ABSTRACT

Bioanalysis for the quantitative determination of drugs in biological fluids plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic and toxicokinetic studies. It is an integral part of characterization of drug from the time of its discovery and during various stages of drug development leading to its market authorization. Thus development of prominence bioanalytical method is of paramount importance. Knowledge of drug concentration levels in body fluids, such as whole blood, plasma, serum and urine, allows the optimization of pharmacotherapy and provides a basis for studies of patient compliance, bioavailability, pharmacokinetics and the influences of co-medications. Selective and sensitive analytical method development become necessary during the quantitative and qualitative analysis of drugs and their metabolites that are purported to display pharmacological activity, determination of multiple drugs in combating a disease, biotransformation investigation, drug monitoring for therapeutic benefits and for in-vitro experiments\textsuperscript{1-3}.

Determination of drugs and their metabolites is complicated in biological matrix compared to in formulations. Biological matrix (e.g. blood, plasma, serum and urine) samples contain mostly water and other components like dissolved proteins, glucose, clotting factors, mineral ions, hormones and acids\textsuperscript{4-5}. Drug absorption in body depends upon the properties of drugs and also some patient related factors therefore it’s not possible all the time to avail high drug concentration in biological samples. Also, above mentioned components may interfere at the time of quantification of analyte of interest if matrix interference free sample solution is not injected\textsuperscript{6}.

There are a number of techniques used for the separation of drugs from biological matrices for analytical purposes. Methods generally used in the bioanalysis of drugs and their metabolites are radioimmunoassay (RIA), capillary electrophoresis (CE), gas chromatography (GC), GC-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) with UV, fluorescence, refractive index and mass spectrometry detection (MS)\textsuperscript{7-9}. The most widely used are based on chromatography.

HPLC is the noteworthy technique for chemical and pharmaceutical analysis with the ability to separate, analyze, and/or purify any sample. The separation of analytes is based on differences in relative rates of migration through the column arising from different partition of the analytes in the stationary and the mobile phase. Reverse phase HPLC having hydrophobic stationary phase and polar mobile phase is generally used for the analysis of most of the compounds\textsuperscript{10}.
Mass spectrometry (MS) combined with the separation power of chromatography has revolutionized the way chemical analysis is done today. Liquid chromatography – Tandem mass spectrometry (LC-MS/MS) is an extremely versatile and highly selective instrumental technique. LC-MS/MS has become an ideal and extensively used method in the analysis of drugs and their metabolites due to its supreme sensitivity, extraordinary selectivity and rapid speed of analysis\textsuperscript{11}. Analytes, easily separated by liquid chromatography can be detected even at lower concentration by MS/MS detection using different ionization techniques like electro-spray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI)\textsuperscript{12-15}.

MS provides a fingerprint mass spectrum, which contains the molecular weight and structure-specific fragmentation ion information. The major drawback of MS is its limitation in handling mixtures of compounds. Tandem mass spectrometry (MS/MS) can alleviate this problem to certain extent. Interfacing of HPLC with MS thus benefits mutually from the high resolution separation capability of HPLC and highly sensitive and structure specific detection capability of MS. MS comes very close to being a universal detector for LC\textsuperscript{16}.

Sample preparation plays momentous role in attaining supreme selectivity and sensitivity. It is mandatory to clean the biological sample as much as possible to get matrix interferences free sample solution. A proficient extraction procedure is necessitate to develop that can deliver quantitative and reproducible recovery. Sometimes, concentrating the sample after extraction, derivatization at sample processing step or at chromatography phase and adduct ion formation can enhance the sensitivity of method.

Thus, development of selective and sensitive analytical methods for the quantitative evaluation of drugs are critical for the successful accomplishment of pre-clinical and clinical pharmacology studies\textsuperscript{17-18}. These requirements are generally met with HPLC, especially if combined with an advanced detection technique such as mass spectrometry (MS). Now-a-days, analysis time of biological samples can be decreased sharply using variety of analytical columns, but the choice of an appropriate sample preparation method is crucial for reliability and accuracy of the analysis as separation of analytes from other matrix components on column takes a short time.

Prior to LC analysis, biological samples must be ‘cleaned up’. This involves isolation of the drug to be analyzed from its matrix with consistent recovery. Three sample preparation techniques are commonly used to extract analytes from biological matrices: protein precipitation, liquid-liquid extraction and solid phase extraction.
Protein precipitation technique (PPT) is a simplest procedure to remove proteins from biological matrix. It is a rapid and cost effective extraction method, but can give the sample with lots of matrix interferences that causes column clogging, ion suppression/enhancement and requires frequent system clean-up due to deposition of sticky and non-volatile matrix component in the chromatographic system and ion source.

Liquid-liquid extraction (LLE) is still one of the most popular extraction techniques. This method is used to separate compounds based on their relative solubility in two different immiscible liquids, usually water and organic solvent. Sometimes it is required to back extract the compounds or multiple extractions to remove the interferences from sample. It is cost effective method compared to solid phase extraction (SPE) but is tedious and time consuming, often requiring drying followed by reconstitution. LLE is a simple and proficient method for the separation and concentration of relatively hydrophobic compounds. For some polar compounds, it is not possible to get matrix free clean sample using this extraction procedure.

Both these techniques (PPT and LLE) used for extraction of drug from biological matrix is being superseded by SPE. This technique employs a solid phase and a liquid phase to isolate one or more analyte(s) from biological samples. It is used to clean up the sample before using a chromatographic analytical method and to quantitate the amount of drug in the sample. Many of the problems associated with LLE can be prevented, such as incomplete phase separations, less-than-quantitative recoveries, use of expensive and breakable specialty glassware and disposal of large quantity of organic solvents. SPE methods are easy to perform, rapid and can be automated. Drug can be extracted using small volume of samples, thus solvent use and processing time are significantly reduced. This method has advantage over PPT as clean and matrix interference free sample can be obtained by washing undesired components. Different types of SPE cartridges are available to extract different type of drugs.19-21.
ATAZANAVIR

Atazanavir is chemically methyl N-[(1S)-1-[(2S,3S)-3-hydroxy-4-[(2S)-2-[(methoxycarbonyl) amino]-3, 3-dimethyl- N' - [(4-(pyridin-2-yl) phenyl)methyl] butanehydrazido] -1-phenylbutan- 2-yl] carbamoyl] -2,2-dimethylpropyl] carbamate. It is an antiretroviral drug of the protease inhibitor (PI) class. Atazanavir is an azapeptide HIV-1 protease inhibitor (PI) with activity against human immunodeficiency virus type 1 (HIV-1). HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1.

DARUNAVIR

Darunavir, formerly known as TMC114, is a new and Protease Inhibitor (PI) active against HIV strains resistant to other currently available PIs. Darunavir is chemically (1R, 5S,6R)-2,8- dioxabicyclo [3.3.0] oct-6-yl] -N- [(2S,3R)-4-[(4-aminophenyl) sulfonyl- (2-methylpropyl) amino] -3-hydroxy- 1- phenyl-butan-2-yl] carbamate is a HIV peptidic protease inhibitor, with high levels of antiviral activity against wild-type virus and stains with phenotypic resistance to other protease inhibitors (PIs).

RITONAVIR

Ritonavir is chemically designated as 10-hydroxy -2- methyl- 5- (1-methylethyl)-1- [2-(1-methylethyl)- 4thiazolyl]- 3,6-dioxo-8,11-bis (phenyl methyl)-2,4,7,12-tetraazatridecan- 13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*,8R*,10R*,11R*)]. Ritonavir is a peptidomimetic inhibitor of the HIV-1 protease. Ritonavir is used to inhibit a particular liver enzyme that normally metabolizes protease inhibitors, cytochrome P450-3A4. The drug's molecular structure inhibits CYP3A4, so a low dose can be used to enhance other protease inhibitors.

LOPINAVIR

Lopinavir is chemically (2S)-N- [(2S,4S, 5S) -5- [2-(2,6- dimethylphenoxy) acetamido] -4-hydroxy-1,6- diphenylhexan -2-yl]- 3- methyl-2- (2-oxo-1,3-diazinan-1-yl) butanamide. It is used against HIV infections as a fixed-dose combination with another protease inhibitor- ritonavir. Administered alone, lopinavir has insufficient bioavailability; however, like several HIV protease inhibitors, its blood levels are greatly increased by low doses of ritonavir, a potent inhibitor of cytochrome P450 3A4.
TENOFOVIR

Tenofovir disoproxil fumarate ([(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl] oxy) methyl) phosphonic acid) belongs to nucleotide analogue reverse transcriptase inhibitors (NRTIs), which block reverse transcriptase, a crucial virus enzyme in human immunodeficiency virus 1 (HIV-1) and hepatitis B virus infections.32

EMTRICITABINE

Emtricitabine (4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one), is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults and children.33

RALTEGRAVIR

Raltegravir is an integrase inhibitor class drug which is chemically N-(4-Fluorobenzyl)-5-hydroxy-1-methyl-2-(2-[(5-methyl-1,3,4-oxadiazol-2-yl) carbonyl] amino)-2-propyl)-6-oxo-1, 6-dihydro-4-pyrimidinecarboxamide. Raltegravir targets integrase, an HIV enzyme that integrates the viral genetic material into human chromosomes, a critical step in the pathogenesis of HIV. In combination therapy, raltegravir exhibited potent and durable antiretroviral activity.34

KEY WORDS:

HPLC-MS/MS, human plasma, atazanavir, darunavir, ritonavir, lopinavir, tenofovir, emtricitabine, raltegravir, antiretroviral drugs, bioanalytical method development, bioanalytical method validation.