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A REVIEW ON BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION

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Abstract:
In bioanalytical methods are widely used to quantitative drugs and their metabolites in plasma matrices and the methods should be applied to studies in areas of human clinical and nonhuman study. Bioanalytical method employed for the quantitative estimation of drugs and their metabolites in biological media and plays an important role in estimation and interpretation bioequivalence, pharmacokinetic, toxicokinetic, studies. The major bioanalytical role is method development and sample analysis liquid-chromatography coupled with double mass spectroscopy can be used for the bioanalysis of drugs in body. Each of the instruments has its own merits and demerits. Chromatographic methods are HPLC and gas chromatography have been mainly used for the bioanalysis of small/large molecules, with LC/MS. Linearity, accuracy, precision, selectivity, sensitivity, reproducibility, and stability are some of the regularly used parameters. In this review article, we are proposed to add some points regarding bioanalytical method development and validation parameter, beneficial to quality assurance to determine the drug, concentration and its metabolite.

Keywords*: Method development, clinical and nonclinical study, analyte, validation of bioanalysis techniques, validation parameter.*

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1.INTRODUCTION:

Analytical method is a set of techniques that allow us to know qualitatively and quantitively the analyte in sample. Bioanalytical methods are analytical methods followed for determination of drug or drug substance in biological matrices such as urine, serum, plasma etc.

The quantitative determination of drug and their metabolite in biological material in order to establish pharmacokinetic and toxicokinetic studies is known as bioanalytical method development.

- It includes number of steps

1. Collection
2. Storage
3. Sample Preparation
4. Separation
5. Identification and
6. Quantification of analyte.

Bioanalytical method development is important method for identification of analyte, removal of analyte, concentration of analyte, pre-concentration of analyte, dissolution of analyte, quantification of analyte. It is also important for new drug discovery, new drug development, Preformulation studies, formulation studies, validation of product, Analysis of compound, method development studies and bioanalytical research purpose. The official test methods that result from these processes are used by quality control laboratories to ensure the identity purity, potency and performance of drug products.

- ✓ **Bioanalysis**- Bioanalysis is a quantitative determination of xenobiotics (drug and metabolites) and biotics in biological fluids play a significant role in evaluation of pharmacokinetic (PK) and toxicological studies. It is the way of comparative studies between two or more formulations of the similar drug products.

- **Applications of Bioanalysis**
  - ✓ To monitor the concentration of drugs and metabolites in tissue, blood plasma, serum and urine specimens for a better understanding of the pharmacology and toxicology of drugs.
  - ✓ To apply therapeutic drug monitoring in the management of the drug treatment in patients.
  - ✓ To identify and/or quantify illicit drugs in biological samples for the detection of substance to abuse.

BIOANALYTICAL TECHNIQUES:

Generally used bioanalytical techniques involved are:

1) Hyphenated Techniques

a) LCMS (liquid chromatography mass spectroscopy)
b) GCMS (gas chromatography mass spectroscopy)
c) NLC-DAD (liquid chromatography diode array assay)
d) CEMS (capillary electrophoresis mass spectroscopy)

2) Chromatographic techniques

a) HPLC (High Performance Liquid Chromatography)
b) GC (Gas Chromatography)
c) UPLC (Ultra-Performance Liquid Chromatography)
d) Super critical fluid chromatography

3) Nuclear magnetic resonance

4) Electrophoresis

5) Mass spectroscopy

6) Ligand binding assays

a) ELISA (enzyme linked immune sorbent assay)
b) MIA (magnetic immune assay)
c) RIA (radio immune assay)
d) Dual polarization interferometry

7) Bioanalytical Method Validation

It is a process of documenting or providing evidence that an analytical method provides analytical data acceptable for intended use. Validation is of five types,

- Process validation
- Equipment validation
- Full validation
- Partial validation
- Cross validation.

NEED OF BIOANALYTICAL METHOD VALIDATION:

It is essential to employ well-characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactorily interpreted. It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements, and in many instances, they are at the cutting edge of the technology. It is also important to emphasize that each bioanalytical technique has its own characteristics, which will vary from analyte to analyte. In these instances, specific validation criteria may need to be developed for each analyte. moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study. When sample analysis for a given study is conducted at more than one site, it is necessary to validate the bioanalytical method(s) at each site and provide appropriate validation information for different sites to establish inter-laboratory reliability.

2.BIOANALYTICAL METHOD DEVELOPMENT

2.1. SAMPLE COLLECTION:

In bioanalytical method development we generally select four samples such as blood, urine, saliva, and rarely cerebro-spinal fluids.

Blood: Blood is used for analyzing pharmacokinetic profile of a drug. Blood is generally collected from veins and capillaries. Blood collected from veins is known as venous blood and blood collected from capillaries is known as capillary blood.

Venous blood is collected from arms, taken into tubes and anticoagulants like heparin and EDTA are added **(fig 1(a))**. If the blood is centrifuged then plasma is obtained. If this blood is centrifuged without adding anti-coagulant then serum is obtained.

Capillary blood is collected from fingertip by making a puncture with lancelet **(fig 1(b)).** This blood is collected into a capillary pipette of a fixed size and applied on a sampling paper. These papers are dried completely and stored in plastic bags to avoid moisture which may lead to bacterial and fungal growth.

- **Advantages of Capillary Blood:**

In rural areas refrigerators, centrifuges are not present for storage of blood. To overcome this problem capillary blood sample is applied on sampling paper and dried blood spots (DBS) are obtained **(fig 1(c)).**

- It requires minimal training.
- Less prone to infections risk from viruses like HIV, hepatitis.
- Less minimal.

1(a) 1(b) 1(C)

Fig 1: Sample Collection, 1(a)Venous Blood Collection
1(b)Capillary Blood Collection
1(c) Dried Blood Spots

Saliva: It is collected into salivette sampling device by spitting in a tube or by chewing on parafilm or gum to stimulate saliva production. A collection roll is chewed so that it absorbs saliva. Then the roll is placed in sampling device and stored in refrigerator until brought to laboratory for centrifugation.

Advantages of Using Saliva:

- Collection is non-invasive
- Stress free
- Very convenient
- Cost effective

c)Urine: It is selected as sample for determining renal elimination profile of compound.

2.2. SAMPLE PREPARATION

Sample preparation is done for removing interfering compounds. Cleanliness of the sample affects overall performance of the analysis. For example, if the sample is dirty it causes contamination of ion source resulting in variations of detector response. Sample preparation is also required for removing protein related material that may contaminate chromatography column and also for elimination of endogenous substance like phospholipids that are major cause of ion suppression or enhancement in LCMS. It is tedious and time consuming.

Sample preparation is majorly done by three methods, they are:

- Liquid -liquid extraction
- Solid phase extraction
- Protein precipitation

2.2.1 Liquid-Liquid Extraction

In liquid-liquid extraction the analyte present in liquid sample is extracted by addition of immiscible organic solvent. Methyl terbutyl ether, ethyl acetate, hexane, diethyl ether, dichloro methane are the most commonly used extraction solvents. It must contain two immiscible phases like aqueous phase and organic phase **(fig 2).** The required analytes are removed from matrix by using suitable buffer and extraction solvent.

The extraction solvent must be immiscible in water and should be compatible with detection method.

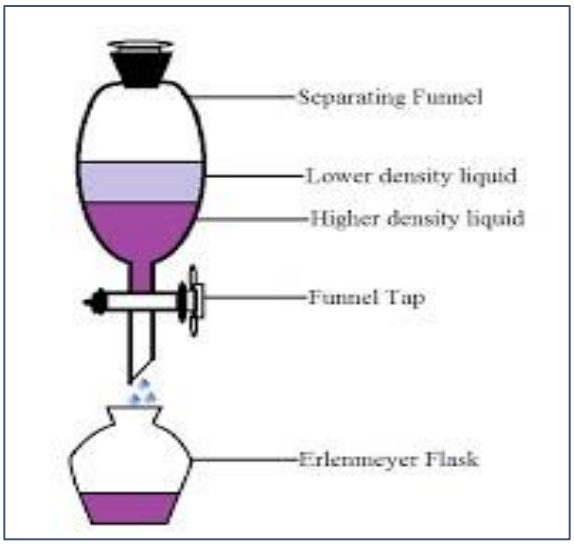


Fig 2: liquid-liquid extraction

2.2.2 Solid Phase Extraction (SPE)

In this method the substances or analytes are separated out according to their physical and chemical properties. The substances that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture. Analytical laboratories concentrate and purify sample for analysis by using solid phase extraction. This technique is mainly used to isolate analytes of interest from urine, blood, water, beverages, soil, animal tissue etc. solid phase extraction is more efficient process when compared to liquid liquid extraction. It is based on selective adsorption method **(fig 3)**. The targeted analyte is adsorbed on the solid phase and it can be selectively removed or eluted by using suitable elution solvent. The recovery of analyte is very high in solid phase extraction.

SPE involves following steps. They are:

- Conditioning
- Sample application
- Washing or rinsing
- Drying
- Elution

Conditioning:

All solid phase extraction tubes are conditioned with appropriate solvents before going to sample application.

- Conditioning is essential for:
  - Removing dust particles
  - Removing moisture and other contaminants
  - To avoid drying which keeps the sites in arising position
  - To activate the stationary phase bed sites.

Some of the examples of conditioning solvents are methyl alcohol, dichloro methane (DCM), methyl terbutyl ether (MTBE), any one organic solvent, water and buffer.

Sample Application:

Sample is applied from top of the cartridges at a slow flow rate so that no sample drop must remain on the inner wall of the cartridges slow rate is necessary to allow analyte to interact with adsorbent, and finally the retention of analyte occurs because of temporary weak bonding.

Rinsing or Washing:

Rinsing or washing is important for the removal of components and other interferences. Cartridges are washed with relative weak dilute solvent or buffer or solvent mixtures and interferences that are weakly retained than the analytes are drained away from the cartridge. Rinsing or washing is done by using water and buffers of different pH.

Drying:

It is done by applying appropriate vaccum for particular time period with help of vaccum pump.
Generally drying is done for about 2-3minutes. Drying also removes excess washing solvents buffers.
Drying also helps in avoiding blockage of cartridges due to air bubble formation during emulsion.

Elution:

It involves passing of strong solvent through cartridge at a slow rate and this finally increases soak time on the packing to reach maximum extraction efficiency. some of the examples of elution solvents are as following

- Methyl alcohol
- Acetonitrile (CAN)
- Small volumes of dichloro methane (DCM)
- Methyl terbutyl ether (MTBE)

It is used for breaking of weak bonds formed in between analyte and solvent. To improve elution multiple small volumes usage is recommended.

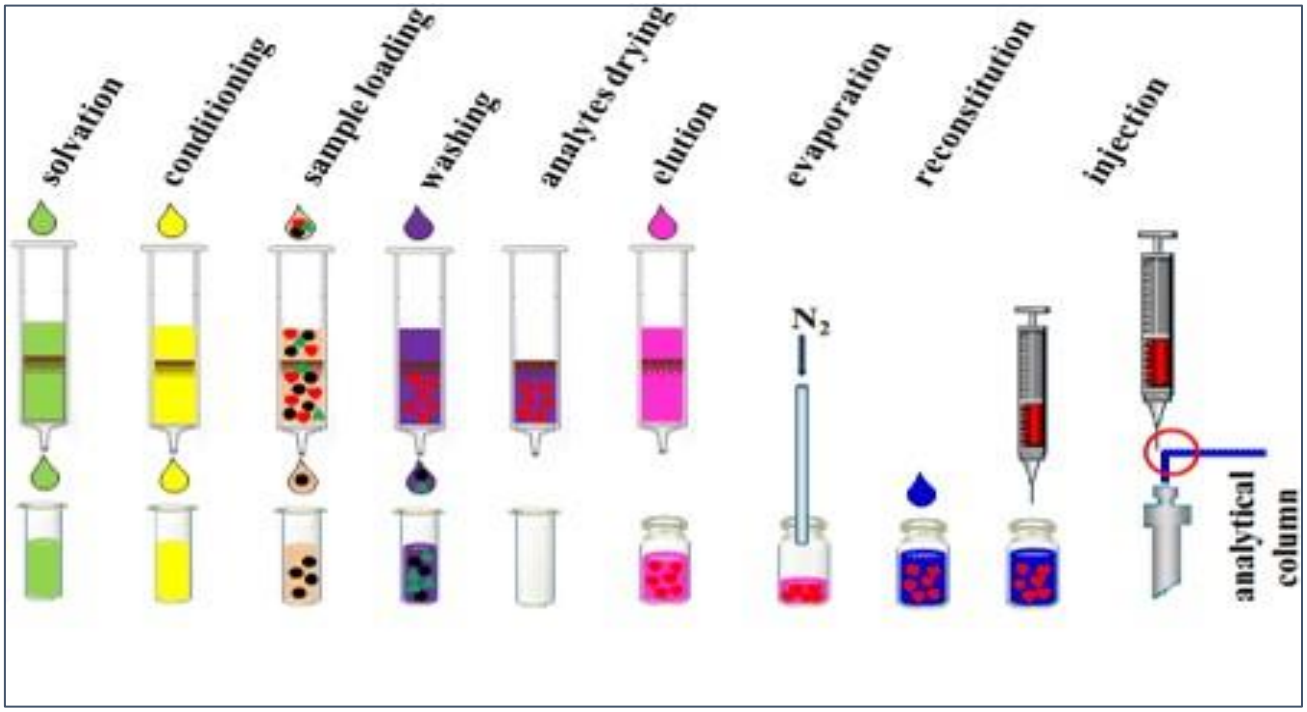


Fig 3 solid Phase Extraction

Types of Solid Phase Extraction

- Reversed phase solid phase extraction
- Normal phase solid phase extraction
- Ion exchange solid phase extraction

1) Reversed Phase Solid Phase Extraction

Polar and moderately polar sample is used as a mobile phase and non-polar stationary phase is used in reverse phase solid phase extraction. Several solid phase materials like acid or alkyl bonded silicas for reversed phase extraction of non-polar to moderately polar compounds such as antibiotics, barbiturates, benzodiazepines, caffeine, dyes, essential oils, fat soluble vitamins and phenol.

2) Normal Phase Solid Phase Extraction

It involves a polar analyte, mid to non-polar matrix and a polar stationary phase. Polar functionalized bonded silicas and polar absorption media typically are used under normal phase conditions. Retention of an analyte under normal phase conditions is due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. These interactions include hydrogen bonding, pi-pi interactions, dipole-dipole interactions, dipole-dipole induced interactions, etc. A compound adsorbed by these mechanisms is eluted by passing a solvent that disrupts the binding mechanisms usually a solvent that is more polar than the samples original matrix. The bonded silicas-LC-CN, LC-NH3 and LC-DIOL have short alkyl chains with polar functional groups bonded to the surface. These silicas, because of their polar functional groups, are much more hydrophilic relative to the bonded reversed phase silicas. As with typical normal phase silicas, this packing can be used to adsorb polar compounds from non-polar matrices. Such SPE tubes have been used to adsorb and selectively elute compounds of very similar structure (e.g. isomers), or complex mixtures or classes of compounds such as drugs and lipid.

3) Ion Exchange Solid Phase Extraction

Ion exchange solid phase extraction can be used for compounds that are charged when in a solution. Anionic compounds can be isolated on LC-SAX or LC-NH2 bonded silica cartridges. The primary retention mechanism of the compound is mainly based on the electrostatic attraction of the charged functional group on the compound to the charged group that is bonded to the silica surface. In order for a compound to retain by ion exchange from an aqueous solution, the pH of the sample matrix must be one at which both the compound of interest compound and the functional group on the bonded silica are charged. Also, there should be few, if any, other species of the same charge as the compound in the matrix that may interfere with the adsorption of the compound of interest. A solution having a pH that neutralizes either the compounds functional group or the functional group on the sorbent

surface is used to elute the compound of interest. When one of these functional groups is neutralized, the electrostatic force that binds the two together is disrupted and the compound is eluted. Alternatively, a solution that has a high ionic strength, or that contains an ionic species that displaces the adsorbed compound, is used to elute the compound.

Advantages of Solid Phase Extraction Over Liquid-Liquid Extraction:

- Convenient method
- Easy to use
- Greater recovery and accuracy
- More easily automated
- Easier collection of total analyte fraction.

2.2.3. PROTEIN PRECIPITATION

Protein precipitation is a very easy method of extraction when compared to the liquid-liquid extraction and solid phase extraction. Acetonitrile is most commonly used as a solvent for protein precipitation due to its complete precipitation of proteins occurs and methanol is the second choice of organic precipitant provided the solubility of the analyte in these solvents. After precipitation of protein the supernatant obtained is injected directly into the HPLC or it is evaporated and reconstituted with the mobile phase (**fig 4)**. After that sample is cleaned by using micro centrifuge at very high speed. Using the biological matrix with the least interference. Three aliquots are prepared at each concentration level, with the concentrations of 1/20 of Cmax, 1/30 of Cmax and 1/40 of Cmax of the analytes. The wash volume and washing pattern of auto injector needle are evaluated to avoid passing previous injections to next injections.

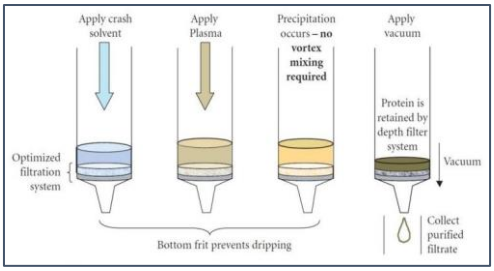


Fig 4: protein precipitation

3. NEW METHODS OR RECENT ADVANCEMENTS-

3.1 Liquid-liquid extraction
3.2 protein precipitation plates
3.3 solid phase extraction technique (SPE)

3.1 Liquid-Liquid Extraction

Salting Out Assisted Liquid- Liquid Extraction-

Water miscible solvent is added to water immiscible solvent. Salt is added to this mixture, and separation of solvent from mixture occurs. This separation is called as salt induced phase separation. salting out depends on type of salt added and physico-chemical properties of analyte **(fig 5)**

Most commonly salts are magnesium, sulphate, calcium sulphate, sodium chloride, calcium chloride, potassium carbonate, ammonium formate, ammonium sulphate, zinc sulphate etc. zinc sulphate is used for salting out lopinavir, ritonavir, ammonium formate is used for salting out simvastatin, simvastatin acid. The principle of salting is commonly used in environmental analysis of various trace elements.

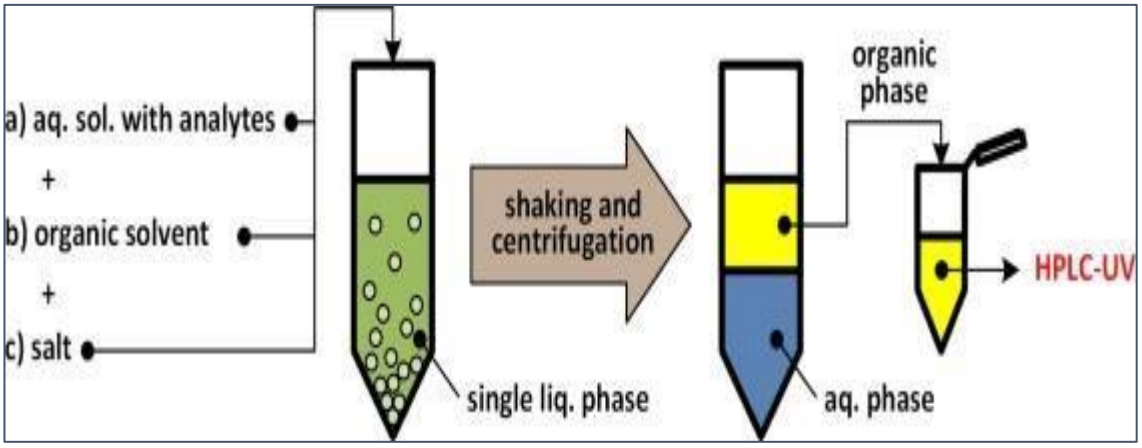


Fig 5: Salting Out Assisted Liquid-Liquid Extraction

3.1 liquid-liquid extraction:

- Single drop micro extraction

✓ Direct immersion single drop micro extraction
✓ Head space single drop micro extraction
✓ Liquid-liquid extraction
✓ Continuous flow micro extraction

- Hollow fiber liquid phase micro extraction
- Dispersive liquid-liquid micro extraction

3.1.1 Cloud-Point Extraction

The cloud point extraction method (CPE) was introduced for the first time by watanabe and co-workers in, 1976 which overcomes the disadvantages associated with the conventional extraction techniques. Recently, the cloud-point extraction (CPE) method has aroused much attention as a convenient alternative to the conventional extraction systems.

Principle-

Cloud point extraction (CPE) is based on the phase behaviour of non-ionic and zwitter ionic surfactants in aqueous solutions, which exhibit phase separation after an increase in temperature or the addition of a salting- out agent. The cloud point is the temperature above which aqueous solutions of non-ionic and zwitter ionic surfactants become turbid. Specifically, above the cloud point the solution is separated into two phases: a rich phase containing a high surfactant concentration in a small volume and a poor phase with a surfactant concentration close to the critical micelle concentration (CMC).

3.2 Protein Precipitation

3.2.1 Protein Precipitation Plates

The use of automated protein precipitation by filtration in the 96-well format as a rapid sample preparation technique for high throughout bioanalysis using liquid chromatography tandem mass spectrometry is reported. A robotic sample processor is used to aspirate sequentially a plasma sample and acetonitrile separated by air gaps. These are then mixed by being dispensed into individual channels of a 96-well filter block. The resulting supernatant is separated from the precipitated plasma proteins by the application of gentle vaccum using a custom manifold. The filtered supernatants are collected into a deep well microlitre plate, evaporated to dryness using a heated 96-well dry down station and reconstituted in water prior to analysis. The efficiency of the extraction procedure is measured by the lowry method for determining protein concentration. This method was used to optimise both the volume and the order of reagent addition, and to compare several prototype 96-well filter blocks. **(fig 6).**

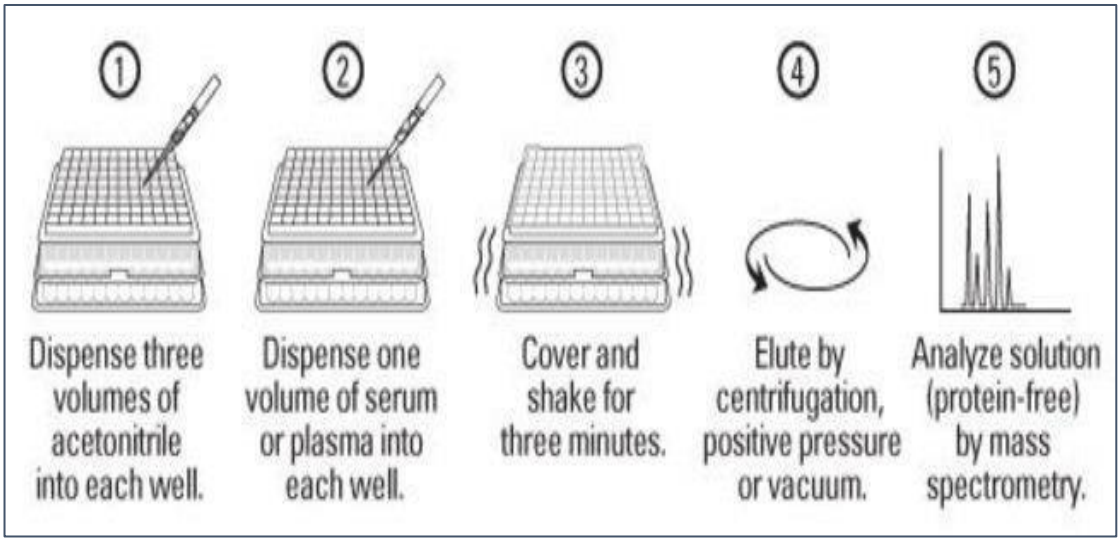


Fig 6: protein precipitation plates

3.3 SPE (Solid Phase Extraction) Technique:

In order to overcome limitation of liquid-liquid extraction in sample preparation SPE technique was developed. Principal involved in SPE is affinity based separation.SPE concentrates on retention and elution of analyte from biological sample. In SPE, interfering substances and contaminants are removed.

Types of Solid Phase Extraction:

Resent SPE techniques:

a) Molecular imprinted polymer SPE
b) Dispersive SPE
c) Disposable pipette extraction
d) Micro extraction by packed sorbent
e) Solid phase micro extraction
f) Stir bar sportive extraction

Other Methods

- On line solid phase extraction
- Monolith spin extraction
- Restricted access materials
- Turbulent flow chromatography
- Aptamers

3.3.1 Molecularly Imprinted Polymer SPE

This technique is based on molecular recognition. Principal in this is that a polymer network is created around a template.

Steps Involved:

Pre-polymerization complex is formed between the template molecule and functional monomers, polymerization of formed template monomer complex in presence of a cross linker occurs. Template molecule is extracted from polymer matrix. **(fig 7)**

the reason for higher selectivity of this method is because of interactions such as ion-exchange reversed phase with polymer backbone and hydrogen bonding between MIP stationary phase and analyte FGs.

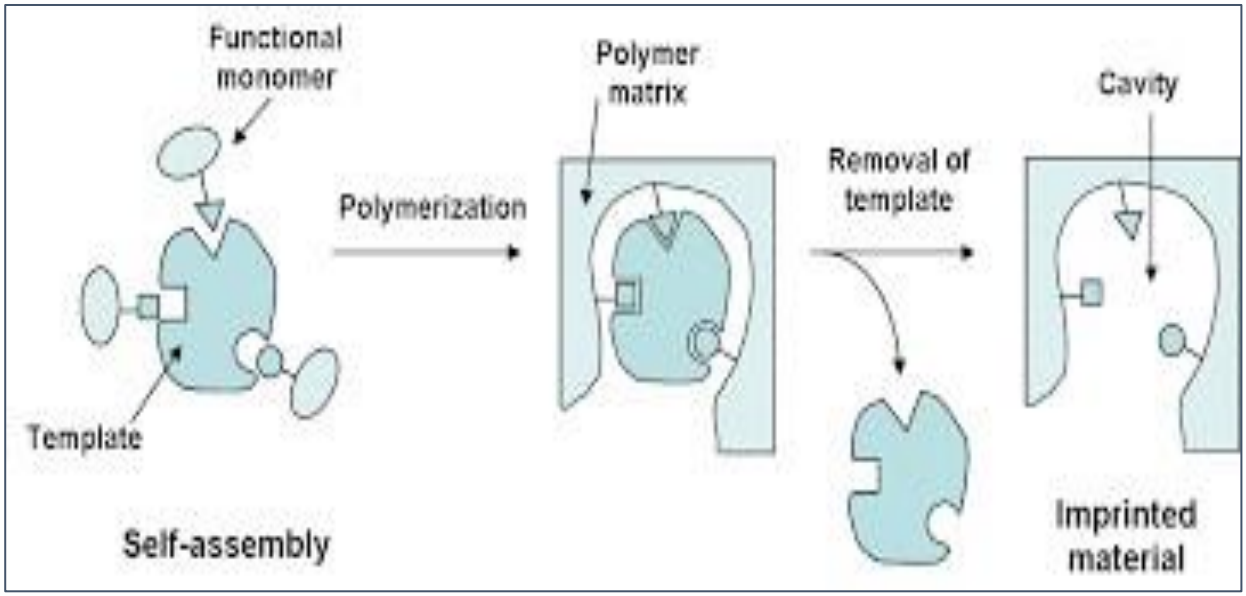


Fig 7: Moderately Imprinted Solid Phase Extraction

Applications:

- Environmental sample analysis
- Food and sample analysis
- Veterinary sample analysis

3.3.2 Dispersive SPE (Solid Phase Extraction)

Biomatrix sample is selected and organic solvent is added. To this add salt and centrifuge. Finally, the supernatant is separated **(fig 8).**

- It is quick, easy, cheap, effective, and safe method.
- It is a novel technique.

Advantages:

- ✓ Applicability to wide variety of analyte chemistries.
- ✓ Usage of solvent is less.
- ✓ Effectiveness of cost is high.
- ✓ Glassware and plastic ware use are also less.

It involves extraction of sample with organic solvent and partitioning with magnesium sulphate alone or in combination with salts like Nacl.

Addition of salt induces phase separation, which results in salting out effect and to influence the analyte partitioning behaviour.

Traditional Applications of DSPE:

- ✓ Food sample analysis.
- ✓ Pesticide sample analysis.
- ✓ Environmental sample analysis.
- ✓ Cosmetic additive sample analysis.

Recent Applications of DSPE:

- ✓ Determination of anti-helminthic drug residues in milk.
- ✓ Quantitative analysis of β-lactam antibiotics in kidney tissue.

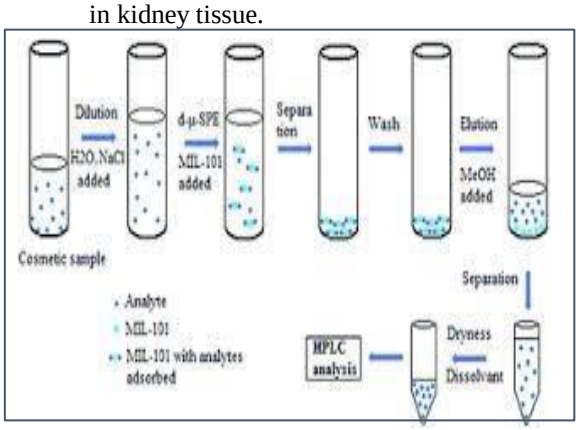


Fig 8: Dispersive Solid Phase Extraction

3.3.3 Disposable Pipette Extraction

DPX is an effective sample preparation technique for separation and extraction of analytes from the various matrices. It is modern pipette (1/5ml), which is loaded with a free-flowing sorbent powder, which is free to disperse. Being a standard tip, it is easy to take the solvent in or out through dispersive sorbent. In its modified form, the standard tip contains a dispersible sorbent loosely placed between two frits (one frit placed on lower end of the tip through which the solvent can be taken up/down and the second placed at the upper end of the tip to avoid solvent contamination into the pipette).

In case of conventional SPE cartridges which contain packed bed sorbent, sample is loaded from the top; every sorbent particle is used once (as sample through the bed under gravity), thus much sorbent materials required to retain analytes. Also, the success of conventional SPE depends on flow control of the sample loading, washing and elution to achieve good repeatabilities. This leads to fast and efficient extraction and not much material needed to retain analytes. Although contact time (in tip mixing/ aspiration) control is important to achieve good repeat abilities **(fig 9).**

The steps involved in DPX are similar to those of traditional SPE with some differences in a typical DPS extraction, first, the sorbent in tip is conditioned with appropriate solvent to activate the sorbent sites. After conditioning the sample is withdrawn/ aspirated through the tip and mixed by withdrawing air via pipette. These steps need optimization as the analyte of interest should spend sufficient time (equilibration time) with sorbent material for effective extraction. The sample is flushed out of the tip is followed by a wash step. The washing can be optimized based on chemical/ nature and type of sorbent, the nature of the analyte and probable matrix interferences. The washing solvent can be withdrawn, aspirated with air and pipette out. Finally, elution solvent is to be withdrawn through the tip and aspirated several times with air so as to ensure complete desorption of analyte from sorbent to solvent. The solvent with extracted analyte can be simply pipetted out and directly injected or evaporated/ reconstituted for sensitivity purposes. All operations involved in DPX extraction are available for both manual and automated platforms.

Similar to traditional SPE cartridges, the DPX tips (capacity 1/5 ml) are available with a variety of sorbents. Commercial DPX tips are available with sorbents such as reverse-phase (DPXRP, polystyrene divinylbenzene copolymer) for non-polar to medium polar compounds, strong cation exchanger (DPX-CX) for cations and non-polar compounds.

And weak anion exchanger (DPX-WAX) for anions and non-polar compounds, DPX tips are also available with sorbents such as graphitized and carbons.

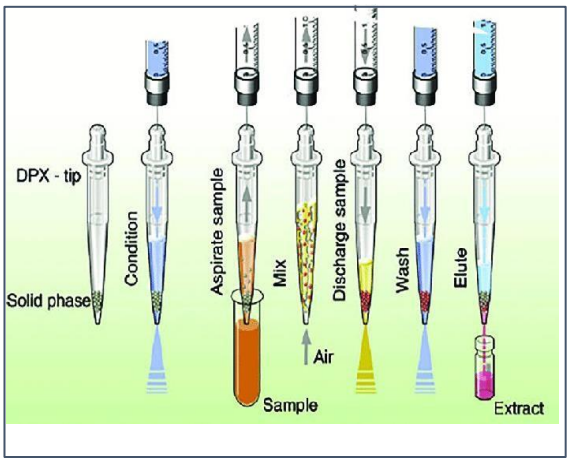


Fig 9: Disposable Pipette Extraction

3.3.4. Micro-Extraction by Packet Sorbent

Miniaturization is trend of today's science. Micro-extraction by paced sorbent (MEPS) is a miniature SPE. The purpose of MEPS is to reduce the sorbent bed volume, making it suitable for large sample volume range (from as low as 10-100ml), reducing the number of steps typically involved in conventional SPE and providing easy automation. Typical MEPS is designed in the syringe format where in approximately 1mg of sorbent is packed inside a syringe(1oo-250ml) as a plug or between the barrel and needle as a cartridge **(fig 10).** Similar other sample preparation techniques such as MSPE/DSPE/DPX, novel sorbent materials such as silica-based material (strong cation exchanger, SCX). MEPS protocol involves pre-treatment of complex sample such as dilution and centrifugation. Dilution with water followed by centrifugation is recommended for plasma/serum and whole blood samples in the ratios of 1:4 and 1:20, respectively, once the sample is ready, it can be directly drawn by MEPS syringe. As the sample travels through the MEPS cartridge, then gets bound to the sorbent. The sample can be drawn and ejected several times from the same vial if pre-concentration of the sample is required to improve the sensitivity of the method. The next step involves washing of sorbent bed (typically only once with a solvent such as water, 50ml). the final step is elution of analytes with suitable solvent. The elution step is elution of analytes with suitable solvent. The elution step uses 20-50ml of organic solvent. The elution step can be directly carried out into the instrument's injector (GC or LC). One of the most significant advantages of the MEPS is that the same syringe (sorbent bed) can be reused many times just by washing with water (three or four cycles) and for four or five cycles with appropriate solvent (e.g. elution solvent). This is also said to reduce carry-over effects. This is also said to reduce carry-over effects. This is in complete contrast to the use of conventional SPE cartridges.

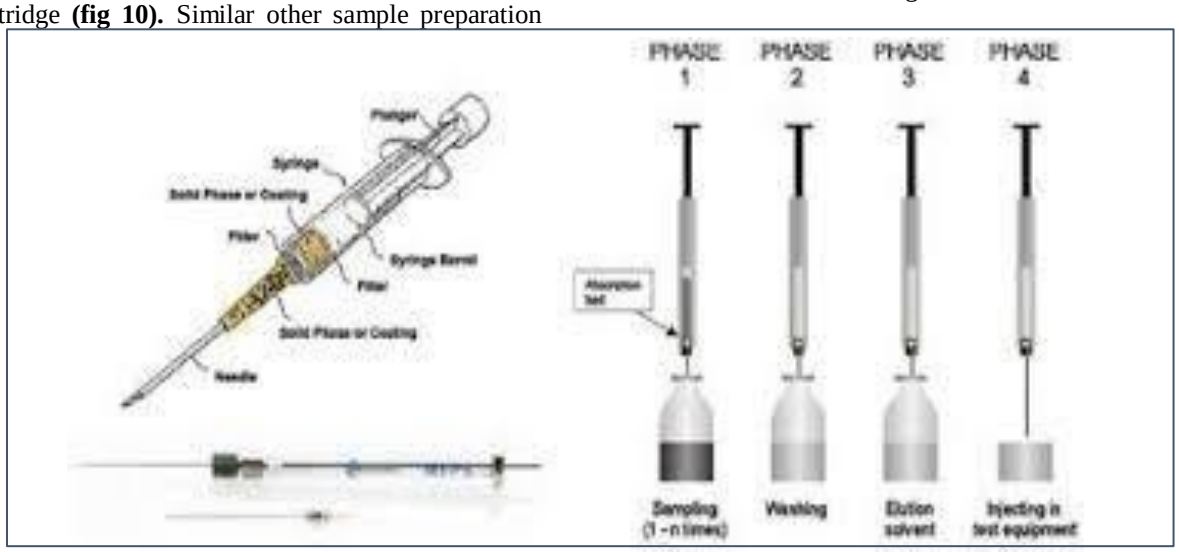


Fig 10: Micro Extraction by Packed Sorbent

3.3.5. Solid-Phase Micro-Extraction (SPME):

A new sample preparation technique using a fused-silica fiber coated on the outside with an appropriate stationary phase and is termed as solid phase micro-extraction (SPME). Physically, it is a modified syringe that contains stainless steel micro tubing within its syringe needle. This micro tubing has about a 1cm fused silica fiber tip which is coated with an organic polymer. This coated silica fiber can be moved backwards and forwards with the plunger of the syringe **(fig 11).**

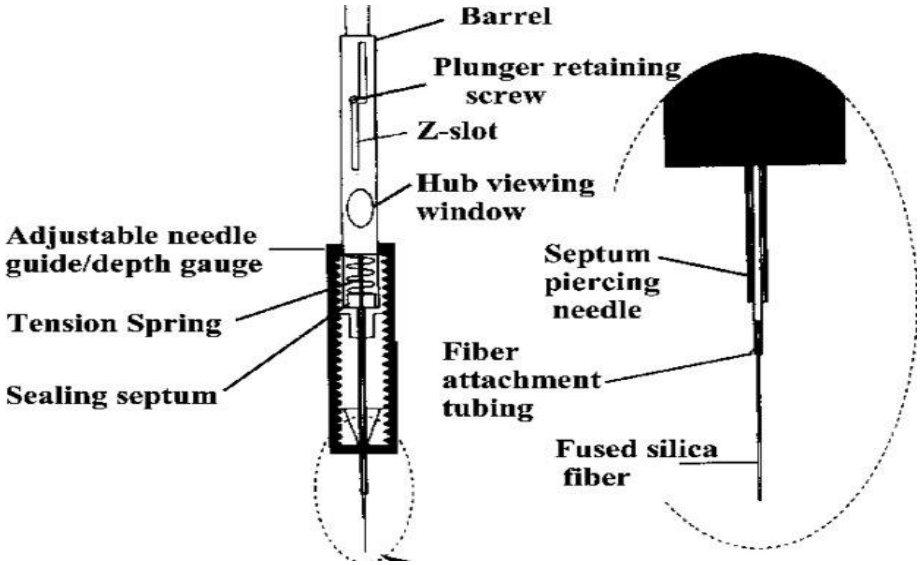
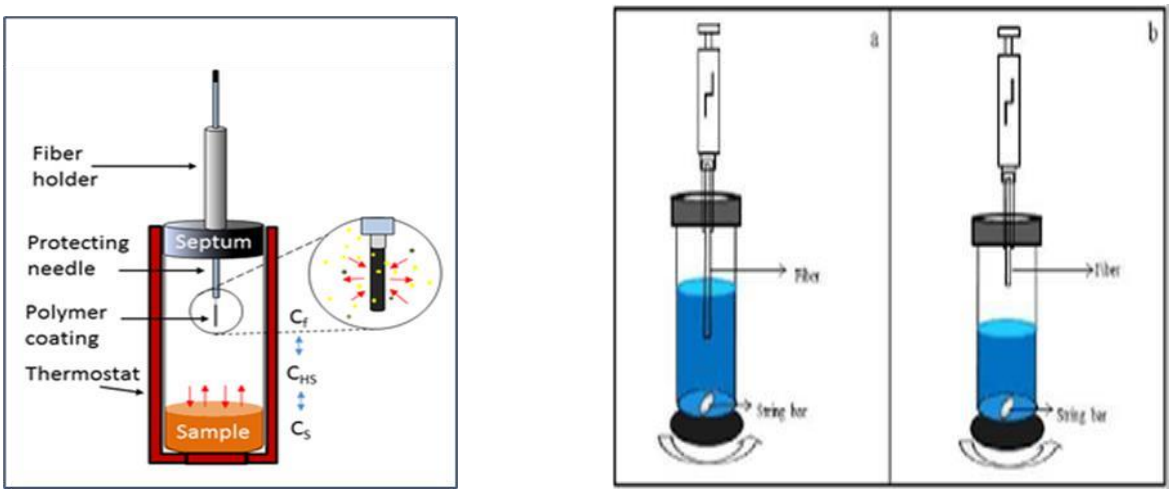


Fig 11: Basic Construction of SPME

In contrast to conventional SPE with packed-bed cartridges, SPME syringe assembly design allows combination of all steps of sample preparation into one step, thus reducing sample preparation time, use of organic solvents and disposal cost. The foremost advantage of the technique is improved detection limits. There are two types of extraction modes for SPME: first, direct immersion (DI) of SPME fiber into liquid sample matrix, simply termed as DI-SPME and second, head-space (HS) extraction in which the liquid sample matrix is heated in a vial to volatilize the analytes and the fiber is placed just above the sample matrix. This process is usually referred to as HS-SPME. Success of SPME is determined by physicochemical properties and the thickness of the fiber coating. Various commercially available fiber coatings are polydimethylsiloxane (PDMS) for extraction of non-polar analytes, polyacrylate (PA, thickness 85mm) for extraction of polar analytes (escipillay phenols), polydimethylsiloxane divinylbenzene (PDMS-DVB thickness of 65 and 60 mm) for extraction of polar analytes (especially amines).



(a) **(b)**

Fig 11': Solid-Phase Micro Extraction

a) Headspace Solid-Phase Micro Extraction
b) Direct Immersion Phase

3.3.6. Stir Bar Sportive Extraction:

Based on the similar principal of SPME, stir bar sportive extraction (SBSE) was introduced as a novel sample preparation technique in the year of 1999, SBSE differs from SPME in such a way that, instead of fibre, the extraction phase (such as PDMS) is coated

onto the magnetic stir bars. The typical stir bar will have magnetic rod with a glass jacket. The polymer is coated onto this glass jacket with a specific thickness. When this stir bar is added to the prepared sample, analytes will partition between the sample matrix and the extraction phase. in SPME, the maximum volume of PDMS coated onto the fiber is 0.5ml (film thickness 100mm); however, in SBSE the amount is 50-250 times larger. Higher amounts of extraction phase in SBSE presumably afford greater extraction efficiency over SPM. Like SPME, SBSE has two modes for extraction, i.e., PDMS, SBSE is mainly used for extraction of hydrophobic analytes SBSE is a highly cost-effective sample preparation technique.

Other Methods:

On-Line Solid Phase Extraction (OLSPE)

On-line solid phase extraction (OLSPE) methods are easier and faster prior to transfer to an analytical column. Two types of online SPE columns are commercially available. The restricted access material [RAM] column and turbulent flow chromatography (TFC) column. RAM'S are used mainly for the analysis of substances with low molecular mass (e.g. drugs, endogenous substances and xenobiotics) in complex matrices containing high molecular substances (most frequently proteins). RAM HPCL, columns eliminate the sample clean-up and can be used as a pre-column for the direct injection of biological samples such as serum and plasma. RAM columns are characterized by hydrophilic/hydrophobic, ion-exchange or size exclusion mechanisms. Similarly, RAM columns have also been used in multi component analysis of antidepressant drugs in biological fluids.

4. BIOANALYTICAL METHOD VALIDATION:

Bioanalytical method validation includes all the procedure that demonstrates that a particular method used for quantitative measurement of analyte in given biological matrix is reliable and reproducible for intended use.

4.1 Types of Validation

There are three types of validation.

- full validation,
- partial validation,
- cross validation.

4.1.1 Full Validation:

Full validation is performed when developing and implementing a bioanalytical method for the first time. Full validation is revised assay and it is done for a new drug molecule. Full validation is important if metabolites are added to an existing assay for quantification.

4.1.2 Partial Validation:

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

Bio analytical method transfers between analysts or laboratories.

- Change in analytical methodology for e.g., change in detection system.
- Change in anticoagulant used for harvesting biological fluid.
- Change in biological sample within species for e.g., human plasma to human urine.
- Change in sample processing procedures.
- Change in species within matrix for e.g., rat plasma to mouse plasma.
- Change in relevant concentration range.
- Changes in instruments and/or software platforms.
- Limited sample volume for e.g., paediatric study.
- Rare matrices
- Selectivity for demonstration of an analyte in the presence of contaminant medications.
- Selectivity demonstration of an analyte in the presence of specific metabolites.
-

4.1.3 Cross Validation:

Cross-validation is a comparison of validation parameters when two or more bio analytical methods are used to generate data within the same study or across different studies. An example of cross-validation would be a situation where an original validated bio analytical method serves as the reference and the revised bio analytical method is the comparator. The comparisons should be done both ways. When sample analysis within a single study are conducted at more than one site or more than one laboratory, cross validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter laboratory reliability.

4.2 Validation Parameters:

They are many parameters in validation of bio-analytical method, some of the Parameters are as following:

1. Accuracy:

Accuracy is defined as closeness of a measured value to a standard or known value. **(fig.12)**

Fig 12: Accuracy

$$\text{Accuracy} = \frac{\text{Measured value - True value}}{\text{True value}} \times 100$$

Acceptance Criteria

The mean recovery will be within 90 to 100% of the theoretical value for non-regulated products. For the U.S, pharmaceutical industry, 100 ± 2% is typical for an assay of an active ingredients in a drug product over a range of 80 to 120% of the target concentration. Lower percent recoveries may be acceptable based on the needs of the methods. HC states that the required accuracy is a bias of ≤ 2% for dosage forms and ≤ 1 % for drug substance.

2. Precision:

Precision is defined as the closeness of agreement [degree of scatter] between a series of measurements obtained from multiple samplings of the same homogenous sample.

Acceptance Criteria

The FDA states that the typical RSD should be 1% for drug substances and drug products, ±2% for bulk drugs and finished products. HC states that the RSD should be 1% for drug substances and 2% for drug products. For minor components, it should be ± 5% but may reach 10% at the limit of quantitation.

$$\text{Precision} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

Considered at 3 levels:

a) Repeatability

b) Intermediate precision

c) Reproducibility

Repeatability:

Express the precision under the same operating conditions over a short interval of time. It is also called as intra-assay precision. It requires minimum 9 determinations [3 concentrations at 3 replicates] or minimum 6 determinations [3 concentrations at 2 replicates].

Intermediate Precision:

It is expressed with in laboratory variations, intermediate precision is expressed in terms of standard deviation and relative standard deviation. It depends on circumstances under which the procedure is intended to be used. Studies should include varying days, analysts, equipment etc.

Acceptance Criteria:

The assay results obtained by two operations using two instruments on different days should have a statistical RSD ≤ 2% .

Reproducibility:

It is the ability to reproduce data within predetermined precision **(fig 13)**. It is determined in terms of standard deviation, relative standard deviation, confidence levels.

Fig 13: Precision

3. Ruggedness:

Ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of same sample under a variety of conditions, such as:

- Different laboratories
- Different analysis
- Different instruments
- Different reagents
- Different elapsed assay timer
- Different assay temperatures
- Different days etc

4. **Sensitivity:**
Sensitivity is defined as the lowest analyte concentration that can be measured with acceptable accuracy and precision.

5. **Robustness:**
Robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of its reliability during normal usage.

6. **Specificity:**
The specificity is defined as the ability of an analytical method to measure the analyte free from interferences due to other components.

7. **Linearity:**
It is the ability of an assay to elucidate a direct and proportional response to change in the analyte concentration.
- Linear regression (y=mx+b)
Where b = y-intercept, m=slope
- Acceptance criteria = linear regression r^2>0.95
• It requires a minimum of 5 concentration level.

Acceptance criteria:
The correlation coefficient for six concentrations levels will be ≥0.999 for the range of 80-120% of the target concentration **(fig 14).** The y-intercept must ≤ 2% of the target concentration response. A plot of response factor versus concentration must show all values within 2.5% of the target level response factor, for concentrations between 80 and 120% of the target concentration. 10 HC states that the coefficient of determination for active ingredients should be ≥ 0.997, for impurities 0.98 and for biologics 0.95

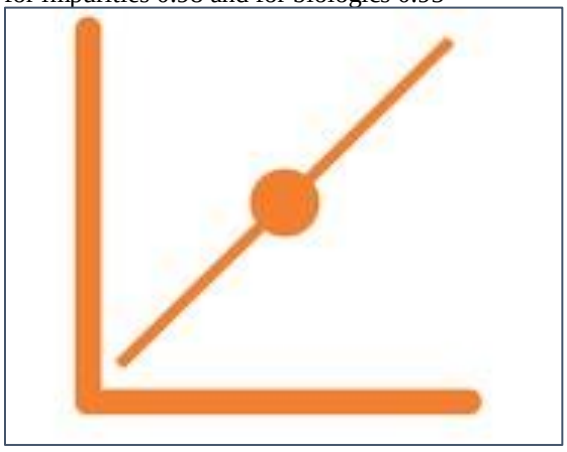


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Fig 14: linearity

6. Limit of Detection (LOD):
The limit of detection of an analytical method is the lowest amount of analyte in a sample that can be detected but not necessarily quantified under stated experimental conditions.

The limit of detection is usually expressed as a percentage, parts per million, or parts per billion.

7. Range:
The range of an analytical procedure is the interval between the upper and lower concentration (amount) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

8. 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. 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 other systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the 7 intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution. All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

$$\textbf{Percentage stability} = \frac{\textbf{mean response of stability samples}}{\textbf{mean response of comparison samples}}$$

5. CONCLUSION:
Bioanalysis and the production of pharmacokinetic, toxicokinetic, and metabolic data plays a fundamental role in pharmaceutical research, development involved in the drug discovery and development process. An attempt has been made to understand and explain the bioanalytical method development and validation from a quality assurance department point view. Some of the method and how is validation carried out were

described in different situations encountered in the study sample analysis has been reported in this article. These various essential development and validation characteristics for bioanalytical methodology has been discussed with a view to improving the standard and acceptance in this area of research.

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