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A REVIEW: BIOANALYTICAL EXTRACTION TECHNIQUES AND ADVANCES IN LIQUID-LIQUID EXTRACTION TECHNIQUE.

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ABSTRACT
This review focus on sample collection (blood, plasma, urine, serum, saliva), sample preparations, pre-treatment of sample for better recoveries and physicochemical properties to be considered in selection of sample for bioanalysis. In sample preparations brief on traditional methods as Solid phase extraction, Protein precipitation technique and Liquid-Liquid extraction and detailed on advanced liquid-liquid extraction techniques i.e. salt assisted Liquid-Liquid extraction technique (SALLE). Nowadays SALLE is a growth of interest in bioanalysis because of its fast, reliable and simple experimental execution at laboratory level. Recent developments on miniaturization, micro extraction and automation are future perspective for bioanalytical application in biological sampling.

KEYWORDS: Extraction techniques, Liquid-Liquid extraction, SALLE, micro extraction, miniaturization, automization.

INTRODUCTION

Analysis of drugs/metabolites/biomarkers (qualitative/quantitative) in biological matrices such as plasma, whole blood, saliva, tissues, urine, serum, cerebrospinal fluid, etc., is termed as bioanalysis. Bioanalysis is a sub discipline of analytical chemistry covering the quantitative measurement of xenobiotic (drugs, their metabolites and biomolecules in unnatural concentrations) and biotic (macromolecules, DNA, larger molecule drug, metabolites, proteins) in biological systems.[1] Bioanalysis is an essential part of overall drug development process starting with in vivo/in situ tests, preclinical studies to clinical studies.[3]

The main aim of bioanalysis in the pharmaceutical industry is to provide quantitative measurement of active drugs and/or its metabolites for the purpose of pharmacokinetic, toxicokinetic, bioequivalence, bioavailability and exposure response i.e. pharmacokinetics/pharmacodynamics studies.[1]

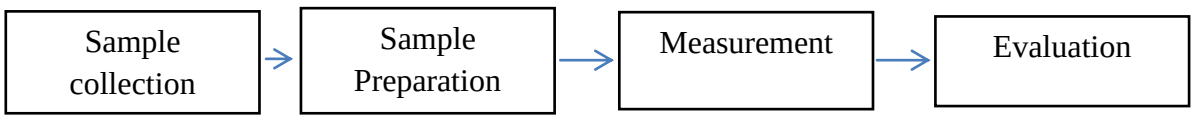
Bioanalytical method development is generally employed to demonstrate that an analytical method used for quantification of analytes in biological matrix is reliable and reproducible to achieve its purpose. Both HPLC and LC-MS/MS are utilized for bioanalysis of drugs in plasma.[2]

AIM AND OBJECTIVE

The strategies of bioanalytical studies is to understand how to prepare, collect, separate and detect sample by different method for analysis of drugs and their metabolites from biological fluids.[1]

1. To provide information about various sample collection and preparation techniques.
2. Advanced LLE technique.

BIOANALYTICAL TOOL



SAMPLE COLLECTION[1]

The first step in the analytical chain is to decide which matrix to utilize. Frequently venous blood withdrawn from arm/capillary blood, blood withdrawn from fingertip or urine is utilized. Rarely, saliva and cerebrospinal fluid are utilized. Blood is collected in tubes containing anticoagulant e.g., EDTA or heparin. Plasma can be obtained when the tubes are centrifuged.

If blood without anticoagulant is centrifuged, serum is obtained. Saliva is sampled either by spitting in saliva tubes or by chewing on parafilm or gum (stimulate salivary production) thereafter spitting in the saliva collecting tubes.

Different biological samples are used such as.

- Plasma/serum- to determine the pharmacokinetic profile of analyte and therefore, drug clearance, half-life, and bioavailability can be analyzed.
- Urine- to determine the renal elimination profile of the compound.

PHYSICOCHEMICAL PROPERTIES[2]

1. **Water miscibility and water immiscibility:** drugs which consists several aromatic rings will be poorly soluble in strong intermolecular dispersive forces of solid drug will encourage the ready solubility in organic solvents.
2. **Molecular phenomena for solubility and miscibility:** to dissolve a drug, a solvent must break the bonds like ionic bond, hydrogen bond, Van der Waals forces which inter links the compound to its neighbour and must not break substantial intermolecular bonds of the solvent without replacing them with the drug solvent interaction.
3. **Distribution coefficient:** drugs which are in ionized form are hydrophilic in nature than the unionized form because of the hydration of the ions, therefore the ionized form are difficult to extract into organic solvents whereas the unionized forms will dissolve in the organic solvents which can be extracted into organic solvents.
4. **Plasma proteins:** the proteins can be precipitated by addition of 10-20% Trichloroacetic acid or a water miscible solvent like acetonitrile.
5. **Choice of solvent:** several factors are to be considered while choosing a solvent to extract a drug from the matrix in addition to its powder to dissolve the required compounds which includes selectivity, density, toxicity, volatility, reactivity, physical hazards and miscibility with aqueous media.
6. **Mixed solvents:** alcohols are excellent solvent but those with lower boiling points are too soluble in water whereas less miscible one are having high boiling points, but the use of mixed solvents containing alcohols can overcome the problem.
7. **Role of pH for solvent extraction:** extraction of bases into an organic solvent should be carried out at high pH usually about pH 2 above the pKa and extraction of acids should be carried out at low pH.

PRE-TREATMENT OF SAMPLE[2]

1. **Serum, plasma and whole blood**

To disrupt protein binding in these biological fluids following methods are used

- Precipitate the proteins using a polar solvent (two parts solvent per one part biological fluid) mix and centrifuge, remove the supernatant and dilute with water or an aqueous buffer.
- Sonicate the biological fluid for 15 minutes, add water or buffer, centrifuge, remove the supernatant and dilute with water or an aqueous buffer.
- Shift pH of the sample to extremes with acids or bases, use the resultant supernatant.
- To precipitate proteins, treat the biological fluid with acids or inorganic salts. The pH of the resultant supernatant may be adjusted prior to use.

2. **Urine:** urine is exposed to heat for 15-20 minutes and then cooled and diluted with a buffer, adjust pH approximately.

SAMPLE PREPARATION

Sample preparation also known as sample treatment; sample clean-up; sample extraction[1]. Sample preparation is a process which targets at selective isolation of analyte of interest from the matrix, minimization/elimination of matrix components in the processed sample. Sample preparation is a process which aims at selective isolation of analyte of interest from matrix, minimization/elimination of matrix components in processed samples and, if required concentration of analyte of interest.[3] Conventionally, Liquid-Liquid extraction (LLE), Protein precipitation (PPT) and Solid phase extraction (SPE) techniques have been used as sample preparation techniques.[3]

a.Solid phase extraction

Principle: SPE is based selective adsorption technique. It is based on adsorption or partitioning on to a solid sorbent (adsorbent). Selective retardation of analyte using a solid sorbent under specific condition. Target analyte is adsorbent on solid surface and can be selectively removed or eluted using an appropriate eluting solvent. Various types of cartridges are used (HLB, MCX, MAX etc.).[1]

Steps involved in SPE[1, 2]

Step 1. Conditioning: conditioning is generally done prior to sample application. Appropriate solvent is passed through the SPE bed (avoid drying, remove dust and activate the bed) to condition the bonded functional groups (Methanol, water, buffers).

Step 2. Equilibration: Sorbent is treated with a solution that is similar polarity, pH etc. to the sample matrix to maximize the retention. Use the same aqueous solution that the sample is prepared in)

Step 3. Load: Allow the sample into the cartridge from top at a slow flow rate (interaction) so analyte of interest are extracted onto the sorbent.

Step 4. Washing: Use the strongest aqueous solution that will not elute the target compounds and remove the matrix and other interfering substances. Washing solutions are water, buffers at different pH.

Step 5. Elution: Organic solvent are used to remove all the target analyte. Polar target compounds elute best in polar solvents so in order of polarity:

methanol>acetonitrile>ethyl acetate>acetone>THF.
Modifying the pH, increase the ionic strength.

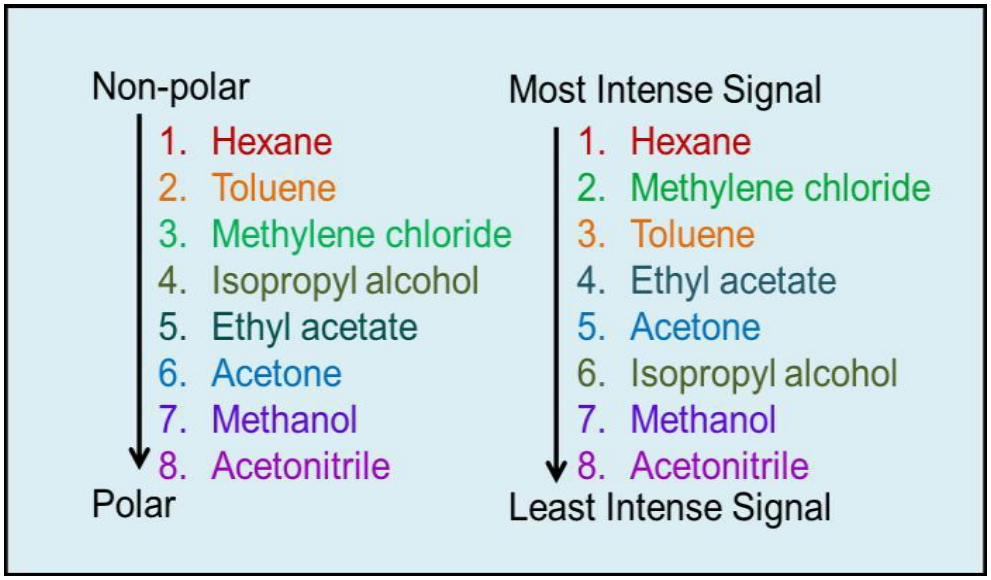


Figure No.1: Solvent Selection.

Merits

- High reproducibility
- Very Selective
- Concentration effect
- High recoveries
- Effective with variety of matrix

Demerits

- Multiple steps: Greater complexity
- Lengthy method development
- Costly[2]

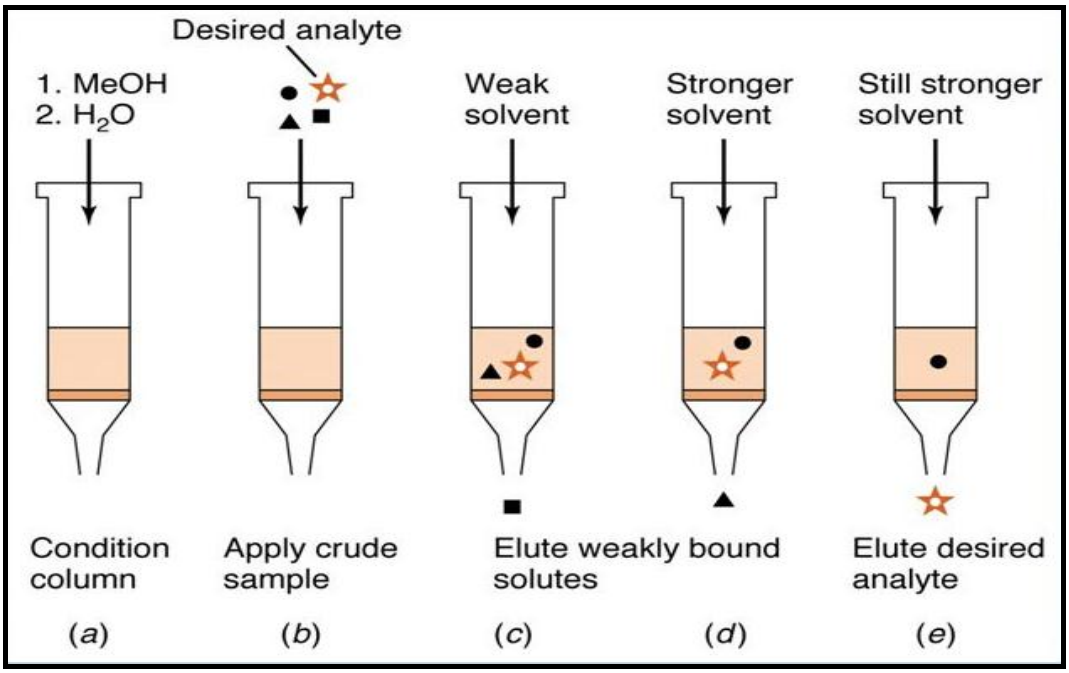


Figure No.2: Solid Phase Extraction technique

b. Protein Precipitation Technique

Principle: It basically is denaturing the proteins. It depends on the solubility of analyte in particular solvent present in biological matrix i.e., blood, plasma, serum.
Solvent used: Methanol, Acetonitrile etc.[1]

Protein precipitation can be achieved by one of following methods

By changing the pH of sample- by mixing inorganic reagents e.g. per chloric acid, Trichloroacetic acid etc. In

iso-electric pH, proteins have no net charge, which causes insolubility thus proteins precipitates.

By addition of Salts: salts used for precipitates of proteins are citrates, phosphates, acetates, etc.

By addition of organic solvents: it decreases the dielectric constant of the medium, leads to insolubility thus causes precipitates in another case high affinity for the hydrophobic surfaces of the proteins leads to denaturing of proteins e.g. Methanol, Acetonitrile[1].

Merits

- Removal of unwanted plasma proteins from plasma samples prior to analytes.
- PPT can be used in wide range of aqueous and organic sample preparation.

Demerits

- Increase the back pressure of HPLC system.
- Some components of plasma which are soluble in diluting solvents that bound to stationery phase permanently and will affect the column performance.[2]

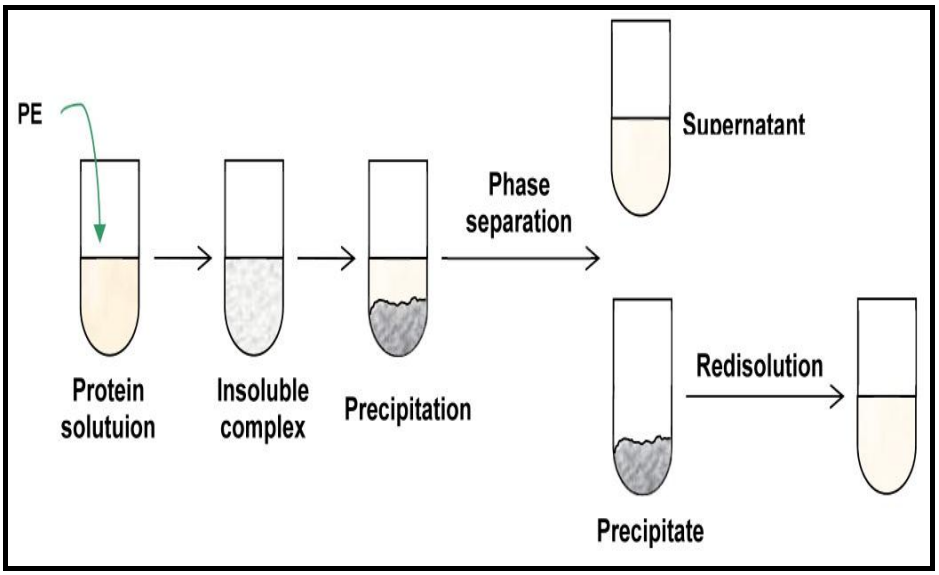


Figure No.3: Protein Precipitation Technique.

c. Liquid-Liquid Extraction

Principle: it is based on selective extraction of intended analyte present in a liquid sample through immiscible organic solvent. LLE is based on differential solubility and partitioning of two immiscible liquid phases i.e. aqueous phase and organic phase. Solvent used- Tertiary butyl methyl ether (TBME), methanol, n-hexane, diethyl ether (DEE), Dichloro methane (DCM), ethyl acetate (EA).

LLE is a common sample preparation choice in regulated bioanalysis. The concept like dissolves like works well in LLE. The ability to separate compounds in a mixture using the technique of LLE depends upon how differently the compounds of the sample mixture partition themselves between the two immiscible solvents. In this technique sample is distributed in two phases in which one phase is immiscible to other. LLE separates analytes from interferences by partitioning the sample between two immiscible liquids or phases.

Procedure: First, the component mixture is dissolved in a suitable solvent and second solvent that is immiscible with the first solvent added. Mix the content thoroughly and further the immiscible solvents are allowed to separate in to layers. The less dense solvent will be the upper layer while the more dense solvent will be the lower layer.

The components of mixture will be distributed amongst the two immiscible solvents as determined by partition coefficient. The relative solubility that a compound has in two given solvents can provide an estimation of the extent to which compound will be partitioned between them. A compound that is more soluble in the less dense solvent will be preferentially reside in the upper layer and more dense solvent will reside in the lower layer. Lastly, the two immiscible layers are separated, transferred and the component in that is isolated. The residue is reconstituted with small volume of an appropriate solvent preferably mobile phase while analyte extracted into the aqueous phase can be directly injected into a column. Several equations can illustrate the extraction process. The Nernst distribution law states that any neutral species will distribute between two immiscible solvents so that the ratio of concentration remains constant,

KD = Co/Caq

Where KD is the distribution constant,

Co is the concentration of the analyte in the organic phase and

Caq is the concentration of the analyte in the aqueous phase.[1]

Merits

- LLE can generate high analyte recoveries, clean extracts
- Preferably low cost
- Short method development time
- Inorganic salts easily removed

Demerits

- Large volume of organic solvent required
- Labour intensive
- Difficult to automate[2]

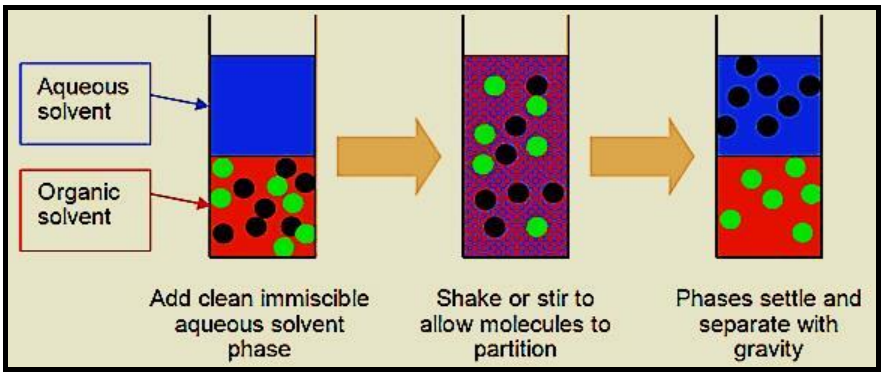


Figure No.4: Liquid-Liquid Extraction Technique.

DEVELOPMENT IN LIQUID-LIQUID EXTRACTION TECHNIQUE

SALTING-OUT ASSISTED LIQUID-LIQUID EXTRACTION TECHNIQUE (SALLE)[3,4]

Traditionally LLE is one of the oldest sample preparation technique applied in bioanalytical technique. Though it has several limitations. Moreover, conventional LLE limits the extraction of polar compounds from aqueous media. As in organic polar solvents such as acetone, ethanol, or acetonitrile, the solubility of compounds is higher but are water miscible solvents cannot be applied in conventional LLE technique. In such situations, an alternate SALLE is used for extracting of high polarity, low mass which are not efficiently applied in LLE. SALLE is a homogeneous LLE technique.

Procedure: the SALLE consists of adding a water miscible organic solvent to aqueous sample, proceeded by addition of salt/mixture of salt for phase separation between water and organic solvents, that is due the resultant salting out effect. The addition of salt reduces the miscibility of water and selected water miscible organic solvents under suitable controlled condition can induce phase separation. The effect is holding attention for sample preparation since simultaneous extraction of compounds into the organic solvent can take place, allowing analysis to be performed over organic phase. Though salting out has been widely utilized in LLE, which make use of water immiscible organic solvents to enhance extraction recoveries, the term SALLE terms to homogenous extractions with water miscible organic solvents.[4]

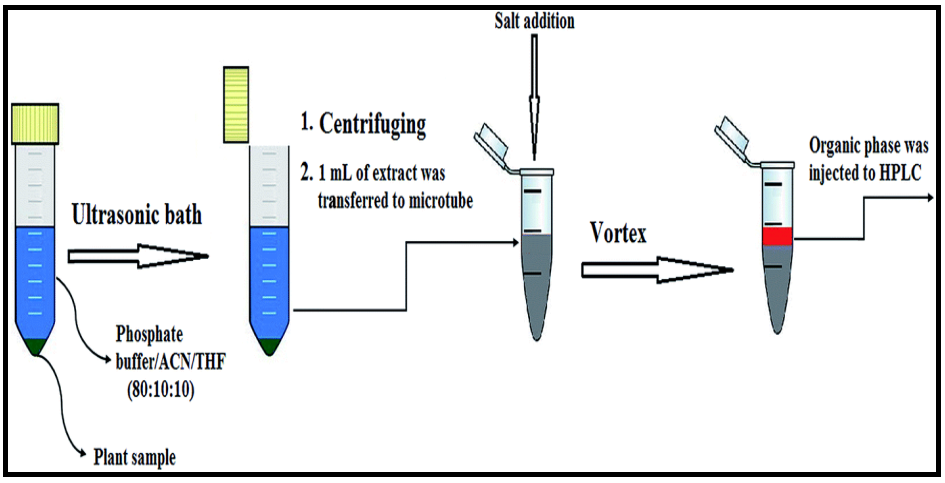


Figure No. 5: General experimental procedure followed for SALLE.

AUTOMIZATION, MINIATURIZATION AND MICROEXTRACTION

Nowadays, 'decrease/reduce' is a basic keyword in all analytical processes, bioanalytical is not an exceptional. Decrease in experimental steps, decrease in cost, reduce time of analysis, minimizing sample, solvents etc. To meet these requirements several works has been carried to achieve automization and miniaturization of SALLE.

Automization: a fast and simple methodology was developed[5] describing the flow injection coupled to MS/MS analysis of pesticides in blood and urine samples. Compared to present LC-MS/MS method for the analysis of the same compound (SPE). The flow injection procedure importantly reduce the work load, the time of analysis, the operating steps, work and costs, the solvent consumption was reduced thereby reducing the waste generation. Automization of extraction procedures was also developed by coupling microwave assisted extraction and SALLE for determination of steroids in tissues of fish.[6]

MINIATURIZATION: this method of SALLE[7] has accomplished minimization of cost analysis of Sulphonamides utilizing syringe for extraction purpose. For miniaturizing the procedure for multiple matrices like urine, plasma serum, blood, 96-well plates have been employed.[8, 9]

MICRO EXTRACTION: this method is still under investigation. It is an important step in exploring the extraction techniques, as in biological samples some analytes are present in very low concentrations. The first development reported[10] a SALLE procedure for micro extraction, anti-depressant drugs in serum, blood plasma using LC-UV. Firstly the analytes were derivatized in sample then the resulting derivatives were extracted further from sample using SALLE with acetonitrile (less volume of acetonitrile) extraction in which the concentration of analytes increases in the resultant extract. Along with which the analytes are converted into more hydrophobic compounds by derivatizing. Therefore, the extraction of these derivatized to acetonitrile is more efficient than that of underivatized analytes. Resulting in lowering the limit of detection compared to other methods. The results obtained by this method overlook new developments in SALLE with micro extraction. When a very small volume of the organic solvent was used, due to high miscibility of acetonitrile with water lead into difficulty in liquid phase separation. Therefore, minimizing the acetonitrile: sample volume ratio targeting the concentration factor increase cannot be viable. The use of derivatization in SALLE can be more encouraging. The presence of derivatizing reagents in extracting solutions will replace the chemical equilibrium to organic phase, resulting in enhancing the extraction efficiency. This effect has been widely employed in many extraction techniques.

CONCLUSION

The review covers sample preparation, sample collection used in bioanalysis. Various sample preparation tools are describes in which primarily plasma, whole blood, serum and urine is utilized. Mainly LC-MS, LC-MS/MS is used for detection purpose. Also covers pre-treatment applied to matrix and physicochemical properties affect the biological matrix. Proper pre-treatment and physicochemical properties affect on matrix results in good recoveries. SALLE employed in liquid-liquid extraction technique to achieve fast, simple and reliable separation.

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