2.0. LITERATURE REVIEW

2.1. BACKGROUND INFORMATION AND BIOANALYTICAL ASSAY METHODS

OVERVIEW OF DRUGS

2.1.1. Aceclofenac

Aceclofenac [2-[2-[2-[2, 6-dichlorophenyl] amino] phenyl] acetyl] oxyacetic acid] \(^{97-98}\) was invented by Daniel L. Simmons at Brigham Young University in 1991. The original product is listed as AIRTAL 100mg tablets licensed in May 1991 in Spain, licensed to Almirall Prodesfarma SA and first granted in the EU [Spain] in May 1991. It was first granted in the UK in 1995, UCB Pharma Ltd UK is the company responsible for placing the product in the UK.

The empirical formula of aceclofenac is \(\text{C}_{16}\text{H}_{13}\text{Cl}_{2}\text{NO}_{4}\) and its molecular weight is 354.2 g/mol. The structural formula is as follows:

![Structural formula of aceclofenac]

Aceclofenac is a white to almost white crystalline powder with practically insoluble in water, freely soluble in acetone, soluble in alcohol. Aceclofenac is a widely used non-steroidal anti-inflammatory drug. It is a phenylacetic acid derivative and is indicated for the relief of pain and inflammation in osteoarthritis, rheumatoid arthritis and ankylosing spondylitis\(^{99-101}\).
Aceclofenac is a novel NSAID known to exhibit multifactor mechanism of action. Aceclofenac was developed in order to provide a highly effective pain relieving therapy with a reduced side effect profile.

After oral administration, aceclofenac is rapidly absorbed and the bioavailability is almost 100%. Peak plasma concentrations are reached approximately 1.25 to 3 hrs following ingestion. $T_{\text{max}}$ is delayed with concomitant food intake whereas the degree of absorption is not influenced.

Aceclofenac is highly protein-bound (>99.7%). Aceclofenac penetrates into the synovial fluid where the concentrations reach approximately 60% of those in plasma. The volume of distribution is approximately 30L.

Aceclofenac is probably metabolized via CYP2C9 to the main metabolite 4-hydroxyaceclofenac. The mean plasma elimination half-life is 4-4.3 hrs.

Approximately two-thirds of the administered dose is excreted via the urine, mainly as conjugated hydroxyl metabolites. Only 1% of an oral single dose is excreted unchanged. A slower rate of elimination of aceclofenac has been detected in patients with decreased liver function after a single dose of aceclofenac. In a multiple dose study using 100 mg once daily, there was no difference in the pharmacokinetic parameters between subjects with mild to moderate liver cirrhosis and normal subjects. In patients with mild to moderate renal impairment, no clinically significant differences in the pharmacokinetics were observed after a single dose.

Several chromatographic methods have been reported for the quantification of the Aceclofenac are HPLC-UV detection, HPTLC
method\textsuperscript{111-112}, HPLC-PDR detection\textsuperscript{113-115} in combined tablet dosage formulation.

The spectrophotometric method\textsuperscript{105} for simultaneous estimation of aceclofenac utilized the spectrum mode of analysis of Jasco V-530 spectrophotometer. The method utilized 274 nm & 248 nm as analytical wavelengths for estimation of aceclofenac and PARA in the tablet formulation.

Dual wavelength spectrophotometric method\textsuperscript{106} was used for estimation aceclofenac in the combined tablet dosage form by using the wavelengths 301.5 nm and 311.0 nm.

Simultaneous determination of drotaverine hydrochloride and aceclofenac in combined tablet dosage form was used RP-HPLC method\textsuperscript{107}. In this method separation was carried out on Jasco HPLC system equipped with Hypersil GOLD C18 column [250 x 4.6 mm i.d.] and UV/VIS detector using methanol: 10 mM potassium dihydrogen phosphate buffer in ratio of [80:20, v/v] as mobile phase and detection was carried out at 231 nm.

Reverse phase HPLC method\textsuperscript{108} was used for the simultaneous estimation of paracetamol and aceclofenac from pharmaceutical dosage forms. The method was carried out on a Hichrom C18 [25 cm x 4.6 mm i.d., 5 µ] column with a mobile phase consisting of acetonitrile: 20 mM phosphate buffer [pH 5.0] [60:40 v/v] at a flow rate of 0.8 mL/min. Detection was carried out at 265 nm.

A high-performance liquid chromatographic [HPLC] method\textsuperscript{109} was developed for quantification of aceclofenac in rat plasma. Ibuprofen was used as an internal standard [IS]. The method used protein precipitation for
extraction of aceclofenac from rat plasma. Separation was carried out on reversed-phase C18 column [250 mm × 4.6 mm, 5 μ] and the column effluent was monitored by UV detector at 282 nm. The mobile phase used was methanol-triethylamine [pH 7.0; 0.3% v/v in Milli-Q water] [60:40%, v/v] at a flow rate of 1.0 mL/min.

Three methods\textsuperscript{110} for quantitative determination of aceclofenac [AC] in pure form and in pharmaceutical formulation were described. The first method is based on the reaction between the drug via its secondary aromatic amino group and p-dimethyl amino cinnam aldehyde [PDAC] in acidified methanol to give a stable coloured complex after heating at 75°C for 20 min. Absorption measurements were carried out at 665.5 nm. Beer’s law is obeyed over concentration range 20–100 μg/mL with mean recovery 100.33±0.84. The other two methods are high performance liquid chromatography [HPLC] and densitometric methods by which the drug was determined in the presence of its degradation products over concentration range of 20–70 μg/mL and 1–10 μg per spot and mean recoveries are 99.59±0.90 and 99.45±1.09, respectively.

High performance thin layer chromatographic method\textsuperscript{111} was developed for the determination of diacerein and aceclofenac in the combined pharmaceutical tablet dosage form. The developed method demonstrates extraction of diacerein and aceclofenac by solid–liquid extraction and densitometric determination of them. Paracetamol was used as an internal standard [IS]. The precoated silica gel 60F254 aluminum plate was selected as the stationary phase and the mixture of ethyl acetate: methanol: glacial acetic acid in the ratio of [12: 0.5:
0.2 v/v/v was used as developing solvents. The detection of diacerein and aceclofenac was carried out at 268 nm by TLC scanner-3 [Camag].

HPTLC method has been developed for the determination of paracetamol, aceclofenac and rabeprazole in combined tablet dosage form. Determination was performed on aluminium backed silica gel 60F254 washed with methanol. The mobile phase used is ethyl acetate- methanol- glacial acetic acid [9:1:0.1]. The spots were scanned at 275nm. The linearity of aceclofenac was found to be 20-100μg/mL respectively.

A narrowbore high performance liquid chromatography with column-switching was developed for the simultaneous determination of aceclofenac and diclofenac from human plasma samples. Plasma sample [100 μL] was directly introduced onto a Capcell Pak MF Ph-1 column [20 x 4 mm i.d.] where primary separation was occurred to remove proteins and concentrate target substances using acetonitrile–potassium phosphate [pH 7, 0.1 M] [14:86, v/v]. The drug molecules eluted from MF Ph-1 column were focused in an intermediate column [35 x 2 mm i.d.] by the valve switching step. The substances enriched in intermediate column were eluted and separated on the narrowbore phenyl–hexyl column [100×2 mm i.d.] using acetonitrile: potassium phosphate [pH 7, 0.02M] [33:67, v/v] when the valve status was switched back to A position.

HPLC method was developed for quantitation of aceclofenac and paracetamol from bulk drug and pharmaceutical formulations using a mobile phase consisting mixture of methanol and water [70:30 v/v] at the flow rate of 1mL/min. An ODS C-18 [Intersile 25 cm x 4.6 mm, 10 μ] column was used as
stationary phase. The retention time of aceclofenac and paracetamol were 1.8 min. and 2.7 min. respectively. Linearity was observed in the concentration range of 2-50 μg/mL for aceclofenac and 5-50 μg/mL for paracetamol.

RP-HPLC-PDA method\textsuperscript{115} was used for simultaneous estimation of drotaverine and aceclofenac in a combined dosage form.

Here we are reporting a highly sensitive Liquid chromatography/ Tandem Mass spectrometry [LC-MS/MS] method developed and validated for the quantification of aceclofenac in human plasma.

\textbf{2.1.2. Artemether}

Co-Artemether is an oral fixed-dose combination tablet of artemether [a derivative of artemisinin] and lumefantrine, an antimalaria\textsuperscript{116-127} synthesized and developed by the academy of military medical sciences in Beijing, People’s Republic of China. This combination was registered in China in 1992 for the treatment of plasmodium falciparum malaria, and has been subsequently further developed by Novartis Pharmaceuticals.

The chemical name of artemether is [3R, 5aS, 6R, 8aS, 9R, 10S, 12R, 12aR]-decahydro-10-methoxy-3, 6, 9-trimethyl-3, 12-epoxy-12H-pyrano [4, 3-j]-1, 2-benzodioxepine. It has the empirical formula C\textsubscript{16}H\textsubscript{26}O\textsubscript{5} with a molecular weight of 298.4 g/mol, and the following structural formula:
Artemether is a white, crystalline powder that is freely soluble in acetone, soluble in methanol and ethanol, and practically insoluble in water.

Artemether and lumefantrine are active against the erythrocytic stages of plasmodium falciparum. Strains of P. falciparum with a moderate decrease in susceptibility to artemether or lumefantrine alone can be selected in vitro or in vivo, but not maintained in the case of artemether.

Artemether is rapidly metabolized into an active metabolite dihydroartemisinin. The anti-malarial activity of artemether and dihydroartemisinin [DHA] has been attributed to endoperoxide moiety. The exact mechanism by which lumefantrine, exerts its anti-malarial effect is not well defined. Available data suggest lumefantrine inhibits the formation of β-hematin by forming a complex with hemin. Both artemether and lumefantrine were shown to inhibit nucleic acid and protein synthesis.

Artemether is absorbed with peak plasma concentrations reached about two hrs after oral drug administration. Absorption of lumefantrine, a highly lipophilic compound, starts after a lag-time of up to two hrs, with peak plasma concentrations about 6 to 8 hrs after administration.
Artemether and lumefantrine are both highly bound to human serum proteins in vitro [95.4% and 99.7%, respectively]. Dihydroartemisinin is also bound to human serum proteins [47% to 76%]. Protein binding to human plasma proteins is linear.

The metabolism of artemether was catalyzed predominantly by CYP3A4/5. Dihydroartemisinin [DHA] is an active metabolite of artemether. The metabolism of artemether was also catalyzed to a lesser extent by CYP2B6, CYP2C9 and CYP2C19.

Artemether and DHA are cleared from plasma with an elimination half-life of about two hrs. Lumefantrine is eliminated more slowly, with a terminal half-life of 3-6 days in healthy volunteers and in patients with falciparum malaria.

The pharmacokinetics of artemether [A] and its active main metabolite dihydroartemisinin [DHA] have previously been characterized using a high performance liquid chromatography with electro chemical detection in the reductive mode\textsuperscript{136}. However, this analytical method is difficult to use routinely due to very rigorous conditions that should be applied to prevent dissolved oxygen from entering the flow cell.

Several chromatographic methods have been reported for the quantification of the artemether and its metabolite in human plasma, are High-performance liquid chromatography method\textsuperscript{137-138}, in saliva\textsuperscript{138}, high-performance liquid chromatography and an evaporative light scattering detector\textsuperscript{139}, plasmodium falciparum-based bioassay for measurement of artemisinin derivatives in plasma or serum\textsuperscript{140}, supercritical fluid chromatography with electron-capture detection\textsuperscript{141}, gas chromatography–mass spectrometry-selected ion monitoring\textsuperscript{142}, HPLC-Electrospray ionization mass spectrometric analysis\textsuperscript{143-145}, in rat serum\textsuperscript{146}. 
An analytical method for the determination of artemether [A] and its metabolite dihydroartemisinin [DHA] in human plasma is based on high-performance liquid chromatography [HPLC] and electrochemical detection in the reductive mode. A, DHA and artemisinin, the internal standard [I.S.], were extracted from plasma [1 mL] with 1-chlorobutane-iso-octane [55:45, v/v]. The solvent was transferred, evaporated to dryness under nitrogen and the residue dissolved in 600 μL of water-ethyl alcohol [50:50, v/v]. Chromatography was performed on a Nova-Pak CN, 4μ analytical column [150 mm x 3.9 mm I.D.] at 35°C. The mobile phase consisted of pH 5 acetate-acetonitrile [85:15, v/v] at a flow-rate of 1 mL/min. The analytes were detected by electrochemical detection in the reductive mode at a potential of -1.0 V.

A method was described for the separation of artemether [ARM] from its metabolite dihydroartemisinin [DHA] and determination by HPLC. The basis of the separation is differential extraction of the drugs from plasma as a function of plasma pH. Hexane extracted ARM from basified plasma and both ARM and DHA from normal plasma. Derivatized extracts were chromatographed on a 5 μ ODS column with water-acetonitrile [40:60 v/v] as mobile phase and detected at 254 nm.

Direct analysis was performed in plasma and saliva for artemisinin using coupled-column high-performance liquid chromatography with a restricted-access material pre-column.

The high-performance liquid chromatographic method was developed for artemisinin and several analogues of artemisinin using a readily available evaporative light scattering detector.
Plasmodium falciparum-based bioassay\textsuperscript{140} was developed for measurement of artemisinin derivatives in plasma or serum by high-performance liquid chromatography [HPLC] with electrochemical detection.

Packed-column supercritical fluid chromatography\textsuperscript{141} was developed for measurement of artemisinin [qinghaosu] with electron-capture detection.

A GC–MS–SIM method\textsuperscript{142} was developed for determination of artemether [ARM] and dihydroartemisinin [DHA] in plasma using artemisinin [ART] as internal standard. Solid phase extraction was performed using C Bond Elut cartridges. The analysis was carried out using a HP-5MS 5% phenylmethylsiloxane capillary column.

A HPLC–Electrospray ionization mass spectrometric method\textsuperscript{143} was developed for analysis of antimalarial drug artemisinin.

A method\textsuperscript{144} was used for the determination of artemether and its active dihydroartemisinin metabolite in human plasma using artemisinin as internal standard. The method consists of a liquid–liquid extraction with subsequent evaporation of the supernatant to dryness followed by the analysis of the reconstituted sample by liquid chromatography–mass spectrometry [LC–MS] in single ion monitoring mode using atmospheric pressure chemical ionization [APCI] as an interface. Chromatography was performed on a C18 reversed-phase column using acetonitrile–glacial acetic acid 0.1% [66:34 v/v] as a mobile phase.

A method\textsuperscript{145} for the determination of artemether [ART] and its main metabolite dihydroartemisinin [DHA] in plasma employing liquid-phase microextraction [LPME] for sample preparation prior to liquid chromatography-tandem mass spectrometry [LC-MS-MS] was developed. The analytes were extracted from 1mL of plasma utilizing
a two-phase LPME procedure with artemisinin as internal standard. Using the optimized LPME conditions, mean absolute recovery rates of 25 and 32% for DHA and ART, respectively, were achieved using toluene-n-octanol [1:1, v/v] as organic phase with an extraction time of 30 min. After extraction, the analytes were resolved within 5 min using a mobile phase consisting of methanol-ammonium acetate [10 mmol/L, pH 5.0, 80:20, v/v] on a laboratory-made column based on poly[methyltetradecylsiloxane] attached to a zirconized-silica support. MS-MS detection was employed using an electrospray interface in the positive ion mode.

Liquid chromatographic-mass spectrometric method\textsuperscript{146} was developed for the determination of \( \alpha \)-, \( \beta \)-arteether in rat serum.

We now report a highly sensitive liquid chromatography-tandem mass spectrometry [LC-MS/MS] method developed and validated for the quantification of artemether in human plasma. It was essential to establish an assay capable of quantifying artemether at concentrations down picograms level. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of artemether.

\subsection*{2.1.3. Alverine and P-Hydroxy Alverine}

Alverine citrate [N-ethyl-3-phenyl-N-[3-phenylpropyl]propan-1-amine dihydrogen 2-hydroxy propane-1, 2, 3-tricarboxylate N-ethyl-N-[3-phenylpropyl]-benzene propanamine, citrate] and P-Hydroxy Alverine are an anticholinergic [antispasmodic] agents\textsuperscript{147} used as a smooth muscle relaxants in disorders of the gastrointestinal and genitourinary tracts and utilized in the treatment of abdominal manifestations, is particularly useful in treating irritable bowel syndrome, dysmenorrhoea and similar
conditions. Empirical formula for Alverine citrate is $\text{C}_{20}\text{H}_{27}\text{N.C}_6\text{H}_8\text{O}_7$, with a molecular weight = 473.56 g/mol. Empirical formula for P-Hydroxy Alverine is $\text{C}_{20}\text{H}_{27}\text{NO}$, with a molecular weight = 297.43 g/mol. The following are structural formulas for Alverine and P-Hydroxy Alverine.

![Structural formulas for Alverine and P-Hydroxy Alverine](image)

Alverine is white to pale yellow fine powder, slightly soluble in water and methylene dichloride. Sparingly soluble in ethanol. It has melting point about 104°C.

It can also be used to help relieve period pain and cramps without affecting the heart, blood vessels and tracheal muscle at therapeutic doses. The mode of action of alverine is different from tricyclic antidepressants and specific or non-specific inhibitors of the recapture of serotonin, since alverine interacts marginally with serotonin or noradrenaline recapture systems. However, the exact mechanisms of alverine inhibitory actions are still not clear, due to the lack of information of its effects on isolated smooth muscle in vitro. After oral administration, alverine is rapidly converted to its primary active metabolite, which is then further converted to two secondary metabolites. There is a high renal clearance of all metabolites indicating that they are eliminated by active renal secretion. The peak plasma level of the most
active metabolite occurs between 1 and 1.5 hrs after oral dosing. Alverine has a very low toxicity and side effects, which are highly limited, as compared to the classic antidepressants.

Analytical methods so far reported for quantification of alverine and metabolite PHA are tandem mass spectrometric detection\textsuperscript{156-158}.

The liquid chromatography-tandem mass spectrometry method\textsuperscript{156} developed and validated for simultaneous analysis of Alverine [ALV] and one of its hydroxy metabolites, para hydroxy Alverine [PHA] in human plasma. The analytes were extracted from the matrix using a simple solid-phase extraction procedure. Mebeverine was used as the internal standard for both analytes. A Kromasil C8 column provided chromatographic separation of analytes followed by detection with mass spectrometry. The method involves simple isocratic chromatography conditions and mass spectrometric detection in the positive ionization mode using an API 5000 MS/MS system. The method has been validated with a linear range of 100-10,000 pg/mL for both ALV and PHA. The interrun and intrarun precision values are within 6.3%, 3.7% for ALV and 6.3%, 3.2% for PHA at LOQ levels. The intrarun accuracy in terms of % accuracy was within the range of -7.0% to -0.1% and -8.1% to -1.7% for ALV and PHA, respectively whereas the interrun accuracy was within the range of -5.1% to -0.5% for ALV and -8.6% to 0.4% for PHA, respectively. The overall recoveries for ALV and PHA were 83.5% and 86.2% respectively. Total elution time was about 4 min which allowed quantitation of more than 150 plasma samples per day.

A LC-MS/MS method\textsuperscript{157} for the determination of alverine [ALV] and its major metabolite, monohydroxy alverine [MHA], in human plasma using imipramine as an internal standard was developed. The analytes were extracted from 0.5 mL aliquots of
human plasma by solid phase extraction, using oasis cartridge. Chromatographic separation was carried on Thermo Gold C18 column [50 x 4.6 mm, 5 μ] at 30 °C, with isocratic mobile phase, a flow rate of 0.4 mL/min and a total run time of 3.5 min. Detection and quantification were performed using a mass spectrometer in the selected reaction-monitoring mode with positive electrospray ionization at m/z 282.3 → 91.11 for alverine, m/z 298.3 → 106.9 for mono-hydroxy-alverine, and m/z 281.0 → 86.0 for internal standard [IS] respectively. This assay was linear over a concentration range of 0.060-10 ng/mL with a lower limit of quantification of 0.060 ng/mL for both alverine and monohydroxy alverine. The coefficient of variation for the assay precision were <9.18% and <8.44%, the accuracy were >104.66% and >100.38% for alverine and monohydroxy alverine respectively.

A method for the determination of alverine [ALV] and its metabolite, para hydroxy alverine [PHA], in human plasma using LC-MS/MS in positive ion electrospray ionization [ESI] in multiple reactions monitoring [MRM] mode was developed. The procedure involves a simple solid phase extraction [SPE]. Chromatographic separation was carried out on a Hypersil GOLD C[18] column [50 mm x 4.6 mm, 5 μm] with an isocratic mobile phase. The standard calibration curves showed linearity within the range of 0.060-10.051 ng/mL for ALV and 0.059-10.017 ng/mL for PHA [r or ≥ 0.990].

Therefore, the development of a new, easier to use and at least equally sensitive method is necessary. A combined LC-tandem mass spectrometric method [LC-MS/MS] was found to be the favorite choice.

The present work describes a sensitive and selective first automated high performance liquid chromatography tandem mass spectrometric method with liquid-
liquid extraction developed and validated for the simultaneous quantification of alverine and its metabolite PHA in human plasma using Ticlopidine as an internal standard.

2.1.4. Clopidogrel and its Metabolite

Clopidogrel bisulfate\textsuperscript{159} is a thienopyridine class inhibitor of P2Y12 ADP platelet receptors. Chemically it is methyl [+]\textsuperscript{S}-α-[2-chlorophenyl]-6, 7-dihydrothieno [3, 2-c] pyridine-5[4H] acetate sulfate [1:1]. The empirical formulae for clopidogrel bisulfate and its metabolite carboxylic acid metabolite are C\textsubscript{16}H\textsubscript{16}ClNO\textsubscript{2}S.H\textsubscript{2}SO\textsubscript{4} and C\textsubscript{15}H\textsubscript{14}ClNO\textsubscript{2}S; their molecular weights are 421.547 g/mol and 307.924.

The structural formulae are as follows:

Clopidogrel bisulfate is a white to off-white powder. It is practically insoluble in water at neutral pH but freely soluble at pH 1. It also dissolves freely in methanol,
dissolves sparingly in methylene chloride, and is practically insoluble in ethyl ether. It has a specific optical rotation of about +56°. Clopidogrel carboxylic acid is a Pale Yellow Solid and melting point 100 to 105°C.

Clopidogrel is an inhibitor of platelet activation\textsuperscript{160-162} and aggregation through the irreversible binding of its active metabolite to the P2Y12 class of ADP receptors on platelets.

Clopidogrel is extensively metabolized by two main metabolic pathways: one mediated by esterases and leading to hydrolysis into an inactive carboxylic acid derivative [85% of circulating metabolites] and one mediated by multiple cytochrome P\textsubscript{450} enzymes\textsuperscript{163}. Cytochromes first oxidize clopidogrel to a 2-oxo-clopidogrel intermediate metabolite. Subsequent metabolism\textsuperscript{164-165} of the 2-oxo-clopidogrel intermediate metabolite results in formation of the active metabolite, a thiol derivative of clopidogrel. The active thiol metabolite binds rapidly and irreversibly to platelet receptors, thus inhibiting platelet aggregation for the lifespan of the platelet.

The $C_{\text{max}}$ of the active metabolite is twice as high following a single 300 mg clopidogrel loading dose as it is after four days of 75 mg maintenance dose. $C_{\text{max}}$ occurs approximately 30 to 60 minutes after dosing. In the 75 to 300 mg dose range, the pharmacokinetics of the active metabolite deviates from dose proportionality: increasing the dose by a factor of four results in 2.0- and 2.7-fold increases in $C_{\text{max}}$ and AUC, respectively.

Following an oral dose of 14C-labeled clopidogrel in humans, approximately 50% of total radioactivity was excreted in urine and approximately 46% in feces over the 5 days post-dosing. After a single, oral dose of 75 mg, clopidogrel has a half-life of approximately 6 hrs. The half-life of the active metabolite is about 30 minutes.
Analytical methods so far reported for quantification of clopidogrel, are tandem mass spectrometric detection\textsuperscript{166-168} and for quantification of carboxylic acid metabolite, it is by employing HPLC coupled ultraviolet detection in human plasma\textsuperscript{169}, in rat plasma\textsuperscript{170} and in human serum\textsuperscript{171}, mass spectrometric detection\textsuperscript{172} and tandem mass spectrometric detection\textsuperscript{173-174}. No method was reported for simultaneous quantification of clopidogrel and its metabolite. In literature some of the automated methods were reported using high-throughput technique\textsuperscript{175} described below.

A LC-MS/MS method\textsuperscript{166} for the determination of unchanged clopidogrel in human plasma has been developed over the range of 10-12,000 pg/mL [$r^2$ 0.9993]. Samples [0.3 mL] were buffered [pH 6.8], extracted using diethyl ether and 10 µL of the sample extract was injected onto the LC-MS/MS system. Analysis was performed using a C8 column [temperature controlled to 50°C] by gradient elution at a flow rate of 0.9 mL/min over a 3 min run time. Retention times of 1.61 and 1.59 min were observed for clopidogrel and 2H3-clopidogrel [IS], respectively. Detection was achieved using a Sciex API 4000, triple quadrupole mass spectrometer, in positive Turbolonspray [electrospray] ionisation mode. Ion transitions were monitored using MRM [multiple reaction monitoring] for clopidogrel [m/z 322-212] and for 2H3-clopidogrel [m/z 327-217].

A LC-MS/MS assay\textsuperscript{167} was developed for the determination of clopidogrel in human plasma. Clopidogrel was extracted by single liquid-liquid extraction with pentane, and chromatographic separations were achieved on a C-18 column. The method was validated to demonstrate the specificity, linearity, recovery, lower limit of quantification [LLOQ], stability, accuracy and precision. The multiple reaction monitoring was based on m/z transition of 322.2/ 211.9 for clopidogrel and
264.1/125.1 for ticlopidine [internal standard]. The total analytical run time was relatively short [3 min], and the LLOQ was 10 pg/mL using 0.5 mL of human plasma. The assay was linear over a concentration range from 10 to 10,000 pg/mL [r > 0.999]. The intra- and inter-day accuracies were 101.3-108.8 and 98.4-103.5%, respectively, and the intra- and inter-day assay precisions were 1.9-5.5 and 4.4-8.1%, respectively.

A high-performance liquid chromatography/positive electrospray ionization tandem mass spectrometry method was developed for the assay of clopidogrel in human plasma. Following liquid-liquid extraction, the analytes were separated using an isocratic mobile phase on a reversed-phase column and analyzed by mass spectrometry in the multiple reaction monitoring mode using the respective [M+H]+ ions, m/z 322/212 for clopidogrel and m/z 264/154 for the internal standard. The assay exhibited a linear dynamic range of 5–6000 pg/mL for clopidogrel in human plasma. The lower limit of quantification was 5 pg/mL with a relative standard deviation of less than 8%. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. A run time of 2.5 min for each sample made it possible to analyze more than 400 human plasma samples per day.

A method for determination of carboxylic acid metabolite of clopidogrel in human plasma has been developed. After liquid-liquid extraction in acidic medium with chloroform, samples were quantified on a Nova-pak C-8, 5 μm column using a mixture of 30 mM K2HPO4-THF-acetonitrile [pH = 3, 79:2:19, v/v/v] as mobile phase with UV detection at 220 nm. The flow rate was set at 0.9 mL/min. Ticlopidine was used as internal standard and the total run time of analysis was about 12 min. The method was linear over the range of 0.2-10 μg/mL of clopidogrel metabolite in plasma [r [2] >
A HPLC method was developed for the estimation of carboxylic acid metabolite of clopidogrel bisulfate in rat plasma using atorvastatin as internal standard. Plasma samples were extracted with a mixture of ethyl acetate and di-chloro methane [80:20, v/v] followed by subsequent reconstitution in a mixture of water: methanol: acetonitrile [40:40:20, v/v/v]. The chromatographic separation was achieved with gradient elution on Kromasil ODS [250 mm x 4.6 mm i.d., 5 µm] analytical column maintained at 30°C. Carboxylic acid metabolite of clopidogrel and the internal standard were detected at a wavelength of 220 nm.

A method is developed for determination of clopidogrel carboxylic acid [CCA], the inactive metabolite of anti platelet agent, clopidogrel, in human serum. The analytical procedure involves liquid–liquid extraction of the analyte and an internal standard [phenytoin] with ethyl acetate. A mobile phase consisting of 0.05 M phosphate buffer containing triethylamine [0.5 mL/L; pH 5.7] and acetonitrile [56:44 v/v] was used and chromatographic separation was achieved using C18 analytical column at detector wavelength of 220 nm. The calibration curves were linear over a concentration range of 0.05–10 µg/mL of CCA in human serum. The total run time of analysis was 5.5 min and the lower limits of detection [LOD] and quantification [LOQ] were 0.02 and 0.05 µg/mL, respectively.

A method for determination of clopidogrel metabolite [SR26334] in human plasma has been developed. After liquid–liquid extraction on Chem Elut cartridges with dichloromethane, samples were quantified using reversed-phase high performance liquid chromatography with mass detection. The determination was
performed on a Luna C18, 3 μm [75 mm x 4.6 mm i.d.] column with an acetonitrile-water-formic acid mixture [60:40:0.1, v/v/v] as a mobile phase. The flow rate was set at 0.2 mL/min. Repaglinide was chosen as an internal standard and the time of analysis was 12 min. For SR26334 the limits of detection and quantification were 7.5 ng/mL and 20 ng/mL, respectively, and the calibration curve was linear up to 3000 ng/mL.

A quantitative method for the determination of clopidogrel active metabolite [AM] in human plasma was developed and validated using liquid chromatography-tandem mass spectrometry [LC-MS/MS]. The alkylating reagent 2-bromo-3'-methoxyacetophenone was used to stabilize clopidogrel AM in blood. An analog of the derivatized clopidogrel AM was used as the internal standard [IS]. The derivatized samples were subjected to solid-phase extraction with a C2 disk plate and the overall procedure exhibited good reaction [more than 90%] and recovery efficiencies [from 85% to 105%]. The derivative of clopidogrel AM [MP-AM] and IS were separated on an ODS column and quantified by tandem mass spectrometry with electrospray ionization.

An ultra-performance LC-MS method was developed for the quantification of clopidogrel active metabolite in human plasma, with clopidogrel D4 as internal standard. Plasma pretreatment involved a one-step protein precipitation with acetonitrile. The separation was performed by reverse-phase chromatography on a C-8 column. The method was linear over the concentration range of 1–15 0ng/mL. The intra- and inter-day precision values were below 17% and accuracy was from 1.7 to 7.5% at all quality control levels. The lower LOQ was 0.8 ng/mL. Sample analysis time was reduced to 5 min including sample preparation [limited to protein precipitation].
A generic method\textsuperscript{175} was developed for on-line extraction of drug substances in the presence of biological matrices using turbulent flow chromatography.

We now report a first automated high-throughput liquid chromatography tandem mass spectrometric method developed and validated for the simultaneous quantification of clopidogrel and its metabolite carboxylic acid derivative of clopidogrel in human plasma using ticlopidine as an internal standard. Robotic liquid handling systems are employed in all liquid transfer steps including the sample preparation procedure as well as in the addition/removal of the organic solvent. The current method includes a simple and rapid sample preparation as a result of robotic systems utilization that enabled parallel processing as well as significantly shorter analysis run time compared to previously published methods.

\textbf{2.1.5. Carvedilol and 4-Hydroxyphenyl Carvedilol}
Carvedilol phosphate is chemically described as [2RS]-1-[9H-Carbazol-4-yloxy]-3-[[2-[2-methoxyphenoxy] ethyl] amino] propan-2-ol phosphate salt [1:1] hemihydrate. The molecular weights for Carvedilol phosphate \([C_{24}H_{26}N_2O_4\cdot H_3PO_4\cdot 1/2H_2O]\) and 4-Hydroxyphenyl Carvedilol \([C_{24}H_{27}N_2O_5]\) are 513.5 g/mol and 423.856 g/mol, their structural formulae are:

![Structural formulae of Carvedilol and 4-Hydroxyphenyl Carvedilol](image)

Carvedilol phosphate is a white solid. Carvedilol\(^{176-177}\) is a racemic mixture in which nonselective \(\beta\)-adrenoreceptor blocking activity is present in the S [-] enantiomer and \(\alpha_1\)-adrenergic blocking activity is present in both R [+] and S [-] enantiomers\(^{178-179}\) at equal potency. Carvedilol has no intrinsic sympathomimetic activity.

Carvedilol is more than 98% bound to plasma proteins, primarily with albumin. The plasma-protein binding is independent of concentration over the therapeutic range. Carvedilol is a basic, lipophilic compound with a steady-state volume of distribution, indicating substantial distribution into extra vascular tissues.

Carvedilol\(^{180}\) is extensively metabolized and the 4′-hydroxyphenyl metabolite is approximately 13 times more potent than carvedilol for \(\beta\)-blockade\(^{181-183}\).
Compared to carvedilol, the three active metabolites exhibit weak vasodilating activity. Plasma concentrations of the active metabolites are about one-tenth of those observed for carvedilol and have pharmacokinetics\textsuperscript{184} similar to the parent. Carvedilol undergoes stereoselective first-pass metabolism with plasma levels of R [+]-carvedilol approximately 2 to 3 times higher than S [-]-carvedilol following oral administration of carvedilol in healthy subjects.

Therefore, the quantification of carvedilol in plasma requires a bioanalytical method with high sensitivity. The actual plasma concentrations of parent drug and/or metabolite[s] are of major interest in pharmacokinetic studies. However, the metabolite of carvedilol, which is the most abundant species circulating in blood, was used to document the pharmacokinetic profile of carvedilol.

Carvedilol had been determined in plasma and other biological fluids such as high performance liquid chromatography coupled to ultra-violet detection\textsuperscript{185-189}, and capillary electrophoresis coupled to ultra-violet detection\textsuperscript{190-191}, enantiomers detection\textsuperscript{192-194}, and high performance liquid chromatography coupled to tandem mass spectrometry \textsuperscript{195-197}.

Quantitative determination\textsuperscript{185} of serum concentrations of carvedilol was obtained using HPLC with spectrofluorometric detection. Carvedilol was extracted from alkalinized serum with ether and was subsequently back extracted with diluted phosphoric acid.

Liquid chromatographic method\textsuperscript{186} was developed for determination of carvedilol in human plasma using UV detector.
An enantioselective high-performance liquid chromatographic method\textsuperscript{187} for the analysis of carvedilol in plasma and urine was developed using [-] menthyl chloroformate [MCF] as a derivatizing reagent. Chloroform was used for extraction, and analysis was performed by HPLC on a C18 column with a fluorescence detector. The quantitation limit was 0.25 ng/mL for S[-]-carvedilol in plasma and 0.5 ng/mL for R[+]-carvedilol in plasma and for both enantiomers in urine.

A high-performance liquid chromatography with spectrofluoro-metric detection\textsuperscript{188}, using a solid-phase extraction for a simple, was used for determination of plasma carvedilol levels in rats. Extracted aliquots were analyzed by HPLC, using a reversed-phase octadecyl silica column.

Quantitative methodology\textsuperscript{189} for the simultaneous high-performance liquid chromatographic [HPLC] resolution and determination of the enantiomers of carvedilol, and its active metabolite, O-desmethylcarvedilol, in human plasma is described. The method involves reversed-phase solid-phase extraction of the analytes, followed by derivatization of the extract with the chiral reagent, 2,3,4,6-tetra-O-acetyl-\(\beta\)-d-glucopyranosyl isothiocyanate and injection of the resultant diastereoisomers onto a reversed-phase HPLC column coupled to a fluorescence detector. Both pairs of diastereoisomers formed are completely resolved within 12 min.

Comparison between capillary electrophoresis and high-performance liquid chromatography\textsuperscript{190} was done for the stereoselective analysis of carvedilol in serum.

Comparison of the Hummel-Dreyer method\textsuperscript{191} in high-performance liquid chromatography and capillary electrophoresis conditions was done for study of the interaction of [RS]-, [R]- and [S]-carvedilol with isolated plasma proteins.
Measurement of carvedilol enantiomers\textsuperscript{192} in human plasma and urine was done by using S-naproxen chloride for chiral derivatization.

Quantitative determination of the enantiomers\textsuperscript{193} in human plasma by HPLC was carried out after formation of diastereoisomers with the chiral reagent 2,3,4,6-tetra O-acetyl-\(\beta\)-d-glucopyranosyl isothio cyanate [GITC].

A normal phase high performance liquid chromatographic method\textsuperscript{194} was developed to study the steady-state pharmacokinetics of carvedilol and its enantiomers in patients with congestive heart failure.

A method\textsuperscript{195} to quantify carvedilol in human plasma using metoprolol as the internal standard [IS] is developed. The analyte and the IS were extracted from plasma by liquid–liquid extraction using a diethyl-ether solvent. After removed and dried the organic phase, the extracts were reconstituted with a fixed volume of acetonitrile–water [50/50; v/v]. The extracts were analyzed by a high performance liquid chromatography coupled to electrospray tandem mass spectrometry [HPLC–MS/MS]. Chromatography was performed isocratically on Alltech Prevail C18 5\(\mu\)m analytical column, [150mm x 4.6mm i.d.]. The method had a chromatographic run time of 3.5 min and a linear calibration curve over the range 0.1–200 ng/mL \([r^2 > 0.997992]\).

We now report a first automated high-throughput liquid chromatography tandem mass spectrometric method developed and validated for simultaneous quantification of carvedilol and its metabolite 4-hydroxyphenyl carvedilol in human plasma using propranolol as an internal standard. Robotic liquid handling systems are employed to all liquid transfer steps including the sample preparation procedure as well as to the addition/removal of the organic solvent. The current method includes a simple, rapid sample preparation and extraction as a result of robotic systems
utilization that enabled parallel processing as well as shorter analysis run time with simultaneous quantification of carvedilol and its metabolite 4-hydroxyphenyl carvedilol in human plasma when compared to previously published methods.

2.1.6. Clonidine

Clonidine hydrochloride\textsuperscript{198} is a centrally acting alpha-agonist hypotensive agent available as tablets for oral administration in three dosage strengths: 0.1 mg, 0.2 mg and 0.3 mg. The 0.1 mg tablet is equivalent to 0.087 mg of the free base.

Clonidine hydrochloride is an imidazoline derivative and exists as a mesomeric compound. The chemical name is 2-[2, 6-dichlorophenylamino]-2-imidazoline hydrochloride. The following is the structural formula: $\text{C}_9\text{H}_9\text{Cl}_2\text{N}_3\cdot\text{HCl}$ with molecular weight 266.56 g/mol and the following structural formula:

Clonidine hydrochloride is an odorless, bitter, white, crystalline substance soluble in water and alcohol.

Clonidine acutely stimulates growth hormone release in both children\textsuperscript{199-202} and adults, but does not produce a chronic elevation of growth hormone with long-term use.

The plasma level of clonidine peaks in approximately 3 to 5 hrs and the plasma half-life ranges from 12 to 16 hrs. The half-life increases up to 41 hrs in patients with
severe impairment of renal function. Following oral administration about 40-60% of
the absorbed dose is recovered in the urine as unchanged drug in 24 hrs. About 50%
of the absorbed dose is metabolized in the liver. Neither food nor the race of the
patient influences the pharmacokinetics of clonidine.

Clonidine had been determined in plasma and other biological fluids such as high
performance liquid chromatography coupled to UV Detector\textsuperscript{203-204}, gas-liquid
chromatography\textsuperscript{205}, liquid chromatography-tandem mass spectrometry method\textsuperscript{206-207}.

A reversed-phase high performance liquid chromatographic method\textsuperscript{203} was
described for the determination of chlorthalidone and clonidine hydrochloride
combinations in tablets. Individual tablets or composite samples were sonicated in
water, diluted with methanol, and filtered prior to chromatographing. Chlorthalidone,
formulated at 15 mg/tablet, was chromatographed on octadecylsilyl-bonded, 5 to 6-
µm, spherical silica with 50% methanol in water mobile phase. Clonidine
hydrochloride, formulated at 0.1 or 0.2 mg/tablet, was chromatographed on
trimethylsilyl-bonded, 5 to 6-micrometers, spherical silica with 65% methanol in pH
7.9 phosphate buffer mobile phase. Both were determined with a spectrophotometric
detector at 254 nm.

The method\textsuperscript{204} was developed for determination of submicrogram quantities of
Clonidine in biological fluids.

A gas-liquid chromatographic method\textsuperscript{205} with electron capture detection of
clonidine in rat plasma is developed. The alkaline samples [100-200µL] are extracted
into cyclohexane-butanol [9:1 v/v], re-extracted into 0.1 N sulphuric acid, made
alkaline and back-extracted into cyclohexane-butanol. The pentafluorobenzyl
derivatives of clonidine and the internal standard 2,-[2, 4-dichlorophenylamino]-2-
imidazoline are then formed, and excess of reagent is removed by solvent extractions. The minimum detectable quantity [MDQ] of clonidine and the internal standard are $2.5 \times 10^{-16}$ mol/sec. [3.3 pg] and $2.8 \times 10^{-16}$ mol/sec [5.9 pg], respectively. The smallest amount of clonidine which can be determined in plasma samples with a precision of less than 20 per cent S.D. is 200 pg.

A liquid chromatography/tandem mass spectrometric method\textsuperscript{206} in human serum was developed for the quantification of clonidine [CLD], used. Sample preparation consisted of precipitation of serum proteins by adding acetonitrile and centrifugation of the sample subsequently. [\[2\text{H}4]\text{Clonidine} [CLD4] served as internal standard. Chromatographic separation of the supernatant was achieved using a 100mm x mm, 5\text{\mu}m Thermo Electron BetaBasic C4 column with isocratic flow and elution consisting of 0.1% formic acid/acetonitrile [85/15, v/v] and a flow-rate of 350 \text{\mu}L/min resulting in a column pressure of 280-420 kPa. LC-MS/MS detection was performed by using a triple-stage quadrupole mass spectrometer [TSQ Quantum, Thermo Electron] working in selected reaction monitoring mode with positive electrospray ionization. The analyte was quantified in a single run within 5min. Linearity was demonstrated over the expected concentration range 0.15-50 \text{\mu}g/L CLD. The lower limit of quantification [LLOQ] and the limit of detection were 0.1 \text{\mu}g/L and 0.01 \text{\mu}g/L, respectively.

A liquid chromatography-tandem mass spectrometry method\textsuperscript{207}, using silica column and aqueous-organic mobile phase was developed for the analysis of clonidine as low as 10 pg/mL in human serum.

The assays used relatively large plasma sample volumes [up to 2 mL] and either multiple-step liquid or solid-phase extraction procedures. In this report, we describe a highly sensitive liquid chromatography/tandem mass spectrometry [LC-MS/MS]
method developed and validated for the quantification of clonidine in human plasma and utilizing a single-step extraction and a chromatographic separation. It is essential to establish an assay capable of quantifying clonidine at lower concentrations. At the same time, it is expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after administration of therapeutic doses of clonidine.

2.1.7. Lumefantrine

The chemical name of lumefantrine is [±]-2-dibutylamino-1-[2, 7-dichloro-9-[4-chlorobenzylidene]-9H-fluorene-4-yl] ethanol. It has the empirical formula C₃₀H₃₂Cl₃NO with a molecular weight of 528.9 g/mol, and the following structural formula:

![Lumefantrine structural formula]

Lumefantrine is a yellow, crystalline powder that is freely soluble in N, N-dimethylformamide, chloroform, and ethyl acetate; soluble in dichloromethane; slightly soluble in ethanol and methanol; and insoluble in water.

Lumefantrine is active against the erythrocytic stages of Plasmodium falciparum. Strains of P. falciparum with a moderate decrease in susceptibility to lumefantrine alone can be selected in vitro or in vivo.
The exact mechanism by which lumefantrine\textsuperscript{212}, exerts its anti-malarial effect is not well defined. Available data suggest lumefantrine inhibits the formation of β-hematin by forming a complex with hemin. Lumefantrine were shown to inhibit nucleic acid and protein synthesis.

Absorption of lumefantrine\textsuperscript{213-214}, a highly lipophilic compound, starts after a lag-time of up to 2 hrs, with peak plasma concentrations about 6 to 8 hrs after administration.

Lumefantrine\textsuperscript{215} has highly bound to human serum proteins in vitro [99.7\%, respectively]. Protein binding to human plasma proteins is linear. Lumefantrine is eliminated more slowly, with a terminal half-life of 3-6 days in healthy volunteers and in patients with falciparum malaria.

The anti malarial lumefantrine/benflumetol was first synthesized and registered in China and is now commercially available in a co-formulated product with artemether as Co-artemether\textsuperscript{®}/Riamet\textsuperscript{®}. This combination has proved very well tolerated and highly efficacious in children and adults, even against multi-drug resistant strains of Plasmodium falciparum\textsuperscript{216-217}. Lumefantrine is a highly lipophilic compound, which is more than 99.9\% bound to plasma proteins\textsuperscript{218}. Absorption is very variable. The day 7 plasma Lumefantrine level has been shown to be the most important determinant of cure following treatment with the coformulation.

Several chromatographic methods have been reported for the quantification of the Lumefantrine in human plasma, are High throughput assay\textsuperscript{219}, LC-UV method\textsuperscript{220}.

A high throughput bioanalytical assay\textsuperscript{219} for the determination of lumefantrine in plasma was described using 96-well plate format.
A bioanalytical method\textsuperscript{220} for the determination of lumefantrine [LF] and its metabolite desbutyl-lumefantrine [DLF] in plasma by solid-phase extraction [SPE] and liquid chromatography has been developed. Plasma proteins were precipitated with acetonitrile: acetic acid [99:1, v/v] containing a DLF analogue internal standard before being loaded onto an octylsilica [3 M Empore] SPE column. Two different DLF analogues were evaluated as internal standards. The compounds were analysed by liquid chromatography UV detection on a SB-CN [250 mm x 4.6 mm] column with a mobile phase containing acetonitrile–sodium phosphate buffer pH [2.0; 0.1 M] [55:45, v/v] and sodium perchlorate 0.05 M.

We now report a highly sensitive liquid chromatography-tandem mass spectrometry [LC-MS/MS] method developed and validated for the quantification of Lumefantrine in human plasma. It was essential to establish an assay capable of quantifying Lumefantrine at nanograms concentration level. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of Lumefantrine.

\subsection*{2.1.8. Levetiracetam}

Levetiracetam\textsuperscript{221-222} is an antiepileptic drug available as 500 mg and 750 mg extended-release tablets for oral administration. The chemical name of levetiracetam, a single enantiomer, is [−]-[S]-α-ethyl-2-oxo-1-pyrrolidine acetamide. Levetiracetam is chemically unrelated to existing antiepileptic drugs [AEDs]. It has the empirical formula C\textsubscript{8}H\textsubscript{14}N\textsubscript{2}O\textsubscript{2} with a molecular weight of 170.21 g/mol, and the following structural formula:
Levetiracetam is a white to off-white crystalline powder with a faint odor and a bitter taste. It is very soluble in water. It is freely soluble in chloroform and in methanol, soluble in ethanol, sparingly soluble in acetonitrile and practically insoluble in n-hexane.

The exact mechanism by which levetiracetam acts to treat epilepsy is unknown. However, the drug binds to a synaptic vesicle protein, SV2A which is believed to impede nerve conduction across synapses.

Absorption of levetiracetam is rapid and peak plasma concentrations occur in about four hrs. The time to peak plasma concentrations is about three hrs longer with extended-release levetiracetam than with immediate-release tablets.

Intake of a high fat, high calorie breakfast before the administration of extended-release levetiracetam tablets resulted in a higher peak concentration, and longer median time to peak. The median time to peak \[T_{\text{max}}\] was two hrs longer in the fed state.

Levetiracetam is not extensively metabolized in humans. The major metabolite is inactive in animal seizure models. Two minor metabolites were identified as the product of hydroxylation of the 2-oxo-pyrrolidine ring [2% of dose] and opening of the
2-oxo-pyrrolidine ring in position 5 [1% of dose]. There is no enantiomeric interconversion of levetiracetam or its major metabolite.

Levetiracetam plasma half-life in adults is 7±1 hour and is unaffected by either dose or repeated administration. Levetiracetam is eliminated from the systemic circulation by renal excretion as unchanged drug which represents 66% of administered dose.

There are only a few papers published reporting therapeutic drug monitoring methods of Levetiracetam. Several methods to determine Levetiracetam with HPLC or gas chromatography-mass spectrometry have been previously described. The assays used relatively large plasma sample volumes [up to 2 mL] and either multiple step liquid or solid-phase extraction procedures.

A liquid chromatographic ultra violet method was used for the quantization of levetiracetam in human plasma using liquid-liquid extraction.

Two assay methods were developed and compared levetiracetam [ucb L059] in human serum. A solid-phase extraction procedure was followed by either reversed-phase HPLC separation-UV-detection or GLC separation using cold on-column injection on a megabore column and nitrogen-phosphorous detection. Absolute recovery of the drug exceeded 97%. Precision and accuracy values for the 16.0 micrograms/mL quality control sample were 2.4% and 101 ± 5% [n = 10], respectively, for the GLC method. Precision and accuracy values for the 12.1 µg/mL quality control sample were 1.0% and 100 ±1% [n = 7], respectively, for the HPLC method.

A method for the routine quantification of the novel levetiracetam in human serum by HPLC-UV. The sample preparation consists only in the precipitation of
serum proteins by perchloric acid and extraction of unpolar components by cyclohexane. The aqueous phase containing the analyte levetiracetam is injected onto a porous graphitic carbon analytical HPLC-column and separated by gradient elution with diluted phosphoric acid/acetonitrile. Detection is carried out at a wavelength of 205 nm. The calibration function is linear in the range of 1-75 µg/mL.

A gas chromatographic-mass spectrometric method\textsuperscript{232} was developed for the enantioselective analysis of levetiracetam and its enantiomer \([\text{R}]\)-alpha-ethyl-2-oxopyrrolidine acetamide in dog plasma and urine. A solid-phase extraction procedure was followed by gas chromatographic separation of the enantiomers on a chiral cyclodextrin capillary column and detection using ion trap mass spectrometry. The fragmentation pattern of the enantiomers was investigated using tandem mass spectrometry. For quantitative analysis three single ions were selected from the enantiomers, enabling selected ion monitoring in detection.

In this report, we describe a highly sensitive liquid chromatography-tandem mass spectrometry [LC-MS/MS] method developed and validated for the quantification of Levetiracetam in human plasma and utilizing a single-step extraction and a chromatographic separation. It is essential to establish an assay capable of quantifying Levetiracetam at lower concentrations. At the same time, it is expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after administration of therapeutic doses of Levetiracetam.

\section*{2.1.9. Telmisartan}

Telmisartan\textsuperscript{233}, a nonpeptide molecule, is chemically described as \(4^\prime\)-[[1, 4\textsuperscript{\prime}-dimethyl-2\textsuperscript{\prime}-propyl \ [2, 6\textsuperscript{\prime}-bi-1H-benzimidazol]-1\textsuperscript{\prime}-yl] \text{methyl}\]-[1, 1\textsuperscript{\prime}-biphenyl] -2-
carboxylic acid. Its empirical formula is $\text{C}_{33}\text{H}_{30}\text{N}_{4}\text{O}_{2}$, its molecular weight is 514.63 g/mol, and its structural formula is:

![Telmisartan structure](image)

Telmisartan is a white to slightly yellowish solid. It is practically insoluble in water and in the pH range of 3 to 9, sparingly soluble in strong acid [except insoluble in hydrochloric acid], and soluble in strong base.

Telmisartan$^{234-235}$ is an orally active, AT1 selective angiotensin II receptor antagonist. Following oral administration, telmisartan$^{236}$ is well absorbed with a mean absolute bioavailability of about 50%. Mean peak plasma concentrations [$\text{C}_{\text{max}}$] of telmisartan are reached in 0.5-1.0 hour after dosing. The pharmacokinetic profile is characterized by greater than proportional increases in plasma concentrations [$\text{C}_{\text{max}}$ and $\text{AUC}$] with increasing doses greater than 40 mg. Telmisartan shows bi-exponential decay kinetics with terminal elimination half life of approximately 24 hrs, and does not accumulate in plasma upon repeated once daily administration. Food slightly reduces the bioavailability of telmisartan.

Telmisartan is extensively bound to plasma proteins [$>99.5\%$] at concentrations achieved at the recommended dosage. The apparent volume of distribution is approximately 500 L, suggesting extensive tissue binding sites.
Telmisartan\textsuperscript{237} is metabolized by conjugation to form a pharmacologically inactive acylglucuronide; this is the only metabolite that has been detected in human plasma and urine. Following both oral dosing and intravenous administration of radiolabelled telmisartan, the parent compound represented approximately 85\%, and the glucuronide approximately 11\% of total radioactivity in plasma. Total plasma clearance of telmisartan is >800 mL/min. Biliary excretion is the predominant route of elimination of telmisartan and its metabolite.

Several methods have been previously described to determine Telmisartan in human plasma or other biological fluids are Immunoassay method\textsuperscript{238}, HPLC method\textsuperscript{239-241}, Liquid chromatography -tandem mass spectrometry\textsuperscript{242-246}. The assays used relatively large plasma sample volumes [up to 2 mL] and either multiple-step liquid or solid-phase extraction procedures.

Immunoassay\textsuperscript{238} was described for Determination of telmisartan in human blood plasma.

The method\textsuperscript{239-241} was developed for determination of telmisartan in human blood plasma using high performace li quid chromatography.

Characterization\textsuperscript{242} of conjugated metabolites of a new angiotensin II receptor antagonist, Candesartan Cilexetil, in rats was done by using liquid chromatography/electrospray tandem mass spectrometry following chemical derivatization.

Determination of telmisartan in human blood plasma was done with Liquid chromatography-tandem mass spectrometry method\textsuperscript{243} and Comparison was made with immunoassay and pharmacokinetic studies.
Simultaneous quantitation of telmisartan and hydrochlorothiazide in human plasma was described with using liquid chromatographic-tandem mass spectrometric method.

A liquid-chromatography-tandem mass spectrometry method was developed for quantitation of angiotensin II receptor antagonists [ARA-II] in human plasma using minimum sample clean-up and investigation of ion suppression.

A method for the determination of the telmisartan, in human plasma has been developed. Telmisartan and the internal standard, diphenhydramine, were extracted from plasma using diethyl ether-dichloromethane [60:40, v/v], and separated on a Zorbax extend C[18] column using methanol-10mM ammonium acetate [85:15, v/v] adjusted to pH 4.5 after mixing with formic acid as mobile phase. Detection was carried out by multiple reaction monitoring on a Q-trap LC-MS/MS system with an ESI interface.

In this report, we describe a highly sensitive liquid chromatography-tandem mass spectrometry [LC-MS/MS] method developed and validated for the quantification of telmisartan in human plasma and utilizing a single-step extraction and a chromatographic separation. It is essential to establish an assay capable of quantifying telmisartan at lower concentrations. At the same time, it is expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of telmisartan.

2.1.10. Ursodeoxycholic Acid
Ursodeoxycholic acid [UDCA] is a naturally occurring bile acid derived from cholesterol, found in small quantities in normal human bile and in larger quantities in the biles of certain species of bears. It is a bitter-tasting white powder consisting of crystalline particles freely soluble in ethanol and glacial acetic acid, slightly soluble in chloroform, sparingly soluble in ether, and practically insoluble in water. The chemical name of ursodeoxycholic acid is 3α, 7β-dihydroxy-5β-cholan-24-oic \( [\text{C}_{24}\text{H}_{40}\text{O}_4] \). It has a molecular weight of 392.56 g/mol. Its structure is shown below.

Following oral administration, the majority of ursodeoxycholic acid is absorbed by passive diffusion and its absorption is incomplete. Once absorbed, Ursodeoxycholic acid undergoes hepatic extraction to the extent of about 50% in the absence of liver disease. As the severity of liver disease increases, the extent of extraction decreases. In the liver, Ursodeoxycholic acid is conjugated with glycine or taurine, and then secreted into bile. These conjugates of ursodeoxycholic acid are absorbed in the small intestine by passive and active mechanisms. The conjugates can also be deconjugated in the ileum by intestinal enzymes, leading to the formation of free Ursodeoxycholic acid that can be reabsorbed and reconjugated in the liver. Nonabsorbed Ursodeoxycholic acid passes into the colon where it is mostly 7-dehydroxylated to lithocholic acid. Some ursodeoxycholic acid is epimerized to
chenodiol [CDCA] via a 7-oxo intermediate. Chenodiol also undergoes 7-dehydroxylation to form lithocholic acid. These metabolites are poorly soluble and excreted in the feces. A small portion of lithocholic acid is reabsorbed, conjugated in the liver with glycine, or taurine and sulfated at the 3 position. The resulting sulfated lithocholic acid conjugates are excreted in bile and then lost in feces. In healthy subjects, at least 70% of ursodeoxycholic acid [unconjugated] is bound to plasma protein. No information is available on the binding of conjugated ursodeoxycholic acid to plasma protein in healthy subjects or patients. Its volume of distribution has not been determined, but is expected to be small since the drug is mostly distributed in the bile and small intestine. Ursodeoxycholic acid is excreted primarily in the feces. With treatment, urinary excretion increases, but remains less than 1% except in severe cholestatic liver disease. During chronic administration of urso [ursodiol], it becomes a major biliary and plasma bile acid. At a chronic dose of 13 to 15 mg/kg/day, Ursodeoxycholic acid constitutes 30-50% of biliary and plasma bile acids.

Several analytical methods have been developed and published for the determination of bile acids in biological fluids are HPLC-UV detector\(^2\)\(^{50-253}\), HPLC-Fluorescence\(^2\)\(^{54-256}\), gas chromatography-mass spectrometry\(^2\)\(^{257-258}\), liquid chromatography-electrospray tandem mass spectrometry\(^2\)\(^{259-261}\).

High-performance liquid chromatographic method\(^2\)\(^{50}\) was developed for determination of ursodeoxycholic acid after solid phase extraction of blood serum and detection-oriented derivatization.

HPLC Procedure\(^2\)\(^{51}\) was developed for determination of UDCA in blood serum. A higher homologue of UDCA used as an internal standard [IS]. Serum samples with IS were diluted with a buffer [pH=7]. The bile acids and IS were captured using solid
phase extraction [C18 cartridges]. The carboxylic group of the analytes was derivatized using 2-bromo-2*-acetonaphthone [a detection-oriented derivatization], and reaction mixtures were analyzed [HPLC with UV 245 nm detection; a 125-4 mm column containing Lichrospher 100 C18, mobile phase: acetonitrile-water, 6:4 [v/v]].

Separation and quantitation\(^{252}\) of glycine and taurine conjugates of commonly occurring bile acids in bile, i.e. lithocholic, deoxycholic, Chenodeoxycholic, ursodeoxycholic and cholic acids in their naturally occurring states have been accomplished using high-performance liquid chromatography. No preliminary purification of bile acids is required except ethanol extraction of bile. A muBondapak C18 column and acetonitrile, methanol, phosphate buffer and ultraviolet detector at 200 nm were used. Detection limit was 0.05 µg and linearity was observed in the range up to 16 µg. Bile acid composition of ten randomly chosen normal human gallbladder bile samples is given. A large difference in bile acid composition between glycine and taurine conjugates was found to be present.

The reversed-phase high-performance liquid chromatographic method\(^{253}\) determine the taurine and glycine conjugates of five different bile acids in human bile using a mobile phase gradient of acetonitrile and water, modified with tetrabutylammonium hydrogen sulphate [0.0075 mol/L],

A method\(^{254}\) for the quantitative analysis of unconjugated and conjugated bile acids [BA] in serum of patients with primary biliary cirrhosis [PBC] before and after therapy with antibiotic or ursodeoxycholic acid [UDCA] was developed. After separation of the free, glycine and taurine conjugated [F, G and T conjugated] fractions by solid-phase extraction, the isolated T conjugates were hydrolysed enzymatically using cholyglycine hydrolase. The BA fractions were derivatized using 2-bromoacetyl-6-
methoxynaphthalene [Br-AMN] and detected fluorimetrically [lambda exc = 300 nm, lambda em = 460 nm]. The derivatization reaction was performed under mild conditions [10 min at 40°C] in an aqueous medium in the presence of tetrakis [decyl] ammonium bromide [TDeABr]. The HPLC separation was achieved using an ODS column and with a mobile phase gradient mixture of A-B, where A is water and B is acetonitrile: methanol [60:40 v/v] for elution at a flow-rate of 1.2 mL/min.

2-Bromoacetyl-6-methoxynaphthalene\(^{255}\) was used as a pre-chromatographic fluorescent labelling reagent for the high-performance liquid chromatographic [HPLC] analysis of bile acids. The derivatization reaction was performed in an aqueous medium in the presence of tetrahexylammonium bromide by ultrasonication at 40°C to give fluorescent esters which were separated by reversed-phase HPLC and detected fluorimetrically [lambda ex = 300 nm, lambda em = 460 nm].

A fluorometric enzymatic method\(^{256}\) for the determination of ursodeoxycholic acid [UDCA] and its glycine and taurine conjugates in human serum has been developed. A simple and fast purification and preconcentration procedure using Sep Pak C18 cartridges was employed for the UDCA extraction from human serum. UDCA and its conjugates were determined in the extracted sample by an equilibrium method based on the enzymatic conversion of the 7α-hydroxy group into 7-oxo group by β-nicotinamide adenine dinucleotide phosphate in the presence of 7β-hydroxysteroid dehydrogenase [7β-HSD] and the produced NADPH was monitored fluorometrically. The 7β-HSD, which is not yet commercially available, was isolated from clostridium absonum cultures [ATCC No. 27555] and purified by affinity chromatography. The method has a limit of detection of 0.8 μm in serum and the precision varied from 6.1 to 2.0% for low and high concentrations, respectively.
A derivatization method\textsuperscript{257} for the trace analysis of bile acids by gas chromatography [GC] in combination with negative ion chemical ionization [NICI] mass spectrometry is developed. Lithocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid and cholic acid were distinctly separated by GC on a cross-linked methyl silicone fused-silica capillary column. The detection limit for the derivatives of dihydroxylated bile acids was 2 fg when the fragment ion was monitored at m/z 563 in the NICI mode using isobutane as a reagent gas.

A gas-liquid chromatography-mass spectrometry method\textsuperscript{258} was used for characterization of serum and urinary bile acids in patients with primary biliary cirrhosis.

Bile acids in the rat bile\textsuperscript{259} were fractionated into unconjugated, glycine- and taurine-conjugated fractions by employing piperidino-hydroxypropyl Sephadex LH-20 ion-exchange chromatography. Subsequently, these fractions were analyzed by gas-liquid chromatography [GLC] and GLC-mass spectrometry using a Silicone AN-600 column.

A reverse phase high-performance liquid chromatographic method\textsuperscript{260} for a simultaneous analysis of free, glycine- and taurine-amidated bile acids was developed. The resolution of ursodeoxycholic, cholic, chenodeoxycholic, deoxycholic, and lithocholic acids, either free or amidated with glycine and taurine, was achieved using a C-18 octadecylsilane column [30 cm length, 4 µ particle size] with a gradient elution of aqueous methanol [65-75% v/v] containing 15 mM ammonium acetate, pH 5.4, at 37°C. The separated bile acids are detected with a new evaporative light-scattering mass detector and by absorbance at 200 nm. A complete resolution of the 16 bile
acids, including the internal standard nor-deoxycholic acid, was obtained within 55 min.

A liquid chromatography-electrospray tandem mass spectrometry method\textsuperscript{261} for the determination of bile acids in human bile has been developed. The bile acids were extracted with a C-18 [octadecyl] reversed-phase column and identified and quantified by simultaneous monitoring of their parent and daughter ions, using the multiple reaction monitoring mode. Identification and quantification of conjugated bile acids in bile was achieved in 5 min. The percent recovery of the same standard bile acids was determined by gas chromatography-mass spectrometry [GC-MS], using the selected ion monitoring mode, and averaged 66\% to 96\%. A biliary bile acid profile of human gallbladder bile was obtained by LC-MS/MS and GC-MS.

In this report, we describe a highly sensitive liquid chromatography/tandem-mass spectrometry [LC-MS/MS] method developed and validated for the quantification of ursodeoxycholic acid in human plasma and utilizing a single-step extraction and a chromatographic separation. It is essential to establish an assay capable of quantifying ursodeoxycholic acid at lower concentrations. At the same time, it is expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of ursodeoxycholic acid.