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A REVIEW ON BIOANALYTICAL SAMPLE PREPARATION METHOD AND METHOD DEVELOPMENT AND VALIDATION

Rachana Sanjay Khairnar* and Prashik Sudhir Shimpi

B. Pharm, R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur.

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***Corresponding Author**
Rachana Sanjay Khairnar
B. Pharm, R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur.

ABSTRACT

The development of sound bioanalytical method(s) is of paramount importance during the process of drug discovery and development, culminating in a marketing approval. Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacology studies. 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. 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 and solid phase extraction. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix. Applications of bioanalytical method in routine drug analysis also take into consideration in this article. These various essential development and validation characteristics for bioanalytical methodology have been discussed with view to improving the standard and acceptance in this area of research.

KEYWORDS: Bioanalytical Method, Development, Validation.

Terminology

Bioanalyses can be defined as the measurement of an analyte(s) in a biological matrix. The type of analytes can range from small molecules (200 to 500 mw), to peptides or large proteins (>100 kD) and can include xenobiotics such as drug candidates and their

metabolites, endogenous molecules such as biomarkers, and environmental pollutants.For the purpose of this article, the scope will be limited to bioanalyses related to drug discovery and drug development.

Typical biological matrices include plasma, urine, cerebral spinal fluid (CSF), organs such as liver & brain and cell-based in vitro samples. To date, the techniques used to support these analyses fall in to one of three categories.[1] Chromatography based: gas chromatography with electrochemical detector (GC-ECD), high pressure liquid chromatography with ultra violet detector (HPLC-UV),[2] ligand based : radio-immuno assay (RIA), enzyme linked immunosorbent assay (ELISA), and[3] mass spectrometry based: GC-MS, LC-MS.

The generation of increasing number of chemical leads in today's pharmaceutical industry has directly translated to the increasing demand for bioanalyses. Coinciding, the wide spread implementation of LC-MS methods during the past decade has revolutionized the industry standards and as a result the use of HPLC systems coupled to triple-quadrupole mass spectrometers (LC-MS/MS) has emerged as the default method for pharmaceutical bioanalyses.

Need for biopharmaceutical analysis

Methods of measuring drugs in biological media are increasingly important related to following studies are highly dependent on biopharmaceutical analytical methodology.

- Bioavailability & Bioequivalence studies
- New drug development
- Clinical pharmacokinetics
- Research in basic biomedical and pharmaceutical sciencesTherapeutic drug monitoring

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION

Bioanalytical chemistry is the qualitative and quantitative analysis of drug substances in biological fluids (mainly plasma, serum and urine) or tissue. It plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. The main analytical phases that comprise bioanalytical services are method development, method validation and sample analysis (method application).

A bioanalytical method is a set of all of the procedures involved in the collection, processing, storing, and analysis of a biological matrix for an analyte. Analytical methods employed for

quantitative determination of drugs and their metabolites in biological fluids are the key determinants in generating reproducible and reliable data that in turn are used in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetics.

Method development is a trial and error procedure. It involves evaluation and optimization of the various stages of sample preparation, chromatographic conditions, detection and quantification. To start these works an extensive literature survey is done on the analyte. After literature survey select the instrument of interest for example HPLC, LC-MS, LC-MS/MS and GC-MS. Based on the information from this survey, the following can be done.

Strategy for method development on LC-MS/MS

Various stages involved in method development are,

- Literature survey
- Stock solution preparation and tuning of analyte
- Selection and optimization of Chromatographic conditions
- Selection and optimization of Extraction method
- Selection and tuning of Internal standard

Literature survey

In general the process of Method development begins with literature survey of the drug/metabolite with the help of available books, references, published articles, internet search.

Literature survey should contain following information about the drug/metabolite.

- Molecular weight, molecular formula
- Physicochemical properties such as solubility, pKa, Cmax, LLOQ, protein binding, strength and dosage
- Pharmacology & pharmacokinetic properties
- Analytical method if any, specifying the chromatographic conditions such as mobile phase, flow rate, column, Internal standard.

Stock solution preparation and tunning of analyte

Prepare the stock solution and its dilution of suitable concentration by using appropriate solvent and diluent. These stock dilution for ex. 500ng/ml, 1000ng/ml are used for setting tunning process.

Select an ion source (ESI or APCI) and ion mode (positive or negative) based on chemical properties of the compound.

Infuse suitable stock dilution in Full Scan Mode. Select the m/z of the parent ion based on the molecular weight of the compound. (If molecular weight of the compound is 200 then m/z of the parent ion in positive mode is 201 and 199 in negative mode).

Select the m/z of parent ion. Then for fragmentation of parent ion, inject suitable stock dilution in Product Ion Mode and check for m/z of various daughter ions obtained. Select the prominent and suitable daughter ions by altering the various parameters.

For ex. Compound dependent parameters- DP, FP, EP, CE, CXP, CEP Source dependent parameters- Nebulizer Gas, Curtain Gas,

Temperature and, Ion spray voltage.

Then inject suitable concentration using m/z of selected parent and daughter ions in multiple reactions monitoring (MRM) mode. MRM conditions can be optimized by quantitative optimization then by using above parameters stock dilution is injected with the help of different mobile phase.

Selection and optimization of Chromatographic conditions

Column choosing is according to polarity of drug. For polar drugs non-polar stationary phases such as C-18, C-8, -CN, -NH_2 are used, while for nonpolar drugs polar stationary phases such as porous silica columns are used.

Different size column should also be tried i.e. 50mm, 75mm, 100mm, 150mm. Internal diameter of the column is also important, mostly 3.5µm, 4.6µm are used. Select the best column based on the retention time, peak shape and response.

Optimize the mobile phase composition by altering the followings

- Buffer salt
- Buffer concentration
- Buffer pH
- Solvent, Solvent proportion

Ionic samples (acidic or basic) can be separated only, if they are present in undissociated form. Dissociation of ionic samples can be suppressed by selection of proper pH.

Check the response of linearity using selected chromatographic conditions. Analyze a serially diluted calibration curve of 6-8 points. Accept the linearity if regression coefficient is (r) 0.9800.

Selection and optimization of Extraction method

Process blank matrix samples along with spiked middle point of calibration curve range by following extraction techniques,

- Liquid-liquid extraction
- Solid-phase extraction

Analyze the processed samples using optimized chromatographic conditions and compare the sample processing techniques for the interference at analyte retention time, peak shape of spiked sample and recovery of spiked sample.

If the chromatography and/or response are poor re-optimize the chromatographic conditions and processing method. Choose a column and sample processing techniques which gives best possible retention time, peak shape, response, maximum recovery and no significant interference.

Process and inject blank matrix sample to check for any late eluting interference Optimize the run time to avoid presence of late eluting interference in consecutive injections. Check the linearity of spiked sample using selected chromatographic conditions and processing method. Calibration curve should be linear for a required regression coefficient (r) 0.9800.

Selection and tuning of Internal standard

Select the I.S. based on following criteria

- Detectable under chromatographic conditions and getting extracted in extraction procedure of the main compound.
- No significant interference at the retention time of internal standard in the processed blank matrix sample

Check the linearity of spiked sample using selected internal standard. Select the Quality Control (QC) concentration and spiked six sets of QC sample of each concentration in blank

matrix.

Performance of selected method is checked by running three or more precision & accuracy batches and evaluating the results for meeting acceptance criteria. Finally selected method is validated to see whether it does what it was intended to do. Then the validated method is applied for quantitation of drug.

BIOANALYTICAL SAMPLE PREPARATION

The bioanalytical sample preparation is step in the bioanalytical process typically consists of an extraction procedure that results in the isolation and enrichment of components (analyte/metabo lite) of interest from a biological matrix. (e.g. Plasma, Urine, Skin, Saliva, etc).

OBJECTIVE

Sample preparation/treatment is essential step of chromatographic analysis and intended for.

- Clean up by Remove unwanted sample matrixcomponents
- Improve Detection Limits
- Improve Specificity
- Improve Reproducibility
- Improve Recovery
- Improve Instrument life
- Reduce backpressure and LC system fouling

Too dirty- Contains other sample matrix components that interfere with the analysis.

Too dilute- Analyte(s) not concentrated enough for quantitative detection.

- Present sample matrix incompatible with or harmful to the chromatographic column/system.

PRIMARY TREATMENT OF BIOLOGICAL SAMPLES

Biological matrices e.g. Plasma, Serum, Urine, Saliva, Bile and Sweat are associated with diverse physiological characteristics and texture. Each of these samples has its own set of factors that must be considered before an appropriate pretreatment method can be selected.

Such factors as texture and chemical composition of the sample, degree of drug protein-binding, chemical stability, types of interferences can affect the final measurement step.

Sample preparation prior to chromatographic separation has the major objectives

- The dissolution of analyte in suitable solvent
- Removal of interfering compound as possible
- Pre-concentration of the analyte

For primary treatment of biological samples various methods used are

- Protein precipitation
- Hydrolysis of conjugates
- Homogenization

Sample Preparation Precaution

- Lab should be equipped with all necessary contingency arrangements.
- Personnel should perfectly be aware of Good Laboratory Practices (GLP) and all relevant Standard Operating Procedures (SOP) to be followed while sample Preparation.
- Personnel should be trained, and with all required safety measurements.
- Handle biological matrices with taking proper care to avoid any health hazard.
- Don't rush while preparing samples as samples are precious.

Classification Of Sample Preparation Techniques

1. Protein Precipitation(PPT)
2. Liquid-Liquid Extraction(LLE)
3. Solid Phase Extraction(SPE)
4. Hybrid ExtractionTechniques i.e.Combination of two or more techniques.

e.g. PPT and SPE or PPT and LLE.

Selection of the technique

While selecting the method, following criteria's should be noticed

- Composition of matrix
- Chemical structure of analyte
- Lower Limit of Quantitation (LLOQ)
- Specificity
- Reproducibility
- Recovery

- Time required for sample preparation

1) Protein Precipitation(PPT): Principle: Denaturing of proteins Protein precipitation is the method of denaturing of the proteins which can be done by:

A) Changing the pH of the medium

B) Using Organic Solvents. (Denaturing agents)

C) Using Salts (Denaturing agents)

- The denaturing process causes disabling of protein's ability of binding to analyte molecules.
- Analytes are typically released from the proteins and remained in the supernatant liquid.

Methods of Precipitation

I) Changing the pH of the medium Reagents: Perchloric Acid(PCA),

Trichloroacetic acid (TCA),

Trifluoroacetic acid (TFA), etc.

- At low pH, proteins have net Positive (+) charge.
- At high pH, proteins have net negative (-) charge.
- At Isoelectric pH proteins are having zero net charge which leads to insolubility and it helps in precipitation.

II) Addition of organic solvents

Organic Solvents: Methanol, Acetonitrile, chloroform, Acetone, etc.

- By adding organic Solvent the protein losses their structure and this leads to release of binde drug from the proteins in the added organic solvent.
- Decrease the di-electric constant of the medium, leads to insolubility thus precipitates. High affinity for the hydrophobic surfaces of the protein leads to denaturing of proteins.

III)Salt induced precipitation

Salts: Citrates, Phosphates, Sulphates, Acetates, etc

- At low concentrations, solubility of proteins increases.
- At high concentration, solubility of proteins drop sharply, thus precipitates.

Protein Precipitation Steps involved

Sample (With Internal standard)

↓

Addition of denaturing agent(s) to the sample

↓

Vortex

↓

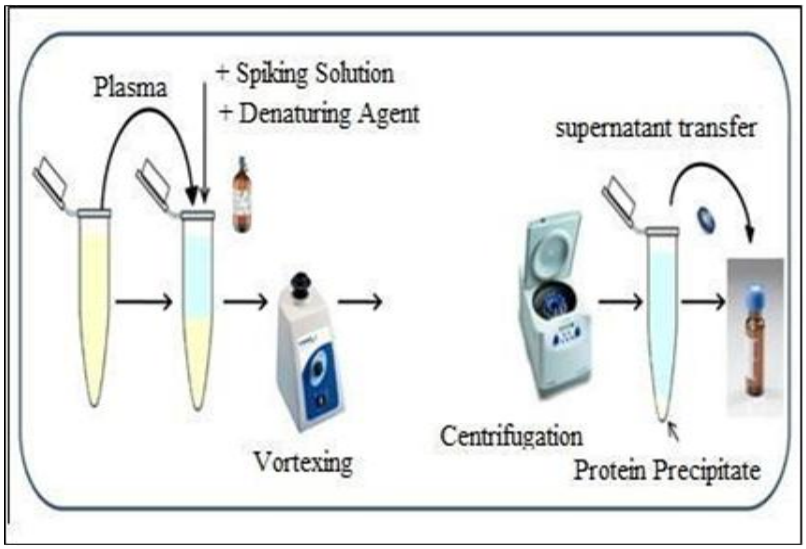
Centrifugation

↓

Phase separation (Supernatant Transfer)

↓

Analysis



Advantages

- Less steps involved, easy to implement
- Simple and time saving process.
- Excellent Reproducibility
- No evaporation, in most of the cases
- Cost effective (Less usage of Manpower or chemicals or (Consumables)

2) Liquid Liquid Extraction (LLE)

Liquid–liquid extraction is a method to separate compounds, based on their relative solubilities in two different immiscible liquids, usually aqueous and anorganic solvent.

"Selective extraction of intended analyte present in liquid sample through immiscible organic solvent"

Principle: Partitioning coefficient and Differential Solubility.

"LLE provides efficient removal of analyte with desired specificity /selectivity required for intended bioanalysis"

- LLE based on Differential Solubility and Partitioning equilibrium.
- LLE required to immiscible phases, in most of the cases one aqueous and second organic phase, both phases must be immiscible.
- Analyte can be removed from matrix selectively by choosing a suitable Extraction Solvent and Buffering (pH adjustment of sample to get analyte in unionized form) of sample, if required.

Log P= 1 10:1 (Organic:Aqueous)

Log P= 0 1:1 (Organic:Aqueous)

Log P= -1 1:10 (Organic:Aqueous)

- Log P value is more than 1 indicates Drug is present in Unionized form, as Solubility in the Non Polar Solvent (prefer LLE Extraction method).
- Log P value is Less than 1 indicates Drug is having more Solubility in the Aqueous Phase (Water/Plasma) (prefer SPE Extraction method).
- LLE can be carried out by using any one of the methods mentioned below:

I. Single Step LLE
II. Double Extraction
III. sBack Extraction

Selection of Extraction Solvent

Selection of extraction solvent depends on.

- **Analyte Characteristics**
- Structure
- *pKa*

- Solubility

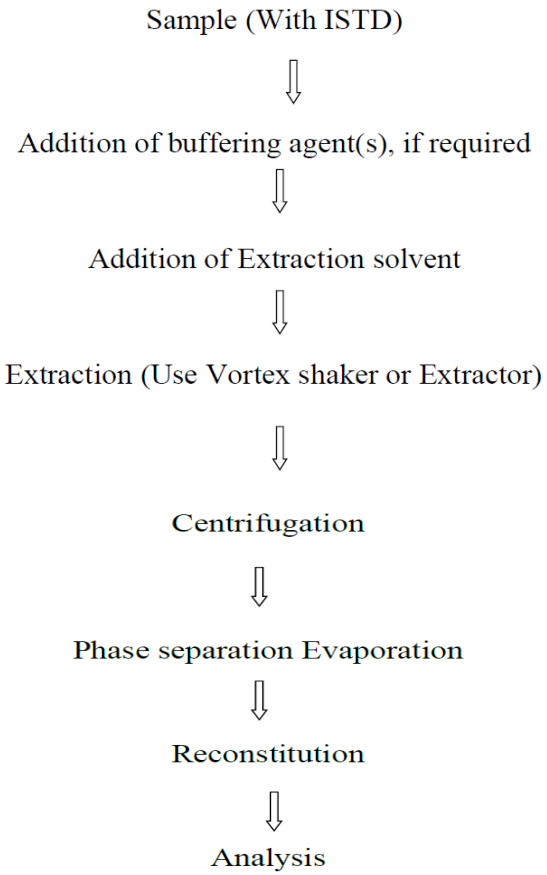
- **SolventCharacteristics**
- Miscibility •Inertness
- Purity •Volatility
- Efficiency •Viscosity
- Polarity •Density

Commonly used Extraction Solvents Tertiary-Butyl Methyl Ether (TBME) Dichloromethane (DCM)

Ethyl Acetate Diethyl Ether Hexane etc.

Any solvent as individual or in combination with any other suitable solvent can be used as an extraction solvent.

Steps involved



Back Extraction

Steps involved:

Sample (With ISTD)

↓

Addition of buffering agent(s), if required.

↓

Addition of Extraction solvent

↓

Extraction (Use Vortex shaker or Extractor)

↓

Centrifugation

↓

Phase separation

↓

Addition of Back Extraction agent, Vortex

↓

Centrifugation

↓

Phase separation, partial evaporation, if required

↓

Analysis

Advantages

- Specific
- Sensitive
- Reproducible
- Reasonably, Good Recovery
- Increase no. of injections
- Improve column life
- Improve Instrument life

3) Solid phase extraction(SPE)

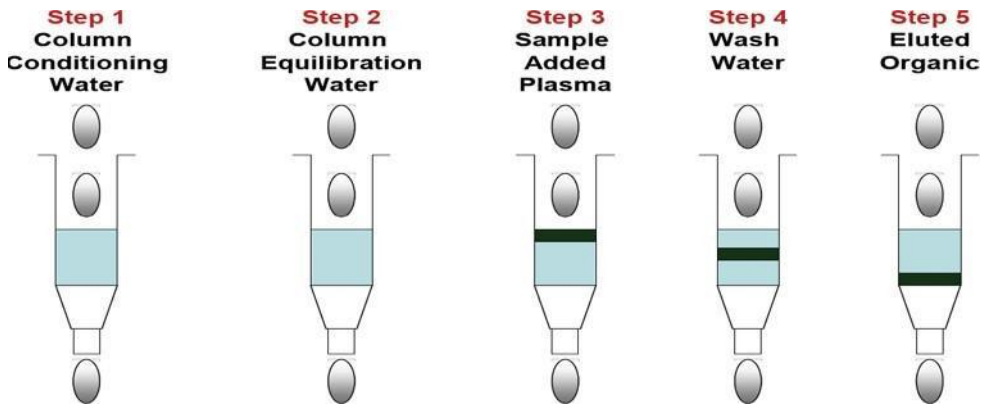
Solid-phase extraction is an extractive technique by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties.

"Selective retardation of analyte using solid sorbent under specific conditions" Principle: Adsorption (Absorption on surface)

Universal sorbent for acidic, basic and neutral compounds

Waters OASIS HLB (hydrophilic lipophilic balance) is water wettable reverse phase sorbent. It is made from balanced ratio of two monomer the hydrophilic N-vinyl pyrrolidine and lipophilic divinyl benzene.

These sorbents are resistant to pH extremities, show extraordinary retention of polar compounds, three times relative retention capacity as compared to traditional silica based SPE sorbent like C-18. In the adsorption process the hydrophobic portion of the solute adsorbed on the resin while the hydrophilic portion of the solute remain in the aqueous phase. The sample is passed through the resin bed where drug adsorbed and finally eluted with a suitable solvent.



Advantages of solid phase extraction

Effective in selective removal of interferences enabling selective sensitive and robust LC-MS/MS.

SPE enables the enrichment of selected analytes without concentrating interference.

SPE technology combined with robotic automation makes not only cost effective but also a time efficient sample preparation technique which improves analytical system performance by introducing analyte in a MS compatible solvent.

Extending the analytical column life, reduced system maintenance, minimizing ion suppression while improving signal response

BIO-ANALYTICAL METHOD VALIDATION

Bioanalytical method validation selectivity depends upon stage of development of the drug

Development stage	Assay Objectives	Validation requirement
1.Discovery	Comparison of some related compounds	Similar selectivity towards all compound examined
2. Pre Clinical	Toxicology/metabolism support regulatory standard	Reliable selective; for blood /plasma/other animal matrices
3.Cinical Healthy volunteers	Definitive clinical studies; high throughput; usable in other labs	Excellent selectivity in human biofluids-plasma, urine, saliva, CSF, perhaps facial homogenates
4.Clinical Patients	Worldwide routine monitoring of patients varying in medication and dietary background	Reliable selectivity in the presence of numerous substances

Method validation can be defined (as per ICH) “Establishing documented evidences, which provides a high degree of assurance that a specific method or activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics”.

Method validation is an integral part of method development, it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity and potency of the drug substances and drug products. Simply method validation is the process of proving that an analytical method is acceptable for its intended purpose.

Selective and sensitive analytical methods for the quantitative evaluation of drug and their metabolites (analytes) are critical for the successful conduct preclinical and/or biopharmaceuticals and pharmacology studies. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum and urine is reliable and reproducible for the intended use.

Types of method validation

Full Validation

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

Partial Validation

Partial validations are modifications of already validated bioanalytical methods. Partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation. Typical bioanalytical method changes that fall into this category include, but are not limited to.

- Bioanalytical method transfers between laboratories or analysts
- Change in analytical methodology (e.g., change in detection systems)
- Change in anticoagulant in harvesting biological fluid
- Change in matrix within species (e.g., human plasma to human urine)
- Change in sample processing procedures
- Change in species within matrix (e.g., rat plasma to mouse plasma)
- Change in relevant concentration range
- Limited sample volume (e.g., pediatric study)
- Selectivity demonstration of an analyte in the presence of concomitant medications or of specific metabolites.

Cross validation

Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross validation would be a situation where an original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator.

Each method developed is validated for the following fundamental parameters

- Selectivity
- Sensitivity
- Linearity
- Accuracy

- Precision
- Matrix effect, anticoagulant effect
- Carryover
- Recovery
- Stability
- Ruggedness
- Dilution integrity

Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the target analyte in the presence of other components in the sample. It is also defined as the lack of significant interfering peaks at the retention time of analyte and internal standard.

Selectivity is checked by injecting extracted blank biological matrix (plasma, serum, urine, etc) and comparing any interference at the retention time of analyte peak by proposed extraction procedure and chromatographic conditions. Blank matrix lots are compared with LLOQ samples processed with internal standard.

Sensitivity

According to IUPAC as cited in Roger Causon (Causon, 1997), a method is said to be sensitive if small changes in concentration cause large changes in the response function. Sensitivity of an analytical method is determined from the slope of calibration curve. The sensitivity required for a specific response depends on the concentration to be measuredin the biological specimens generated in the specific study.

Acceptance Criteria

Any significant interference peak corresponds to analyte and internal standard peak should not be present. The significant interference peak can be considered if it meets the either of the criteria.

- The retention time of interference peak corresponds to the analyte peak and the internal standard peak.
- The response of interference peak is greater than or equal to 20% of the response of LLOQ.
- The response of interference peak is greater than or equal to 5% of the response of internal standard.

- Any blank sample and zero samples that present significant interference in the retention time of the analyte and internal standard shall be rejected. If one or more of the analyzed samples presents such interference, new samples of six other individuals must be tested. If one or more of the samples of this group present significant interference in the retention time of the analyte and internal standard, the method shall be modified.

Linearity (Calibration curve)

Calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample and also for analytical tools such as methods, reagents, equipments, instrumentation and procedures for quality control and verification of results.

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. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. 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 obtained values of slope "**m"** and intercept "**c"** are used in the Linear regression equation.

y = mx + c

To calculate the concentration of the quality controls (**x**) by interpolating the peak area ratios (y), from the corresponding standard curve. As an appropriate weighing model, the standard curves were calculated with $1/x^2$ weighing factor.

Acceptance Criteria

The calibration curve should meet the following acceptance criteria.

- The deviation of the LLOQ from nominal concentration should not be more than20 %.
- The deviation of the standards, other than LLOQ, the nominal concentration should not be more than 15 %.
- The correlation coefficient of determination (r) should not be less than 0.98.
- The deviation of slope of inter day linearity shall be not more than 20 %.
- The response of interference peak in blank sample shall be less than or equal to 20 % of the response of LLOQ at retention time of analyte(s) and 5 % of the response of

internal standard at the retention time of internal standard.

- The response of interference peak in zero sample shall be less than or equal to 20 % of the response of LLOQ at retention time of analyte(s).

Accuracy

The ***accuracy*** of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy should be measured using a minimum of five determinations per concentration.

Precision

The ***precision*** of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration.

Acceptance Criteria

- Accuracy and precision should be measured at minimum of four concentrations in the range of expected concentrations (LLOQ, LQC, MQC and HQC) and minimum of six determinations per concentration.
- **Precision:** The % CV of each concentration level should not be more than 15% except for the LLOQ, for which it should not be more than 20%.
- **Accuracy:** The mean value should not deviate by 15% of the actual concentration except for the LLOQ where it should not deviate by more than 20% of the actual concentration.
- For both intra- and inter-run precision and accuracy, all QC samples, including those that failed with no assignable cause, should be used for calculation. Only those QC samples that failed for an assignable cause (e.g. rejected chromatography or sample extraction problem) should be excluded from the calculation of precision and accuracy.

Recovery

The ***recovery*** of an analyte in an assay is the detector response obtained from an amount of the analyte added and extracted from the biological matrix, compared to the pure authentic standard. 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.

It may be desirable to intentionally sacrifice high recovery in order to achieve better selectivity with some sample extraction procedure. Solvents such as ethyl acetate normally give rise to high recovery of analyte; however these solvents simultaneously extract many interfering compounds.

Therefore provided that an adequate sensitive detection limit is attained with good precision and accuracy, the extent of recovery should not be considered an issue in bioanalytical method development and validation.

Acceptance Criteria

Recovery of the analyte and internal standard should be consistent, precise and reproducible, need not to be 100%. The mean % CV of recovery at each concentration level should within 15 %.

Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. Stability of analyte in biological samples is sometimes critical due to degradation of analyte in storage period.

Therefore it is important to verify that there is no sample degradation between the time of collection of the sample and their analysis that would compromise the result of the study. Stability evaluation is done to show that the concentration of analyte at the time of analysis corresponds to the concentration of the analyte at the time of sampling.

Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze-thaw cycle should be repeated two more times, then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -70C during the three freeze and thaw cycles.

Short-Term Temperature Stability

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

Long-Term Stability

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

Stock Solution Stability

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

CONCLUSION

Bioanalysis and the production of pharmacokinetic, toxicokinetic and metabolic data play a fundamental role in pharmaceutical research and development; therefore the data must be produced to acceptable scientific standards. For this reason and the need to satisfy regulatory authority requirements, all bioanalytical methods should be properly validated and documented. The lack of a clear experimental and statistical approach for the validation of bioanalytical methods has led scientists in charge of the development of these methods to propose a practical strategy to demonstrate and assess the reliability of chromatographic methods employed in bioanalysis. The aim of this article is to provide simple to use approaches with a correct scientific background to improve the quality of the bioanalytical method development and validation process. Despite the widespread availability of different bioanalytical procedures for low molecular weight drug candidates, These various essential development and validation characteristics for bioanalytical methodology have been discussed with view to improving the standard and acceptance in this area of research.

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