such as Solid phase extraction, Liquid-liquid extraction, Protein precipitation, etc.

A) Solid Phase Extraction (SPE)

Solid phase extraction is the most widespread and commonly used sample preparation technique. Solid phase extraction occurs between a solid phase and liquid phase. Solid phase extraction is a straightforward method that uses sorbent of 50-200 mg as a cartridge to separate required analyte from biological matrix.

Steps involved in Solid phase extraction:

i) Conditioning

All the cartridge are required to be conditioned with suitable solvents prior to sample application. Conditioning is to achieve the sites of stationary phase bed and keep the sites in arising position.

ii) Sample application

Application of sample from the top of the cartridge at a slow rate is necessary to allow the interaction of analyte with adsorbent to achieve the retention of analyte of temporary weak bonding.

iii) Washing / Rinsing

Washing is intended for removal of matrix components or other interferences with weak dilute solvents or solvent mixtures. Rinsing solvents are water, buffers having different pH.

iv) Drying

It can be done by application of appropriate vaccum for required time period using suitable vaccum pump. It is intended for removal of excess washing solvents.

v) Elution

It involve passing of strong solvent through cartridge at a slow rate thus allowing more soak time on the bed of adsorbent. It is used for cleavage of weak bonds formed between analyte and sorbents. The eluted analyte is either injected directly or evaporated to dryness followed by dissolution in the HPLC mobile phase.

Advantages of Solid Phase Extraction
1. High extraction efficiency.

2. Relatively green and cost effective technique.

3. High reproducibility.

4. Easy to operate and automate.

5. Removal of particulates.

6. Reduced organic solvent consumption, etc.

B) Liquid Liquid Extraction (LLE)

Principle: "The technique is based on selective extraction of analyte of interest present in liquid sample through the bed of immiscible organic solvent "

Despite of development in sample preparation techniques, Liquid liquid extraction is still an attractive technique. It has been commonly used for the preparation of aqueous and biological samples. LLE is useful for separating analyte from interferences by partitioning the sample between two immiscible phases. One phase is aqueous and other is organic solvent. Hydrophilic components prefers the aqueous phase while hydrophobic components prefer the organic phase.

Analyte extracted in organic phase are easily recovered by evaporation of solvent. On the other hand, analyte extracted into aqueous phase can be injected directly onto a reversed phase column. The extraction containing analyte can be evaporated to dryness and the residue is then reconstituted with suitable solvent in a smaller volume.

Advantages of LLE :

1. Flexibility with physical properties and various parameters.

2. Facilitate stage wise phase contact, etc.

C) Protein precipitation

Principle: "It depends on the solubility of analyte in a particular solvent present in biological matrix"

It is simple method which is performed by using suitable organic solvent having good solubility of the analyte and protein precipitating properties. It basically denaturating the proteins. Precipitation of protein can be done by using one of the following method:

i) By changing the pH of sample

By mixing inorganic reagent e.g. perchloric acid. In isoelectric pH, protei8n have no net charge which causes insolubility thus precipitation.

ii) By addition of organic solvents

It decreases the dielectric constant of medium leads to insolubility that result in precipitation.

iii) By addition of salts

Commonly used salts are citrate, acetate, phosphate, etc.

The supernatant obtained after protein precipitation can be directly injected into HPLC or it can be evaporated followed by reconstitution with the mobile phase and further clan up of sample can be carried out by using microcentrifuge at very high speed.

When the sensitivity of the drug is more, prefer protein precipitation. When the sensitivity of drug is less, prefer LLE. When the recovery and reproducibility is less in LLE, prefer solid phase extraction for better sensitivity and recovery.

Typical applications of LC-MS/MS in drug bioanalysis using different sample processing methods:

Drug	Biological matrix	Sample preparation technique	LLOQ	Reference
Nicotine and its metabolites	Human blood	SPE	15 ng mL−1	41
Fluoxetine	Human plasma	SPE	2.0 ng mL−1	42
Bupropion and its metabolites	Human plasma	PPT	0.5–2.0 ng mL−1	43
Amisulpride	Human plasma	LLE	2.0 ng mL−1	44
Ketamine	Human hair	LLE	100 pg g−1	45

5) Processing the analytical method in the biological matrix

Checking the developed method with matrix sample for Precision, Accuracy and Recovery is essential before starting the Method Validation. Minimum three aliquots each of HQC (Higher Quality Control), LQC (Lower Quality Control) and LLOQ (Lower Limit of Quantification) matrix samples are analysed with one set of extracted calibration standard along with the matrix blank and blank + IS (Internal standard) and the result shall be compared with aqueous Quality control samples (QC's) of equivalent concentration. The method is accepted if it meets the criteria of Precision, Accuracy and Recovery. If required method shall be modified.

Parameters to be optimized

The parameters to be optimized during Method Development includes:

1. Mode of separation

The nature of analyte is important factor in the selection of mode of separation. For the separation of polar compound, the suitable mode is reverse phase in which mobile phase is more polar than stationary phase.

2. Selection of Column

Selection of column is most important step in Method Development as column is the heart of separation process. Selection of column depends on following parameters:

a) Column dimensions

The optimum length of column is required for efficient separation. If the column is too short, then it will not have enough resolving power and if it is too long, analysis time is extended. Three most common column length are 10, 12.5,15 and 25 cm.

b) Nature and Shape of particles

The spherical forms gives superior column packaging properties to the non-spherical forms.

c) Particle size

Most HPLC separation are carried out with 5µm diameter packaging material. Columns with 5µm particle size gives the best compromise of efficiency, reliability and reproducibility.

3. Selection of Mobile phase

The objective in selection and optimization of mobile phase is to achieve optimum separation. Following parameters to be considered during selection of mobile phase:

a) Buffer

Buffer plays an significant role in the peak symmetries and separation. Retention time depends on molar strength of buffer. In order to achieve better separation, the strength of buffer can be increased. Commonly used buffers are ammonium acetate, sodium acetate, etc.

b) pH of buffer

pH plays an important role in achieving the chromatographic separation as it controls the elution properties by controlling the ionization characteristics. It is important to maintain the pH of mobile phase in the range of 2.0 to 8.0 as Siloxane linkage are cleaved below pH 2 and silica get dissolves above pH 8.

Bioanalyical Method Validation

Method validation is a part of GLP study and it is to ensure the quality of analytical method or it is a process of demonstrating that analytical method is suitable for its intended use. Method validation is applied to ensure the method reliability, quality and reproducibility.

The different types and levels of Bioanalytical Method Validation are defined and characterized as follows:

1. Full Bioanalytical Method validation:

Full Bioanalytical Method Validation shall be done when developing and implementing a Bioanalytical Method for the first time. It should be done for a new drug entity. A Full Bioanalytical Method Validation of the revised assay should be done if metabolites are added to an existing assay for quantification.

2. Partial Bioanalyical Method Validation:

Partial validations are modifications of already validated Bioanalytical method. A validation performed to substantiate the modification of a validated method. Partial validation parameters to be performed can range from as little as one intra-assay accuracy and precision determination to a nearly full validation.

3. Cross validation

Cross validation is the comparative study of validation parameters, when two or more bioanalytical method are intended to produce data within the same or across different studies. Cross validation is considered when data is generated using different analytical techniques.

A typical Full Bioanalytical Method Validation should include determination of:

1. System suitability
2. Selectivity
3. Sensitivity
4. Precision and Accuracy
   i) Inter-Day Precision and Accuracy
   ii) Intra-Day Precision and Accuracy
5. Recovery
6. Calibration Curve
7. Matrix effect
8. Haemolyzed effect
9. Lipemic effect
10. Dilution integrity
11. Stability
12. Auto sampler carry over
13. Reinjection reproducibility
14. Ruggedness

1. System Suitability

System suitability tests are used to verify that the system (HPLC/LC-MS/MS) is suitable for the analysis. For performing System Suitability Test (SST), Middle Quality Control (MQC) sample and Lower Limit of Quantification (LLOQ) sample should be extracted as per the selected extraction procedure. After extraction, MQC sample should be injected in six replicates along with one extracted LLOQ sample. More than one MQC sample can be used after pooling as per the requirement. Equivalent aqueous MQC and aqueous LLOQ can be used to perform SST experiment where followed by experiment are prepared in aqueous samples. Six

replicates of MQC sample are injected to check the signal to noise (S/N) ratio at LLOQ. System suitability should be performed before start of each analytical day of method validation or after changing the chromatographic parameters e.g. mobile phase, column, etc.

Acceptance criteria:

% Relative Standard Deviation (RSD) of area ratio of analyte to IS for six consecutive injections of MQC sample.	Not more Than (NMT) 5.00%
Signal to noise ratio for LLOQ sample.	Not Less Than (NLT) 5.00

2. Selectivity

Selectivity should be assessed to show that the intended analyte is measured and their quantitation is not affected by the presence of the endogenous matrix components such as metabolites, degradation products, etc. the definition of Selectivity is little bit 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. For performing selectivity, Blank and LLOQ level sample from six different lots along with one Haemolyzed lot and Hyperlipidemic lot should be processed and extracted as per the extraction procedure. These sample should be run at least thrice of total run time.

Acceptance Criteria:

If any peak area is present at the retention time of analyte in blank matrix, its area response should be < 20.00% of analyte area response of an extracted LLOQ standard of the same lot.

If any peak area is present at the retention time of internal standard in blank matrix, its area response should be <5.00% of the IS area response of an extracted LLOQ standard of the same lot.

3. Sensitivity

Sensitivity is determined by using Lower Limit of Quantification (LLOQ). The LLOQ is the lowest concentration of an analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision. Analyte response should be 5 times the response compared to blank response. Analyte peak should be identifiable and reproducible with a precision of 20% and accuracy of 80-120%. For performing sensitivity, six samples of blank biological matrix from different lots should be taken. These samples should be spiked with solution “SS STD A” (LLOQ) and should be extracted along with Precision and Accuracy batches.

Acceptance Criteria:

Paramter	Limit
Signal to Noise Ratio of Sensitivity samples	Not Less Than 5.00
% RSD of Sensitivity samples	Not More Than 20.00%
% Nominal of sensitivity samples	80.00 to 120.00%

4. Precision and Accuracy

The precision of the bioanalytical method describes the closeness of repeated individual measures of analyte. Precision is expressed in terms of Coefficient of variation (CV). While the accuracy of an analytical method describes the closeness of the determined value obtained by the method to the true concentration of analyte (expressed in percentage). During method validation, Precision and Accuracy should be determined by replicate analysis using a minimum of six determinations at a minimum of five concentration level which are covering the Calibration Curve range. The lowest QC (LLOQ QC) should be near to LLOQ. The Low QC (LQC) should be within three times the LLOQ. Lower MQC (LMQC) should be between LQC and MQC i.e. 15 to 25% of the ULOQ, Middle QC (MQC) near the center of calibration curve range i.e. 45 to 55% of the ULOQ, and High QC (HQC) near the upper boundary of the standard curve i.e. 75 to 85% of the ULOQ. Each Precision and Accuracy batch should consist of one set of Calibration Curve standards and Quality Control samples spiked with analyte at five different concentration levels i.e. LLOQ QC, LQC, LMQC, MQC and HQC.

i) Intra-Day Precision and Accuracy

Intra-Day Precision and Accuracy experiment should be evaluated with Precision and Accuracy batches, which were processed separately on same analytical day.

ii) Inter-Day Precision and Accuracy

Inter-Day Precision and Accuracy experiment should be evaluated with Precision and Accuracy batches, which were processed on different analytical days.

Acceptance Criteria:

Parameter	Limit
% RSD for LLOQ QC level	Not More Than 20.00 %
% RSD for samples other than LLOQ QC	Not More Than 15.00 %
% Accuracy for LLOQ QC level	80.00% to 120%
% Accuracy for samples other than LLOQ QC	85.00% to 115.00%
At least 67% of total QC samples and 50% at each concentration level should comply with above mentioned criteria of % Accuracy	

5. Recovery

Recovery should be performed by comparing the analyte response for extracted samples and unextracted samples at three quality control sample concentrations (low, medium, high) that represent 100% recovery. Recovery can be evaluated in form of absolute recovery or relative recovery. The recovery (%) of analyte and IS from biological matrix should be determined by comparing the mean peak area of six extracted and six un-extracted samples at three different concentrations (LQC, MQC and HQC).

Acceptance Criteria:

Precision (%RSD) of overall % Recovery should be within 15% and % Recovery should be ≥ at individual quality control level (i.e. LQC, MQC, HQC).

6. Calibration Curve

A calibration curve is the relationship between the response of the instrument and known concentrations of the analyte. A sufficient number of concentration level should be used to adequately define the relationship between concentration and the analyte response. The number of standards used in constructing a calibration curve should depends on the anticipated range of analytical concentration and the nature of the analyte response relationship. A calibration curve should be comprised of a “Blank sample” (blank matrix processed without analyte and IS), a “Blank + IS sample” (blank matrix processed only with internal standard) and eight or more calibration standards covering the expected range, including the LLOQ and ULOQ. LLOQ should not be higher than 5% of the reported C_{max} and ULOQ should be 2.2 times of C_{max}. For preparation of Calibration Curve Standards and Quality Control Samples separate weighing should be done. Standard curve fitting is determined by applying the simplest model to the calibration curve standards that adequately describes the concentration response relationship using appropriate weighing (e.g. 1/x, $1/x^2$, etc.), established during the method development.

Acceptance Criteria:

Parameter	Limit
Correlation coefficient	≥ 0.99
% Accuracy at LLOQ level	80.00 to 120.00%
% Accuracy at standards other than LLOQ	85.00% to 115.00%
At least 6 out of 8 non-zero standards (at least 75% of all) should meet the above criteria including STD A (LLOQ) and STD H (ULOQ)	
For Blank sample and Blank + IS sample : If any peak area is present at the retention time of analyte in Blank or Blank + IS sample, its area response should be <20.00% of analyte area response of LLOQ standard. If any peak area is present at the retention time of Internal standard in Blank sample, its area response should be <5.00% of the IS area response of LLOQ standard.	

7. Matrix Effect

Matrix effect results in ion suppression or ion enhancement and it is common in LC-MS bioanalysis. Matrix effect provide incorrect data affecting the result and performance of study. Biological matrix contains many components such as salts, phospholipids, proteins, etc. All of these substances are the main source of ion suppression in bioanalysis. The quantitative measure of matrix effect can be termed as matrix factor and defined as a ratio of the analyte peak response in the presence of matrix ion to the analyte peak response in the absence of matrix ion. Matrix factor should be performed at Low Quality Control (LQC) and High Quality Control (HQC) concentration in at least 8 different lots of same type of matrix, out of which 06 should be normal buffered and out of other two, one Lipemic matrix and one Haemolysed matrix. This is to ensure that there is no impact of different matrix lots or matrix composition on the method reproducibility with respect to selectivity, precision and accuracy of results.

$$\text{Matrix Factor (MF)} = \frac{\text{Peak response in presence of matrix ions}}{\text{Peak response in absence of matrix ions}}$$

$$\text{IS Normalized MF} = \frac{\text{Matrix factor of Analyte}}{\text{Matrix factor of IS}}$$

Matrix Factor 1 signifies no matrix effect. A value less than 1 suggest ionization suppression. An Matrix factor greater than 1 may be due to ionization enhancement and can also be caused by analyte loss in the matrix during analysis.

8. Haemolysis effect

Haemolysis effect can be described as he rupture of red blood cells and the release of haemoglobin into the surrounding plasma. Plasma containing more than 2% of lysed blood is considered as Haemolysed plasma. Haemolysis constitute a special cause of matrix effect since certain compounds may behave differently in the presence of RBS's. This exercise should be done to assess the Haemolysis effect throughout the application of developed method. Haemolyzed matrix has a lot of inherent variability and can affect the response of analyte during the method validation and subsequently in subject sample analysis.

For Haemolysis effect experiment, LQC and HQC concentration level should be spiked in Haemolyzed plasma (six individual sample preparation). These samples should be extracted as per the extraction procedure and analyse along with precision and accuracy batch.

Acceptance Criteria:

Parameter	Limit
% RSD	Not More Than 15.300%
% Accuracy	85.00 to 115.00%
At least 67% of total QC samples & 50% at each concentration level should comply with above mentioned criteria of % Accuracy	

9. Lipemic effect

This exercise should be done to assess the Lipemic effect throughout the application of developed method. Matrix containing more than 300 mg/dL of triglycerides is considered as Lipemic matrix. Lipemic matrix has a lot of inherent variability and can affect the response of analyte during the method validation and subsequently in subject analysis. The quantification of analyte from plasma can be grossly affected by a significant Lipid content in sample.

For Lipemic effect experiment, LQC and HQC concentration level should be spiked in Lipemic plasma (six individual sample preparation). These samples should be extracted as per the extraction procedure and analyse along with precision and accuracy batch.

Acceptance criteria is same as that of Haemolysed effect.

10. Dilution Integrity

Dilution of samples will affect the accuracy and precision. Dilution integrity is performed in order to check the validity of method in case the sample needs to be diluted during analysis and also to quantify values which

are above the Upper Limit of Quantification (ULOQ). Dilution Integrity Spiking solution (DISS) having concentration of 2 and 10 times of spiking solution of HQC concentration level should be prepared and spiked in biological matrix to make Dilution Integrity standards. Respective Dilution integrity standards should be diluted to 18/2 and 1/10 using screened biological matrix and extracted as per extraction procedure and run with precision and accuracy batch.

Acceptance Criteria:

Parameter	Limit
% RSD	Not More Than 15.00%
% Nominal	85.00 to 115.00%
At least 67% of total DI samples at each concentration level should comply with above mentioned criteria of % Nominal.	

11. Stability

Drug stability in biological matrix is a function of the storage conditions, the chemical and physical properties of the drug, the matrix and container system. All stability determinations should use a set of comparison samples prepared or processed from a freshly prepared stock solution or stock solutions with proven stability of the analyte and internal standard in the appropriate analyte -free, interference-free biological matrix. Stability procedure should evaluate the stability of the analyte during collection of sample and handling, after short term and long term storage, after going through freeze thaw cycles.

a) Freeze-thaw stability

It should be determined after three freeze-thaw cycles. At least three aliquots at each of the low and high concentration 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 analyse on third cycle. Standard deviation of error should be <15%.

b) 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 analysed. Percent deviation should be <15%.

c) Long term stability

At least three aliquots of each of low and high concentration at same conditions as study samples. Analyse on three separate time intervals. Storage time should exceed the time between the date of first sample collection and the date of last sample analysis.

d) Bench Top stability

Bench Top stability will be evaluated by analysing biological matrix samples containing analyte at LQC and HQC concentration levels. These samples should be kept on work bench at ambient temperature for at least 24 hours. After relevant stability period, fresh biological matrix samples containing analyte at LQC and HQC concentration level should be prepared and extracted along with stability sample and one prepared calibration curve standards as per extraction procedure. These ix samples of LQC and HQC levels each of Stability and Fresh samples should be injected along with one set of Calibration Curve standards.

Acceptance Criteria:

Parameter	Limit
% Difference	± 15.00%
% RSD	Not More Than 15.00%
% Nominal	85.00 to 115.00%

12. Autosampler carry over

Autosampler carry over test should be performed to confirm that there is no carry over of analyte from the previous injection. If carry over is unavoidable specific measures should be taken during the validation. This include the injection of blank sample after high concentration samples before the analysis of the next study.

Autosampler carry over test should be carried out using aqueous and extracted samples in the sequence as given below:

Sr. No.	Autosampler carry over test samples	
1.	Aqueous (Aq)	Reconstitution solution / Mobile phase (RS/MP)
2.		Aq. STD H
3.		Reconstitution solution / Mobile phase (RS/MP)
4.		Aq. STD A
5.	Extracted	Blank
6.		STD H
7.		Blank
8.		STD A

The carryover response indicated by a peak in the Reconstitution solution or Mobile phase as well as in Blank sample at the Retention time of analyte and IS should be evaluated.

Acceptance criteria:

If any peak area is present at the Retention time of the analyte in Mobile phase or Reconstitution solution or blank sample, its area response should be <20.00% of analyte area response obtained in an aqueous STD A or extracted STD A respectively.

If any peak area is present at the Retention time of the IS in Mobile phase or Reconstitution solution or blank sample, its area response should be <5.00% of IS area response obtained in an aqueous STD A or extracted STD A respectively.

13. Reinjection Reproducibility (RIR)

This parameter is performed to assess any change occurring due to reacquisition of the same sample in vial placed in autosampler. RIR should be evaluated by preparing and extracting a set of calibration curve standards and six replicates of LQC and HQC samples, these sample should be subjected to initial instrumental analysis. After initial analysis, the processed sample should allow to remain in autosampler al least for 12 hours. The sample should then reanalysed. After reanalysis, the result for the reanalysed Quality Control sample should be calculated using both the calibration curve standards derived from initial analysis as well as that derived from the reanalysed calibration curve standards.

% RSD and % Nominal of back calculated concentration of analyte should be calculated.

Acceptance Criteria:

Parameter	Limit
% RSD	Not More Than 15.00%
% Nominal	85.00 to 115.00%
At least 67% of total QC samples and 50% at each concentration level should comply with above mentioned criteria of % Nominal.	

14. Ruggedness

Ruggedness test should be performed for different column, different equipment as well as different analyst.

Different Analyst: The precision & Accuracy batch will be processed by different analyst.

Different Equipment: The precision & Accuracy batch will be analysed on different equipment with the same hardware configuration.

Different Column: The set of passing Precision and accuracy batch sample will be analysed on same equipment by using different column of same make and the specification.

A single run should consist of Calibration curve standards and biological matrix samples spiked with analyte at five different concentration levels i.e. LLOQ QC, LQC, LMQC, MQC, and HQC. The calibration curve will be generated using applicable regression and weighting factor. % RSD, % Bias and % Nominal of back calculated concentration of analyte should be calculated.

Acceptance Criteria for Quality Control Samples:

Parameter	Limit
% RSD for LLOQ QC level	NMT 20.00 %
% RSD for samples other than LLOQ QC	NMT 15.00 %
% Bias for LLOQ QC level	± 20.00 %
% Bias for samples other than LLOQ QC	± 15.00 %
% Nominal for LLOQ QC level	80.00 to 120.00 %
% Nominal for samples other than LLOQ QC	85.00 to 115.00 %
At least 67 % of total QC samples and 50 % at each concentration level should comply with the above mentioned criteria of % Nominal.	

Conclusion:

The effective development and validation of Bioanalytical method's are critical elements in the development of pharmaceuticals. In this review, the basic required concepts and terminologies in Bioanalytical method development and validation were discussed. The objective of this paper is to provide practical approaches for determining precision, accuracy, selectivity, sensitivity, matrix effect, stability of chromatographic method to support Bioavailability, Bioequivalence, pharmacokinetic and toxicokinetic studies.

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