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Extraction of Drug from the Biological Matrix: A Review

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1. Introduction

The assessment of therapeutic compliance is commonly done by either indirect method or direct method. In indirect method, the assessments are done by indirect measurement of parameters such as discussion with patients and pill counts at intervals during the treatment which don't measure the drug concentration in a matrix such as blood or urine. In direct method, the assessments which rely upon evidence provided by the patients or care giver on the presumptive compliance based upon electronic medical event monitoring system and this is based upon either the qualitative or quantitative measurement of the drug under investigation in a biological matrix provided by the system.

A more objective assessment of patient compliance has its own limitations. The most widely used matrix is plasma, but the major limitation with this approach is that it provides the clinician with the concentration of drug within the systematic circulation at the time of sample collection. This concentration is primarily related to the time interval between the administration of the last dose and the sample collection time although other pharmacokinetic parameters can also influence the concentration. Plasma samples should be collected prior to the administration of the next dose. Plasma monitoring is a useful adjunct for the assessment of therapeutic compliance (Williams, 1999).

Bioanalysis is a sub-discipline of analytical chemistry covering the quantitative measurement drugs and their metabolites in biological systems. 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, toxicokinetics, bioequivalence and exposure-response (pharmacokinetics /pharmacodynamics studies). Bioanalysis also applies to drugs used for illicit purposes, forensic investigations, anti-doping testing in sports, and environmental concerns. Bioanalytical assays to accurately and reliably determine these drugs at lower concentrations. This has driven improvements in technology and analytical methods.

Some techniques commonly used in bioanalytical studies include:

- Hyphenated techniques
 - LC-MS (liquid chromatography-mass spectrometry)
 - GC-MS (gas chromatography-mass spectrometry)

- LC-DAD (liquid chromatography–diode array detection)
- CE-MS (capillary electrophoresis–mass spectrometry)
- Chromatographic methods
 - HPLC (high performance liquid chromatography)
 - GC (gas chromatography)
 - UPLC (ultra performance liquid chromatography)
 - Supercritical fluid chromatography

The area of bioanalysis can encompass a very broad range of assays which support the clinical and nonclinical studies. Many factors influence the development of robust bioanalytical methods for analyzing samples from clinical and nonclinical studies which includes the matrices of interest, the range over which analytes need to be measured, the number and structures of the analytes, the physicochemical properties of the analytes, and their stability in the biological matrices from the time of sample draw to analysis also needs to be measured.

Because biological samples are extremely complex matrices comprised of many components that can interfere with good separations and or good mass spectrometer signals, sample preparation is an important aspect of bioanalytical estimation. This is important whether samples originate as tissue extracts, plasma, serum or urine. The question sometimes arises as to whether serum or plasma should be collected for analysis. In general, from a bioanalytical perspective, there are few advantages to choosing serum over plasma except where chemical interferences in a given assay might require processing blood all the way to serum.

Nearly all bioanalytical assays involve the use of an internal standard processed along with the sample. The area ratio of analyte to internal standard is used in conjunction with a standard curve to back calculate the concentration of analyte in the sample. The internal standard is expected to behave similarly to the analyte with respect to extraction efficiency across the range of concentration, which helps compensate for sample to sample differences in sample preparation. Often, an analogue or similarly behaving compound is used as an internal standard.

To produce adequate and precise results, certain criteria like minimal loss or degradation of sample during blood collection, the appropriate sample cleanup and internal standard, chromatographic conditions that minimize the interference and ion suppression, and sufficient signal to noise to allow for reproducible peak integration.

However, an examination of the individual plasma samples could show one or more with an unacceptable interference that is effectively washed out when the samples are pooled. Thus, it is important to use several individual matrix samples when evaluating matrix effects. If unacceptable matrix interferences are observed, it is necessary to further clean up the sample to eliminate the interference.

A key component of the sample preparation is the emphasis on analyte stability which needs to be carefully assessed from the time of sample drawn till the analysis is complete. It is important to study the analytes stability in blood at the appropriate temperatures from the time the sample is drawn until after centrifugation when plasma or serum are separated and stored in a new vial. It is also important to ensure that the anticoagulant or other

components of the blood collection do not interfere with the sample preparation (Destefano & Takigiku, 2004).

Blood is the transporter of many vital substances and nutrients for the entire body and thus contains many endogenous and exogenous compounds in different concentrations. Drug determination in human plasma is often complicated by low concentrations (0.1 – 10ng mL⁻¹ level). An extra problem posed by blood sample is the complex sample matrix due to proteins, which can lead to protein binding of the analyte and by limited sample volumes normally 0.5 – 1ml will be available for the determination. The magnitude of the challenge of protein purification becomes clearer when one considers the mixture of macromolecules present in the biological matrices. Hence, sample preparation is crucial in drug analysis which includes both analyte pre-concentration and sample cleanup (Ho *et al.*, 2002).

To produce meaningful information, an analysis must be performed on a sample whose composition faithfully reflects that of the bulk of material from which it was taken. Biological samples cannot normally be injected directly into the analyzing system without sample preparation. Sample pretreatment is thus of utmost importance for the adequate analysis of drugs. However, as sample pretreatment can be a time consuming process, this can limit the sample throughput. The proper selectivity can be obtained during the sample preparation, the separation and the detection. A major differentiation between the analyte of interest and the other compounds is often made during the first step. Sensitivity is to a large extent obtained by the detector. Thus, sample pretreatment is required for achieving sufficient sensitivity and selectivity, whereas the time should be kept to a minimum in order to obtain adequate speed. Therefore, there is a clear trend towards integration of sample pretreatment with the separation and the detection. Numerous sample preparation techniques have been developed for bioanalytical purpose. Sample preparation is a difficult step, especially in clinical and environmental chemistry and generally involves filtration, solid phase extraction with disposable cartridges, protein precipitation and desalting. Sample preparation prior to chromatographic separation is performed to dissolve or dilute the analyte in a suitable solvent, removing the interfering compounds and pre-concentrating the analyte.

The principle objectives of sample preparation from biological matrix are;

- a. Isolation of the analytes of interest from the interfering compounds
- b. Dissolution of the analytes in a suitable solvent and pre-concentration.

In an analytical method sample preparation is followed by a separation and detection procedure. In spite of the fact that sample preparation, in most of the analytical procedures, takes 50-75% of the total time of the analysis, most technical innovations of the last 5 years are related to separation and detection.

Extraction is one of humankind's oldest chemical operations. Extraction is the withdrawing of an active agent or a waste substance from a solid or liquid mixture with a liquid solvent. The solvent is not or only partially miscible with the solid or the liquid. By intensive contact between analyte and the extraction medium this leads the analyte transfers from the solid or liquid mixture into the extraction medium (extract). After thorough mixing the two phases can be separated either by gravity or centrifugal forces (Gy, 1982; Arthur & Pawliszyn, 1990; Zief & Kiser, 1990).

2. Sampling

The sampling and sample preparation process begins at the point of collection and extends to the measurement step. The proper collection of sample during the sample process (called primary sampling), the transport of this representative sample from the point of collection to the analytical laboratory, the proper selection of the laboratory sample itself (called secondary sampling), and the sample preparation method used to convert the sample into a form suitable for the measurement step can have a greater effect on the overall accuracy and reliability of the results than the measurement itself.

Primary sampling is the process of selecting and collecting the sample to be analyzed. The objective of sampling is a mass or volume reduction from the parent batch, which itself can be homogeneous or heterogeneous. If the wrong sample is collected or the sample is not collected properly, then all the further stages in the analysis become meaningless and the resulting data are worthless. Unfortunately, sampling is sometimes left to people unskilled in sampling methodology and is largely ignored in the education process, especially for non-analytical chemists (Smith & James, 1981). Once the primary sample is taken, it must be transported to the analytical laboratory without a physical or chemical change in its characteristics. Even if a representative primary sample is taken, changes that can occur during transport can present difficulties in the secondary sampling process. Preservation techniques can be used to minimize changes between collection and analysis. Physical changes such as adsorption, diffusion and volatilization as well as chemical changes such as oxidation and microbiological degradation are minimized by proper preservation.

Common preservation techniques used between the point of collection and the point of sample preparation in the laboratory are

- Choice of appropriate sampling container
- Addition of chemical stabilizers such as antioxidants and antibacterial agents
- Freezing the sample to avoid thermal degradation
- Adsorption on a solid phase

In secondary sampling, if the sample has made it to the laboratory, a representative subsample must be taken. Statistical appropriate sampling procedures are applied to avoid discrimination, which can further degrade analytical data. Clearly speeding up or automating the sample preparation step will reduce analysis time and improve sample throughput (Keith, 1990).

Before sample preparation, solid or semisolid substances must be put into a finely divided state. Procedures to perform this operation are usually physical methods, not chemical methods. The reasons for putting the sample into a finely divided state are that finely divided samples are more homogeneous, so secondary sampling may be carried out with greater precision and accuracy, and they are more easily dissolved or extracted because of their large surface-to-volume ratio.

2.1 Sample preparation

Sample preparation is necessary for at least two reasons:

- a. To remove as many of the endogenous interferences from the analyte as possible
- b. To enrich the sample with respect to the analyte, thus maximizing the sensitivity of the system.

It also serves to ensure that the injection matrix is compatible with the selected column and mobile phase.

2.2 Goal and objectives of sample preparation

Two of the major goals of any sample pretreatment procedure are

- Quantitative recovery
- A minimum number of steps.

Successful sample preparation has a threefold objective.

- In solution
- Free from interfering matrix elements
- At a concentration appropriate for detection and measurement

The most common approach in analyte separation involves a two phase system where the analyte and interferences are distributed between the two phases. Distribution is an equilibrium process and is reversible. If the sample is distributed between two immiscible liquid phase, the techniques is called liquid-liquid extraction. If the sample is distributed between a solid and a liquid phase, the technique is called liquid-solid adsorption.

Often, when analysis involves the measurement of trace amounts of a substance, it is desirable to increase the concentration of the analyte to a level where it can be measured more easily. Concentration of an analyte can be accomplished by transferring it from a large volume of phase to a smaller volume of phase. Separation can be carried out in a single batch, in multiple batches or by continuous operation.

2.3 Types of samples

Sample matrices can be classified as organic and inorganic. Biological samples are a subset of organic samples but often require different sample preparation procedures in order to preserve biological integrity and activity. Compared to volatile compounds or solid, liquid samples are much easier to prepare for analytical measurement because a dissolution or an extraction step many not be involved. The major consideration for liquid samples is the matrix interferences, the concentration of analyte, and compatibility with the analytical techniques. When a sample is a solid, the sample pretreatment process can be more complex. There are two specific cases: the entire sample is of interest and must be solubilized or only a part of the solids is of interest and the analytes must be selectively removed. If the solid is a soluble salt or drug tablet formulation, the only sample preparation that may be required is finding a suitable solvent that will totally dissolve the sample and the components of interest. If the sample matrix is insoluble in commonly solvents but the analytes of interest can be removed or leached out, then sample preparation can also be rather straightforward. In these cases, techniques such as filtration, Soxhlet extraction, supercritical fluid extraction, ultrasonication or solid-liquid extraction may be useful. If both the sample matrix and the sample analytes are not soluble in common solvents, then more drastic measures may be needed.

3. Physicochemical properties of drug and their extraction from biological material (Chang 1977; Moore 1972; Barrow 1996; Wiltshire 2000)

3.1 Molecular phenomena for solubility and miscibility

To dissolve a drug, a solvent must break the bonds like ionic bond, hydrogen bond and Van der Waals forces which inter links the compound to its neighbors and must not break substantial intermolecular bonds of the solvent without replacing them with drug solvent interaction. Because breaking of bonds is an endothermic process, requires energy and causing an increasing in enthalpy. Similarly, if the gain in entropy from the dissolution of two solvents in each other is insufficient to counteract any reduced amount of intermolecular bonding, they will not be completely miscible.

3.2 Water miscibility and water immiscibility

Commonly alcohols can have hydrogen bonding with water and also dipole-dipole interactions will aid miscibility. On the other hand, presence of alkyl groups will reduce the solubility with water and the interaction may be by means of dispersive force. Hydrocarbons are hydrophobic in nature, which dissolves the compounds by dispersive forces. Whereas halogenated hydrocarbons are more polar and dissolve the compounds by dispersive forces and dipolar interactions.

Hydrophilic groups, which are polar in nature, will encourage the solubility in water, whereas C-C, C-H and C-X bonds are hydrophobic in nature will encourage the solubility in organic solvents. Drug with several aromatic rings will have poor solubility in water due to lack of hydrogen bonding with water and the strong intermolecular dispersive forces of the solid drug will encourage the ready solubility in organic solvents.

3.3 Distribution coefficient

Drug which are in ionised forms are hydrophilic in nature than the unionized form because of the hydration of the ions, therefore the ionized forms are difficulty to extract into organic solvents whereas the unionized forms will dissolve in the organic solvents which can be extracted into organic solvents.

3.4 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.

- Ethyl acetate is a powerful solvent for many organic compounds and will therefore extract a considerable amount of endogenous material with the required drug.
- If the drug is relatively non-polar, a more selective extraction could be obtained by using a hydrocarbon solvent.
- Halogenated hydrocarbons like chloroform and dichloromethane are excellent, volatile solvents. However they are denser than water which makes them difficult to use for analysis.

- Benzene is a useful solvent, reasonably volatile, inert and immiscible with water, but its toxicity precludes its use.
- Toluene has similar properties as a solvent to benzene is not particularly toxic, however its boiling point is 111°C and it is not really sufficient volatile for use as a solvent in bio-analysis.
- Chloroform is an excellent solvent but reactivity with bases reduces its uses with basic drugs that need to be extracted at high pH.
- Di-isopropyl ether is less miscible with water than di-ethyl ether but is much more likely to form explosive peroxides and is best avoided.
- Diethyl ether is a good, volatile solvent but it is quite soluble (~4%) in water and difficult to blow to complete dryness.

3.5 Mixed solvents

In some cases pure solvents will not be satisfactory for the extraction of the compound of interest. 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 solve the problem. A 1:1 mixture of tetrahydrofuran and dichloromethane is a powerful solvent for the extraction of polar compounds from aqueous solutions.

3.6 Plasma proteins and emulsions

Presence of proteins can cause difficulties in extracting the drug from plasma. Emulsions are often formed and partial precipitation can unclear the interface between the two layers. The proteins can be precipitated by addition of 10-20% trichloroacetic acid or five volumes of a water-miscible solvent like acetonitrile.

3.7 Role of pH for solvent extraction

Organic acids and bases are usually much less soluble in water than its salts. As a general rule, extraction of bases into an organic solvent should be carried out at high pH usually about 2 pH units above the pKa and extraction of acids carried out at low pH. If the drug is reasonably non-polar base, it could be back extracted from the organic solvent into acid, basified and re-extracted into the organic solvent and vice versa for the acidic drugs.

4. Sample pretreatment in different biological matrices (Horne, 1985; Christians & Sewing, 1989; McDowall *et al.*, 1989; Ingwersen, 1993; Krishnan & Ibrahim 1994; Simmonds *et al.*, 1994; Allanson *et al.*, 1996; Plumb *et al.*, 1997)

4.1 General concern with biological samples

Extraction of biological samples before injection into an HPLC system serves a number of objectives.

- Concentration
- Clean-up
- Prevention of clogging of analytical columns

- Elimination of protein binding
- Elimination of enzymatic degradation of the analyte

4.2 Serum, plasma, and whole blood

Serum and plasma samples may not need to be pretreated for SPE. In many cases, however, analytes such as drugs may be protein-bound, which reduces SPE recoveries. To disrupt protein binding in these biological fluids, use of one of the following methods for reversed phase or ion exchange SPE procedures.

- Shift pH of the sample to extremes (pH<3 or pH>9) with acids or bases in the concentration range of 0.1M or greater. Use the resulting supernatant as the sample for SPE
- Precipitate the proteins using a polar solvent such as acetonitrile, methanol, or acetone (two parts solvent per one part biological fluid is typical). After mixing and centrifugation, remove the supernatant and dilute with water or an aqueous buffer for the SPE procedure
- To precipitate proteins, treat the biological fluid with acids or inorganic salts, such as formic acid, perchloric acid, trichloroacetic acid, ammonium sulfate, sodium sulfate, or zinc sulfate. The pH of the resulting supernatant may be adjusted prior to use for the SPE procedure
- Sonicate the biological fluid for 15 minutes, add water or buffer, centrifuge, and use the supernatant for the SPE procedure

4.3 Urine

Urine samples may not require pretreatment for reversed phase or ion exchange SPE, but often is diluted with water or a buffer of the appropriate pH prior to sample addition. In some cases, acid hydrolysis (for basic compounds) or base hydrolysis (for acidic compounds) is used to ensure that the compounds of interest are freely solvated in the urine sample. Usually a strong acid (e.g. concentrated HCl) or base (e.g. 10M KOH) is added to the urine. The urine is heated for 15- 20 minutes, then cooled and diluted with a buffer, and the pH adjusted appropriately for the SPE procedure. Enzymatic hydrolysis that frees bound compounds or drugs also may be used.

4.4 Solid samples

Solid samples ex. tissues, faeces are normally homogenized with a buffer or an organic solvent, then remaining solids removed by centrifugation, and the diluted sample applied to the cartridge.

5. Methods of extraction

The aim of the sample preparation process is to provide a suitable sample, usually for chromatographic analysis, which will not contaminate the instrumentation and where the concentration in the prepared sample is reflective of that found in the original. The method of sample preparation selected is generally dictated by the analytical technique available and the physical characteristics of the analytes under investigation (Watt *et al.*, 2000). The two main

sample preparation methods are matrix cleanup or direct injection. In a matrix cleanup procedure, the aim is to remove as much endogenous material as possible from the drug sample.

Sample preparation is traditionally carried out (a) by liquid-liquid extraction, (b) solid-phase extraction or (c) by precipitation of the plasma proteins, while the final analysis in most cases is accomplished by liquid chromatography interfaced with mass spectrometry or tandem mass spectrometry or capillary gas chromatography.

5.1 Liquid-Liquid Extraction (LLE) (Christian & O'Reilly, 1986; Harris, 1994; Majors & Fogelman, 1993; Wells, 2003)

One of the most useful techniques for isolating desired components from a mixture is liquid-liquid extraction (LLE). LLE is a method used for the separation of a mixture using two immiscible solvents. In most LLEs, one of the phases is aqueous and the other is an immiscible organic solvent. 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. Selective partitioning of the compound of interest into one of two immiscible or partially miscible phases occurs by the proper choice of extraction of solvent. 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. First, the component mixture is dissolved in a suitable solvent and a second solvent that is immiscible with the first solvent is added. Next, the contents are thoroughly mixed (shaking) and the two immiscible solvents allowed separating into layers. The less dense solvent will be the upper layer, while the more dense solvent will be the lower layer. The components of the initial mixture will be distributed amongst the two immiscible solvents as determined by their partition coefficient. The relative solubility that a compound has in two given solvents can provide an estimation of the extent to which a compound will be partitioned between them. A compound that is more soluble in the less dense solvent will preferentially reside in the upper layer. Conversely, a compound more soluble in the more dense solvent will preferentially reside in the lower layer. Lastly, the two immiscible layers are separated, transferred and the component in that solvent is isolated. Generally after extraction hydrophilic compounds are seen in the polar aqueous phase and hydrophobic compounds are found mainly in the organic solvents. Analyte is extracted into the organic phase are easily recovered by evaporation of the solvent, the residue reconstituted with a small volume of an appropriate solvent preferably mobile phase while analyte extracted in to the aqueous phase can be directly injected into a RP column. LLE technique is simple, rapid is relative cost effective per sample as compared to other techniques and near quantitative recoveries (90%) of most drugs can be obtained by multiple continuous extraction (Fig.1).

Several useful equations can help illustrate the extraction process. The Nernst distribution law states that any neutral species will distribute between two immiscible solvents so that the ratio of the concentration remains constant.

$$K_D = C_o / C_{aq}$$

Where K_D is the distribution constant, C_o is the concentration of the analyte in the organic phase, and C_{aq} is the concentration of the analyte in the aqueous phase.

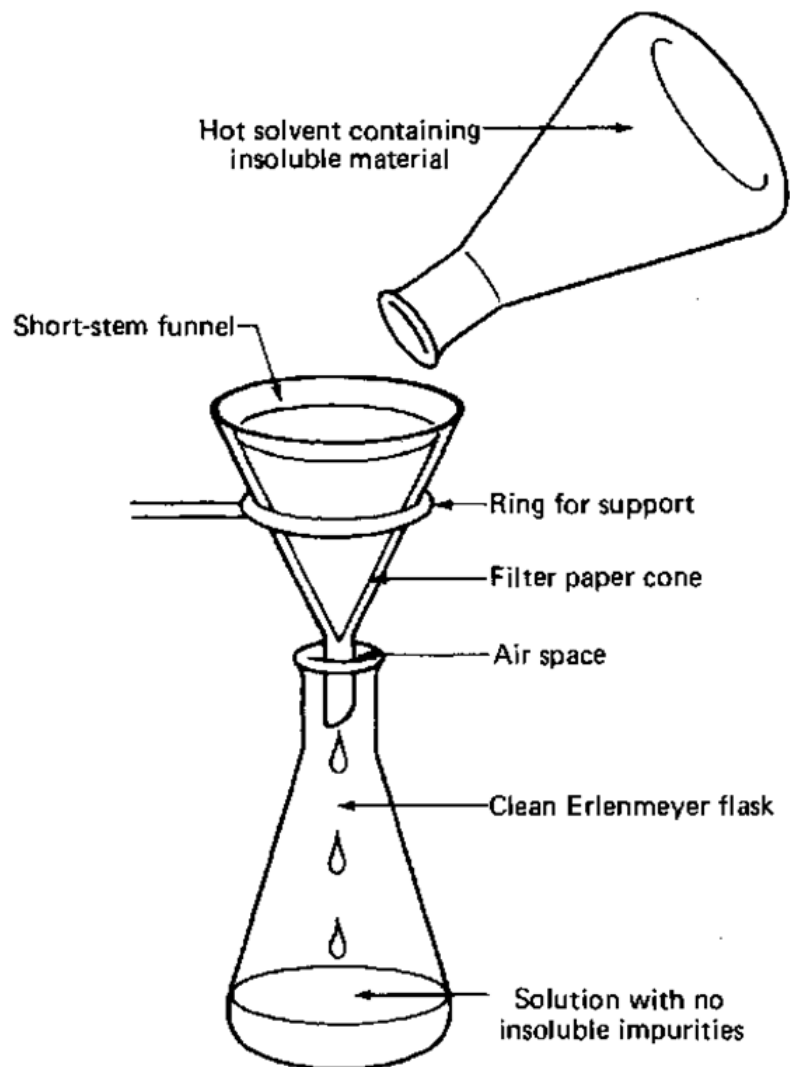


Fig. 1. Gravity Filtration Setup

To increase the value of K_D , several approaches may be used:

- The organic solvent can be changed to increase solubility of the analyte
- If the analyte is ionic or ionizable, its K_D may be increased by suppressing its ionization to make it more soluble in the organic phase. It can also be extracted into the organic phase by the formation of an ion pair through the addition of a hydrophobic counter-ion.
- Metal ions can form a complex with hydrophobic complexing agents.
- The salting out effect can be used to decrease analytes concentration in the aqueous phase.

If the K_D value is unfavorable, additional extraction may be required for better solute recovery. In this case, a fresh portion of immiscible solvent is added to extract additional solute. Normally, the two extracts are combined. Generally, for a given volume of solvent, multiple extractions are more efficient in removing a solute quantitatively than a single extraction. Sometimes, back extractions can be used to achieve a more complete sample cleanup.

If K_D is very low or the sample volume is high, it becomes nearly impossible to carry out multiple simple extractions in a reasonable volume. Also, if the extraction rate is slow, it may take a long time for equilibrium to be established. In these cases, continuous liquid-liquid extraction is used, where pure solvent is recycled through the aqueous phase.

Benefit of this technique is that, with a judicious choice of solvents and pH, very clean extracts can be obtained with good selectivity for the targeted analyte. The drug is extracted from the aqueous phase to the organic phase. One point of note is that LLE system unlike solid-phase systems, are more likely to give consistent results year after years, as there is usually less batch to batch variation with solvents.

The extraction of drug from the aqueous phase is mainly depends on the following factors:

- Solubility of analyte in the organic solvent
- Polarity of the organic solvent
- pH of the aqueous phase

In some cases there is a possibility that interferences may present in the extracted sample. In that case back liquid-liquid extraction can be performed, this gives a clear extracts. Here two times organic solvent is used for the extraction of analyte from the matrix. Often, however, it is not possible to find the optimum condition that provides both high recovery and purity of the analyte in one extraction step. Low recoveries may require further extraction to achieve acceptable value. About the purity it may require second extraction procedure with different solvent or pH of the aqueous phase. Each successive extraction increase the analytical time, also the resulting large volume of extraction solvent must be evaporated to recover the product. If extraction requires many steps, techniques such as Craig Counter Current distribution can be used to increase recovery and purity. However this technique increases the cost and time of the analysis.

5.1.1 Selection of the solvent

There are also practical concerns when choosing extraction solvents. As mentioned previously, the two solvents must be immiscible. The properties of an ideal solvent is that it should withdraw the active agent from a mixture by liquid-liquid extraction are

- Selectivity: Only the active agent has to be extracted and no further substances which mean that a high selectivity is required.
- Capacity: To reduce the amount of necessary solvent, the capacity of the solvent has to be high.
- Miscibility: To achieve simple regeneration of the solvent the miscibility of solvent and primary solvent has to be low.
- Difference in density: After extraction, the two phases have to be separated in a separator and for this a high positive difference in density is required.
- Optimal surface tension: σ low \rightarrow low amount of energy for dispersing required; if surface tension < 1 mN/m stable emulsions are produced; $\sigma > 50$ mN/m \rightarrow high amount of energy for dispersing and high tendency to coalesce.
- Recovery: The solvent has to be separated from the extract phase easily to produce solvent free active agents.

- Corrosion: If the solvent is corrosive prices for construction increases.
- Low price
- No or low toxicity and not highly flammable
- Flame temperature: 25°C higher than operating temperature
- Vapour pressure: To prevent loss of solvent by evaporation a low vapour pressure at operating temperature is required.
- Viscosity: A low viscosity of the solvent leads to low pressure drop and good heat and mass transfer.
- Chemical and thermal stability
- Environmentally acceptable or easily recoverable
- Convenient specific gravity
- Suitable volatility
- High chemical stability and inertness
- Not prone to form an emulsion
- Dissolves the neutral but not the ionized form of the analyte

The stoichiometric ratio of the analyte in the organic phase compared to that in the aqueous phase is known as the distribution ratio D . Ideally, this ratio should approach 100% in order to minimize the losses through the effects of small changes in sample composition, temperature and pH. Reproducibility also increases with increasing extraction efficiency, although a consistent low recovery may be acceptable if an internal standard is used to compensate for changes in efficiency.

5.1.2 Extraction under basic and acidic conditions

As mentioned above, the ability to separate compounds of a mixture using liquid-liquid extraction procedures depends upon the relative solubility that each compound has in the two immiscible solvents. A change in the pH of the solvent (the addition of acid or base) can change the solubility of an organic compound in a solvent considerably.

Liquid/liquid extraction is the most common technique used to separate a desired organic product from a biological matrix. The technique works well if your target compound is more soluble in one of two immiscible solvents. Extraction usually involves shaking a solution that contains the target with an immiscible solvent in which the desired substance is more soluble than it is in the starting solution. Upon standing, the solvents form two layers that can be separated. The extraction may have to be repeated several times to effect complete separation.

In general liquid-liquid extractions can separate four different classes of compounds:

- a. **Organic bases:** Any organic amine can be extracted from an organic solvent with a strong acid such as 1M hydrochloric acid
- b. **Strong acids:** Carboxylic acids can be extracted from an organic solvent with a weak base such as 1M sodium bicarbonate
- c. **Weak acids:** Phenols can be extracted from an organic solvent with a strong base such as 1M sodium hydroxide
- d. **Non-polar compounds** stay in the organic layer

5.1.3 Disadvantages

- Large solvent consumption is needed for extraction of drug.
- LLE is time consuming process when compare to other methods.
- LLE require an evaporation step prior to analysis to remove excess of organic solvent.
- LLE technique is not a suitable one for the estimation of several analytes.
- Emulsion formation may be possible when two immiscible phases were used in the extraction procedure.

5.2 Solid Phase Extraction (Zwir Ferenc & Biziuk, 2006; James, 2000; Krishnan & Abraham, 1994; Moors *et al.*, 1994; Plumb *et al.*, 1997; Arthur & Pawliszyn, 1990; Zief & Kiser, 1990; MacDonald & Bouvier, 1995; Wells, 2003; Scheurer & Moore, 1992)

Since the 70's SPE has become a common and effective technique for extracting analytes from complex samples. Solid phase extraction is the very popular technique currently available for rapid and selective sample preparation. Many sample preparation methods today rely on solid-phase extractions, an advantage being that SPE is amenable to automation and parallel processing. SPE evolved to be a powerful tool for isolation and concentration of trace analysis in a variety of sample matrices. The versatility of SPE allows use of this technique for many purposes, such as purification and trace enrichment (Rawa *et al.*, 2003).

The objectives of SPE are to reduce the level of interferences, minimize the final sample volume to maximize analyte sensitivity and provide the analyte fraction in a solvent that is compatible with the analytical measurement techniques. As an added benefit, SPE serves as a filter to remove sample particulates.

The degree of enrichment achievable for a particular sample is dependent upon:

- a. The selectivity of the bonded phase for the analyte
- b. The relative strength of that interaction

SPE prepares multiple samples in parallel (typically 12-24) and uses relatively low quantities of solvents and the procedures can be readily automated. As the name implies the principle of SPE is an extraction technique similar to that of LLE, involving a partitioning of solutes between two phases. However, instead of two immiscible liquid phases, as in LLE, SPE involves partitioning between a liquid (sample matrix or solvent with analytes) and a solid (sorbent) phase (Table 1).

SPE is a more efficient separation process than LLE, easily obtains a higher recovery of analyte by employing a small plastic disposable column or cartridge, often the barrel of a medical syringe packed with 0.1 to 0.5 g of sorbent which is commonly RP material (C₁₈-silica). The components of interest may either preferentially adsorbed to the solid, or they may remain in the second non-solid phase. Once equilibrium has been reached, the two phases are physically separated by decanting, filtration, centrifugation or a similar process. If the desired analyte is adsorbed on the solid phase, they can be selectively desorbed by washing with an appropriate solvent. If the component of interest remains in a liquid phase, they can be recovered via concentration, evaporation and or recrystallization. When SPE is performed in this single step equilibrium batch mode, it will be similar to LLE, where the solid sorbent simply replaces one of the immiscible liquids. By passing a liquid or gas

through the uniform solid bed, the liquid solid phase extraction technique becomes a form of column chromatography, now commonly called phase extraction that is governed by liquid chromatographic principle.

Polarity	Solvent	Miscible in Water?		
Non-polar ↓	Strong Reversed Phase	Hexane	No	
	↑	Isooctane	No	
		Weak Normal Phase	Carbon tetrachloride	No
	↓	Chloroform	No	
		Methylene Chloride	No	
		Tetrahydrofuran	Yes	
		Diethyl ether	No	
		Ethyl acetate	Poorly	
		Acetone	Yes	
		Acetonitrile	Yes	
	Polar	Weak Reversed Phase	Isopropanol	Yes
		Strong Normal Phase	Methanol	Yes
			Water	Yes
	Acetic Acid	Yes		

Table 1. Characteristics of Solvents Commonly Used in SPE

Liquid samples are added to the cartridge and wash solvent is selected to either strongly retain or un-retain the analyte. Interferences are eluted or washed from the cartridge, even as the analyte is strongly retained, so as to minimize the presence of interferences in the final analyte fraction, the analyte is then eluted with a strong elution solvent, collected and either injected directly or evaporated to dryness followed by dilution with the HPLC mobile phase. Conversely, when interferences are strongly held in the cartridge, the analyte can be collected for the further treatment. Advantages of SPE over LLE include a more complete extraction of the analyte, more efficient separation of interferences from analyte, reduced organic solvent consumption, easier collection of the total analyte fraction, more convenient manual procedures, better removal of particulates and more easy automation.

5.2.1 Mechanism of Solid Phase Extraction process (Font *et al.*, 1993; Sabik *et al.*, 2000)

The ideal process is to create a digital chromatography system on the SPE cartridge such that the analyte is at first fully bound, then the interferences are completely eluted, and then the analyte is entirely eluted; it is either all on or all off the cartridge. The separation mechanism is the function due to the intermolecular interactions between analyte and the functional groups of the sorbent.

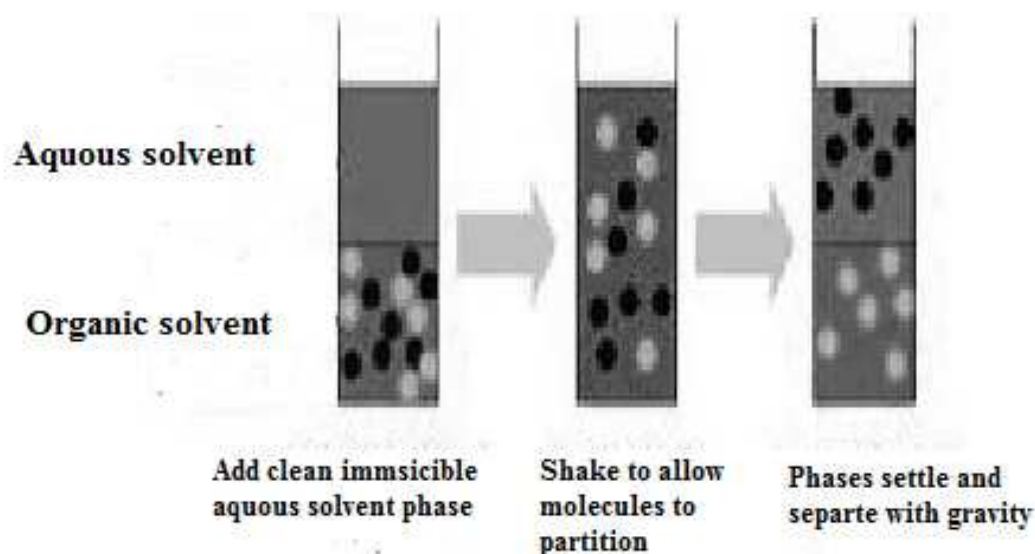


Fig. 2. Partitioning of drugs towards aqueous and organic solvent

The selection of an appropriate SPE extraction sorbent depends on understanding the mechanism(s) of interaction between the sorbent and analyte of interest. That understanding in turn depends on knowledge of the hydrophobic, polar and inorganic properties of both the solute and the sorbent. The most common retention mechanisms in SPE are based on van der Waals forces (“non-polar interactions”), hydrogen bonding, dipole-dipole forces (“polar” interactions) and cation-anion interactions (“ionic” interactions).

Each sorbent offers a unique mix of these properties which can be applied to a wide variety of extraction problems (Fig 2). Four general theory interactions exist (Yu *et al.*, 2004):

- a. **Reversed phase** involves a polar or moderately polar sample matrix (mobile phase) and a non-polar stationary phase. The analyte of interest is typically mid- to non- polar.

Retention occurs via non-polar interaction between carbon-hydrogen bonds of the analyte and carbon-hydrogen bonds of the sorbent function groups due to Van der Waals or dispersion forces.

The materials that are used as reversed phases are carbon-based media, polymer-based media, polymer-coated and bonded silica media.

Carbon-based media consist of graphitic, non-porous carbon with a high attraction for organic polar and non-polar compounds from both polar and non-polar matrices. Retention of analytes is based primarily on the analyte’s structure, rather than on interactions of functional groups on the analyte with the sorbent surface.

Polymer-based sorbents are styrene/divinylbenzene materials. It is used for retaining hydrophobic compounds which contain some hydrophilic functionality, especially aromatics.

Polymer-coated and bonded silica media is hydrophobic-bonded silica that is coated with a hydrophilic polymer. The pores in the polymer allow small, hydrophobic organic

compounds of interest (e.g. drugs) to reach the bonded silica surface, while large interfering compounds (e.g. proteins) are shielded from the bonded silica by the polymer and are flushed through the SPE tube.

Several SPE materials, such as the alkyl- or aryl-bonded silicas (LC-18, ENVI-18, LC-8, ENVI-8, LC-4, and LC-Ph) are in the reversed phase category.

- b. **Normal phase** involve a polar analyte, a mid- to non-polar matrix (e.g. acetone, chlorinated solvents and hexane) and a polar stationary phase. 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. Ex. Hydrogen bonding, dipole-dipole, induced dipole-dipole and pi-pi.

These phases can offer a highly selective extraction procedure capable of separating molecules with very similar structures. The main drawback is that the analyte must be loaded onto the sorbent in a relatively non-polar organic solvent such as hexane.

Polar-functionalized bonded silicas (e.g. LC-CN, LC-NH₂, and LC-Diol), and polar adsorption media (LC-Si, LC-Florisil, ENVI-Florisil, and LC-Alumina) typically are used under normal phase conditions.

- c. The **bonded silicas** have short alkyl chains with polar functional groups bonded to the surface. These silicas, because of their polar functional groups, are much more hydrophilic relatively to the bonded reversed phase silicas.

The bonded silicas LC-CN, LC-NH₂, and LC-Diol - have short alkyl chains with polar functional groups bonded to the surface.

- d. **Ion exchange SPE** can be used for compounds that are in a solution. Anionic (negatively charged) compounds can be isolated on an aliphatic quaternary amine group that is bonded to the silica surface. Cationic (positively charged) compounds are isolated by using the silica with aliphatic sulfonic acid groups that are bonded to the surface.

Biofluids can usually be applied directly to ion-exchange sorbents following dilution of the sample with water or a buffer, and possibly adjustment of pH. However, elution from strong ion-exchange sorbents can be a problem as high ionic strength or extremes of pH, may be required which may affect analyte stability or further processing of sample.

Anionic (negatively charged) compounds can be isolated on **LC-SAX** or **LC-NH₂** bonded silica cartridges. Cationic (positively charged) compounds are isolated by using **LC-SCX** or **LC-WCX** bonded silica cartridges.

5.2.2 General properties of bonded silica sorbents

Although other materials are available ex. polymeric resins and alumina, the vast majority of SPE extractions are carried out by using bonded silica materials similar to those used in HPLC columns except the particle size and diameter. Bonded silica materials are rigid and do not shrink or swell like polystyrene-based resins in different solvents. Use of too high flow rate when processing cartridges may affect retention of analytes, particularly during the sample loading and elution steps. Potentially the capacity of the cartridge will be affected by all the components from the sample not only the analytes of interest.

5.2.3 Steps of Solid Phase Extraction

Generally following steps are followed for developing the method for extracting the analyte from plasma (Fig 3).

- Pretreatment of sample - which includes dilution of sample or pH adjustment, filtration to avoid the blocking of the SPE cartridge and for better adsorption.
- Conditioning of the cartridge - which is the main step in case of reverse phase SPE cartridges. Preconditioning is mainly done by solvent such as methanol, acetonitrile, isopropyl alcohol or tetrahydrofuran which is necessary to obtain reproducible result. Without this step, a highly aqueous solvent cannot penetrate the pores and wet the surface. Thus, only a small fraction of the surface area is available for interaction with the analyte. For the same reason, it is important not to let the cartridge dry out between the salvation step and addition of the sample.
- Loading the sample - Sample size must be scaled to suit the size of the cartridge bed. A typical reverse phase cartridge may have capacity for up to 100 mg of very strongly retained substances.
- Wash - very important step in case of the sample treatment by SPE. In this step a suitable solvent or water mixture is passed through SPE bed to remove the contaminants.
- Elution of fraction - in this a suitable solvent or buffer is used to elute the analyte from the SPE bed for analysis.

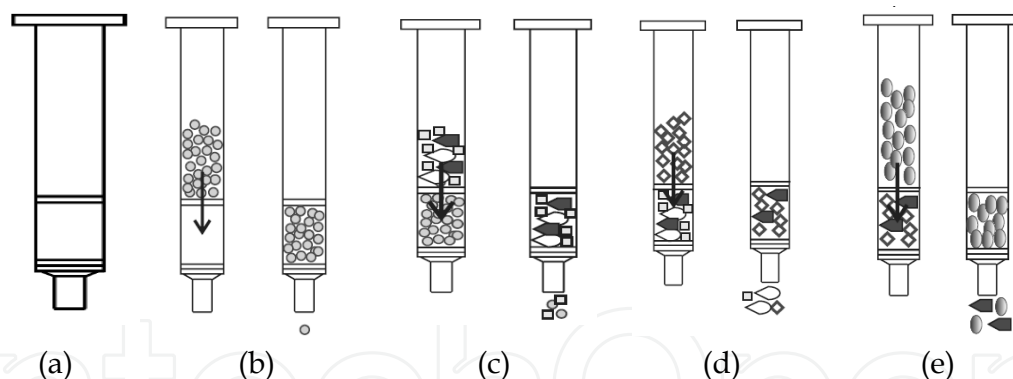


Fig. 3. Five steps of SPE: (a) selection of tube, (b) conditioning of tube, (c) addition of sample, (d) washing and (e) elution

5.2.4 A strategy for method development for plasma samples

- Rationale
- Practical consideration
- Pretreatment of samples and cartridges
- Screening sorbents and pH values
- Optimizing the washing procedure
- Optimizing the elution procedure
- Final optimization and simplification
- Strategies for removing persistent interferences

5.2.5 Developing SPE methods

In developing a SPE method, the properties of the isolate, sorbent and the matrix should be considered. The mechanisms of retention and the various secondary interactions may occur, and that a selective extraction from biofluid also requires the sorbent not to extract a large number of unknown compounds from the matrix. The best conditions are not easily predicted and the method needs to be developed experimentally in combination with the analytical method being used.

The following stages are recommended for method development.

- a. Consider physic-chemical properties of analyte, nature of matrix and known chromatographic properties of analytes and the possible interaction with SPE sorbents.
- b. Screen a range of cartridges (ex. C₁₈, C₈, C₂, Ph, CBA, SCX, SAX, PRS, NH₂, DEA, CH, Si and polymeric sorbent) under simple conditions (ex. from aqueous buffer solutions) looking for good retention of the analyte. If radiolabelled analyte is available this can conveniently be used to track analyte in such screening experiments. This experiment should not only identify likely cartridges for use in the assay, but should be used to try to confirm possible mechanism of interaction between the analyte and sorbent.
- c. Select a more limited number of sorbents and examine conditions for loading/wash/elution (consider if pH control is needed, possible strength (% organic solvent) of wash solvents). Try the extraction from bio-fluid and select sorbent for final development.
- d. Final development of extraction conditions and chromatographic analysis. Consider the robustness of the assay when finalizing extraction conditions. Ex. Do not select a wash solvent where a small change in composition could lead to elution of analyte from the cartridge.

The choice of different cartridges, manufactures and formats is becoming so extensive that it can appear almost overwhelming. It is generally better to build experience with a more limited set of sorbents, perhaps concentrating on cartridges from only one manufacturer. Also concentrate on investigating the use of cartridges with different functional groups (i.e. test C₁₈, C₈, C₂, Ph, CBA, SCX, SAX, PRS, NH₂, DEA, CH, Si, etc.), and those that use a contrasting mechanism to the analytical separation.

5.2.6 Characteristic features of SPE

- Complete flexibility
- Longer column lifetimes
- Powerful contaminant removal
- Greater recovery
- Better reproducibility
- More sensitivity

5.2.7 Advantages of SPE over LLE

- In SPE by choosing selective adsorbent analyte can be driven completely by adsorption and desorption
- In single stage LLE each extraction step equivalent to one chromatographic plate on the other hand by SPE in single step one can generate 10-50 plates

- Higher plate numbers in SPE leads to higher recoveries and purer of the analyte as compared to LLE
- For extracting more than one component from a mixture of component of different desorption solvent are required in case of SPE. To achieve similar results with LLE, one must perform several liquid extractions
- SPE is less time consuming and not tedious as compare to LLE

5.2.8 Limitation

- Depending on the nature of the analyte, SPE may not always be the method of choice, and liquid-liquid extraction may be a more viable solution.

6. Protein precipitation method (Backes, 2000; Wells, 2003)

This method is least one in bioanalytical. This is a very simple technique for extraction of the analyte from the matrix. If protein binding is suspected, then protein precipitation prior to sample extraction may be considered. Reagents to evaluate include perchloric, trichloroacetic and tungstic acids, and organic solvents such as acetonitrile or methanol. With all of these it is necessary to bear in mind the ability of the analytes and the matrix requirements of the extraction procedures. If protein binding is believed to be through a covalent linkage, then there is very little change of breaking it since this is the strongest of the intermolecular forces.

The main requirement for this technique is that the analyte should be freely soluble into reconstituting solvent. Preparation of sample through protein precipitation achieves separation by conversion of soluble proteins to an insoluble state by salting out or by addition of water miscible precipitation solvent or organic solvents such as acetone, ethanol, acetonitrile or methanol. Ideally, precipitation results in both concentration and purification, and is often used early in the sequence of downstream purification, reducing the volume and increasing the purity of the protein prior to any chromatography steps. In addition, precipitating agents can be chosen that provide a more stable product than found in the soluble form.

Proteins might stick to each other through one of three forces: electrostatic, hydrophobic, and van der Waals. The last one is difficult to distinguish from hydrophobic and operates over only in a very short range. Electrostatic forces operate at long range, but between like molecules are repulsive rather than attractive, so molecules have the same charge and repel each other.

Proteins are made insoluble by altering their surface properties, charge characteristics or changing the solvent characteristics; but changing the solvent characteristics being preferred. Greater the initial concentration of the desired protein, greater is the efficiency of precipitation; proteins are least soluble as its isoelectric point (pI) ranges from 4 - 10. The selection of a buffer at or near the pI of the protein is recommended. However some proteins may denature at their pI and above the pI. The solubility of a protein increases with the addition of salt and reaches a maximum after which there is a rapid linear decrease in solubility. There are several methods to reduce the solubility of proteins, which are ionic precipitation ex. ammonium sulphate, sodium chloride; metal ions ex. Cu^{+2} , Zn^{+2} and Fe^{+2} ;

non-ionic polymers ex. polyethylene glycol; organic solvents ex. ethanol, acetone; tannic acids, heparin, dextran sulphates, cationic polyelectrolytes ex. protamines; short chain fatty acids ex. caprylic acid; trichloroacetic, lecithins ex. concanavalin A and group specific dyes ex. procion blue. The use of temperature, pH or organic solvents can lead to denaturation and should be performed with care to minimize any decrease in yield or activity.

6.1 Type of protein precipitation

Salting out: Ammonium sulphate is the salt usually used for salting out, because of its high solubility and high ionic strength (which is proportional to the square of the charge on the ion, so that the ionic strength of 1M $(\text{NH}_4)_2\text{SO}_4$ is 3 times that of 1M NaCl). Neither ion associates much with proteins, which is good since such association usually destabilizes proteins. Its solubility changes little with temperature, it is cheap, and the density of even a concentrated solution is less than that of protein, so that protein can be centrifuged down from concentrated solutions.

Solvent Precipitation: When large amounts of a water-miscible solvent such as ethanol or acetone are added to a protein solution, proteins precipitate out. The conventional wisdom is that this is due to decrease of the dielectric constant, which would make interactions between charged groups on the surface of proteins stronger. Water miscible solvents associates with water much more strongly than do proteins, so that its real effect is to dehydrate protein surfaces, which then associate by van der Waals forces, at least if they are isoelectric or reasonably close to it. Removal of water molecules from around charged groups would also deshield them and allow charge interactions to occur more strongly, if there are areas of opposite charge on the surfaces of two proteins.

In practice, solvent precipitation is usually performed at low temperature. The condition for the protein is at 0°C and the solvent colder, -20°C in an ice-salt bath, because proteins tend to denature at higher temperatures though if sufficient control can be achieved and your protein is more stable than others, this can be selective and achieve greater purification.

Solvent precipitation can be done with polyethylene glycol at concentrations between 5 and 15%. It probably works the same way, by competing with the protein for water, but is less likely to inactivate the protein and does not require such low temperatures, but it tends to give an oily precipitate.

Commonly the sample is centrifuged at high speed for sufficient time, all the precipitated components of plasma will be settled at the bottom and clear supernatant liquid will be separated out. The obtained supernatant liquid can be injected directly into the HPLC or it can be evaporated and reconstituted with the mobile phase and further clean up of the sample can be carried out by using micro centrifuge at very high speed.

6.2 Advantage

- Now protein precipitation plates are available, able to remove the unwanted plasma proteins from plasma fluid samples prior to analysis
- Protein precipitation plates can be used in a wide range of aqueous and organic sample preparation including total drug analysis and sample preparation prior to HPLC or LC-MS/MS

- Protein precipitation plates are compatible with small volume of solvent
- Protein precipitation plate contains hydrophobic PTFE membrane as a prefilter removes the unwanted precipitated proteins prior to analysis
- Traditionally in this method plasma is mixed with protein precipitating agent and diluting solvent then the whole mixture is vortex, mixed, centrifuge and filter
- By using the new protein precipitate filter plate, precipitating solvent is added first followed by the plasma sample. This method does not require any mixing. Generally these plates are fitted to 96 well extraction plates. This new process showed 90% removal of plasma proteins when compare to the old method 60-65%

6.3 Disadvantage

- May increase the back pressure of the HPLC system
- Some components of plasma which are soluble in diluting solvent that bound to stationary phase permanently that will affect the column performance.

7. Solid Phase Microextraction (SPME) (Pawliszyn *et al.*, 1997; Pawliszyn, 1995, 1998, 1999, 2003; Wercinski 1999)

Miniaturization of sorbent technology and the concomitant decrease in solvent purchase, exposure and disposal has also taken a further giant step with the development of SPME. Solid-Phase Microextraction (SPME) is a very simple and efficient, solventless sample preparation method. SPME integrates sampling, extraction, concentration and sample introduction into a single solvent-free step. In this technique a fused silica fiber coated with polyacrylate, polydimethylsiloxane, carbowax or other modified bonded phase is placed in contact with a sample, exposed to the vapour above a sample (solid, liquid) or placed in the stream of a gaseous sample to isolate the analyte and concentrate analytes into a range of coating materials. After extraction, the fibers are transferred with the help of syringe like handling device to analytical instrument like gas chromatography (GC) and GC/mass spectrometry (GC/MS) for separation and quantification of the target analyte. In such a manner SPME has been used frequently to analyze volatile and semi-volatile compounds and may be used to purge and trap procedures. The method saves preparation time and disposal costs and can improve detection limits. The most interesting aspect of this technology involves the ability to use the exposed fiber as extraction and sample delivery device. Phases for SPME are available in a range of polarities and properties for analyses of volatile and semi-volatile compounds as well as application to extraction of analytes from liquid samples. One of the limitations of SPME is the low capacity of the fiber and the perturbation of equilibria that can occur in the presence of sample components or analytes at very high concentration versus those of lesser concentration. Dilution of the sample can overcome some of these problems but not all, as limits of detection for trace analytes are compromised. Formaldehyde, Triton X-100, Phenylurea, pesticides and amphetamines are some of the analytes which are successfully extracted using SPME.

7.1 SPME basics (Vas & Vekey, 2004)

The concept of SPME may have been derived from the idea of an immersed GC capillary column. The SPME apparatus looks like modified syringe containing a fiber holder and a fiber assembly, the latter containing a 1-2 cm long retractable SPME fiber. The SPME fiber

itself is a thin fused-silica optical fiber, coated with a thin polymer film conventionally used as a coating material in chromatography. In SPME the amount of extraction solvent is very small compared to the sample volume. As a result, exhaustive removal of analytes to the extracting phase does not occur; rather equilibrium is reached between the sample matrix and the extracting phase. In practical, the extracting phase is a polymeric organic phase commonly poly(dimethylsiloxane) and polyacrylate which is permanently attached to rod. The rod consists of an optical fiber made of fused silica, which is chemically inert. A polymer layer is used to protect the fiber against breakage. Poly(dimethylsiloxane) behaves as a liquid, which results in rapid extraction compared to polyacrylate, which is a solid. When the coated fiber is placed into an aqueous matrix the analyte is transferred from the matrix into the coating. The extraction is considered to be complete when the analyte has reached an equilibrium distribution between the matrix and fiber coating. The amount of extracted analyte is independent of the volume of the sample. Therefore, there is no need to collect a defined amount of sample prior to analysis. Thus, the fiber can be exposed directly to the ambient air, water, production stream, etc., and the amount of extracted analyte will correspond directly to its concentration in the matrix.

7.2 Principle modes of SPME

7.2.1 Direct extraction

In the direct-extraction mode, the coated fiber is inserted directly into the sample, and analytes are extracted directly from the sample matrix to the extraction phase. For gaseous samples, natural air convections and high diffusion coefficients are typically sufficient to facilitate rapid equilibration. For aqueous matrices, more efficient agitation techniques are required, such as forced flow, rapid fiber or vial movement, stirring or sonication.

7.2.2 Headspace configuration

In the headspace mode, the vapor above the bulk matrix is sampled. Thus, analytes must be relatively volatile in order to be transported from the bulk matrix to the fiber coating.

7.2.3 Advantages

Sampling of the headspace protects the fiber coating from damage by hostile matrices, such as those at very high or low pH, or those with large molecules, such as proteins which tend to foul the coating.

7.2.4 Types of extraction in SPME

- Fiber extraction
- In-tube extraction
- Stir BAR sorptive extraction (SBSE)

7.2.5 Advantage of SPME

- In SPME volatile, semi-volatile and non-volatile organic and inorganic analytes can be used for analyzed

- During desorption of the analyte, the polymeric phase is cleaned and therefore ready for reuse. The absence of solvent in SPME is an important feature, as it is not only environmentally friendly but makes the separation faster
- Important feature of SPME is its small size, which is convenient for designing portable devices for field work
- Solvent free environment, fast extraction, convenient automation and easy hyphenation with analytical instrument

8. Matrix Solid-Phase Dispersion (MSPD) (Barker *et al.*, 1989, 1993; Walker *et al.*, 1993)

Matrix solid phase dispersion is a sample preparation technique for use with solid sample matrices. MSPD is a microscale extraction technique, typically using less than 1g of sample and low volumes of solvents. It has been estimated to reduce solvent use by up to 98% and sample turnaround time by 90%.

Conventional extraction of organic analytes from tissue usually begins with a homogenization of a small amount of sample tissue with bulk bonded silica based sorbent in a pestle and mortar. The mechanical shearing forces produced by the grinding process disrupt the structure of the tissue, dispersing the sample over the surface of the support sorbent by hydrophilic and hydrophobic interaction which produces the mixture to become semi-dry and free-flowing, and a homogenous blend of sample. The bound solvent in the sorbent will aid complete sample disruption during the sample blending process and the sample disperses over the surface of the bonded phase-support material to provide a new mixed phase for isolating analytes from various sample matrices. The blend is then transferred into a pre-fitted SPE cartridge and elution of interference compounds and analytes of interest. This technique has recently been applied, using acid alumina, to extract the organic analyte. However, MSPD procedure needs longer analytical time generally and its limit of determination (LOD) is limited.

In method development using MSPD sorbents, the following points are to be considered

- Sample pre-treatment
- Interference elution
- Analyte elution
- Sample clean up

9. Supercritical fluid extraction (Mohamed & Mansorri, 2002; Antero, 2000)

Supercritical fluid extraction is becoming a very popular technique for the removal of non-polar to moderately polar analytes from solid matrices. Supercritical fluids (SCFs) are increasingly replacing the organic solvents that are used in industrial purification and recrystallization operations because of regulatory and environmental pressures on hydrocarbon and ozone-depleting emissions. SCF processes eliminate the use of organic solvents, so it has attracted much attention in the industrial sectors like pharmaceuticals, medical products and nutraceuticals. Pharmaceutical chemists have found SCFs useful for extraction of drug materials from tablet formulation and tissue samples.

Supercritical fluids exhibit a liquid-like density, while their viscosity and diffusivity remain between gas and liquid values. The recovery of a supercritical solvent after extraction can be carried out relatively simply by reducing the pressure and evaporating the solvent. Above the critical temperature the liquid phase will not appear even the pressure is increased. The compressibility of a supercritical fluid just above the critical temperature is large compared to the compressibility of ordinary liquids. A small change in the pressure or temperature of a supercritical fluid generally causes a large change in its density. The unique property of a supercritical fluid is that its solvating power can be tuned by changing either its temperature or pressure. The density of a supercritical fluid increases with pressure and becomes liquid-like, the viscosity and diffusivity remain between liquid-like and gas-like values. Additionally, supercritical fluids exhibit almost zero surface tension, which allows facile penetration into microporous materials leads to more efficient extraction of the analyte than the organic solvents. Carbon dioxide is a relatively good supercritical solvent will dissolve many relatively volatile polar compounds. In the presence of small amounts of polar co-solvents like water and short-chain alcohols to the bulk, the carbon dioxide gas can enhance the solubility of polar, non-volatile solutes in supercritical carbon dioxide. Supercritical fluids can be used to extract analytes from samples.

The main advantages of using supercritical fluids for extractions is that they are inexpensive, extract the analytes faster and more environmental friendly than organic solvents. For these reasons supercritical fluid CO₂ is the reagent widely used as the supercritical solvent.

9.1 Advantages

- SCFs have solvating powers similar to organic solvents, with higher diffusivity, lower viscosity and lower surface tension
- The solvating power can be changed by changing the pressure or temperature for effective extraction
- Separation of analytes from solvent is fast and easy
- Polarity can be changed by using co-solvent leads to have more selective separation of the analyte
- Products are free from residual solvents
- SCFs are generally cheap, simple and safe
- Disposal costs are less
- SCF fluids can be recycled

10. Column switching (Falco *et al.*, 1993, 1994; Henion *et al.*, 1998; Lee & Kim 2011)

Column switching techniques afford an interesting and creative form of sample preparation. This approach depends on the selectivity of appropriately chosen HPLC stationary phase to retain and separate the analyte of interest while allowing unretained components to be eliminated from the column. The benefits of this technique include total automation and quantitative transfer of targeted compounds within the column switching system. In the heart cut mode a narrow retention time region containing a desired components is cut from the chromatogram and transferred onto another HPLC column for further separation. In this

instance, quantitative transfer of the components without adsorptive or degradative losses can be assured.

10.1 Advantages

It is capable of having increased selectivity by the judicious choice of two or more HPLC stationary phases. A limitation of column switching system is that sample throughput will likely not be as high as for other sample clean up methods. A second limitation of the column switching approaches includes restricted sample enrichment because of the limited amount of original, untreated, crude sample that can be loaded onto the first column of the HPLC separation. These limitations can be overcome by combining online SPE and a column switching system. In this method the recovery is more but very expensive one.

11. Future directions

Bio-equivalency is an important one for the Pharmaceutical Formulations especially in the Pharmaceutical Regulatory Market. The availability of methodology for the extraction procedure of the interested analyte coupled along with the analytical method for the quantification of the interested analyte in GC-MS/MS or LC-MS/MS will greatly facilitate future studies to rigorously establish a bio-equivalency of the Pharmaceutical formulations in the Pharmaceutical regulatory market.

12. Conclusion

The subject area of bioanalysis is a sub-discipline of analytical chemistry which covers a very broad range of assays like quantitative measurement of drugs and their metabolites in various biological systems like whole blood, plasma, serum, urine, faeces and tissues. The biological samples cannot be analyzed directly into the analytical system for the quantification of the interested analyte. Estimation of the analyte in the biological matrix can be done after the isolation of the interested analyte from the interferences in the biological sample. Isolation and estimation of the analyte is based on the sample preparation and extraction of the analyte from the biological matrix. The process adopted for the isolation of the interested analyte like sample preparation procedure and isolation steps must ensure the stability of the analyte until the completion of the analytical estimation. 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, toxicokinetics, bioequivalence and exposure-response (pharmacokinetics/pharmacodynamics) studies. In this chapter, various extraction methods of the analyte from the biological matrix have been described with its advantages and disadvantages.

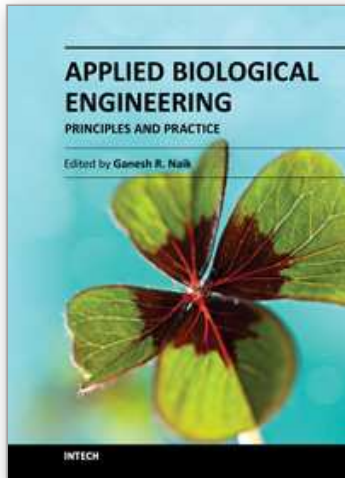
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Biological engineering is a field of engineering in which the emphasis is on life and life-sustaining systems. Biological engineering is an emerging discipline that encompasses engineering theory and practice connected to and derived from the science of biology. The most important trend in biological engineering is the dynamic range of scales at which biotechnology is now able to integrate with biological processes. An explosion in micro/nanoscale technology is allowing the manufacture of nanoparticles for drug delivery into cells, miniaturized implantable microsensors for medical diagnostics, and micro-engineered robots for on-board tissue repairs. This book aims to provide an updated overview of the recent developments in biological engineering from diverse aspects and various applications in clinical and experimental research.

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