Sample Preparation In Bioanalysis: A Review

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Abstract: Bioanalysis is sub-discipline of analytical chemistry covering the quantitative measurement of drugs and their metabolites in biological systems. Whereas Liquid chromatography mass spectrometry is a technique which is widely used for the quantification of drug from biological fluid. This article reviews the most recent advances in sample preparation, separation analysis and different type of cartridge used in analysis of biological fluid. Further, this paper also discuss about the merits and demerits of solid phase extraction (SPE), liquid-liquid extraction (LLE) and precipitation.

Key words: Bioanalysis, Liquid chromatography mass spectrometry, Solid phase extraction, Liquid-Liquid extraction, Precipitation

1.0 INTRODUCTION

Bioanalytical method validation employed for the quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic study data.(VP Shah 2007;[1]) Bioanalytical method validation is a procedure employed to demonstrate that an analytical method used for quantification of analytes in a biological matrix is reliable and reproducible to achieve its purpose: to quantify the analyte with a degree of accuracy and precision appropriate to the task.(C.C.Chan et al (2004)[2]) Both HPLC and LCMS-MS can be used for the bioanalysis of drugs in plasma. The main advantages of LCMS/MS include low detection limits, the ability to generate structural information, the requirement of minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities. (S. Murugan et al (2013) [3] Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for the intended use. (E Reid and ID Wilson (1990) [4] As per bioanalytical method validation guidelines Measurement of drug concentrations in biological matrices is an important aspect of medicinal product development. Such data may be required to support applications for new actives substances and generics as well as variations to authorised drug products. (EMA (2011) [5]) Guideline also provides assistance to sponsors of investigational new drug applications(INDs), new drug applications(NDAs), abbreviated new drug applications (ANDAs), biologic license applications and supplements in developing bioanalytical method validation information used in human clinical pharmacology, bioavailability (BA) and Bioequivalence studies that require pharmacokinetic (PK) or biomarker concentration evaluation. (FDA (2013) [6])

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2.0 SAMPLE PREPARATION

The sample preparation portion of the analysis is often the most critical and difficult part, both in terms of time involved and the difficulty of extracting the desired analyte from the matrix. In addition, each matrix has its own unique challenges. For example, urine has a high salt content, plasma contains a great deal of phospholipids, whole blood contains red blood cells that usually must be lysed and so forth. The different characteristics of each analyte and matrix often dictate the type of extraction approach that should be used. (C. Singleton (2012) [7]) Good bioanalysis starts with good sample collection procedures. Therefore, the integrity of samples must be maintained from the time of collection to the moment of analysis, such that the determined concentration closely reflects the *in vivo* concentrations. Various different types of matrices may be collected for subsequent LC-MS/MS bioanalysis. The most common matrix is plasma; however, depending on the characteristics of the drug and its metabolic behavior, analysis of blood or serum may be more appropriate. Sometimes it is also useful to measure the concentration of the drug in urine, to help further understand the behavior of the drug under investigation, especially if a significant amount of unchanged drug is excreted by this particular route. In general, the key point in biological sample collection is to collect them quickly and store them at the correct temperature, stabilize an unstable drug in the matrix, and ensure that the samples are labeled correctly. (M. Pawula (2013)[8])

3.0 PHYSICOCHEMICAL PROPERTIES OF DRUG AND THEIR EXTRACTION FROM BIOLOGICAL FLUID (SL PRABU AND T SURIYAPRAKASH (2012)[9])

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.

3.2 Water miscibility and water immiscibility

Drug 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.

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.

3.5 Mixed solvents
Alcohols are excellent solvent but those with lower boiling points are too soluble in water whereas less miscible one are having high boiling points, but the use of mixed solvents containing alcohols can solve the problem.

3.6 Plasma proteins
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
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.

4.0 SAMPLE PRE-TREATMENT (S. SIGMA-ALDRICH (BULLETIN 910) [10])

4.1 Serum, plasma, and whole blood
To disrupt protein binding in these biological fluids, use of the following methods:
- Shift pH of the sample to extremes with acids or bases. Use the resulting supernatant.
- Precipitate the proteins using a polar solvent (two parts solvent per one part biological fluid). After mixing and centrifugation, remove the supernatant and dilute with water or an aqueous buffer.
- To precipitate proteins, treat the biological fluid with acids or inorganic salts. The pH of the resulting supernatant may be adjusted prior to use.
- Sonicate the biological fluid for 15 minutes, add water or buffer, centrifuge, and use the supernatant.

4.2 Urine
The urine is heated for 15-20 minutes, then cooled and diluted with a buffer, and the pH adjusted appropriately.

5.0 METHOD OF EXTRACTION

5.1 Solid phase extraction
SPE is a sample preparation technology that uses solid particle, chromatographic packing material, usually in a cartridge type device, to chemically separate the different components of a sample.(Waters(2016)[11]) in its broadest sense, solid-phase extraction as defined same as physico-chemical principles that influence a wide variety of sorptive processes.(M. Henry (2000)[12])

5.1.1 Mechanism
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 ionogenic 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).

Reversed phase It involves a polar or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase. The analyte of interest is typically mid- to nonpolar. The following materials are used as reversed phase: 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 nonpolar compounds from both polar and nonpolar matrices.

Normal phase It involve a polar analyte, a mid- to non-polar matrix (e.g. acetone, chlorinated solvents and hexane) and a polar stationary phase. As with typical normal phase silicas, these sorbents can be used to adsorb polar compounds from nonpolar matrices.

Ion exchange it can be used for compounds that are in a solution. Anionic compounds can be isolated on an aliphatic quaternary amine group that is bonded to the silica surface. Cationic compounds are isolated by using the silica with aliphatic sulfonic acid groups that are bonded to the surface.(M. Biziuk(2006)[13])

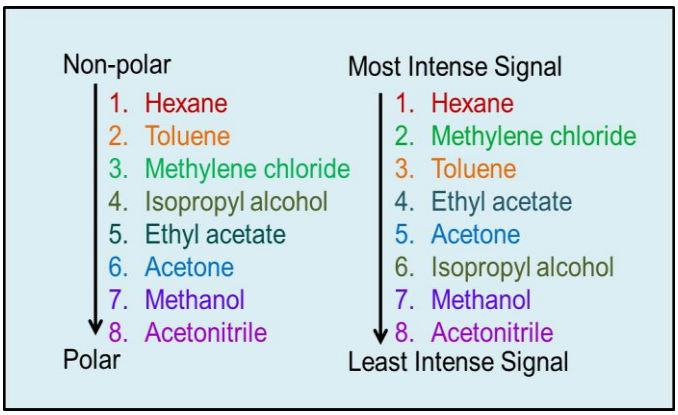

Fig.: 1: *Solvent Selection in SPE*

5.1.2 Types of cartridge

HLB cartridge
HLB is Hydrophilic-Lipophilic balanced, water- wetteble, reversed phase sorbent. It is made up from specific ratio of two monomers, the hydrophilic N-vinylpyrrolidone and the lipophilic divinylbenzene. Universal for acidic, basic and neutral compound. (Waters(2016)[14])

MCX Cartridge
MCX is a novel, mixed-mode polymeric sorbent that has been optimized to achieve higher selectivity and

sensitivity for extracting basic compounds with cation-exchange groups. Since the MCX is water wettable, it maintains its capability for higher retention and excellent recoveries even if the sorbent runs dry, which means there is no need to take extraordinary steps to keep the sorbent beds from drying out during the critical steps prior to sample loading. (Waters(2016)[15])

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

5.1.5 Demerits
Greater complexity
Lengthy method development
Costly (J Stevens (QuEChERS 101)[20])

5.2 Liquid-Liquid extraction

MAX Cartridge
MAX is a mixed-mode polymeric sorbent that has been optimized to achieve higher selectivity and sensitivity for extracting acidic compounds with anion-exchange groups. (Waters(2016)[16])

WCX Cartridge
WCX is a polymeric reversed-phase, weak ion exchange mixed-mode sorbent that has been optimized for fast, simple, and highly selective sample preparation of strong basic compounds and quaternary amines. (Waters(2016)[17])

WAX Cartridge
WAX is a polymeric reversed-phase, weak anion exchange mixed-mode sorbent that has been optimized for fast, simple, and highly selective sample preparation of strong acidic compounds. (Waters (2016)[18])

5.1.3 Extraction steps in SPE

Conditioning Solvent is passed through the SPE material to wet the bonded functional groups (Use methanol)

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

Sample Load Introduction of the sample so that analytes of interest are extracted onto the sorbent. Must be an aqueous solvent.

Washing Use the strongest aqueous solution that will not elute the target compounds. Increasing the % organic, increasing or decreasing the pH, changing the ionic strength for increasing clean-up. Dry the cartridge to remove all water.

Elution Use the weakest organic solvent that will remove all of the target analyte. Polar target compounds elute best in polar solvents so in order of polarity try: methanol>acetonitrile>ethylacetate>acetone>THF.
Modify the pH, increase the ionic strength.

Solvent exchange The organic elution solvent should be evaporated and the sample reconstituted in starting mobile phase. If the next analysis is GC, then methanol is the reconstitution solvent. In all cases the reconstitution must be to the same volume.(CH Ball(2009)[19]

Liquid-liquid extraction (LLE) is a common sample preparation choice in regulated bioanalysis. LLE can generate high analyte recoveries, clean extracts, and is perceived as low cost. Extraction solvents may need to be acidified, basified or low percentages of more polar solvents may be required to simultaneously achieve high recoveries for metabolites and related compounds, as well as the primary analyte. In many cases, the choice of LLE solvent may lead to extracts particularly saturated with phospholipids (PLs). As well as contributing to matrix effects, residual PLs can build-up on the analytical column and the LC system. PL build-up may cause analyte signal variability, suppression of MS response, and potentially lead to instrument down-time. Similar to LLE, phospholipid removal (PLR) plates also provide high analyte recoveries and clean extracts. [21] 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 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 and vice versa.. Lastly, the two immiscible layers are separated, transferred and the component in that solvent is isolated. 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. 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.

KD = Co/Caq

Where KD is the distribution constant, Co is the concentration of the analyte in the organic phase, and Caq is the concentration of the analyte in the aqueous phase. If the KD value is unfavorable, addition 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 KD is very low or the sample volume is high, it becomes nearly impossible to carryout 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. 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 (SL Prabu and T Suriyaprakash (2012)[9]))

Merits
Inorganic salts easily removed
Short method development time
Low cost

Demerits
Labor intensive
Large volume of Organics
Difficult to automate(J Stevens (QuEChERS 101)[20])

5.3 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 (NH4)2SO4 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**.**

Merits
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 LCMS/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 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%

Demerits
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. (SL Prabu and T Suriyaprakash (2012)[9])

6.0 CONCLUSION

The new concepts and new progress made in sample preparation discussed in this review article. The concept covered techniques used for preparation of sample in Bioanalysis, Pretreatment given to the matrix and physicochemical properties affect to the biological matrix. Good Recovery obtained by taking consideration of physicochemical properties and proper sample pretreatment and preparation.

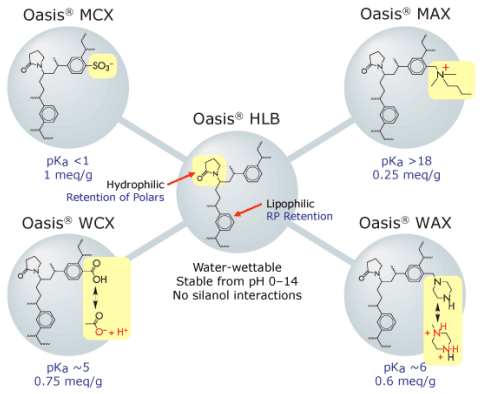

Fig.: 2: *Types of Cartridge*

Reference

[1] VP Shah, "The history of bioanalytical method validation and regulation", American Association Pharma.Science, Vol.9, No. 1, pp.43-47, Feb 2007

[2] C.C. Chan, "Analytical Method Validation and Instrument Performance Verification", A John Wiley and sons, pp. 105, 2004.

[3] S Murugan, N Pravallika, P Sirisha, K Chandrakala. "A Review on Bioanalytical Method Development and Validation By Using Lc-Ms / Ms." J. Chem. Pharma Science, Vol. 6, No. 1, pp. 41-45, March 2013

[4] E Reid and ID Wilson," Analysis for drugs and metabolites,including anti-infective agents: Methodological servey in biochemistry and analysis.", J. Pharma Pharmacol., Vol. 43, no. 2, pp. 1-57, feb.1991

[5] European medicine agency (EMA) –July 2011,1-23

[6] US Food and drug administration (FDA) – Sept. 2013, 1-34

[7] C. Singleton, "Recent advances in bioanalytical sample preparation for LC–MS analysis.", Future Science group, Vol. 4, no. 9, pp. 1123-1140, 2012

[8] M. Pawula, G. Hawthorne,GT Smith, HM Hill. 'Best Practice in Biological Sample Collection , Processing , and Storage for LC-MS in Bioanalysis of Drugs." A John Wiley and sons, pp. 139-164, 2013

[9] SL Prabu and T Suriyaprakash, " Extraction of drug from the biological matrix: A review." Appl Biol Eng Pract, pp.479-507, 2012. Available from: http://www.intechopen.com/books/applied biological-engineering-principlesandpractice/extraction-of-the-drug from-the-biological-matrix assessed on 15/12/2015

[10] Sigma-Aldrich S. Guide to Solid Phase Extraction - bulletin 910. Bull 910 [Internet]. 1998;-. Available from: http://www.sigmaaldrich.com/Graphics/Supelco/objects/4600/4538.pdf

[11] https://waters.com/waters/en_US/Beginner's-Guide-to-SPE-%5BSolid-Phase-Extraction%5D/nav.htm?cid=134721476 assessed on 23/02/2016

[12] M. Henry, SPE technology-Principles and practical consequences. Solid-Phase Extr Princ Tech … [Internet]. 2000; Available from: http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:SPE+Technology+-+Principles+and+Practical+Consequences#0 assessed on 28/09/2015

[13] M. Biziuk, " Solid Phase Extraction Technique – Trends , Opportunities and Applications." Polish J Environ Stud, vol. 15, no. 5, pp. 677–90, 2006

[14] https://www.waters.com/webassets/cms/support/docs/715000109.pdf assessed on 30/03/2016

[15] http://www.waters.com/waters/partDetail.htm?partNumber=186000252 assessed on 30/03/2016

[16] http://www.waters.com/waters/partDetail.htm?partNumber=186000367 assessed on 30/03/2016

[17] http://www.waters.com/waters/partDetail.htm?partNumber=186002494 assessed on 30/03/2016

[18] http://www.waters.com/waters/partDetail.htm?partNumber=186002493 assessed on 30/03/2016

[19] CH Ball and D P, "Solid Phase Extraction Method Development Tips and Tricks Sources of Error Generated and Time Spent During a Typical Chromatographic Analysis." Scientist. 2009; available from https://www.agilent.com/cs/library/slidepresentation/Public/Food%20Safety%20Analysis%20Using%20SPE_020309.pdf assessed on 29/03/2016