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A REVIEW ON BIOANALYTICAL METHOD DEVLOPMENT AND ITS VALIDATION

Ramanlal N. Kachave [a], Akshay G. Mundhe [b],

[a*] Department of Pharmaceutical Chemistry , Amrutvahini College of Pharmacy, Tal: Sangamner, Dist: Ahmednagar, Maharashtra, India.

[b] Department of Quality Assurance Technique, Amrutvahini College of Pharmacy, Tal: Sangamner, Dist: Ahmednagar, Maharashtra, India.

Abstract:

Bioanalytical method development is the process of creating a procedure to determine a compound of interest to be identified and quantified in a biological matrix. A compound can often be measured by several methods and the choice of analytical method involves many considerations. Analysis of drugs and their metabolites in a biological matrix is carried out using different extraction methods like liquid-liquid extraction (LLE), solid phase extraction (SPE) and protein precipitation from these extraction methods samples are spiked with calibration (reference) standards and using quality control (QC) samples. 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. Bioanalytical validations play a significant role in evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and pharmacodynamic studies. In which different parameters like accuracy, precision, selectivity, sensitivity, reproducibility, and stability are performed.

Keyword: Bioanalytical Method, Biological Extraction, Method Development

Introduction:

Bio-analytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a biological matrix.[1]

Example- bio-analytical method for the quantification of drug in human plasma or saliva.[2]

Bio-analysis plays an important role in drug development & essential for study of following during drug development & drug discovery.

- Pharmacokinetics
- Pharmacodynamics
- Toxicological evaluation

Bio-analytical method mainly used in clinical & non-clinical pharmacology study in evalution and interpretation of bioequivalence, bioavailability,therapeutic drug monitoring pharmacokinetics studies.[3] by quantitative determination of various analyte in biological matrices. It include the sampling , sample preparation, analysis, calibration, data evaluation & reporting of the all activity related to bio-analytical activity. In modern bio-analysis good sample preparation & hyphenated instrumentation are required for better results with their accuracy.eg.LC-MS (use for long time in drug bio-analysis. [3]

The official test methods that result from these processes are used by quality control laboratory ensure the identity, purity, potency and performance of drug products.
[4]

Bio-analytical method validation parameters include selectivity, accuracy, precision, linearity and range, limit of detection, limit of quantification, recovery, robustness and stability. The method validation has an important role in regulatory bio-analysis to ensure quality of applied method & provides assistance to sponsors of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs) [5]

Bio-analytical method validation is to be carried out to ensure validity of the bioanalytical method & the validation is to be carried out in the following condition:

During development & implementation of a newly developed bioanalytical method.

- Analysis of new drug entity
- For revisions to an existing Method .
- Bio-analytical method transfers from lab to lab or analyst to analyst.
- Change in analytical method.
- Change in matrix within species (e.g. human plasma to human urine).
- Change in sample processing steps. [6]
- Needs of bio-analytical method development & validation
- No suitable method for available particular analyte in specific sample matrix.
- Existing method may be too error or contaminated.
- Existing method may be too expensive.
- Existing method may not provide adequate sensitivity or analyte selectivity.[1]

Needs for bio-analytical method validation.

- To submit data regarding the human clinical trial & its non-pharmacological study & toxicological study for applying IND ,NDA, ANDA.
- To yield reliable results of bio-analytical method. [8]

Bio-analytical techniques:

- Hyphenated techniques
- LC-MS
- GC-MS
- CE-MS
- LC-UV
- UPLC-Tandem mass spectrometry
- Supercritical fluid –Tandem mass spectrometry
- Chromatographic techniques
- HPLC
- GC

these are the some techniques commonly used in bio-analytical studies.[8]

Method development:

- There are various steps in the method development are as follows:
- Step 1- Method selection & information of sample
- Step 2- Selection of initial method condition
- Step 3- Checking the analytical method in aqueous standard
- Step 4- Development & optimization of sample processing method
- Step 5- Checking the analytical method in the biological matrix
- Step 6- Pre-validation

These are the steps used for bio-analytical method development.

- **Step 1- Method selection & information of sample**

the collection of information of the physicochemical property of the drug analyte and related compounds for the development of the analytical method is based on the literature survey of the specific drug analyte. For example- solubility, polarity, molecular size, shape, structure, functional groups , partition coefficient, dissociation constant etc.

select the internal standard of the comparable chemical structure and physicochemical property of the analyte.[1]

- **Step 2- Selection of initial method condition**
  The selection of initial method condition include diluent selction based on solubility of drug analyte, drug metabolite , internal standard & compatibility with analytical method. During this phase run time & resolution between peaks is taken. The lowest quantity of analyte is to be determine by using the aqueous solution.
- **Step 3- Checking the analytical method in aqueous standard**
  Before analyzing method in biological matrix, first check the analytical method in aqueous standards. Minimum 4 concentration of aqueous calibration curve standards prepared , including the highest and lowest concentration. The selection of highest concentration standard is to be based on Cmax & lowest concentration standard based on 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 then adjust the mobile phase, mass spectral parameters 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 & 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.
- **Liquid-liquid extraction:**
  The principle of liquid-liquid extraction is based on differential solubility and partioning of analyte in the aqueous (original sample phase) and organic phase. It involves extraction of substance from one liquid phase to another liquid phase. The less dense solvent will be upper layer, while more dense solvent will be the lower layer. More hydrophilic compounds found in polar aqueous phase while the hydrophobic solvent found in organic phase. But now-a-days traditional liquid-liquid extraction has been replaced by advanced techniques like liquid phase micro extraction & supported membrane micro-extraction.
- **Solid phase extraction:**
  Solid phase extraction is the most important technique used in sample pretreatment for HPLC. SPE occur between a solid phase and a liquid phase. SPE is more efficient separation process than LLE. It is easier to obtain a higher recovery of analyte. SPE employs a small plastic disposable column or cartridge, often the barrel of a medical syringe packed with 0.1 to 0.5 g of sorbent. The sorbent is commonly reversed phase material (C18-silica. 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 Injected directly or Evaporated to dryness followed by dissolution in the HPLC mobile phase.
  where analyte is weakly retained, interferences are strongly held on the cartridge and the analyte is collected for the further treatment.
- **Protein precipitation :**
  protein precipitation method is the simple method used for the extraction of analyte from plasma and blood sample. Protein precipitation based on solubility of analyte in organic solvent which is having good precipitation property. For protein precipitation solvent selection first preference solvent is acetonitrile and second preference is to methanol because of its complete protein precipitation property & solubility of analyte in these solvent. After protein precipitation the supernatant liquid is collected & injected directly into the HPLC or it can be evaporated and reconstituted with the mobile phase and further cleanup of the sample can be carried out by using micro centrifuge at very high speed.
  Preliminary evaluation of lower limit of quantification (LLOQ) to be done after fixing the sample processing technique. By using the biological matrix with lowest interference, prepare at least three aliquots of each concentration of 1/20 of Cmax, 1/30 of Cmax and 1/40 of Cmax of the analytes.
- **Solid phase micro extraction:**
  It is simple solvent free method which involve sampling, extraxtion, sample introduction into single step. In this step fused silica fibers are coated with modified bonded phase & it is kept in contact with sample & exposed to vapour or place in stream of gaseous sample to isolate analyte and concentrate analyte into range of coating material.

The separation and quantification of analyte from these fibers by transferring to analytical instruments like GC or GC-MS. The SPME used for routine analysis of volatile and semi-volatile compound.

The SPME is looks like simple device having modified syringe consisting of fiber holder& fiber assembly. analyte in the sample directly extracted and concentrated to extraction fiber. These SPME directly coupled with HPLC & HPLC/MS to anlayse weakly volatile or thermally liable compounds not detected by GC or GC/MS.

- **Matrix-solid phase dispersion:**

Solid matrices are used for sample preparation. It is economic because of its sample requirement Is less than 1gm and solvent requirement is also less so it is also termed as microscale extraction.

- **Step 5- Checking the analytical method in the 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.

Then this bio-analytical method check for validation parameter in pre-validation stage.

- **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.

Bioanalytical Method Validation:

All the procedure require to confirming that particular analytical method for the quantitative analysis of the analyte in biological matrix is reliable for the intended application. These bio-analytical method validation depend on nature of analyte or techniques which is use for the method development & validation. These methods are very demanding for study of bio-availability, bio-equivalence & pharmacokinetic of drug & its metabolites. this validation having different stages which must be need to understand basic requirement of the process.

- **Specificity / Selectivity :**

Selectivity exercise is carried out to assess the ability of bio-analytical method to differentiate and quantify the analyte in the presences of other component in the sample. For selectivity ,analysis of biological sample of appropriate biological matrix obtain from at least 6 from source should be carried out. Each blank sample sample should be tested for interferences and selectivity should be ensured at the lower LOQ.

Guidelines : Blank sample of appropriate biological matrix from at least 6 individual sources.

1.Blank , Blank with Internal standard , calibration standard (CS-1 to CS-8) and QC'S (HQC,MQC,LQC)Sample by appropriately spiking with calibration spiking solution respectively

2. LLOQ analyte spiking solution spiked with 6 individual biological matrix sources and analyze.

Acceptance criteria:

1. blank samples and zero calibrators should be free from interference at the analyte and internal standard retentation time. IS respone with biological matrix should be NMT 5% for CS-1 to CS-8 and QC'S samples.

2. Spiked sample should be +/- 20% of LLOQ

- **Calibration curve (Linearity):**

according to ICH- linearity is define as the ability to obtain test result are directly proportional to the concentration of analyte present in the sample (within given range).

Calibration curve is the simples model to describe concentration-response relationship. The concentration for calibration curve should be prepare in same biological matrix as the sample and calibration curve generated for each analyte. If th total ranges cannot be describe in single calibration curve , two calibration ranges can be validated.

Co-relation co-efficeint are most widely used to show the high degree of relationship between concentration - response data. The deviation should not exceed more than 20% from nominal concentration of LLOQ and NMT 15%b of the other standard curve.

Guidelines:blank , blank+ IS , at least 6 non-zero(CS-1 to CS-6) calibration concentration covering the quantitaion range,including LLOQ .

Acceptance criteria:

1. Non-zero calibration standard (CS-1 to CS-6) should be +/- 15% of their nominal concentration.(75% of minimum six non-zero standard should meet the above criteria)

2. for LLOQ- +/- 20% of their nominal concentration in each validation run.

- **Precision:**
  It is the closeness of individual measure of an analyte when procedure is applied repetedly to multiple aliquots of single homogenous biological matrix. minimum 3 of 5 concntration in the range of expected study sample. There are various parts of precision
- Repetability :how the method perform in one lab, one instrument within a day. Duration of time interval are not defined. Assay run and batch are commonly used to express repetability.
- Reproducibility: how the method perform from lab to lab , day to day from analyst to anlyst from instrument to instrument in qualitative and quantitative way.(kirthi)
- Intermediate precision: intermediate precision express within laboratory variations like different day, different analyst, different equipment. ISO define the different M-factor intermediate precision. M-Factor express the number of factor that differ between successive determination.
  Guidelines:
  1. precision should be established with at least 3 independant runs. 4 QC'S level per run (LLOQ, LQC,MQC,HQC) and its 5 replicates per QC level.
  2. blank , blank+ IS , at least 6 non-zero(CS-1 to CS-6) calibration concentration covering the quantitaion range,including LLOQ .
  Acceptance criteria:
  1.Acceptance criteria for precision:
  +/- 15% coefficient variation- for nominal concentration.
  +/- 20% coefficient variation- for LLOQ.
  precision run has nop acceptance criteria for QC's run.
  2. Non-zero calibration standard (CS-1 to CS-6) should be +/- 15% of their nominal concentration.(75% of minimum six non-zero standard should meet the above criteria). for LLOQ- +/- 20% of their nominal concentration in each validation run.
- **Accuracy :**
  Accuracy is the closeness of mean test result obtain by method to actual concentration of analyte & it is determine by replicate analysis of sample containing known amount of analyte. It is an absolute measurement an accurate method depend on several factors such as specificity & precision.(kirthi)It should be measured using minimum 5 determination per concentration. Minimum 3 concentration in the range of expected study sample is recommended. The mean value should be 15% of the nominal valueexcept at LLOQ.where it should not deviate more than 20%.
  There are 2 methods commonly used to determine accuracy:
- Analyzing control sample spiked with analyte.
- By comparision of analytical method with reference method.
  Guidelines:
  1. Accuracy should be established with at least 3 independant runs. 4 QC'S level per run (LLOQ, LQC,MQC,HQC) and its 5 replicates per QC level.
  2. blank , blank+ IS , at least 6 non-zero(CS-1 to CS-6) calibration concentration covering the quantitaion range,including LLOQ .
  Acceptance criteria:
  1.Acceptance criteria for Accuracy:
  +/- 15% Of their nominal concentration except LLOQ.
  +/- 20% of concentration for LLOQ.
  Accuracy run has not acceptance criteria for QC's run.
  2. Non-zero calibration standard (CS-1 to CS-6) should be +/- 15% of their nominal concentration.(75% of minimum six non-zero standard should meet the above criteria). for LLOQ- +/- 20% of their nominal concentration in each validation run.
- **Lower limit of Quantification & Upper limit of Quantification (LLOQ & ULOQ):**
  It is the lowest amount of analyte that can be detected but not quantified under stated experimental condition. The detection is usually expresses in percentage, parts per million,parts per billion.
  The quantitation uttermost compasses of an individual consistent methodology is portrayed as the most negligible measure of analyte in an illustration, which can be quantitatively chosen with fitting precision and accuracy.
  accuracy: accuracy for sensitivity replicates should be +/- 20% of their nominal concentration.
  precision : precision for sensitivity replicates should be +/- 20% of CV.

- **Recovery:**
  the recovery of analyte is the part of validation to determine the extraction method efficiency and its reproducibility. the recovery of analyte and internal standard not should be exact 100% but it may be consistent and reproducible. as per their extraction efficiency result the extraction method this method is selected for analyte recovery and used for study the bioanalysis of subject samples.
  Guidelines: Extracted sample at lower, middle, higher (LQC,MQC,HQC) concentration versus extract of blank spiked with the analyte post extraction at (LQC,MQC,HQC).
- **Stability studies :**
  Stabilty study are the studies carried out during the method devlopment to determine the analyte chemical stability in given matrix sample , during their sample collection ,sample handling and storage. the satbility studies should be perfoemed in same matrixto that of subject sample.this include the autosampler stability study,benchtop stability, processed and extracted sample stabilty, freeze thaw satbilty, stock solution stabilty, longe-term stabilty studies.
  for fixed combination drugs the stability study should be performed for specific drug in the presence of another drug.Validation of drug stability in a biological fluid is a function of the storage conditions, the physicochemical 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. if the storage condition is changed or the validation studies should be carried outside of the validated storage condition, the stability stydy should be newly study under new condition.

  1. Stock solution stability study:
  stock solution stability of analyte and their internal standard for subject sample anlaysis sholud be evaluted at room temprature for at leastr 6 hrs.the stabiliyty of stock solution is in the refrigerator or the deep freezer then this solution are stored in refrigerator and deep freezer respectively over specific period of time and evaluted stored stock solution with that of the freshely prepared stock solution.

  2. Autosampler tray stability study(extracted sample stabilty study):
  autosampler stability study determine the stability of extract of analyte when the autosampler storage condition and extract processed sample storage condition are different. in these autosampler stability the sample sholud be evaluted for 48-96 hrs to cover anticipated run time for analytical batch .then the extracted QC samples are stored at autosampler temprature for specific time and analyzed with fresh standard QC samples.

  3. Benchtop Stability:
  benchtop Stability of samples is determine the stability of samples under the laboratory handling condition that are expected for the study samples. analyte samples in matrix are analyzed after keeping them at ambient temprature for 4-24 hrs to cover atleast time to extract the samples. the observed samples concentration are compared with their nominal values. the experiment is combinly determine the overall stability of the experiment during the lab processing conditions.

  4. Freeze-thaw satbility:
  freeze-thaw stability is determine the stabilty of samples after minimum freeze-thaw cycles. in these samples are freeze and thawed and analyzed according to standard procedure used for subject study samples.minimum 2 concentrations (LQC & HQC)are frozen overnight,at normal storage temprature(eg.-20 or -70) and thawed these samples unassisted at room temprature.when samples are thawed , samples are again frozen at at same temprature for 12-24 hrs and thawed.these freeze thaw cycle is repeated 2 more times,after 3rd cycle samples are analyzed.the observed cioncentration are observed with their nominal values . if any degradation is observed in these concentration then cycle 1 st and 2 nd is repeat to determine the at which cycle the instability is occur. if needed the number of freeze-thaw cycle it can be extend.

  5. Freezer storage stability:
  During validation , at nominal freezer storage stability temprature should be determine to the extent possible. however ,long-term stability should be determine and appropriately documented.

  6. Long-term stability:
  these study is detemine thelong-term stability of sample over the specific period of time. the time of long term stability should be equal or more than the period of sample collection and the last sample analysis. the sample storage condition is same as that of study sample storage condition. after completion of validation , stability of analyte in the matrix should be determine by storing sufficient number of quality control samplesat the required long term storage temprature and analyzing them in at least triplicate at minimum of two QC samples (LQC & HQC). long-term stability period should be depend on the length of stability required.it can de determine at several period

like 1,3,6,9,12,18 months.in these the long term stability QC (LQC & HQC) should be compared to freshly prepared calibration standard and QC samples.

7. Matrix stability:

at low temprature, if there may be denaturation of matrix protien.therefore matrix stability should be validated. for that purpose,additional stability should be carried out at lower temprature for sample matrix.

8. Bioanalysis of Hemolyzed samples:

As per 2009 EMA draft guidance and the 2003 ANVISA guideline heamolyzed samples are analyzed. these samples are analyzed during method validation but practically it is difficult.therefore it was recommended to perform at the time of method devlopment. data reliability and reproducibility should be monitered according to internal standard (IS) and incurred sample reproducibility response (ISR).aslo we can studied by applying standard addition or standard dilution for further investigation of datd reporting. so there are final recommendation about heamolyzed sample is that there is no standard approach for testing of these type of samples, so there should be the least impact on method devlopment or validation.

9. Whole blood stability evlution:

the immediate spinning down of aliquot of whole blood containing the drug taken immediately following preparation (time zero) followed by the spinning down of another aliquot following the stability period. the whole blood stability should be perform during method validation.However , there are various approches for determination of whole blood stability. it is not applicable for the larger molecules.

Guidelines: for stability studies 3 replicates of lower and higher concentration (LQC & HQC) in same biological matrix and analyze.

Acceptance criteria: Accuracy at each level should be +/- 15% of their nominal concentration.

Conclusion:

To evaluate and interpret bioavailability, bioequivalence, pharmacokinetic and toxicokinetic study data, bioanalytical method validation plays an important role.In this, the quantitative estimation of drug and its metabolites in the biological fluid can be performed. There is a need to discuss issues related to bioanalytical method development and validation and need to follow the guidelines, regulatory aspects that are formed during the tenure of last three decades. Various related authorities are having a keen focus on different aspects of bioanalytical method development and validation. If researchers applied all practical aspects of bioanalytical method development and validation for determination of API or certain chemical entity, that is advantageous for regulatory submissions of particular drug component. While developing the bioanalytical method there should be complete clarity about the nature of the analyte, that whether it is a small molecule or macromolecule. There certain differences in principles of bioanalysis for these types of an analyte. Recent trends related to bioanalytical method development and validation should be followed with GLP requirements for regulatory acceptance of method. There are continuous conferences; workshops are arranged at different sights of the world by CBF, GCC, and other related agencies to discuss, to solve and to improve practical difficulties and additions in the field of bioanalysis.

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