INTERNATIONAL RESEARCH JOURNAL OF PHARMACY

www.irjponline.com

ISSN 2230 – 8407

Review Article

BIOANALYTICAL METHOD DEVELOPMENT: AN UPDATED REVIEW

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Article Received on: 13/08/17 Approved for publication: 20/09/17

DOI: 10.7897/2230-8407.089151

ABSTRACT

In this review techniques used for the Bioanalytical Method Development for the extraction of analyte present in plasma contain K_2EDTA as an anticoagulant from LC/MS/MS or HPLC is discussed. The extraction is performed by using different methods i.e. Liquid-Liquid Extraction, Solid Phase Extraction and Protein Precipitation. This literature mainly focused on the benefit of LC/MS/MS and advantages and disadvantages of different methods used for extraction of analyte in human plasma. This review article will give information to all those researchers who were engaged in bioanalytical research work by LC/M/MS.

Keywords: Analyte, Reversed Phase Liquid Chromatography, Tandem Mass Spectrometry, Liquid - Liquid Extraction.

INTRODUCTION

Liquid Chromatography-Mass Spectrometry (LC/MS/MS)

Liquid chromatography-mass spectrometry (LC/MS/MS) is an analytical chemistry technique which is a combination of liquid chromatography which helps in physical separation (or High Performance Liquid Chromatography) and mass spectrometer is used as a detector. High Performance Liquid Chromatography is a widely used analytical technique before the development of liquid chromatography-mass spectrometry and continues to be used in many laboratories. (LC/MS) is a very powerful technique which is used for many applications which has very high sensitivity and specificity. Following are the advantages of the mass spectrometer when used as a detector[1]

- With the LC/MS interfaces are now available, a wide range of analytes, from low molecular- weight drugs and metabolites (<1000 Da) to high-molecular-weight biopolymers (>100 000 Da), may be studied.
- The mass spectrometer provides the most definitive identification among all of the HPLC detectors as quantization of drugs in bio matrices.
- The high selectivity of the mass spectrometer often provides this identification capability on chromatographically unresolved or partially resolved components.

Following ideal properties of hyphenation techniques are required for efficient analysis

- The interface should cause no reduction in chromatographic performance. This is particularly important for the analysis of complex multi-component mixtures[2].
- Uncontrolled chemical modification of the analyte should not occur during its passage through the interface or during its introduction into the mass spectrometer.
- The interface should be reliable, easy to use.
- Operation of the interface should be compatible with all chromatographic conditions which are likely to be encountered, including flow rates from around 20nl/min to around 2ml/min, solvent systems from 100% organic phase to 100% aqueous phase, gradient elution, which is of particular importance in the biological field in which mixtures covering a wide range of polarities are often encountered and buffers, both volatile and non-volatile.
- Operation of the interface should not affect on the vacuum requirements of the mass spectrometer, so that all capabilities of the mass spectrometer is used at optimum level, i.e. ionization modes, high resolution, etc.
- The interface should provide quantitative information with reproducibility and have a linear response over a wide range of sample sizes (low pg to µg). it should be simple and inexpensive. HPLC separation is based on interaction and differential partition of sample between mobile liquid phase and stationary phase[3-6].

METHODS USED FOR BIOANALYTICAL EXTRACTION

Liquid - Liquid Extraction (LLE)

It is one of the most useful techniques used for isolating the desired components from a mixture. LLE method is used for the separation of a mixture by using two immiscible solvents. In LLEs, there are mainly two phases one phases is aqueous and the other is an immiscible organic solvent. Mainly "like dissolves like" concept in LLE. The ability of separation from a mixture using the technique of LLE is mainly depends upon how fastly and differently the compounds present in a sample mixture divide themselves in between the two immiscible solvents. The Selective partitioning of the required compound into two immiscible or partially miscible phases depends upon proper choice of extraction solvent. In this technique sample is distributed/partitioned in two phases where one phase is immiscible to other. LLE mainly focuses on separation of analytes from interferences by partitioning/dividing the sample in two immiscible liquids or phases[7]. Firstly, the mixture is

dissolved in a suitable extracting solvent and a second solvent which seems to be immiscible with the first solvent is added slowly. In the next step, the contents are mixed and the two immiscible solvents is allowed to separate in layers. The layer which is slightly dense is upper layer , while the layer which is more dense will be the lower layer. The components which are separated from initial mixture will distribute itself amongst the two immiscible solvents which can be determined by their partition coefficient. The solubility of a compound in two given solvents give estimation of the extent to which a compound will be partitioned/divide between them. A compound which is more soluble in the less dense solvent will reside in the upper layer. On the other side, a compound which is more soluble in the more dense solvent will preferentially reside in the lower layer. In the last step, the two immiscible layers are separated, transferred and the required component in that solvent is isolated/extracted. After extraction hydrophilic compounds were present in the polar aqueous phase and hydrophobic compounds are present in the organic solvents. Analyte is extracted from organic phase may be recovered by the evaporation of solvent, the residue obtained is reconstituted with small volume solvent preferably with mobile phase while analyte extracted from aqueous phase is directly injected into a RP column. The LLE technique is simple, rapid , cost effective per sample as compared to any other techniques and the quantitative recoveries (90%) of most drugs which can be extracted by this technique can be obtained by multiple continuous extraction. There are various equations which help to illustrate the extraction process. According to the Nernst distribution law which states that any species which is neutral will distribute between two immiscible solvents until the ratio of the concentration remains constant. KD = Co/Caq here KD is the known as 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.

To increase the value of KD i.e. distribution coefficient, different approaches were used:

Firstly- The organic solvent is changed which helps to increase the solubility of the analyte - If the analyte is ionic or ionisable in nature its KD is increased by decreasing its ionization and to make it more soluble in the organic phase. The analyte can be extracted from organic phase by forming an ion pair by adding hydrophobic counter ion. Metal ions mainly form complex with complexing agents which are hydrophobic. The salting out effect is also beneficial to decrease the analyte concentration in the aqueous phase. If the KD value is not satisfactory, In this case, a fresh portion of immiscible solvent is added to extract additional solute. Normally, the two extracts are combined. Generally, multiple extractions are more efficient in removing a solute quantitatively than single extraction. Many times, back extractions were used to achieve a full complete sample clean up.

If KD is very less or the sample volume is high, it is impossible to perform multiple simple extractions in a reasonable volume. Also, if the extraction process is very slow, then it take long time for equilibrium to be established. In this type of cases mainly continuous liquid liquid extraction should be used, in which pure solvent is recycled by using aqueous phase. LLE system always 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 depends on the following given factors: - the 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. However, it is not possible to find out the optimum condition that gives both high recovery as well as purity of the analyte in one step. Very low and Low recoveries may require further extraction to achieve acceptable value. Each successive extraction increase the analytical time, also the resulting large volume of extraction solvent must be evaporated to recover the product. If the extraction process require many steps, then techniques such as Craig Counter Current distribution is used to increase the recovery and purity. Due to this, the LLE technique increases the cost and time of the analysis.

Selection of the solvent- The main requirement for an ideal solvent is, it can easily withdraw the active agent from a mixture by liquid-liquid extraction. Selectivity- For high selectivity, Only the active agent has to be extracted and no foreign substance. Miscibility-The miscibility of solvent and primary solvent may be low to achieve simple regeneration of the solvent. Difference in density-After extraction, a separator is used so that it can separate both the two phases by using high positive difference in density. Recovery-The solvent is easily separated from the extract phase so, to get solvent free active agents. Corrosion-solvent should be non corrosive as it increases construction charges.

Extraction under basic and acidic conditions- As discussed previously, the ability of separating compounds from a mixture using liquid-liquid extraction mainly depends upon the solubility of each compound in two immiscible solvents. Slightly change in pH of the solvent leads to change in the solubility of an organic compound in a solvent. Liquid-liquid extraction is the most commonly used technique for the separation of organic product from a biological matrix. This technique works better if the target compound is fully soluble in one of two immiscible solvents. Extraction involves mixing/shaking a solution containing target with immiscible solvent where desired/targeted substance is more soluble than it is in the starting solution standing the solvents form two layers. For complete separation the extraction is repeated several times. Generally liquid-liquid extractions help to separate three different categories of compounds: a. Organic bases b. Strong acids c. Weak acids.

Disadvantages - solvent consumption is high for extraction of drug, time consuming process when compared to other methods, LLE require additional step i.e. evaporation step prior to analysis to remove excess of organic solvent. this technique is not suitable for the estimation of multiple analytes, Emulsion formation may be possible if both solvents are immiscible during extraction procedure.

Solid Phase Extraction

SPE has become a common and effective technique for extracting analytes from complex samples. SPE is a popular technique which is used for rapid and selective sample preparation. The main advantage of SPE is automation and parallel processing. SPE is mainly used for the trace analysis of drugs in matrices. Due to the versatility of SPE, allows use of this technique for many purposes like purification and trace enrichment. The major objectives of SPE is to decrease the level of interferences, small decreases the final volume of of which leads to maximize the analyte sensitivity and by providing analyte part in a solvent that is fully compatible with the analytical techniques used for the measurement .The benefit of SPE technique is that it serves as a filter to remove sample particulates. the principle of SPE is similar to that of LLE,

involving a separation of solutes in between two phases. SPE involves separation of liquid (matrix containing sample or solvent with analytes) and a solid (sorbent) phase SPE proves a more efficient separation process than LLE, which yields to high recovery by using small (plastic column or cartridge) which is disposable, & often a medical syringe which is packed with 0.1 to 0.5 g of sorbent which behaves RP material (C18- silica). The required components may be adsorbed to the solid, or it may remain in non-solid phase. As the equilibrium is reached, both of the phases are physically partitioned by similar processes like decanting, filtration, centrifugation. If the required analyte gets adsorbed on the solid phase which can be desorbed by washing using an appropriate solvent. If the analyte is present in liquid phase, it can be recovered by using concentration, evaporation and or recrystallization processes[8]. The sample which is liquid is added to cartridge and for washing solvent is selected which will strongly retain the analyte. Interferences are washed from the cartridge by using washing solvent, even though the analyte is retained which leads to decrease the interferences present in fraction, after that the analyte is eluted with a strong eluting solvent, evaporate the analyte to dryness followed by diluting HPLC mobile phase.

The main Advantages of SPE includes complete extraction of the analyte, efficiently separation of interferences in analyte, less organic solvent consumption, analyte is easily collected from its fraction, procedure is convenient

Mechanism of Solid Phase Extraction process

The separation mechanism is due to the molecule – molecule interactions between analyte and the functional groups which are present in the sorbent.

The selection of an accurate SPE extraction sorbent greatly depends on the idea of mechanism(s) of interaction in between the sorbent and analyte. The knowledge of the hydrophobic, polar and inorganic properties of solute and the sorbent is required. Commonly used retention mechanisms in SPE are van der Waals forces which is also known as ("non-polar interactions"), hydrogen bonding, dipole-dipole forces ("polar" interactions) and cation-anion interactions ("ionic" interactions)[9].

Steps of Solid Phase Extraction

These are the general steps which are used for the method development for extracting the analyte from plasma.

a. Pre treatment of sample – it includes diluting the sample with dilution, solvent followed by filtration for better adsorption.

b. Conditioning of the cartridge – this is the main step when reverse phase SPE Cartriges are used. Preconditioning is mainly done by using the organic solvents that are methanol, acetonitrile, isopropyl alcohol or tetrahydro furan

c. Loading of sample - Sample size must suit the size of the cartridge bed. A typical reverse phase cartridge having capacity of up to 100 mg for very strongly retained substances.

d. Washing- It is also very important in SPE. In this step a suitable solvent sometimes water is passed through SPE bed to remove contaminants.

e. Elution of fraction in this last step the analyte is eluted by using a appropriate elution solvent.

Protein Precipitation Method

This method is not frequently used in bioanalytical but it is very simple technique which is used for extraction of the analyte from the matrix. When there is a chances of protein precipitation ,then protein precipitation before sample extraction may be required. commonly used Reagents includes perchloric, trichloroacetic and tungstic acids, and organic solvents such as acetonitrile or methanol. If protein binding takes place through covalent linkage, then it should not be easily breakable because it is the strongest bond. In this reconstituting solvent is required in which analyte should be freely soluble. The sample Preparation for protein precipitation requires separation by conversion of proteins (which are soluble)to insoluble state by the addition of water miscible precipitation solvent /organic solvents such as acetone, ethanol, acetonitrile or methanol. In addition, precipitating agents is also chosen which provide more stable product than that was found in the soluble form. proteins mainly stick to each other through one of three forces: electrostatic, hydrophobic, and van der Waals[9]. Proteins were made insoluble by changing their surface properties, charge characteristics or altering solvent characteristics; mainly changes in the solvent characteristics mostly preferred. More is the initial concentration of the desired protein, more is efficiency of precipitation; proteins are very less soluble as they have isoelectric point (pI) ranges from 4 - 10.

Type of protein precipitation Salting out: the main salt used for salting is Ammonium sulphate, because of high solubility and high ionic strength.

Solvent Precipitation: the addition of large amounts of solvent such as ethanol or acetone which is water-miscible ,is added to a protein solution, which precipitate protein. Water soluble solvents associates with water more strongly than proteins, which shows the real effect to dehydrate protein surfaces, which is then associated by using vander Waals forces. The Removal of water molecules from charged groups will deshield them and further it allows the charge interactions to occur more strongly. In practice, mainly solvent precipitation is performed at low temperature. The conditioning for the protein is at 0°C and the solvent colder, -20°C in an ice-salt bath, because proteins have the nature of denaturation. Solvent precipitation mainly done with PEG i.e. polyethylene glycol at concentrations between 5 and 15%. It works by competing the protein for water, but is less likely to inactivate the protein and it does not require such low temperatures, but it will always tend to give oily precipitate. Sample centrifugation is required at high speed for sufficient time, the component precipitated in plasma will be settled at the bottom and clear supernatant liquid is separated out. The supernatant liquid which is obtained can be injected directly into the HPLC[10].

Advantage - Protein precipitation plates are used in a wide range of aqueous and organic sample preparation which includes total drug analysis and sample preparation prior to HPLC or LC MS/MS, these plates requires small volume of solvent, these Protein precipitation plates contains hydrophobic PTFE membrane which acts as prefilter & removes the unwanted precipitated proteins prior to analysis.

Disadvantage – it increases the back pressure of HPLC, Some components of plasma that are soluble in diluting solvent may bound to stationary phase permanently which will affect the column performance.

CONCLUSION

An attempt is made to understand and explain the bioanalytical method development. The aim of this article is to provide simple to use approaches with a correct scientific background to improve the quality of the bioanalytical method development. These methods can be used for high as well as low molecular weight drug candidates. These various essential development characteristics for bioanalytical methodology is discussed in order of improving the standard and acceptance in this area of research.

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Cite this article as:

Anandi Kapri *et al*. Bioanalytical method development: An updated review. Int. Res. J. Pharm. 2017;8(9):6-9 http://dx.doi.org/10.7897/2230-8407.089151

Source of support: Nil, Conflict of interest: None Declared