Drug bio analysis, employed for the quantitative determination of drugs and their metabolites 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 and thus development of sound bio analytical method is of paramount importance. Knowledge of drug 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 1-3.

Determination of drugs and their metabolites is difficult 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 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 6. Methods generally used in the analysis 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 spectrometric detection (MS) 7-9. LC-MS/MS has become an ideal and widely used method in the analysis of drugs and their metabolites due to its unmatched sensitivity, extraordinary selectivity and rapid rate of analysis 10. Analytes, easily separated by liquid
Chromatography can be detected even at lower concentration by MS/MS detection using different ionization techniques like electrospray (ESI), atmospheric chemical ionization (APCI) and atmospheric pressure photo ionization (APPI)\textsuperscript{11-14}.

High performance liquid chromatography is the premier technique for chemical and pharmaceutical analysis with an ability to separate, analyze, and/or purify virtually any sample. The principle of 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{15}. Sample preparation plays important role in achieving desired selectivity and sensitivity. It is necessary to clean the biological sample as much as possible to get matrix interferences free sample solution. An efficient extraction procedure need to develop that can give 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 the method. Thus, development of selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites are critical for the successful conduct of preclinical and clinical pharmacology studies\textsuperscript{16-17}. These requirements are generally met with HPLC, especially if combined with an advanced detection technique such as mass spectrometry (MS). Now days, analysis time of biological samples can be decreased sharply using ultra performance liquid chromatography (UPLC), but the choice of an appropriate sample preparation method is essential for reliability and accuracy of the analysis as separation of analytes from other matrix components on column takes a short time.

Protein precipitation (PP) is a simplest procedure to remove proteins from biological matrix. It is a fast 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. Liquid-liquid extraction (LLE) is still one of the most popular extraction techniques for liquid samples. 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 efficient 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 (PP 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 PP 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 \(^{18-20}\).
NICORANDIL

Nicorandil is chemically [N-[2-(Nitrooxy) ethyl]-3-pyridinecarboxamide]. The vasodilator Nicorandil is a nicotinamide derivative used clinically for the treatment of angina. Nicorandil has been proposed to have at least two mechanism of action: opening K+ Channels and also having a nitro vasodilator action, activating guanylyl cyclase (GC) and so increasing guanosine 3’-5’-cyclic monophosphate (cyclic GMP) $^{21-22}$.

A rapid, simple and specific method for estimation of Nicorandil in human plasma was validated using Metaxalone as an internal standard. The analyte and internal standard were extracted from plasma using simple solid phase extraction. The compound were separated on a reverse-phase column at the flow rate of 0.4 ml/min with an isocratic mobile phase consisting of 2 mM ammonium acetate in water and acetonitrile and detected by tandem mass spectrometry in positive ion mode. MS/MS analysis was performed in multiple reaction monitoring (MRM) using mass transition m/z 212.2→136.4 for Nicorandil; m/z 222.2→161.1 for Metaxalone (IS). The retention times for Nicorandil and Metaxalone were ~ 2.99 minutes and ~ 3.46 minutes respectively with total run time of 5min. Linearity in plasma was observed over the concentration range 1 – 500 ngmL-1 for Nicorandil.. The coefficient of variation of the assay was less than 11.80% and accuracy (%RE) of -1.05% to 5.76%. The mean recovery for Nicorandil was 78.29% and Metaxalone was 90.82 %. Different stabilities like Bench top stability, processed (auto sampler) stability, freeze and thaw stability, long term stability in plasma and stock solution stability was performed for Nicorandil. The validated method can be used for the bioequivalence study in healthy human volunteers.
OLANZAPINE

Olanzapine, is chemically \[2\text{-methyl-4-(4-methylpiperazin-1-yl)-5H-thieno[3,2-c][1,5]benzodiazepine}\]. Olanzapine is a (atypical antipsychotic) psychotropic agent that belongs to the thienobenzodiazepine class 23. The first generation and second-generation antipsychotic drugs are US-FDA approved first-line treatment for schizophrenia. However, Patients who receive antipsychotic drugs differ with respect to treatment response and drug induced adverse events 24. Although antipsychotic drugs relieve the positive symptoms of schizophrenia, these drugs have limited utility in the treatment of the negative symptoms and cognitive deficits associated with this disorder 25. Conventional (typical) antipsychotic cause a variety of side effects both acutely [e.g., extra pyramidal side effects (EPS)]26, and with long-term exposure [e.g., tardive dyskinesia (TD)] 27. Such adverse effects may reduce compliance and represent a major drawback of these drugs. Hence, introduction of atypical antipsychotic like Clozapine 28.

A rapid, simple and specific UPLC-MS/MS method for estimation of Olanzapine in human plasma was validated using Clozapine as internal standard. The analyte and internal standard were extracted from plasma using simple solid phase extraction. The compound were separated on a reverse-phase column with an isocratic mobile phase consisting of 5mM Ammonium Acetate in water and acetonitrile (15:85, v/v) and detected by tandem mass spectrometry in positive ion mode. The ion transition recorded in multiple reaction monitoring mode were m/z 313.18 \rightarrow 256.0 for Olanzapine and m/z 327.03 \rightarrow 270.01 for internal standard. Linearity in plasma was observed over the concentration range 0.15 – 15ng/mL for Olanzapine. The mean recovery for Olanzapine was 88.34 % with a lower limit of quantification of 0.15ng/mL. The validated method can be used for bioequivalence study of 10mg Olanzapine tablet in healthy human volunteers.
CYCLOBENZAPRINE

Cyclobenzaprine (Flexeril®) is a tricyclic compound first synthesized in 1971 for use as a centrally acting muscle relaxant, structurally related to first-generation tricyclic antidepressants. The drug is indicated for relief of the acute pain associated with acute muscle spasms 29. The similarity of the chemical structure of Cyclobenzaprine with Amitriptyline and other antidepressants leads to difficulties in differentiation. It may be difficult to discriminate between the two analytes with many analytical methodologies utilized for comprehensive drug detection and identification. Cross-reactivity occurs with all commercially available immunoassay kits that detect the tricyclic antidepressants. For consistent and correct identification of Cyclobenzaprine, a confirmation technique which can accurately distinguish between Cyclobenzaprine and Amitriptyline must be used 30-32.

Simple, sensitive and reproducible UPLC-MS/MS method was developed for estimation of Cyclobenzaprine in Human Plasma using Imipramine as internal standard. Chromatographic separation was achieved with waters Acquity UPLC BEH C18 (50*2.1mm) 1.7µ column. Isocratic mobile phase consist of Buffer 10 mM ammonium formate in water (pH 4.00±0.05) and Acetonitrile (20:80), with the flow of 0.2mL/min and injection volume was 5µL. Sample Preparation involved solid phase extraction using Phenomenex Strata (30mg/1 mL), sample was pretreated with 10 mM Ammonium formate in Water (pH 4.00±0.05) before loading to cartridge, and washing was done with 1mL 0.1% Glacial acetic acid followed by 1 mL 30% Methanol in Water to remove interferences from matrix. Finally Cyclobenzaprine was eluted with 1.0mL of Mobile Phase. Method was validated as per USFDA guideline.
CANDESARTAN AND HYDROCHLOROTHIAZIDE

Angiotensin II receptor blockers (ARBs) are a new class of therapeutic agents for hypertension. The ARBs have a more direct mechanism of action than other drugs affecting the angiotensin converting enzyme inhibitors. Candesartan is a potent, highly selective ARB that is devoid of agonist activity. Candesartan cilexetil (prodrug) which is a racemic mixture containing one chiral center at the cyclohexyloxy-carbonyloxy-ethyl ester group. Candesartan cilexetil has been developed with the aim of identifying a nonpeptide angiotensin II receptor antagonist with a long-lasting and insurmountable effect. It is administered orally as Candesartan cilexetil, which is completely converted during enteric absorption to the active compound, Candesartan. Candesartan cilexetil is rapidly and completely hydrolysed to the active compound Candesartan during absorption from the gastrointestinal tract. Candesartan is a potent, long-acting, selective angiotensin II AT, receptor blocker which is well-tolerated and effective with a once-daily dosing regimen in hypertensive patients.

Hydrochlorothiazide (HCT), a thiazide diuretic, is also used to treat mild to moderate hypertension, usually in combination with other antihypertension agents with different mechanisms of actions.

Simple, sensitive and reproducible UPLC-MSMS method was developed for simultaneous estimation of Candesartan and Hydrochlorothiazide in Human Plasma using Losartan and Hydroflumethiazide as internal standard respectively. Chromatographic separation was achieved with waters Acquity UPLC BEH C18 (100*2.1mm) 1.7μ column. Isocratic mobile phase consisted of Buffer 2 mM ammonium formate in water and Acetonitrile (50: 50), with the flow of 0.3mL/min and injection volume was 1μL. Sample Preparation involved solid phase extraction using Phenomenex Starta (30mg/1 mL).
KEY WORDS:
HPLC-MS/MS, UPLC-MS/MS, Nicorandil, Olanzapine, Cyclobenzaprine, Candesartan, Hydrochlorothiazide, Method Development, Method Validation, Solid phase extraction, Bioequivalence study.