Review Article

OVERVIEW ON RECENT EXTRACTION TECHNIQUES IN BIOANALYSIS

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ABSTRACT

This review is focused on Bioanalytical Strategies and gives update on the Bioanalytical Tools in Drug Discovery. The method used for sample collection, sample preparation. In sample preparation liquid-liquid extraction [LLE], solid phase extraction [SPE] and protein precipitation is traditional method. The recent methods are salting out assisted liquid extraction, protein precipitation plate and development in solid phase extraction in that molecularly imprinted polymer SPE, dispersive SPE, disposable pipette extraction, micro extraction by packed solvent, solid phase micro extraction, stir bar Sportive extraction and on line solid phase extraction ,this developed Bioanalytical methods applied various purposes such as pharmacokinetic studies, pharmacodynamic studies and comparative clinical studies.

Keyword: liquid-liquid extraction [LLE], solid phase extraction [SPE], protein precipitation plate [PPP]

INTRODUCTION

a. Analytical Chemistry: - "It deals with quantitative analysis of composition of substances and complex materials, by measuring a physical or chemical property of a distinctive constituent of the component or components of interest."¹

b. Classifications in analytical chemistry: - Following are the different techniques [tab:1]

c. The nature of analytical chemistry ²

1. Qualitative and Quantitative Analysis
2. Inorganic and Organic Analysis
3. Analysis, Determination and Estimation
4. Major, Minor and trace constituents
5. Complete and Partial Analysis

d. Terminologies encountered in analytical chemistry are²:-

Qualitative Analysis→ reveals the chemical identity of the species in the sample.

Quantitative Analysis→ establishes the relative amount of one or more of the species [analytes] in numerical terms.

Analyte → the components of a sample that are to be determined.

Assay→ the process of determining how much of a given sample is in the material indicated by its name

e. Biological sample in analytical chemistry:- Analytical chemistry is the study of the separation, identification, and quantification of the chemical components of natural and artificial materials. Qualitative analysis gives an indication of the identity of the chemical species in the sample and quantitative analysis determines the amount of one or more of these components. The separation of components is often performed prior to analysis. Analytical chemistry has applications in forensics, bioanalysis, clinical analysis, environmental analysis, and materials analysis³. Bioanalysis is a sub-discipline of analytical chemistry covering the quantitative measurement of xenobiotics (drugs and their metabolites, and biological molecules in unnatural locations or concentrations)

and biotics (macromolecules, proteins, DNA, large molecule drugs, metabolites) in biological systems. Bioanalytical chemistry dependent on accurate quantification of drugs and endogenous substances in biological samples; the focus of bioanalysis in the pharmaceutical industry is to provide a quantitative measure of the active drug and/or its metabolite(s) for the purpose of pharmacokinetics, toxic kinetics, bioequivalence and exposure–response (pharmacokinetics /pharmacodynamics studies) ⁴.

Aims and Objective

The study of Bioanalytical strategies is central to understanding how to collect, prepare, separate, and detect sample by different method for analysis of drug or drugs and their metabolite from biological fluid.

Our objective behind this review

1. To gives information about various sample collection and preparation techniques.
2. To focus on recently developed advanced techniques of sample preparation.
3. To gives information about their application.
4. To gives information about the developed Bioanalytical method used for purpose of pharmacokinetic, pharmacodynamic and comparative clinical studies.

3. Bioanalytical Tools in Drug Discover

The Bioanalytical strategy can be visualized by the following flow-chart. Figure 1

A. sample collection

The first step in the analytical chain is to decide which matrix to utilize. Most often venous blood, withdrawn from the arm, or capillary blood, with drawfrom the fingertip, or urine is used. More rarely, saliva and cerebrospinal fluid are utilized⁵. The venous blood can be withdrawn into tubes with an anticoagulant, e.g. EDTA or heparin. The blood can be used for analysis or plasma can be obtained if the tubes are centrifuged.

If the blood is withdrawn into tubes without anticoagulant, serum is obtained after centrifugation⁶. Plasma is an accepted sampling method and it allows sample volumes in the range of ml. Saliva may be sampled with saliva-collecting tubes, by spitting in a tube or by chewing on parafilm or gum to stimulate saliva production and then spit in a tube. Saliva samples has collected using Salivette sampling device (Figure 2). The procedure is straightforward and involves chewing on a cotton roll that absorbs the saliva. The cotton roll is placed in the sampling device which is stored in refrigerator until brought to the laboratory for centrifugation and later analysis. The use of saliva for biochemical analysis has a number of advantages compared to blood. Collection is non-invasive, stress free, very convenient and cost effective.⁵

When performing therapeutic drug monitoring (TDM) in rural areas, facilities may not be available (i.e. centrifuges or freezers) for storage of blood samples. This problem can be overcome by capillary blood sampling applied onto sampling paper i.e. dried blood spots, [DBS]. In dried blood spots, blood is obtained by puncturing a fingertip with a lancet. The blood is collected with a capillary pipette of fixed size and the blood is applied on to sampling paper (Figure 3). The dried blood spots is dried completely before storing the samples in plastic bags, for transportation to the laboratory, since moisture may cause bacterial or fungus growth. The dried blood spots sampling technique, compared with vein-puncture, requires minimal training to perform, it's less invasive and the dried blood spots represents also a low infectious risk from viruses such as HIV-1 and -2 and hepatitis C as they are dried on the sampling paper. The hepatitis B virus can remain infectious for at least seven days. With dried blood spots normally only 50 or 100 µl blood (sample) is collected, compared to 500-5000 µl for whole blood/plasma, which is an advantage when collecting blood from children⁵

Different biological sample used as:-Plasma/serum:- To determine the pharmacokinetic profile of the analyte and thus drug clearance, half-life, and bioavailability of analyte can be known⁷

Urine:- To determine the renal elimination profile of the compound⁷

B. sample preparation

Sample preparation and chromatographic separation are essential to reduce the effect of the (biological and buffer) matrix. Sample preparation is applied to remove interfering compounds. As a bonus, analytes can be concentrated during the extraction processes. Sample preparation is tedious and time consuming. However, the cleanliness of the samples affects the

overall performance of the analysis. It is common that the mass spectroscopy signal of the analyte decreases after a number of injections. This is caused by saturation of the high performance liquid chromatography column with endogenous compounds, which start to elute from the column causing suppression of the ionization of the analyte. Also, dirty samples can cause contamination of the ion source resulting in variations of the detector response. The sample preparation method of choice will be determined by the sample matrix and the required low limit of quantification. Sample preparation together with the analytical separation will determine the overall robustness of the analytical method. Sample preparation also known as sample treatment/sample clean up/sample extraction⁶.

The sample preparation process fulfils three major roles;⁷

Removal of protein-related materials that may contaminate the chromatography column

Elimination of endogenous compound, such as phospholipids that are major cause of ion suppression/enhancement in LC/MS

Concentration to increase assay sensitivity

Method of sample preparation:-

Sample preparation can be done by following methods:

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

a. liquid-liquid extraction (lle)

Principle: "It is based on selective extraction of intended analyte present in liquid sample through immiscible organic solvent" [Figure 4]

Solvent used: Methyl ter-Butyl Ether (MTBE), Dichloromethane (DCM), Ethyl acetate (EC), Diethyl ether (DEE), Hexane etc. any solvent as individual or in combination with any other suitable solvent can be used as an extraction solvent.

LLE is based on differential solubility and partitioning of two immiscible liquid phases in one, it required two immiscible phases, in most of the cases one aqueous and second organic phase, both phases must be immiscible. Analyte can be removed from the matrix selectively by choosing a suitable extraction solvent and buffering (pH adjustment of the sample to analyte in unionized form) of sample, if required. It is important to select appropriate solvents for this purpose; the solvent should match the analytes polarity while still being immiscible with water and it should preferably be compatible with the detection method. A larger volume of the extraction solvent, compared to the sample, favours partitioning and the enrichment of the analytes is often inefficient.⁶ [Figure 5]

Applications of liquid-liquid extraction:-

Sr.no	Title	Author	Name of journal	Method used	Biological sample
1.	Spectrophotometric determination of methylene blue in biological fluids after ion-pair extraction and evidence of its adsorption on plastic polymers ⁸	L.A. Decosted et al	European Journal of Pharmaceutical Sciences, Volume 5, Issue 6, November 1997, Pages 335-345	Liquid-liquid ion pair extraction	Blood, plasma, urine
2.	Sample preparation for the HPLC analysis of drugs in biological fluids Original ⁹	G.S. Murkitt et al	Journal of Pharmaceutical and Biomedical Analysis, Volume 7, Issue 9, 1989, Pages 1087-1096	LLE	Plasma, Blood
3.	Semi-automated 96-well liquid-liquid extraction for quantitation of drugs in biological fluids ¹¹	Nanyan Zhang et al	Journal of Pharmaceutical and Biomedical Analysis, Volume 22, Issue 1, February 2000, Pages 131-138	Semi-automated LLE	Plasma
4.	Sample preparation for organic acids in biological fluids ¹¹	H.M. Liebich et al	Analytica Chimica Acta, Volume 236, 1990, Pages 121-130	LLE	Serum, urine
5.	Isolation of Drugs and Drug Metabolites from Biological Fluids by Use of Salt-Solvent Pairs ¹²	M. G. Horning et al	Clinical chemistry, Vol. 20, No.2, 1974	Salt solvent pair	Urine, plasma

b. solid phase extraction (SPE)

Principle: It is based on adsorption (or) Partitioning on to solid sorbent (Adsorbent) Selective retardation of analyte using solid sorbent under specific condition. SPE is based on the selective adsorption mechanism. If the targeted analyte are adsorbed on the solid phase, they can selectively be removed or eluted by using an appropriate elution solvent⁶. Different cartridges are used in solid phase extraction.

Steps involved in SPE:-

Step 1. Conditioning: All SPE tubes are required to be conditioned with appropriate solvents prior to sample application.

Condition is must to

Avoid drying and keep the sites in arising position

Remove any dust, moisture or any other process contaminants

Activate the sites of stationary phase bed

Commonly used conditioning solvents are MeOH, small volumes of DCM, MTBE or anyl other organic solvent, water, buffer.

Step 2. Sample Application: Application of sample from the top of the cartridge at a slow flow rate without any brake by taking care that no sample drop should remain on the inner wall of the cartridges, slow rate is necessary to allow analyte to interact with adsorbent thus to achieve the retention of analytes because of temporary weak bonding.

Step3. Rinsing or Washing: It is intended for the removal of matrix components or other interferences by washing the cartridge with relative weak dilute solvent or solvent mixtures or buffer and interferences that are weakly retained than the analytes are drained out from the cartridge. Rinsing or washing solvents are water, buffers of different pH.

Step 4. Drying; Drying can be done by applying appropriate vacuum for recommended time period with help of vacuum pump, recommended drying time is 2-3 minutes.

It is intended for the removal of excess washing solvents/buffers to

Avoid any interaction/precipitation possibility

Avoid any blockage of cartridges due to air bubble formation during elution

Step 5. Elution: It involves passing of strong solvent through cartridge at a slow flow rate thus allowing more soak time on the packing of reach maximum extraction efficiency, elution solvents are MeOH, ACN, acidic or alkaline MeOH or ACN, small volumes of DCM, MTBE or any other organic solvent in combination with MeOH or ACN or any other suitable mixture. It is used for cleavage of weak bonds formed between analyte and sorbent, usage of multiple small volumes is recommended to improve elution⁶. [Figure 6]

Type of SEP:

1. Reversed phase SPE
2. Normal phase SPE
3. Ion exchange SPE

1. Reversed Phase SPE

Reversed phase separations involve a polar (usually aqueous) or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase. The analyte of interest is typically mid - to nonpolar. Several SPE materials, such as the alkyl- or aryl-

bonded silicas (LC-18, ENVI-18).LC-18(octadecyl bonded, endcapped silica) for reversed phase extraction of nonpolar to moderately polar compounds, such as antibiotics, barbiturates, benzodiazepines, caffeine, dyes, essential oils, fat soluble vitamins, phenol¹⁴.

2. Normal Phase SPE

Normal phase SPE procedures typically involve a polar analyte, a mid- to nonpolar matrix (e.g. acetone, chlorinated solvents, and hexane), and a polar stationary phase. Polar-functionalized bonded silicas (e.g. LC-CN, LC-NH₂, and LC-Diol), and polar adsorption media (LC-Si, LC-Alumina) typically are used under normal phase conditions. Retention of an analyte under normal phase conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. These include hydrogen bonding, pi-pi interactions, dipole-dipole interactions, and dipole-induced dipole interactions, among others. A compound adsorbed by these mechanisms is eluted by passing a solvent that disrupts the binding mechanism — usually a solvent that is more polar than the sample's original matrix. The bonded silicas — LC-CN, LC-NH₂, 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, these packings can be used to adsorb polar compounds from nonpolar 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.¹⁴

3. Ion Exchange SPE

Ion exchange SPE 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. Cationic compounds are isolated by using LC-SCX or LC-WCX bonded silica cartridges. The primary retention mechanism of the compound is based mainly 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 compound's 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 compound¹⁴.

Advantages of SEP over LLE^{14, 15}

1. Convenient method, 2. Easy to use, 3. Greater recovery and accuracy
4. More easily automated, 5. Easier collection of the total analyte fraction

Applications of solid phase extraction

Title	Author	Name of journal	Method used	Biological sample
Solid-Phase Extraction and Reverse-Phase HPLC: Application to Study the Urinary Excretion Pattern of Benzophenone-3 and its Metabolite 2,4Dihydroxybenzophenone in Human Urine ¹⁶	Helena Gonzalez et al	Analytical Chemistry Insights 2008:3 1–7	Solid phase extraction	Urine
A Modified Method for Determination of Lumefantrine in Human Plasma by HPLC-UV and Combination of Protein Precipitation and Solid-Phase Extraction: Application to a Pharmacokinetic Study ¹⁷	Liusheng Huang et al	Analytical Chemistry Insights 2010:5.	SPE	Plasma
Development and Validation of a HPLC Method to Determine Griseofulvin in Rat Plasma: Application to Pharmacokinetic Studies ¹⁸	Bo Wei et al	Analytical Chemistry Insights 2008:3 103–109	SPE	Plasma
High-Performance Liquid Chromatographic Method for Determination of Phenytoin in Rabbits Receiving Sildenafil ¹⁹	Alaa Khedr	Analytical Chemistry Insights 2008:3 61–67	SPE	Plasma
Improved Solid-Phase Extraction Method for Systematic Toxicological Analysis in Biological Fluids ²⁰	T. Soriano et al	Journal of Analytical Toxicology, Vol. 25, March 2001.	SEP	blood, urine, bile, and gastric content

c. Protein precipitation

Principle: “It depends on the solubility of analyte in particular solvent present in biological matrix i.e., blood, plasma, serum.”²¹

Solvent used: Methanol, ACN etc.²¹ [Figure 7]

It is basically denaturing the proteins. Precipitation of proteins can be done by using any one of the method mentioned below:²¹

By changing the pH of sample- by mixing inorganic reagents eg. Perchloric acid, Trichloroacetic acid etc. In iso-electric pH, proteins have no net charge, which causes

insolubility thus precipitates.

By addition of organic solvents- It decreases the dielectric constant of the medium, leads to insolubility thus cause precipitation, in another case high affinity for the hydrophobic surfaces of the protein leads to denaturing of proteins. Eg. Methanol, Acetonitrile etc.

By addition of Salts - salts used for precipitation of proteins are citrates, phosphates, acetates, etc.

Applications of protein precipitation

Title	Author	Name of journal	Method used	Biological sample
A Modified Method for Determination of Lumefantrine in Human Plasma by HPLC-UV and Combination of Protein Precipitation and Solid-Phase Extraction: Application to a Pharmacokinetic Study. ¹⁷	Liusheng Huang et al	Analytical Chemistry Insights 2010:5.	Protein precipitation	Plasma
Bioanalytical Method Development Determination of Drug in Biological Fluids. ¹⁵	Pranay Wal et al	Journal of Pharmaceutical Science and Technology Vol.2(10).210,333-347.	Protein precipitation	Plasma

C. Recent Advances In Sample Preparation Techniques Are

1. salting-out assisted LLE [salle]
2. protein precipitation plates
3. solid-phase extraction technique

1. salting-out assisted LLE

When an inorganic salt is added to mixture of water and a water miscible organic solvent, it leads to the separation of the solvent from the mixture and forms a biphasic system, a phenomenon commonly term as ‘salt- induced phase separation’. Salting out technique is not only useful in separating water-miscible organic solvent, but is also used to enhance extraction into non-polar, immiscible organic solvents. The effectiveness of the salting-out depends on the physicochemical properties of the analyte and the type of salt used. The principle of salting is commonly used in environmental analyses of various trace elements .salting-out Assisted LLE method used magnesium/ calcium sulfate, sodium chloride, calcium chloride, potassium carbonate, ammonium formate and ammonium sulfate. The use of zinc sulfate for salting out lopinavir and ritonavir from plasma samples. However, beings non-volatile salts, they pose difficulties in LC-MS-MS analysis of the drugs. The use of ammonium acetate is a mass spectrometry friendly salt for quantitative estimation of hydrophobic drug candidate and its hydrophilic metabolite in human plasma. Ammonium formate used for the estimation of simvastatin and simvastatin acid. Salting-out Assisted LLE method is cost-effective and can be automated²².

2. protein precipitation plates

One of the recent approaches to eliminating the disadvantages of the conventional manual PP method is development of membrane-based PP filter plates. PP plates have been developed so that, after precipitation of protein, filtration can be carried out in the same well without centrifugation and supernatant transfer steps. Structurally, these plates contain tubes with membrane/depth filter and are attachable to vacuum filtration. The filtration collection plates are made to be compatible with most of the modern autosamplers for easy automation of the overall process. Today, PP plates are available from leading vendors. Use of PPT plates in bioanalysis has been compared with manual PP. It has been reported, in an LCMSMS method, results from PPT plates showed more reproducible (accuracy and precision) than manual PP. [Figure 8]

Some of plates show solvent leaking. Among the PP Solvents tested, acetonitrile showed higher leaking than methanol. It has been suggested that the precipitating solvent, filter material, pore size, vacuum strength, non-specific binding of analyte to the plate and matrix effects (ion suppression) should be considered when choosing a protein precipitation filter plate for an assay²².

It can be concluded that, PP plates when compared with manual PP, offers several advantages such as²²:-

- No centrifugation or filtrate transfer steps
- Reduced processing time
- Higher solvent recovery

Usefulness with very low sample volume and

3. solid-phase extraction technique

To overcome the limitations of the LLE and PP, one of the first developments in the field of sample preparation was SPE technique. SPE is based on the same principle of affinity-based separation as liquid chromatography²⁰. Generally, the goals of SPE are retention and elution of analyte from biological fluid and removal of contaminants/interfering substances and sample concentration. Traditionally SPE is available in normal phase, reverse-phase and ion-exchange. Recently SPE are:-

- a. molecularly imprinted polymer spe
- b. dispersive spe
- c. disposable pipette extraction
- d. micro-extraction by packed sorbent
- e. solid phase micro extraction
- f. stir bar sorptive extraction
- g. on line solid phase extraction

a. molecularly imprinted polymer SPE

The technique of molecularly imprinted polymers is based upon molecular recognition and is often referred to as synthetic antibodies. The principle is that a polymer network is created around a template, i.e. the imprint molecule²²[Figure 9] Steps involved Molecularly Imprinted Polymer SPE²³:-

1. The formation of a prepolymerisation complex between the template molecule and the functional monomers.
2. The polymerization of the formed template-monomer complex in the presence of a cross-linker
3. Extraction of the template molecule from the polymer matrix.

The MISPE is claimed as one of the most selective sample preparation techniques among all the available SPE approaches. For MISPE, through careful design of the imprinting site through molecular modelling, experimental design or screening methods, the binding of analytes of interest can be engineered to offer multiple interactions. Interactions such as ion-exchange, reversed-phase with polymer backbone and hydrogen bonding between the MIP stationary phase and analyte functional groups enable MISPE methods to achieve higher selectivity.²²

Applications in the Molecularly Imprinted Polymer SPE :-

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

The classes of compounds for which MISPE cartridges are available are nitroimidazoles, non-steroidal anti-inflammatory drugs (NSAIDs), fluoroquinolones, amphetamines, clenbuterol, b-agonists (class selective), full b-receptors (agonists and blockers), chloramphenicol and triazines. MISPE is powerful tool in sample preparation²².

b. dispersive solid-phase extract

Dispersive solid-phase extraction (dSPE), popularly known as, quick, easy, cheap, effective, rugged and safe, is a novel sample preparation technique which offers unique advantages such as applicability to wide variety of analyte chemistries, low solvent use, little use of glassware/plastic ware, high cost effectiveness and automation. The dSPE sample preparation approach involves the solvent extraction of the sample with organic solvents such as acetonitrile-ethyl acetate-acetone and partitioning with magnesium sulfate alone or in combination with other salts (such as sodium chloride) followed by clean-up using dSPE. Essentially, a typical procedure will involve a homogeneous biomatrix sample being transferred to plastic/glass tube, followed by addition of organic solvent followed by mixing. To this mixture, salt is added. The purpose of salt addition is to induce phase separation, which also results in the salting out effect and to influence the analyte partitioning

behaviour. In this step, if required, pH adjustment based on analyte pKa is also recommended so as to improve the liquid extraction efficiency. The sample, acetonitrile and salt mixture is further vortex mixed followed by addition of internal standard. The whole mixture is then centrifuged and supernatant is transferred to a clean glass/plastic tube²².

Traditionally, the dSPE approach has been very widely used in²²:-

- Pesticide sample analyses
- Food sample analyses
- Environmental sample analyses
- Cosmetic additive sample analyses

Recent applications of dSPE which are²²:-

Determination of antihelmintic drug residues in milk

Extraction of trace b-agonists in pig tissues

Quantitative analysis of b-lactam antibiotics in kidney tissue

c. disposable pipette extraction

DPX is an effective sample preparation technique for separation and extraction of analytes from the various matrices. In its simplest original form, it is modified standard pipette tip (1/5 mL), which is loaded with free flowing sorbent powder, which is free to disperse. Being a standard tip, it is easy to take the solvent in or out through this dispersive sorbent. In its modified form, the standard tip contains a dispersible sorbent loosely placed between two frits (one frit placed on the lower end of the tip through which solvent can be taken up/down and the second placed at the upper end of the tip to avoid solvent contamination in to the pipette)²².

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 travels through the bed under gravity), thus much sorbent material is 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 repeatabilities.

The steps involved in DPX are similar to those of traditional SPE with some differences. In a typical DPX 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. This step needs 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 followed by a wash step. The washing solvent can be optimized based on the type/chemical nature of sorbent, the nature of the analyte and probable matrix interferences. The washing solvent can be withdrawn, aspirated with air and pipetted 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 the 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. Commercially, 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 carbon. Application of DPX in analysis of pesticides²²

d. micro-extraction by packed sorbent

Miniaturization is trend of today's science. Micro-extraction by packed 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 10–1000 mL), reducing the number of steps typically involved in conventional SPE and providing easy automation. Typical MEPS is designed in the syringe format, wherein approximately 1mg of the sorbent is packed inside a syringe (100–250 mL) as a plug or between the barrel and the needle as a cartridge. Similar to other sample preparation techniques such as MISPE/dSPE/DPX, novel sorbent materials such as silica-based material (strong cation exchanger, SCX). MEPS protocol involves pre-treatment of complex samples 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, the analytes 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, 50 mL). The final step is elution of analytes with suitable solvent. The elution step uses 20–50 mL 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 solution). This is also said to reduce carry-over effects. This is in complete contrast to the use of conventional SPE cartridges. The MEPS can be applied to all types of bio-matrices such as plasma, serum, urine, whole blood, hair and saliva.

e. solid-phase micro-extraction (SPME)

A new sample preparation technique using a fused-silica fibre coated on the outside with an appropriate stationary phase and is termed Solid phase micro-extraction (SPME). Physically, it is a modified syringe that contains stainless steel microtubing within its syringe needle. This microtubing has about a 1 cm fused-silica fibre tip which is coated with an organic polymer. This coated silica fibre can be moved backwards and forwards with the plunger of the syringe. In contrast to conventional SPE with packed-bed cartridges, SPME syringe assembly design allows combination of all the 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 fibre 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 fibre 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 fibre coating. Various commercially available fibre coatings are polydimethylsiloxane (PDMS) for extraction of non-polar analytes, polyacrylate (PA, thickness 85 nm) for extraction of polar analytes (especially phenols), polydimethylsiloxane-divinylbenzene (PDMS-DVB, thickness 65 and 60 nm) for extraction of polar analytes (especially amines)²².

f. stir bar sorptive extraction

Based on the similar principle of SPME, stir bar sorptive 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 fibre is 0.5 mL (film thickness 100 nm); 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. direct immersion and head space (HS, special device to hold the stir bar is available). One of the limitations of the SBSE is that only one extraction phase is commercially available, i.e. PDMS. SBSE is mainly used for extraction of hydrophobic analytes. SBSE is a highly cost-effective sample preparation technique²².

g. 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. RAMs 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 HPLC 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 multicomponent analysis of antidepressant drugs in biological fluids²².

D. Measurement

The measurement of a given analyte can often be divided into a separation step and a detection step.

a. Separation

Liquid chromatography (LC) is a separation process in which a sample mixture is injected onto a column and distributed between two phases. One phase is the stationary phase immobilized in a column and the other a mobile solvent phase passing through the column. Other chromatographic techniques are also used in that thin layer chromatography, high performance liquid chromatography Column chromatography.²²

b. Detection of drug in biological fluids by^{24,33}

- UV-Visible spectrometry
- Fluorescence spectrometry
- Electrochemical
- Mass spectrometry
- LC-MS/MS

E. Evaluation And Application Of Bioanalytical Strategies

If a sample has been analysed with a separation method the result will be in form of a chromatogram. The eluting substances will produce signals in the detector separated by time, creating a pattern of peaks. Calibration curves can be constructed from a set of samples with different concentrations and be used for predictions of concentrations in unknown samples. Area or peak height can be used and the choice depends upon the situation¹⁵.

The developed Bioanalytical method can be applied for various purposes:-

- a. pharmacokinetic studies
- b. pharmacodynamic studies
- c. comparative clinical studies

a. pharmacokinetic methods

Pharmacokinetic deals with the changes of drug concentration in the drug product and changes of concentration of a drug and/or its metabolite(s) in the human or animal body following administration of the drug product, i.e., the changes of drug

concentration in the different body fluid and tissues in the dynamic system of liberation, absorption, distribution, body storage, binding, metabolism, and excretion²⁵.

Two major pharmacokinetic methods are

Plasma level-Time studies

Urinary excretion studies¹³

Applications of pharmacokinetic studies

Title	Author	Name of journal	Method used	Biological sample
Solid-Phase Extraction and Reverse-Phase HPLC: Application to Study the Urinary Excretion Pattern of Benzophenone-3 and its Metabolite 2,4-Dihydroxybenzophenone in Human Urine ¹⁶	Helena Gonzalez et al	Analytical Chemistry Insights 2008:3 1–7	Urine excretion studies	Urine
High-Performance Liquid Chromatographic Method for Determination of Phenytoin in Rabbits Receiving Sildenafil ¹⁹	Alaa Khedr et al	Analytical Chemistry Insights 2008:3 61–67	Plasma level time studies	Plasma
Development and Validation of a HPLC Method to Determine Griseofulvin in Rat Plasma: Application to Pharmacokinetic Studies. ¹⁸	Bo Wei et al	Analytical Chemistry Insights 2008:3 103–109.	Plasma level-time studies	Plasma
Phase I and pharmacokinetic study of Yondelis™ (Ecteinascidin-743; ET-743) administered as an infusion over 1 h or 3 h every 21 days in patients with solid tumours ²⁶	C. Twelves et al	European Journal of Cancer, Volume 39, Issue 13, September 2003, Pages 1842-1851	Urine excretion studies	Urine
Pharmacokinetic Study of Recombinant Human Factor IX in Previously Treated Patients with Hemophilia B in Taiwan ²⁷	Hsiu-Hao Chang	Journal of the Formosan Medical Association, Volume 106, Issue 4, 2007, Pages 281-287	Urine excretion studies	Urine

b. pharmacodynamic studies-

It involves direct measurement of drug effect on a physiological process as function of time. The two pharmacodynamic methods involve determination of bioavailability from¹³:-

Acute pharmacological response

Therapeutic response

Applications of Pharmacodynamic studies

Title	Author	Name of journal	Method	Biological sample
Structured multiplicity and confirmatory statistical analyses in pharmacodynamic studies using the quantitative electroencephalogram ²⁸	Georg Ferber et al	Journal of Neuroscience Methods, Volume 201, Issue 1, 30 September 2011, Pages 204-212	Therapeutic studies	Tissue
Accuracy of repeated blood sampling in rats: a new technique applied in pharmacokinetic/pharmacodynamic studies of the interaction between warfarin and co-enzyme ²⁹	Qingyu Zhou et al	Journal of Pharmacological and Toxicological Methods, Volume 40, Issue 4, November 1998, Pages 191-199	Therapeutic studies	Blood
Quantification of uric acid, xanthine and hypoxanthine in human serum by HPLC for pharmacodynamic studies ³⁰	Nancy Cooper et al	Journal of Chromatography B, Volume 837, Issues 1-2, 6 June 2006, Pages 1-10	pharmacodynamic studies	Serum

c. Comparative clinical study

The plasma concentration time profile data may not be suitable to assess equivalence between two formulations. In such case pharmacodynamics studies can an appropriate tool for establishing equivalence, in other instances this type of study

cannot be performed because of lack of meaningful pharmacodynamics parameters which can be measured and a comparative clinical study has to be performed in order to demonstrate equivalence between two formulations.

Applications of comparative studies

Title	Author	Name of journal	Method used	Biological sample
Evidence behind use of intensity-modulated radiotherapy: a systematic review of comparative clinical studies ³¹	Liv Veldeman DrMDa et al	The Lancet Oncology Volume 9, Issue 4, April 2008, Pages 367-375	comparative clinical studies	Tumour cell
Randomized, double-blind, comparative study of dexrabeprazole 10 mg versus rabeprazole 20 mg in the treatment of gastroesophageal reflux disease ³²	Vikas Pai et al	World J Gastroenterol 2007 August 14; 13(30): 4100-4102	comparative clinical studies	Gastric content

Table 1: Different techniques in analytical chemistry

Techniques	Property measured
Gravimetric	Weight of pure analyte or of a stoichiometric compound containing it
Volumetric	Volume of standard reagent solution reacting with the analyte
Spectrometric	Intensity of electromagnetic radiation emitted or absorbed by analyte
Electrochemical	Electrical properties of analyte solutions
Radiochemical	Intensity of nuclear radiations emitted by the analyte
Mass spectrometric	Abundance of molecular fragments derived from the analyte
Chromatographic	Physic-chemical properties of individual analytes after separation
Thermal	Physic-chemical properties of the sample as it is heated and cooled

Figure 1: Flow chart of Bioanalytical strategy

Figure 2: Salivette sampling device

Figure 3: Capillary blood collection and dried blood spots

Figure 4: Principle of liquid-liquid extraction

Figure 5: Liquid-liquid extraction

Figure 6: General solid phase extraction steps

Figure 7: General protein precipitation process

Figure 8: Protein precipitation tube

Figure 9: Schematic illustration of the principle of Molecular Imprinting

CONCLUSION

There are various sample preparation tools which are describe but manly plasma, serum, urine is commonly used than saliva, milk. In detection manly LC-MS, LC-MS-MS techniques are prominently used. Conventional LLE, SPE and protein precipitation techniques are now being considered as methods of past.

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