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SAMPLE EXTRACTION TECHNIQUES USED IN BIOANALYSIS: A REVIEW

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ABSTRACT

Bio-analysis is part of analytical chemistry which generally involves in the quantitative measurement of drugs and their metabolites in biological systems (Blood, plasma, urine etc.,) Whereas Liquid chromatography mass spectrometry (LCMS) is a technique which is most widely used for the quantification of drug from the biological fluids. This article gives detailed information about Bio-analysis and reviews the advanced sample preparation, Extraction techniques like Liquid-liquid extraction, Solid phase extraction, and protein precipitation.

INTRODUCTION

Bio-analysis is generally employed for the quantitative estimation of drugs and their metabolites in biological fluids and plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic studies. Bio-analytical method validation is a procedure employed to quantify the analyte with a degree of accuracy and precision and also to demonstrate that an analytical method used for quantification of an analyte in a biological matrix is reliable and reproducible to achieve its purpose. Both HPLC and LC-MS-MS can also be used for the bio-analysis of drugs in plasma, but most widely LCMS-MS is used because of its low detection limits, ability to generate structural information, the requirement of minimal sample treatment and also the possibility to cover a wide range of analytes differing in their polarities. Bio-analysis applies to drugs used for illicit purposes, forensic investigations, anti-doping testing in sports, and environmental concerns. Bio-analysis supports Both clinical and non-clinical studies by including a wide range of assays. All the bio-analytical assays involve the use of an internal standard processed along with 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 a sample to sample difference in sample preparation. Often, an analog or similarly behaving compound is used as an internal standard. 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. Biological samples cannot be injected directly into the analyzing system without sample preparation. For sample preparation, sample pre-treatment is very important for the adequate analysis of drugs. However, sample pre-treatment is a time-consuming process and can limit the sample throughput. The proper selectivity can be obtained during the sample preparation,

separation, and detection. A major differentiation between the analyte of interest and the other compounds is often made during the first step. Sensitivity to a large extent is obtained by the detector. Thus, sample pre-treatment is required for achieving sufficient sensitivity and selectivity, but time should be kept minimum in order to obtain adequate speed. Sample preparation 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 and removing the interfering compounds and pre-concentrating the analyte.

EXTRACTION: Extraction is the process of removing an active agent or any waste substance from a solid or liquid mixture with a suitable solvent. The solvent is not or only partially miscible with the solid or the liquid. By intensive contact between the analyte and the extraction medium, this leads the analyte transfers from the solid or liquid mixture into the extraction medium. After thorough mixing, the two phases can be separated either by gravity or centrifugal forces.

Role of Physicochemical properties of the drug in their extraction from Biological sample:

Water miscibility and water immiscibility: Drugs with several aromatic rings will have poor solubility in strong intermolecular dispersive forces of the solid drug will encourage the ready solubility in organic solvents. Commonly alcohols can have hydrogen bonding with water and also dipole-dipole interactions will aid miscibility. 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.

Molecular phenomena for solubility and miscibility: To dissolve a drug, a solvent must break the bonds like an ionic bond, hydrogen bond, and Vander Waals forces which interlinks the compound to its neighbours and must not break substantial intermolecular bonds of the solvent without replacing them with drug solvent interaction. As the breaking of bonds is an endothermic process, it requires energy and causes an increase in enthalpy.

Distribution coefficient: The drug which are in ionized forms are hydrophilic in nature than the unionized form because of the hydration of the ions, therefore the ionized forms are difficult to extract into organic solvents whereas the unionized forms will dissolve in the organic solvents which can be extracted into organic solvents.

Choice of solvent: Several factors need to be considered in choosing a solvent to extract a drug from the biological matrix in addition to its powder to dissolve the required compounds which include selectivity, density, toxicity, volatility, reactivity, and miscibility with aqueous media.

For example, Ethyl acetate is a powerful solvent for many organic compounds and will, therefore, extract a considerable amount of endogenous material with the required drug.

Mixed solvents: Alcohols are excellent solvent but those with lower boiling points are too soluble in Water whereas less miscible one are having high boiling points, but the use of mixed Solvents containing alcohol can solve the problem. In some cases, pure solvents will not be satisfactory for the extraction of the compound of interest.

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.

Plasma proteins and emulsions: The 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 the addition of 10-20% trichloroacetic acid or five volumes of a water-miscible solvent like acetonitrile.

Methods of extraction:

Sample preparation is usually carried out by three extraction methods:

- Liquid-liquid extraction
- Solid-phase extraction (or)
- Protein precipitation

The final analysis is accomplished by liquid chromatography interfaced with mass spectrometry or tandem mass spectrometry (LC-MS/MS).

Liquid-Liquid Extraction: Liquid-liquid extraction is the most widely used technique and is useful for separating analytes from interferences by partitioning the sample between two immiscible liquids or phases. One phase in LLE often is aqueous and second phase an organic solvent. The technique is simple, rapid, and has relatively small cost factor per sample when compared to others. 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 and the two immiscible solvents are separated 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. The extract containing the drug can be evaporated to dryness and the residue can be redissolved in a smaller volume of a more appropriate solvent. The extracted material can be re-dissolved in small volumes (eg: 100 to 500µl of solvent) extending the sensitivity limits of an assay.

ADVANTAGES: It is not possible to extract more than one sample concurrently. LLE technique is simple; rapid is a relatively cost-effective per sample as compared to other techniques and near quantitative recoveries (90%) of most drugs can be obtained by multiple continuous extractions.

DISADVANTAGES: Large solvent consumption is needed for the extraction of drugs.

LLE is a time-consuming process when compared to other methods.

LLE require an evaporation step prior to analysis to remove an excess of organic solvent.

Solid Phase Extraction: SPE has become a common and effective technique for extracting analytes from complex samples.SPE is a more efficient separation process than LLE. It is easier to obtain a higher recovery of analyte SPE occur between a solid phase and a liquid phase. 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. SPE employs a small plastic disposable column or cartridge, often the barrel of a medical syringe packed with 0.1 to 0.5 g of the sorbent. Among the solids that have been used successfully in the extraction of drugs from liquid samples are XAD-2 resin, charcoal, alumina, silica gel, and aluminum silicate. Factors governing the adsorption and elution of drugs from the resin column include solvent polarity, a flow rate of the solvent through the column, and degree of contact the solvent has with the resin beads. Biological samples can be prepared for cleanup by passing the sample through the resin bed where the drug components are adsorbed and finally eluted with an appropriate solvent. Thus, this liquid-solid extraction method provides a convenient isolation procedure for blood samples, thus avoiding solvent extraction, protein precipitation, drug losses, and emulsion formulation.

Steps of Solid Phase Extraction:

The following steps are followed for developing the method for extracting the analyte from plasma

- Pre-treatment of the 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 the SPE process. Preconditioning is mainly done by a solvent such as methanol, acetonitrile, isopropyl alcohol, or tetra hydrofuran which is necessary to obtain a 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 the addition of the sample.

- Equilibration-Sorbent is treated with a solution that is similar to the sample matrix to maximize retention. (Use the same aqueous solution that the sample is prepared in).
- Loading the sample - The sample size must be suitable with the size of the cartridge bed. An atypical reverse-phase cartridge may have capacity for up to 100 mg of very strongly retained substances.
- Washing- is a very important step in the 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 - In this a suitable solvent or buffer is used to elute the analyte from the SPE bed for analysis.

Advantages of SPE over LLE:

•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

•SPE is less time consuming and not tedious as compare to LLE.

LIMITATIONS:

•Based 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.

Protein precipitation method: Protein precipitation is the simple method of extraction as compared to the LLE and SPE. This can be carried out by using the suitable organic solvents which have good solubility of the analyte and protein precipitating properties. Acetonitrile is the first choice of solvent for protein precipitation due to its complete precipitation of proteins and methanol is the second choice of organic precipitant provided the solubility of the analyte in these solvents. 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 protein might stick to each other through one of three forces: electrostatic, hydrophobic, and vander walls. Proteins are made insoluble by altering their surface properties, charge characteristics, or changing the solvent characteristics; changing the solvent characteristics is mostly preferred. The solubility of a protein increases with the addition of salt and reaches a maximum after which there is a rapid linear decrease in insolubility. There are several methods to reduce the solubility of proteins, which are ionic precipitation ex. ammonium sulfate, sodium chloride; metal ions ex. Cu^{+2}, Zn^{+2} and Fe^{+2}; non-ionic polymers ex. polyethylene glycol; organic solvents ex. methanol, acetone. 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.

Types of protein precipitation:

- **Salting out:** Ammonium sulfate is the salt usually used for salting out, because of its high solubility and high ionic strength. 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 a 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 vander Waals forces, at least if they are isoelectric or reasonably close to it. Removal of water molecules from around charged groups would also de shield 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 poly ethylene 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.

Advantages:

1. Protein precipitation plates are available, able to remove the unwanted plasma.
2. 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 LCMS/MS.
3. Protein precipitation plates are compatible with a small volume of solvent.

Disadvantage

Some components of plasma that is soluble in diluting solvent that bound to stationary phase permanently that will affect the column performance.

Dehydration Methods: An aqueous biological sample is treated with a sufficient quantity of anhydrous salt (sodium or magnesium sulfate) to create a dried mix. This mix is then extracted with a suitable organic solvent to remove the desired drug or metabolite.

CONCLUSION:

The advanced techniques and concepts about the Extraction methods employed in the bio-analytical process have been discussed in this review article. This article gives detailed information about the pre-treatment of sample in bio-analysis, various Physico-chemical properties of the sample affecting the extraction technique, and a review on advance extraction methods used for drugs or its metabolites in biological samples are discussed.

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