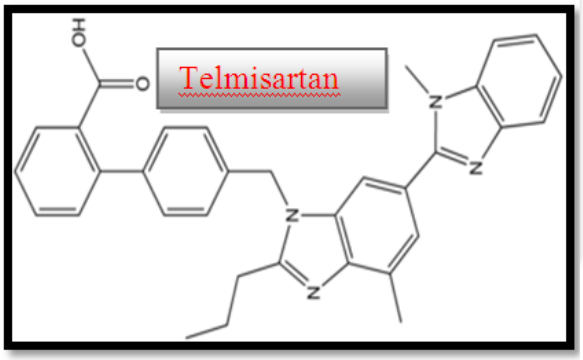
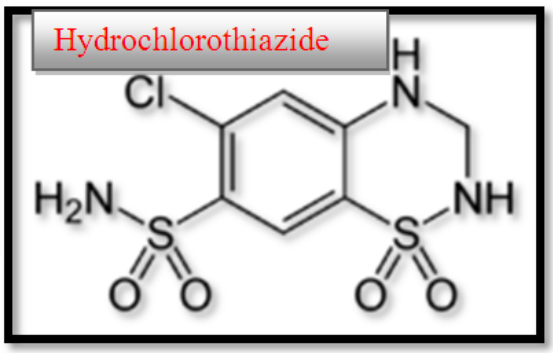
CHAPTER 3





LCMS- MS Method for the Simultaneous Analysis of Telmisartan and Hydrochlorothiazide in Human Plasma (In-vivo)

3.1: Introduction

3.1.1: Telmisartan

Telmisartan is an angiotensin II receptor antagonist (angiotensin receptor blocker, ARB) used in the management of hypertension. Telmisartan is indicated in the treatment of essential hypertension [1.2]. It is also used to reduce the risk of heart attack, stroke, or death due to heart problems in certain patients. Telmisartan is also used sometimes to treat congestive heart failure (condition in which the heart is unable to pump enough blood to the rest of the body) and diabetic nephropathy (kidney disease in people with diabetes and high blood pressure).

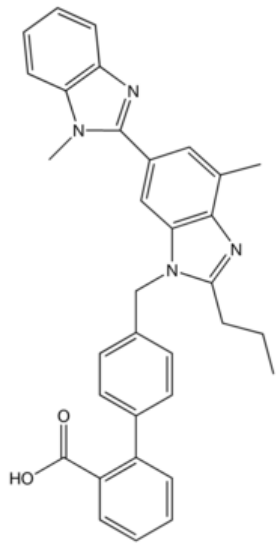


Figure: 3.A: Structure of Telmisartan

It works by relaxing blood vessels, which helps to lower blood pressure. It is an angiotensin II receptor blocker that shows high affinity for the angiotensin II receptor type 1 (AT_1), with a binding affinity 3000 times greater for AT_1 than AT_2. It has the longest half-life of any ARB (24 hours) [1, 3] and the largest volume of distribution. In addition to blocking the RAs, telmisartan acts as a selective modulator of peroxisome proliferator-activated receptor gamma (PPAR-γ), a central regulator of insulin and glucose metabolism. It is believed that telmisartan dual mode of action may provide protective benefits against the vascular and renal damage caused by diabetes and cardiovascular disease (CVD). [3] Telmisartan's activity at the PPAR-γ receptor has prompted speculation around its potential as a sport doping agent as an

alternative to GW 501516. [4]Telmisartan activates PPAR δ receptors in several tissues. [5-8]

Side effects with Telmisartan are similar to other angiotensin II receptor antagonists and include tachycardia and bradycardia (fast or slow heartbeat), hypotension (low blood pressure), edema (swelling of arms, legs, lips, tongue, or throat, the latter leading to breathing problems), and allergic reactions.[9] It should not be taken by breastfeeding women since it is not known whether the drug passes into the breast milk.[9] In rare cases, telmisartan can cause a condition that results in the breakdown of skeletal muscle tissue, leading to kidney failure.

3.1.2: Hydrochlorothiazide

Hydrochlorothiazide is a diuretic drug of the thiazide class that acts by inhibiting the kidneys' ability to retain water. This reduces the volume of the blood, decreasing blood return to the heart and thus cardiac output and, by other mechanisms, is believed to lower peripheral vascular resistance [10]. It is used for the treatment of fluid retention (edema) in people with congestive heart failure, cirrhosis of the liver, or kidney disorders, or edema caused by taking steroids or estrogen. This medication is also used to treat high blood pressure (hypertension). It may also be used to treat patients with diabetes insipidus and certain electrolyte disturbances and to prevent kidney stones in patients with high levels of calcium in their blood, renal tubular acidosis [11].

It is also sometimes used for treatment of hypoparathyroidism, [12] hypercalciuria, Dent's disease and Ménière's disease. For diabetes insipidus, the effect of thiazide diuretics is presumably mediated by a hypovolemia-induced increase in proximal sodium and water reabsorption, thereby diminishing water delivery to the ADH-sensitive sites in the collecting tubules and reducing the urine output. Thiazides are also used in the treatment of osteoporosis. Thiazides decrease mineral bone loss by promoting calcium retention in the kidney, and by directly stimulating osteoblast differentiation and bone mineral formation.[13] It is frequently given together with other antihypertensive agents in fixed combination preparations, such as losartan (an angiotensin II receptor antagonist) as hydrochlorothiazide/losartan.

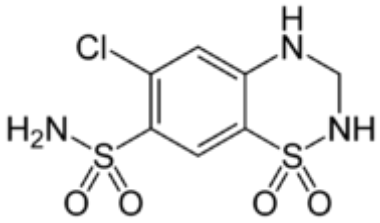


Figure 3.B: Structure of Hydrochlorothiazide

Hydrochlorothiazide belongs to thiazide class of diuretics. It reduces blood volume by acting on the kidneys to reduce sodium (Na) reabsorption in the distal convoluted tubule. The major site of action in the nephron appears on an electro neutral Na^+ Cl^- co-transporter by competing for the chloride site on the transporter. By impairing Na transport in the distal convoluted tubule, hydrochlorothiazide induces a natriuresis and concomitant water loss. Thiazides increase the reabsorption of calcium in this segment in a manner unrelated to sodium transport.[14] Additionally, by other mechanisms, HCTZ is believed to lower peripheral vascular resistance.[15]

Side effects with the Hydrochlorothiazide includes Hypokalemia, an occasional side effect, can be usually prevented by potassium supplements or by combining hydrochlorothiazide with a potassium-sparing diuretic, Hypomagnesaemia, Hyponatremia, Hyperuricemia, High blood sugar, Hyperlipidemia, Hypercalcemia, Headache, Nausea/vomiting, Photosensitivity, Weight gain, Gout, Pancreatitis. World Anti-Doping Agency classified Hydrochlorothiazide as a "specified substance" While Hydrochlorothiazide is not itself a performance-enhancing drug, it may be used to mask the use of performance-enhancing drugs [16-19].

Table 3.1: Properties of Telmisartan

IUPAC	***2-(4-{[4-Methyl-6-(1-methyl-1H-1,3-benzodiazol-2-yl)-2-propyl-1H-1,3-benzodiazol-1-yl]methyl}phenyl)benzoic acid***
Formula	$C_{33}H_{30}N_4O_2$
Mol. mass	514.617 g/mol
Routes	Oral
Excretion	Faecal 97%

Table 3.2: Properties of Hydrochlorothiazide

IUPAC	***6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide***
Formula	$C_7H_8ClN_3O_4S_2$
Mol. mass	297.74 g/mol
Routes	Oral (capsules, tablets, oral solution)
Excretion	Primarily excreted unchanged in urine

Table: 3.3: Telmisartan formulation composition:

Brand name	Dosage form	Composition	Company
ARBITEL	TAB	20mg, 40mg	Micro cardicare
ASTEL	TAB	20mg, 40mg,80mg	AS Pharma
CORTEL	TAB	20mg,40mg,80mg	Corona
HYTEL	TAB	20mg,40mg,80mg	East West
LOVETEL	TAB	40mg	Q Check
MYTEL	TAB	40mg,80mg	Rhine Biogenic
T-PRESS	TAB	20mg,40mg	Talent
TAZLOC	TAB	20mg,40mg	USV
TELDAY	TAB	20mg,40mg,80mg	Torrent(Azuca)
TELEMAR	TAB	20mg,40mg,80mg	Shrrishit HC
TELICAD	TAB	20mg,40mg	Cadomed
TELISTA	TAB	20mg,40mg	Lupin
TELMICHECK	TAB	40mg	(Surge-Radius)
TELMITRUST	TAB	20mg,40mg	Centaur(Sankalp)
TELPRES	TAB	20mg,40mg,80mg	AHPL
TELSAR	TAB	20mg,40mg,80mg	Unichem

Table: 3.4: Hydrochlorothiazide Formulation Composition:

Brand name	Dosage form	Composition	Company
AQUAZIDE	TAB	3.5 mg, 25 mg	Sun
BPZIDE	TAB	3.5 mg, 25 mg	Stadmed
HYZIDE	TAB	3.5 mg,25 mg	East West
KLORZIDE	TAB	25 mg	Zydus(Biogen)
XENIA	TAB	3.5 mg,25 mg	USV

Table: 3.5: Telmisartan Hydrochlorothiazide Combination Formulation:

Brand name	Dosage form	Composition	Company
ARBITEL-H	TAB	Telmisartan-40 mg Hydrochlorothiazide-3.5 mg	Micro cardicare
ASTEL-H	TAB	Telmisartan-40 mg Hydrochlorothiazide-3.5mg	AS Pharma
CORTEL-H	TAB	Telmisartan-40 mg Hydrochlorothiazide-3.5mg	Corona
HYTEL-H	TAB	Telmisartan-40 mg Hydrochlorothiazide-3.5mg	East West
LOVETEL-H	TAB	Telmisartan-40 mg Hydrochlorothiazide-3.5mg	Q Check
MYTEL-H	TAB	Telmisartan-40 mg Hydrochlorothiazide-3.5mg	Rhine Biogenic
NEWTEL-H	TAB	Telmisartan-40 mg Hydrochlorothiazide-3.5mg	Systopic
T-PRESS-H	TAB	Telmisartan-40mg Hydrochlorothiazide-3.5mg	Talent
TELDAY-H	TAB	Telmisartan-40 mg Hydrochlorothiazide-3.5mg Telmisartan-80 mg Hydrochlorothiazide-3.5mg	Torrent(Azuca)
TELEMAR-H	TAB	Telmisartan-40 mg Hydrochlorothiazide-3.5mg Telmisartan-80mg Hydrochlorothiazide-3.5mg	Shrrishit HC
TELICAD-H	TAB	Telmisartan-40mg Hydrochlorothiazide-3.5mg	Cadomed
TELISTA-H	TAB	Telmisartan-40mg Hydrochlorothiazide-3.5mg	Lupin
TELMA-H	TAB	Telmisartan-40 mg Hydrochlorothiazide-3.5mg	Glenmark(Zoltan)

3.2: Review of Literature

Literature review reveals that methods have been reported for analysis of Telmisartan and Hydrochlorothiazide, HPLC method for the determination of Telmisartan in pharmaceutical preparations, RP-HPLC method for determination of Hydrochlorothiazide in combination with Telmisartan and few bio-analytical methods are also reported.

LakshmanaRao et al(20) has described a simple, fast and precise reverse phase, isocratic HPLC method for the separation and quantification of telmisartan and hydrochlorothiazide in pharmaceutical dosage form. The quantification was carried out using ProntoSIL C18- EPS 4.6 x 150mm, 3µm enhanced polar selectivity column and mobile phase comprised of potassium dihydrogen phosphate buffer pH adjusted to 3.2 ± 0.5 with orthophosphoric acid and acetonitrile in proportion of ratio 55:45 and degassed under ultrasonication. The flow rate was 0.8ml/min and the effluent was monitored at 271nm. The retention time of telmisartan and hydrochlorothiazide were 5.01 ± 0.5 and 2.94 ± 0.5 respectively. The method was validated in terms of linearity, precision, accuracy, and specificity, limit of detection and limit of quantitation. Linearity of telmisartan and hydrochlorothiazide were in the range of 15.01 to 75.05µg/ml and 5.02 to 25.10µg/ml respectively. The percentage recoveries of both the drugs were 100.8% and 99.5% for telmisartan and hydrochlorothiazide respectively from the tablet formulation. The proposed method is suitable for simultaneous determination of telmisartan and hydrochlorothiazide in pharmaceutical dosage form.

Gangola R et al (21) developed a simple, sensitive, specific and economic spectrophotometric method and validated for simultaneous estimation of Hydrochlorothiazide and Telmisartan in tablet dosage form. New method based on the simultaneous estimation of drugs in a binary mixture without previous separation was developed. In dual wavelength method, Hydrochlorothiazide and Telmisartan were quantified using principle that absorbance difference between two points on mixture spectra is directly proportional to concentration of component of interest and independent of interfering component. The accuracy and reproducibility of the proposed method was statistically validated by recovery studies. The method permits

simple, rapid and direct determination of Hydrochlorothiazide and Telmisartan commercially available tablet dosage form without previous separations and can therefore be used for routine analysis of both drugs in quality control laboratories.

Sutirtho Mukhopadhyay et al (22) developed and validated a new, accurate, sensitive, precise, rapid, reversed phase high performance liquid chromatography (RP-HPLC) method for determination of related substances of Telmisartan and Hydrochlorthiazide in tablet dosage form. Simultaneous determination of related substances was performed on Kromasil C_{18} analytical column (250 × 4.6 mm; 5µm particle size) column at 40°C employing a gradient elution. Mobile phase consisting of solvent A (solution containing 2.0 g of potassium dihydrogen phosphate anhydrous and 1.04 g of Sodium 1- Hexane sulphonic acid monohydrate per liter of water, adjusted to pH 3.0 with orthophosphoric acid) and solvent B (mixture of Acetonitrile: Methanol in the ratio 80:20 v/v) was used at a flow rate of 1.0 ml min^{-1}. UV detection was performed at 270 nm .During method validation parameter such as precision, linearity, accuracy, specificity, limit of detection and quantification were evaluated, which remained within acceptable limits. HPLC analytical method is linear, accurate, precise, robust and specific, being able to separate the main drug from its degradation products. It may find application for the routine analysis of the related substances of both Telmisartan and Hydrochlorthiazide in this combination tablets.

Ismail Salama et al (23) described a specific, sensitive and rapid method based on high performance liquid chromatography (HPLC) for the simultaneous determination of telmisartan (TELM) and hydrochlorothiazide (HCT) in human plasma using indapamide as internal standard. The method utilizes proteins precipitation with acetonitrile as only sample preparation prior to RP-HPLC. The analytes were chromatographed on shim-pack cyanopropyl column in isocratic elution with methanol: 10 mm ammonium acetate solution (pH 6.0) (35:65 v/v) as mobile phase at a flow rate of 1 ml/min and the wavelength of detection was 270 nm. The method was validated over the concentration range of 1–10 µgml^{-1} for TELM and 0.31–3.12 µgml^{-1} for HCT in human plasma. Inter- and intra-run precision of TELM and HCT were less than 3.60% and the accuracy was less than 1.868%. The linearity, recovery, matrix effect and stability were validated for TELM/HCT in human plasma.

SB Wankhede et al(24) described a validated reverse phase high performance liquid chromatographic method for simultaneous estimation of telmisartan and hydrochlorothiazide in tablet formulation. Chromatography was performed on a ODS Hypersil C18 (25 cm×4.6 mm I.D) column from thermo in isocratic mode with mobile phase containing acetonitrile: 0.05 M KH_2PO_4 pH 3.0 (60:40). The flow rate was 1.0 ml/min and the eluent was monitored at 271 nm. The selected chromatographic conditions were found to effectively separate telmisartan (RT- 5.19 min) and hydrochlorothiazide (RT- 2.97 min). Linearity for telmisartan and hydrochlorothiazide were found in the range of 4.1-20.48 µg/ml and 1.28-6.4 µg/ml, respectively. The proposed method was found to be accurate, precise, reproducible and specific and can be used for simultaneous analysis of these drugs in tablet formulation.

Leena R. Bhat et al(25)developed a simple, selective, and precise reverse phase high performance liquid chromatographic method for the simultaneous determination of Telmisartan and hydrochlorothiazide from pharmaceutical formulation. The mobile phase consisted of methanol and acetonitrile (70:30 v/v) at a flow rate of 1 ml/min and the wavelength of detection was 270 nm. Rabeprazole was used as an internal standard. The retention times of Telmisartan, hydrochlorothiazide and rabeprazole were 1.79±0.01, 2.80±0.01, and 3.19±0.01 minutes, respectively. The developed method was validated according to ICH guidelines. The proposed method can be used for determination of these drugs in combined dosage forms.

J. Kavithaet al [26] described a very simple, rapid and sensitive RP-HPLC method and validated for the analysis of Telmisartan and Hydrochlorothiazide in tablet formulation. Best chromatographic resolution was achieved on a reverse-phase Princeton SPHER C_{18} column using acetonitrile: 50mM potassium dihydrogen ortho phosphate (pH 3.5) ratio 50:50 as mobile phase with a flow rate of 1ml/min and isocratic elution with a total run time of 10 minutes. Sulphadoxine was selected as internal standard. The retention time of Telmisartan, Hydrochlorothiazide and Internal Standard was found to be 4.71, 7.06 and 9.56 respectively. Detection of the multi compounds was carried out at 270 nm. The present newly developed method was found to be accurate, precise and can be useful for routine Quality control analysis.

Shravan Bankey et al(27)described a simple, accurate and precise RP-HPLC method for simultaneous determination of ramipril, hydrochlorothiazide and telmisartan in combined formulation (tablet). The chromatographic separation was achieved on a 250 x 4.6 mm, 5µ Inertsil C18 column. Eluents were monitored by absorbance at 218 and 270 nm using a mixture of methanol: water (pH adjusted to 4.5 using dilute orthophosphoric acid) in the ratio of 72:28 (v/v) at a flow rate of 1.0 ml/min. The elution time for ramipril, hydrochlorothiazide and telmisartan were found 6.34, 4.55 and 7.39 min, respectively. Ramipril showed linearity in the range of 0.5-10 µg/ml at the wavelength 218nm with correlation coefficient (r^2) 0.9987. Hydrochlorothiazide and telmisartan showed linearity in the range of 2.5-25 µg/ml and 4-40 µg/ml, respectively, at 270nm with correlation coefficient (r^2) 0.9992 and 0.9996, respectively. The proposed method was validated by testing its linearity, accuracy, precision, selectivity and LOD/LOQ values and it was successfully employed for the determination of ramipril, hydrochlorothiazide and telmisartan in pharmaceutical tablet formulations

P.Shanmugasundaram et al(28) has developed and validated HPLC method for simultaneous estimation of telmisartan and hydrochlorothiazide in tablet formulations. Separation was achieved on a gemini C_{18} column (4.6mm x 25 cm), in isocratic mode, with phosphate buffer (pH 2.5), acetonitrile and tetrahydrofuran(6:3:1v/ v/v) as mobile phase at a flow rate of 1 ml/minute. Quantitation was carried out by the use of UV detector in absorbance mode at 225 nm. The retention times of telmisartan and hydrochlorothiazide were found to be 5.612 and 4.23 minutes, respectively. Linearity of detector response for telmisartan and hydrochlorothiazide were found to be from 0.32 to 0.48 mg/ml and 0.10 to 0.15mg/ml, respectively. The amounts of drug estimated in the average weight of the tablet were found to be 40.75 and 13.00 mg, respectively. The proposed method was validated and was found to be suitable, precise, accurate and reproducible, and can be adopted for routine analysis of telmisartan and hydrochlorothiazide in tablet formulation

MA Ping et al(29) Telmisartan and hydrochlorothiazide in human plasma were determined by high performance liquid chromatography-tandem mass

spectrometry. Telmisartan and Hydrochlorothiazide and internal standard were extracted from plasma using V (diethyl ether): V (dichloromethane) = 60:40, and separated on a Zorbax Eclipse XDB-C 18 column using acetonitrile 5 m mol/; ammonium acetate as mobile phase by gradient elution. Detection was carried out by multiple reaction monitoring (MRM) on a 3200Q- Trap LC-MS/MS system. The assay is linear over the range 1.0-1000µg/L with a lower limit of quantitation of 5.0µg/ml for telmisartan, and linear over the range 0.6-200.0µg/L with a lower limit of quantitation of 0.6µg/l for hydrochlorothiazide. Intra and interday precision are less than 15%. The relative deviations are in the range of 5.6% - 4.3% for telmisartan, and 6.3%-2.7% for hydrochlorothiazide. The recoveries of telmisartan and hydrochlorothiazide are more than 50%, and stabilities are good.

***Vasanth PM et al* (30)** *described a* new reverse phase HPLC method and validated for the simultaneous estimation of Enalapril maleate & Hydrochlorothiazide. The proposed method should be simple, economic, accurate, and precise. The method was optimized by using Symmetry C_{18} column (4.6 x 150mm, 5µm, Kromosil) or equivalent, Phosphate buffer and Methanol were used as a Mobile Phase in the proportion of 70%:30%.Phosphate buffer pH was adjusted with Orthophosphoric acid. Precision shows that %Relative standard deviation of Enalapril Maleate and Hydrochlorothiazide are about 0.42 and 0.07.Accuracy of this method shows that the % Recovery for each level should be between 98.0 to 102.0%, Calibration curve shows good Linearity and Range .The Correlation Coefficient of Enalapril and Hydrochlothiazide is 0.999.And the results obtained for LOQ, LOD, Robustness, Ruggedness were well within the acceptance Criteria

Rajesh nakum et al(31)reported a RP-HPLC method for simultaneous estimation of Telmisartan with Hydrochlorothiazide in its solid dosage forms as per the ICH guidelines. A tablet formulation combines Telmisartan, a Anti- Hypertensive Drug, with Hydrochlorothiazide, a Diuretic drug. Advantage expected from this combination is synergistic effect of Blood pressure in the therapy of Anti-Hypertensive. Here Telmisartan and Hydrochlorothiazide are non-compendial. It was found that though individually Telmisartan and Hydrochlorothiazide have been analyzed by many methods, but no few method have been reported for the simultaneous determination of Telmisartan and Hydrochlorothiazide in combination.

The peak purity data of standard solution, test solution and spiked sample solution prove the specificity of the proposed methods. The linearity of developed method for simultaneous estimation was achieved in the range of 38.43–115.28 µg/ml (r2=0.9999) for Telmisartan and 3.22–36.67 µg/ml (r2=0.9998) for Hydrochlorothiazide. The results of precision, recovery and all other validation parameters are within acceptance criteria. From the validation results, we can conclude that developed methods are simple, sensitive, rapid, linear, precise, rugged, accurate, and robust and hence they can be used for the routine analysis of simultaneous estimation of Telmisartan with Hydrochlorothiazide in quality control department.

SHI Qiao-Juan et al (32) described a HPLC method for determination of telmisartan and hydrochlorothiazide in compound telmisartan capsules. A Hypersil-BDS C18 column was used with the mobile phase of tetra butyl ammonium hydroxide phosphate buffer solution (pH 2.26)-acetonitrile (78∶22) at the detection wavelength of 230nm and the column temperature of 30℃. The calibration curves of telmisartan and hydrochlorothiazide were linear in the concentration ranges of 5 – 200mg/ml(r=0.9999) and 1.6 – 64mg/ml(r=0.9998), respectively. The recoveries were 98.8% – 99.8%, and99.6% – 99.9%, respectively. The detection limits were 2.5 and 3.9ng, respectively.

Thirupathaiah sanikommu et al(33)developed a simple, specific, accurate and precise reverse phase high performance liquid chromatographic method for the simultaneous determination of Telmisartan, Amlodipine and Hydrochlorothiazide from combined dosage form by RP-HPLC method utilizes a Column: X-Terra RP8, 250 mm × 4.6 mm, 5 µm, at ambient temperature. The separation is achieved on a simple gradient method, the mobile phase A contains a mixture of Sodium perchlorate buffer pH 2.5 adjusted with 2.5 with Orthophosphoric acid: acetonitrile in the ratio 700:300, v/v, and mobile B contains a mixture of pH 2.5 buffer, acetonitrile and methanol in the ratio of 300:300:400, v/v. The flow rate is 1.0 ml min−1 .The gradient program (T/%B) is set as 7/30, 15/55, 17/75, 19/100, 22/100 and 23/0. Detection wavelength 237 nm for Amlodipine, 271 nm for Hydrochlorothiazide and Telmisartan. The retention times of HCTZ is 4.9 min, Amlodipine is 13.06 min and

15 min for Telmisartan. The described method is validated with respect to system suitability, specificity, linearity, precision and accuracy as per ICH guidelines.

P Mandlekar et al(34)described a simple, precise, and reproducible RP-HPLC method for the simultaneous determination of antihypertensive pharmaceutical tablet dosage form containing ramipril, hydrochlorothiazide and telmisartan. RP-HPLC separation of three drugs was achieved on a Chromatopak peerless basic C18 column (250 mm × 4.6 mm, 5 µm) using UV detection at 215 nm. The optimised mobile phase consisted of acetonitrile and 0.01 M phosphate buffer pH 3 adjusted with o-phosphoric acid (2%, v/v) in a proportion of 60:40 v/v. The flow rate was 0.8 ml min-1. The three drugs were satisfactorily resolved with retention time values of 3.56, 4.75 and 9.23 mins for ramipril, hydrochlorothiazide and telmisartan respectively. The method was validated for linearity, accuracy, precision, robustness, and specificity, as per ICH recommended guidelines. The method was found to be linear over concentration ranges of 9-19, 22.5-47.5 and 72-152 µg ml-1 for ramipril, hydrochlorothiazide and telmisartan respectively. Recovery studies indicated more than 99% of recovery for the three drugs. The relative standard deviation values for intra-day and inter-day precision studies were found to be less than 2% for ramipril, hydrochlorothiazide and telmisartan respectively. No chromatographic interference from any tablet excipients was observed. The method proved to be simple and rapid for routine simultaneous estimation of ramipril, hydrochlorothiazide, and telmisartan in the bulk drug and in a tablet formulation.

R. A. Mhaske et al (35)developed and validated a simple, precise and stability-indicating HPLC method was for the simultaneous determination of anti-hypertensive drugs Amlodipine Besylate, Valsartan, Telmisartan and diuretics Hydrochlorothiazide and Chlorthalidone. The separation was achieved on Cosmosil PAQ (150 mm × 4.6 mm) 5 µm column with gradient flow. The mobile phase at a flow rate of 1.0 ml min−1 consisted of 0.05 M sodium dihydrogen phosphate buffer and acetonitrile (Gradient ratio). The UV detection was carried out at 220 nm. The method was successfully validated in accordance to ICH guidelines. Further, the validated method was applied for commercially available pharmaceutical dosage form

Dhanalakshmi K et al(36)reported a new and simple RP-HPLC method for the analysis of Telmisartan and Hydrochlorothiazide in tablet formulation. The process of dissolution has been carried out with apparatus USP Type II, RPM-75 and Time point - 60min. pH 7.5 phosphate buffer is the dissolution media as well as

diluent. Telmisartan and Hydrochlorothiazide is analyzed using HPLC whose optimized chromatographic conditions include: column- C8 (250mm x 4.6 x 5µ particle size), column oven temperature of 40°C over a run time of 7mins, injection temp 25°C with volume of 50µL at a flow rate of 1.5ml/min. Ammonium phosphate buffer and Acetonitrile (60:40) is used as mobile phase. Validation parameters selectivity, precision, linearity, accuracy, Robustness all are within the limit so method was validated it is use full to pharmaceutical analysis.

B. Kalyan Kumar et al(37)described a new simple, accurate, rapid and precise isocratic High performance liquid chromatographic (HPLC) method and validated for the determination of Hydrochlorothiazide (HCTZ), Ramipril (RAM) and Telmisartan (TEL) in tablet formulation. The Method employs Waters HPLC system on XTerra RP8 Column (4.6 x 150 mm and 3.5 µm) and flow rate of 0.8 ml/min with a load of 20µl. Acetonitrile and Phosphate buffer was used as mobile phase in the composition of 45:55. The Detection was carried out at 215 nm. Linearity ranges for Hydrochlorothiazide, Ramipril and Telmisartan were 3.5-22.5 µg/ml, 5-9µg/ml and 40-72µg/ml respectively. Retention Time of Hydrochlorothiazide, Ramipril and Telmisartan were found to be 2.83 min, 3.65 min and 5.03 min respectively. Percent Recovery study values of HCTZ, RMP and TEL were found to be within 98-102 %.This newly developed method was successfully utilized for the Quantitative estimation of Hydrochlorothiazide, Ramipril and Telmisartan in pharmaceutical dosage forms. This method was validated for accuracy, precision, linearity and Robustness as per ICH guidelines.

Subhakar Nandipati et al(38)developed a simple RP-HPLC method in bulk and formulation dosage form for Estimation of Telmisartan. Mobile phase potassium di-hydrogen phosphate and acetonitrile (60:40) pH adjust with ortho phosphoric acid , C18 sun fire column (250mmx4.6mmx5µm) flow rate 1ml/min, wave length 243 nm, column temperature 45ºc, injection volume10 µl. System suitability parameters of Telmisartan retention time 3.4, plate count 8968, tailing 1.086, % RSD 0.1 those all are within the limit method is suitable for analysis. Validation parameters selectivity, precision, linearity, accuracy, Robustness all are within the limit so method was validated it is use full to pharmaceutical analysis

Jabir Aboobacker O et al(39) has reported simple, selective, rapid, precise and economical reverse phase high-pressure liquid chromatographic method as per ICH nomination for the simultaneous estimation of Hydrochlorothiazide, Amlodipine

besylate and Telmisartan in tablet dosage form. The separation method was carried out by using a mobile phase consisting of 0.02M sodium dihydrogen phosphate, methanol and acetonitrile in the ratio 30:35:35.The detection was carried out by using UV – Visible SPD 20 A at 240nm.The column was phenominex Gemini C18 (250×4.6mm×5µ).The flow rate was selected as 1.5ml/min. The retention time of Hydrochlorothiazide, Amlodipine besylate and Telmisartan was found to be 2.2, 3.7 and 6.1 respectively. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantification and system suitability. The proposed method can be used for the estimation of these drugs in combined dosage forms.

Susheel John Varghese et al(40) described a validated reversed phase high performance liquid chromatography (HPLC) method for the simultaneous determination of ramipril(RPL), telmisartan(TLM), and Hydrochlorothiazide((HTZ) in combined tablet dosage forms. The isocratic reverse phase HPLC analysis was done on a Merck C18 4.0 mm× 250 mm, column with mobile phase consisting of 0.1%phosphoric acid(pH adjusted to 2.5 with triethylamine) – acetonitrile (58:42,v/v) at a flow rate of 1 ml/min. Quantification was carried out using a photo-diode array UV detector at 210 nm. The developed analytical method was validated according to International Conference on Harmonization guidelines and the acceptance criteria for parameters like accuracy, precision, linearity, specificity and system suitability were met in all cases. The method is simple, precise, and sensitive, and hence applicable for simultaneous determination of RPL, TLM, and HTZ in pure powder and tablets.

CVN. Prasad et al(41)developed a simple, precise and rapid stability-indicating High-performance liquid chromatography (HPLC) method for the simultaneous quantitative determination of Telmisartan, Amlodipine besylate and Hydrochlorothiazide from their innovative poly pill combination drug product in the presence of degradation products. It involves a 150 mm x 4.6 mm, 5 µm C-8 column. The separation is achieved on a simple Isocratic method. The mobile phase contains a mixture of sodium perchlorate buffer pH 2.4 (0.05M): acetonitrile in the ratio 60:40 v/v. The flow rate is 1.0 ml min−1 and the column temperature is maintained at 25°C. The detector wavelength is 271 nm for Hydrochlorothiazide and Telmisartan and 237 nm for Amlodipine. The retention times of Telmisartan, Amlodipine and Hydrochlorothiazide are 4.8 minutes, 3.8 minutes and 2.4 minutes respectively. The total runtime for the separation of the three active compounds and their degradation

products is 8 minutes. The described method is validated with respect to system suitability, specificity, linearity, precision and accuracy. The precision of the assay method is evaluated by carrying out six independent assays of Telmisartan, Amlodipine and HCTZ (0.032 mg ml−1 of Telmisartan, 0.004 mg ml−1 of Amlodipine, 0.01 mg ml−1 of HCTZ). The accuracy of the method is evaluated in triplicate at three concentration levels i.e. 50%, 100% and 150% of target test concentration (0.64 mg ml−1 of Telmisartan, 0.08 mg ml−1 of Amlodopine, 0.2 mg ml−1 of HCTZ). The described method is linear over the range, 16 to 48 µg ml−1 for Telmisartan, 2 to 6 µg ml−1 Amlodipine and 5 to 15 µg ml−1 for HCTZ.

SantajiNalwade et al(42)reported a chromatography (UPLC) method for the simultaneous quantitative determination of Telmisartan, Amlodipine besylate and Hydrochlorothiazide from their innovative poly pill combination drug product in the presence of degradation products. It involves a 100 mm x 2.1 mm, 1.7 µm C-18 column. The separation is achieved on a simple gradient method. The mobile phase A contains a mixture of sodium perchlorate buffer pH 3.2 (0.053M): acetonitrile in the ratio 90:10 v/v, and mobile B contains a mixture of sodium perchlorate buffer pH 3.2 (0.053M): acetonitrile in the ratio 20:80 v/v. The flow rate is 0.6 ml min−1 and the column temperature is maintained at 55°C.The gradient program (T/%B) is set as 0/5, 1.2/5, 1.6/40, 4/40, 4.1/5 and 4.5/5. The detector wavelength is 271 nm for Hydrochlorothiazide and Telmisartan and 237 nm for Amlodipine. The retention times of Telmisartan, Amlodipine, and Hydrochlorothiazide are 3.6 minutes, 3.2 minutes and 0.9 minutes; respectively. The total runtime for the separation of the three active compounds and their degradation products is 4.5 minutes. The described method is validated with respect to system suitability, specificity, linearity, precision and accuracy. The precision of the assay method is evaluated by carrying out six independent assays of T, A and H (0.032 mg ml−1 of T, 0.004 mg ml−1 of A, 0.01 mg ml−1 of H). The accuracy of the method is evaluated in triplicate at three concentration levels, i.e. 50%, 100% and 150% of target test concentration (0.64 mg ml−1 of T, 0.08 mg ml−1 of A, 0.2 mg ml−1 of H). The described method is linear over the range, 16 to 48 µg ml−1 for T, 2 to 6 µg ml−1A and 5 to 15 µg ml−1 for H. The method is fast and suitable for high-throughput analysis allowing the analysis of about 250 samples per working day.

N. Delhiraj et al(43)described two new, rapid, precise, accurate and specific chromatographic methods for the simultaneous determination of Telmisartan,

Amlodipine besylate and Hydrochlorothiazide in combined pharmaceutical dosage forms. The first method based on reverse phase liquid chromatography by using Qualisil BDS C18 column (250 mm X 4.6 i.d., 5µm). Mobile phase consists of 1.0 ml of triethylamine in one litre water and the pH was adjusted to 2.5 with orthophosphoric acid and Acetonitrile (60:40) with a flow rate of 1ml/min, with a detection wavelength of 281nm.The second method involved silica gel 60F254 high performance thin layer chromatography and densitometric detection at 281 nm using chloroform: methanol: formic acid (85:15:5) as the mobile phase.

AjitPandey et al(44)described a simple, precise and accurate UV spectrophotometric method has been developed and validated for the estimation of Telmisartan in bulk and tablet dosage form. The zero order spectra of Telmisartan in 0.1N NaOH shows λmax at 234.0 nm and estimation was carried out by A(1% 1cm) and by comparison with standard. Calibration graph was found to be linear (r2 =0.999) over the concentration range of 4-24 µg/ml. The proposed method was validated for its accuracy, precision, specificity, ruggedness and robustness. The method can be adopted in its routine analysis.

ZHOU Su-qin et al (45) established a method for simultaneous determination of telmisartan and hydrochlorothiazide in human plasma. The concentrations of telmisartan and HCTZ were analysed on a Zorbax 80 Extend-C18(4.6 mm×150 mm,5 µm)column with a mobile phase consisting of tetrabutyl ammonium hydroxide phosphate buffer solution(pH 2.26)-acetonitrile(76∶24) at a flow rate of 1 ml·min-1,the wavelengths were 230 and 272 nm respectively. The calibration curve of TMST was linear with the range of 31.8~1 591.2 µg·L-1.The minimum concentration was 20 µg·L-1.The calibration curve of hydrochlorothiazide was linear with the range of 30~240 µg·L-1.The minimum concentration was 20 µg·L-1. It is a rapid, reproducible method. Clinical application demonstrated that this method was practical and it can be completely used in the study of pharmacokinetics and pharmacodynamics.

3.3 Materials and methods

3.3.1 Instrumentation

An HPLC system (Shimadzu, Kyoto, Japan) consisting of an advance C18 column, a binary LC-20AD prominence pump, an auto-sampler (SIL-HTc) and a solvent degasser (DGU-20A3) was used for the study. Aliquots of the processed samples (20 ml) were injected into the column, which was kept at 30 1 C. The isocratic mobile phase was delivered into the electro-spray ionization chamber of the mass spectrometer. Quantitation was achieved with MS–MS detection in positive ion mode for both the analytes using a MDS Sciex API-4000 mass spectrometer equipped with a Turbo ion spray TMinterface at 500 1 C. The ion spray voltage was set at 5500 V. The source parameters, viz.the nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 45, 20, 45 and 10 psi, respectively. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM)

3.3.2 Chemicals and standard drugs:

The working standard drug Telmisartan having a purity of 99.36% and Hydrochlorothiazide with 98.91% pure were kindly provided by Medley Pharmaceuticals Ltd, Mumbai; Maharashtra, India. All the chemicals used were of laboratory reagent grade and were purchased from Merck chemicals private limited, Mumbai; Maharashtra, India.

3.3.3Preparation of solutions:

3.3.3.1 pH 4.4 Acetate buffer (USP):weigh accurately dissolve 136 g of sodium acetate and 77 g of ammonium acetate in water and dilute to 1000.0ml with the same solvent; add 250.0 ml of glacial acetic acid and mix well to get a buffer solution of pH 4.4.

3.3.3.2Preparation of mobile phase:

Measure accurately buffer solution, Methanol and Acetonitrile in the ratio of 600:200:200 (v/v) and sonicate the solution for ten minutes mix the contents. The content was mixed and degased using ultrasonic sonicator, and then it was filtered through 0.45µ nylon membrane filter paper using vacuum filtration set. The solution was stored at room temperature and used within 7 days from the date of preparation.

3.3.3.3Preparation of diluent:

An equal ratio of Methanol and Acetonitrile was used as diluent in the analysis. For the preparation of diluent, 50mL of methanol was transferred into a 100mL reagent bottle and 50mL of Acetonitrile was added, mixed and sonicated for 5 minutes. The solution was stored at room temperature and use within 7 days from the date of preparation.

3.3.3.4 Rinsing solution:

7:3 ratios of methanol and Acetonitrile were used as rinsing solution. To this 70ml of methanol was mixed with 30ml of acetonitrile in a 100ml beaker. Mix the solution well and then it was filtered through membrane filter paper. The solution was used as rinsing solution to rinse useful things. The solution was stored at room temperature and used within 7 days from the date of preparation.

3.3.3.5 Preparation of extraction solution:

Diethyl ether and dichloromethane in the ratio of 60:40 (v/v) was used for the extraction of drugs from the biological matrix. 60 ml of Diethyl ether was added to 40ml of dichloromethane. Mix the solution well and then it was filtered and used for the extraction. The solution was stored at room temperature and used within 7 days from the date of preparation.

3.3.3.6 Preparation of standard stock solution for Telmisartan:

A stock solution of mg/ml (1000mcg/ml) was prepared by accurately weighing25.16mg of the standard drugs Telmisartan and was dissolved in 25ml of methanol. The standard stock solution was prepared as per the potency of Telmisartan. A standard concentration of 1001.59mcg/ml was obtained. The solution was filtered and was used as standard stock solution. The solution was preserved safely and was used when it required.

3.3.3.7 Preparation of aqueous calibration curve dilutions for Telmisartan:

From the standard stock solution of Telmisartan, pre-calculated dilutions were made accurately and a working standard stock solution concentration of 500.795ng/ml was prepared. From the working standard stock solution, aqueous calibration dilutions were prepared as per the table given bellow 3.6.

Table 3.6: Preparation of aqueous calibration curve dilutions for Telmisartan

S.No	Concentration (ng/ml)	Volume taken (ml)	Volume added (ml)	Final volume (ml)	Final concentration (ng/ml)	Vial code
1	500.795	6.0	4.0	10	300.477	AQ-CC 1
2	500.795	4.0	6.0	10	200.318	AQ-CC 2
3	500.795	3.0	7.0	10	150.238	AQ-CC 3
4	500.795	1.5	8.5	10	75.119	AQ-CC 4
5	500.795	0.8	9.2	10	40.064	AQ-CC 5
6	500.795	0.2	9.8	10	10.016	AQ-CC 6
7	250.400	0.1	9.9	10	2.504	AQ-CC 7

3.3.3.8 Preparation of standard stock solution for Hydrochlorothiazide:

A stock solution of mg/ml (1000mcg/ml) was prepared by accurately weighing 10.20mg of the standard drugs Hydrochlorothiazide and was dissolved in 10ml of methanol. The standard stock solution was prepared as per the potency of Hydrochlorothiazide. A standard concentration of 1008.91mcg/ml was obtained. The solution was filtered and was used as standard stock solution. The solution was preserved safely and was used when it required.

3.3.3.9 Preparation of aqueous calibration curve dilutions for Hydrochlorothiazide:

From the standard stock solution of Hydrochlorothiazide, pre-calculated dilutions were made accurately and a working standard stock solution concentration of 504.455ng/ml was prepared. From the working standard stock solution, aqueous calibration dilutions were prepared as per the table given bellow 3.7.

Table 3.7: Preparation of aqueous calibration curve dilutions for Hydrochlorothiazide

S. No	Concentration (ng/ml)	Volume taken (ml)	diluent added (ml)	Final volume (ml)	Final concentration (ng/ml)	Vial code
1	1008.91	7.0	3.0	10	706.237	AQ-CC 1
2	1008.91	5.0	5.0	10	504.455	AQ-CC 2
3	504.455	7.0	3.0	10	353.118	AQ-CC 3
4	504.455	3.0	7.0	10	151.336	AQ-CC 4
5	504.455	1.5	8.5	10	75.668	AQ-CC 5
6	504.455	0.5	9.5	10	25.223	AQ-CC 6
7	504.455	0.1	9.9	10	5.044	AQ-CC 7

3.3.3.10Plasma spiked calibration curve for Telmisartan:

The prepared aqueous dilutions were used to spike the screened blank human plasma matrix to prepare plasma calibration curve standards. The plasma spiked calibration curve was prepared with in the concentration range of 801.272ng/ml-40.064ng/ml. The preparation of solution was given in table 3.8.

Table 3.8: Preparation of plasma spiked calibration curve dilutions for Telmisartan

S. No	Concentration (ng/ml)	Volume taken (ml)	Volume added (ml)	Final volume (ml)	Final concentration (ng/ml)	Vial code
1	1001.59	8.0	2.0	10	801.272ng/ml	PS-CC 1
2	1001.59	5.0	5.0	10	500.795ng/ml	PS-CC 2
3	1001.59	3.5	6.5	10	350.557ng/ml	PS-CC 3
4	1001.59	1.7	8.3	10	170.27ng/ml	PS-CC 4
5	1001.59	0.9	9.1	10	90.143ng/ml	PS-CC 5
6	1001.59	0.7	9.3	10	70.111ng/ml	PS-CC 6
7	1001.59	0.4	9.6	10	40.064ng/ml	PS-CC 7

3.3.3.11 Plasma spiked calibration curve for Hydrochlorothiazide:

The prepared aqueous dilutions were used to spike the screened blank human plasma matrix to prepare plasma calibration curve standards. The plasma spiked calibration curve was prepared with in the concentration range of 908.019ng/ml-20.178ng/ml. The preparation of solution was given in table 3.9.

Table 3.9: Preparation of plasma spiked calibration curve dilutions for Hydrochlorothiazide

S. No	Concentration (ng/ml)	Volume taken (ml)	Volume added (ml)	Final volume (ml)	Final concentration (ng/ml)	Vial code
1	1008.91	9.0	1.0	10	908.019	PS-CC 1
2	1008.91	6.0	4.0	10	605.346	PS-CC 2
3	1008.91	4.0	6.0	10	403.564	PS-CC 3
4	1008.91	1.0	9.0	10	100.891	PS-CC 4
5	1008.91	0.7	9.3	10	70.624	PS-CC 5
6	1008.91	0.5	9.5	10	50.445	PS-CC 6
7	1008.91	0.2	9.8	10	20.178	PS-CC 7

3.3.3.12 Extraction of drugs from plasma:

Prior to sample analysis, 100µL of each solution was extracted using 300µL of diethyl ether: dichloromethane (60:40% v/v) for protein precipitation. Further, each of the mixtures was vortex for a period of 5 min in a vortex mixer with subsequent centrifugation at 10000 rpm, for a period of 10 min at 4°C using a centrifuge. For each sample, an aliquot of a supernatant was isolated and subjected to dryness. The residue was reconstituted in 100µL of mobile phase and subsequently centrifuged at 10000 rpm for10 min at 4°C in a centrifuge. The supernatant was finally collected and directly injected for analysis. This procedure was followed for all samples of calibration curve plasma spiked dilutions and plasma spiked samples.

3.4 Method Development:

The development of a bio-analytical method for the simultaneous Telmisartan and hydrochlorothiazide includes the optimization of various parameters like mobile phase, flow rate and mass parameters.

The separation of the analytes was carried out on an Aquasil-C18 (250×4.6mm×5µm) column. Temperature was set to 20^0C. The mobile phase composed of buffer solution, Methanol and Acetonitrile in the ratio of 60:20:20 (v/v) in isocratic condition at a flow rate of 0.5m L/min for 10min and the isocratic mobile phase comprised

The full scan MS and M S/M S spectra of each analyte were obtained by direct infusion of the respective sample solution at a concentration of801.272ng/ml of Telmisartan and 908.019ng/ml of hydrochlorothiazide solution prepared in the mobile phase. The flow rates of sheath gas and auxiliary gas were optimized and set to 30 psi and 5 psi, respectively. The needle spray voltage was set to 4.5 k V. Helium was used as collision gas tuned for each analyte to obtain good signal intensity in MS2experiment. The drugs were analyzed using multiple react ions monitoring (MRM) mode.

Mass parameters were tuned in both positive and negative ionization modes for the analytes. Good response was achieved in positive ionization mode. Data from the MRM mode were considered to obtain better selectivity. Protonated form of each analyte ion was the parent ion in the Q 1 spectrum and was used as the precursor ion to obtain Q 3 product ion spectra. The most sensitive mass transition was monitored from m/z 298.12→204.36 (Q1/Q3) for hydrochlorothiazide and m/z of 513.56→469.19(Q1/Q3) for Telmisartan.

At the optimized conditions, the standard drug Telmisartan elute at a retention time of 4.39min and Hydrochlorothiazide elutes at 5.73min. The separation was found to be accurate and symmetric peaks with high resolution was observed. The optimized chromatographic and mass parameters were given in table 3.10

Table 3.10 optimized chromatographic conditions

S. No	Parameters	Conditions
1	API	Telmisartan Hydrochlorothiazide
2	Mobile phase	Methanol and Acetonitrile in the ratio of 60:20:20 (v/v)
3	column	Aquasil-C18 (250×4.6mm×5µm)
4	Flow rate	0.5 m L/min
5	Run time	10 min
6	Retention time	Telmisartan - 4.39min Hydrochlorothiazide - 5.73min

Figure 3.C: Standard LC chromatogram of Telmisartan and Hydrochlorothiazide

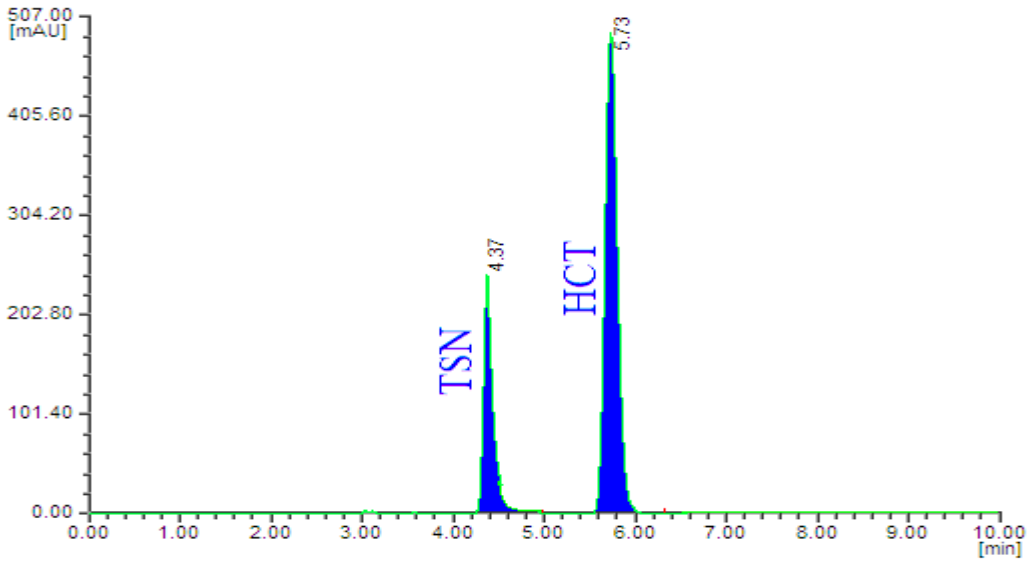


Figure 3.D: Blank chromatogram of Telmisartan and Hydrochlorothiazide

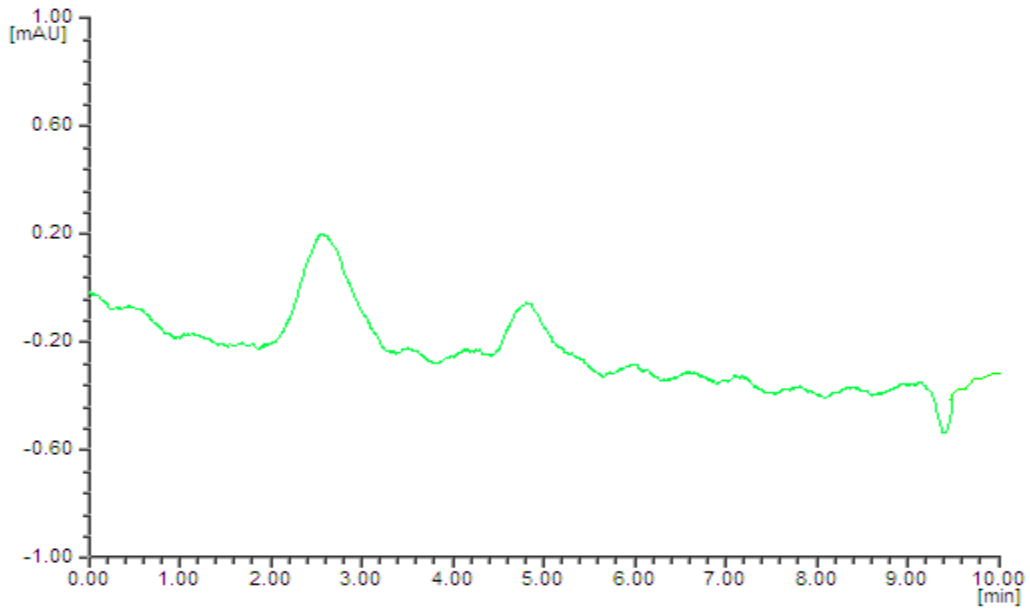


Figure 3.E: Standard LC chromatogram of Telmisartan

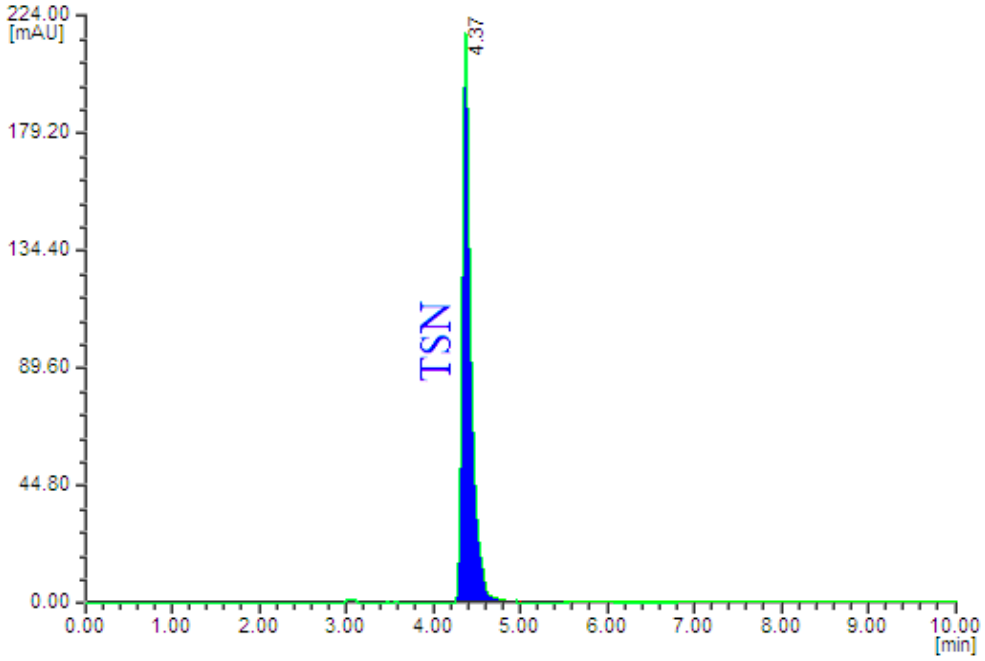


Figure 3.F: Standard LC chromatogram of Telmisartan

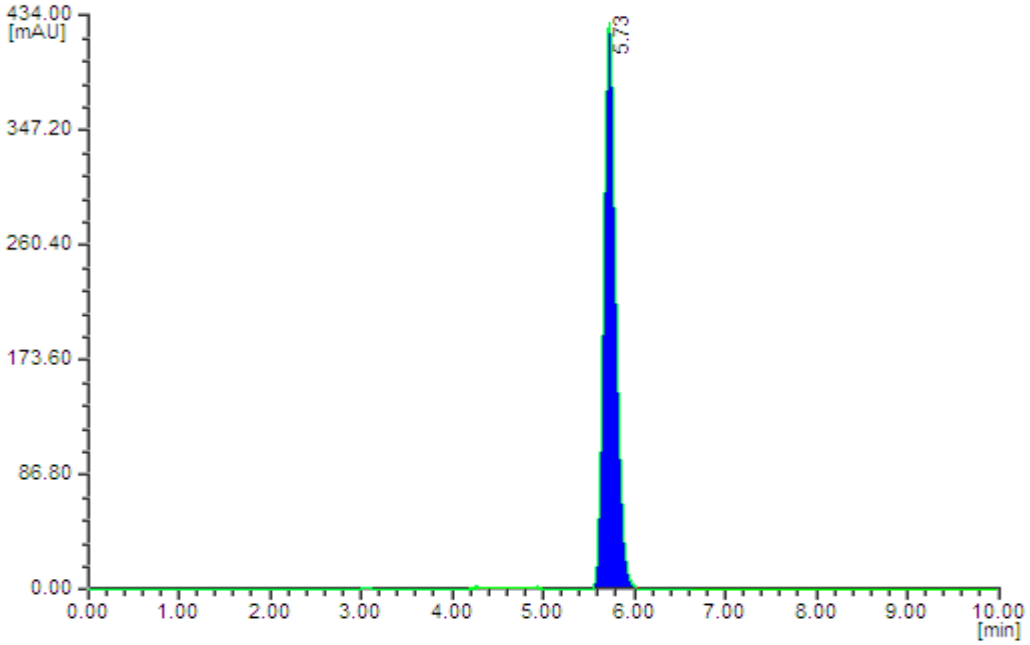


Figure 3.G: Mass spectrum of Telmisartan

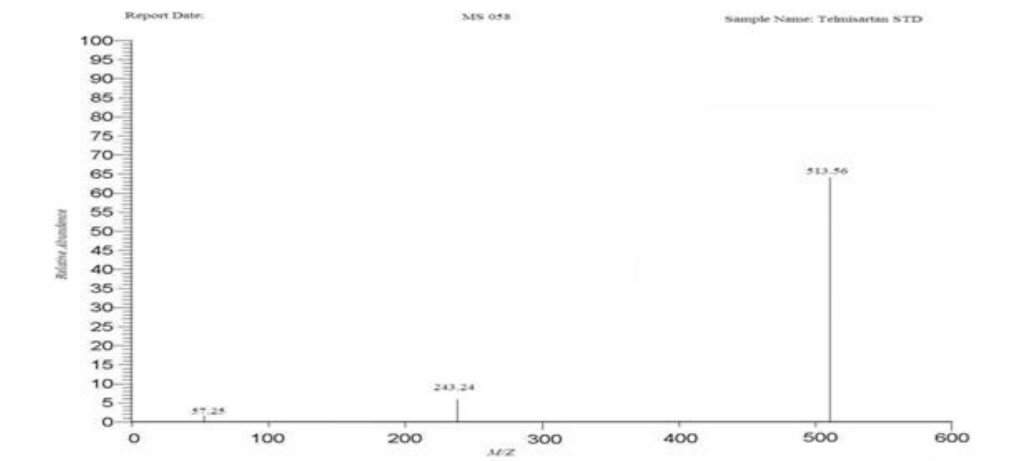
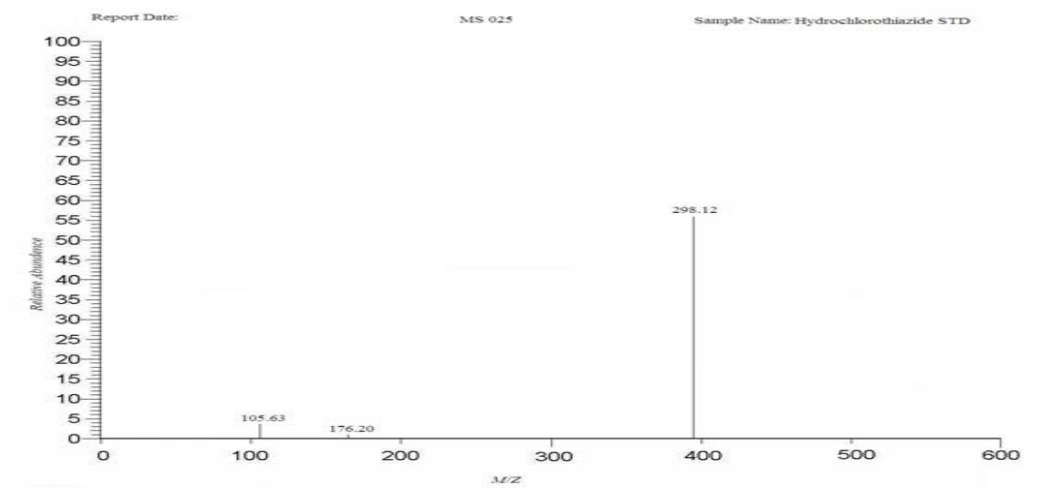


Figure 3.H: Mass spectrum of Hydrochlorothiazide



3.5 Method Validation

A thorough validation of the method was carried out as per the US FDA guidelines. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability.

3.5.1Selectivity:

Selectivity of the method was assessed by analyzing six blank human plasma matrix samples. The responses of the interfering substances or background noises at the retention time of the Telmisartan and hydrochlorothiazide acceptable if they are less than 20% of the response of the lowest standard curve point or LLOQ. There is no remarkable noise was observed at the retention time of Telmisartan and hydrochlorothiazide and hence the proposed method was selective for the standard drugs Telmisartan and hydrochlorothiazide only and hence the method selective.

3.5.2 Linearity:

The plasma spiked calibration curve dilution for both the drugs were prepared. The prepared plasma spiked dilutions were used for the determination of the linearity of the method. Linearity was tested for Telmisartan and Hydrochlorothiazide in the concentration range of 40.064ng/ml-801.272ng/ml for Telmisartan and 20.178ng/ml-908.019ng/ml for Hydrochlorothiazide in the method. For the determination of linearity, standard calibration curves containing at least 6 points (non-zero standards) were plotted and checked. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences, but these data were not used to construct the calibration curve. The samples were run in the order from low to high concentration. The results were given in table.3.11 .and linearity graphs were given in figure 3.I.

Table 3.11: Plasma spiked calibration curve

S.NO	Telmisartan		Hydrochlorothiazide		Sample vial code
	Concentration	Area at the retention	Concentration	Area at the retention time	
1	40.064ng/ml	88251	20.178ng/ml	42814	PSCC 001
2	70.111ng/ml	97123	50.445ng/ml	63817	PSCC 002
3	90.143ng/ml	141935	70.624ng/ml	93871	PSCC 003
4	170.27ng/ml	187936	100.891ng/ml	115827	PSCC 004
5	350.557ng/ml	360369	403.564ng/ml	298710	PSCC 005
6	500.795ng/ml	478251	605.346ng/ml	436107	PSCC 006
7	801.272ng/ml	712258	908.019ng/ml	652814	PSCC 007
	Slope Intercept r^2	831.27 54897 0.9976	Slope Intercept r^2	671.61 36274 0.9984	

Figure 3.I: Linearity graph for Telmisartan

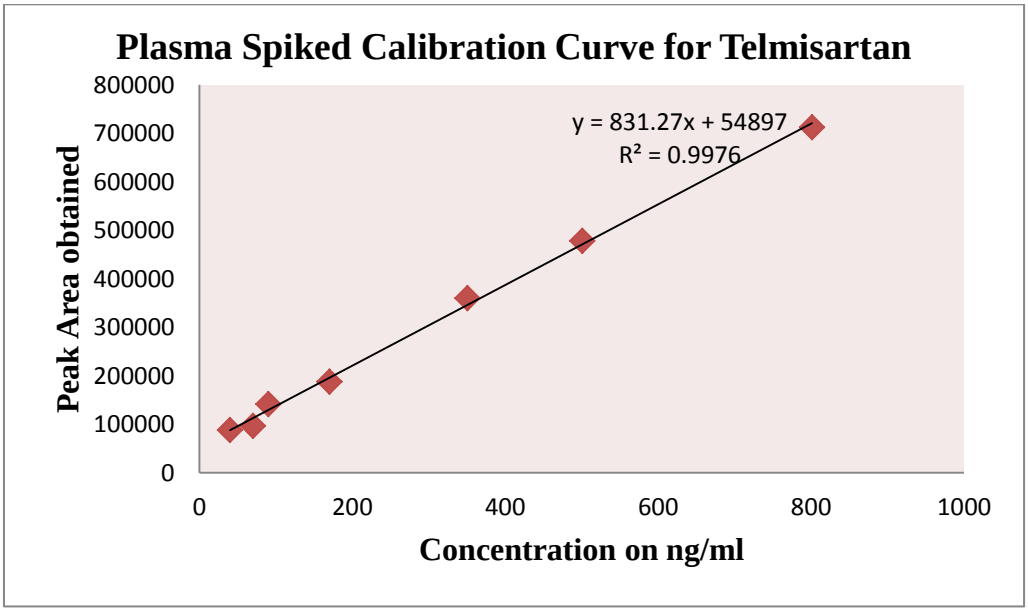
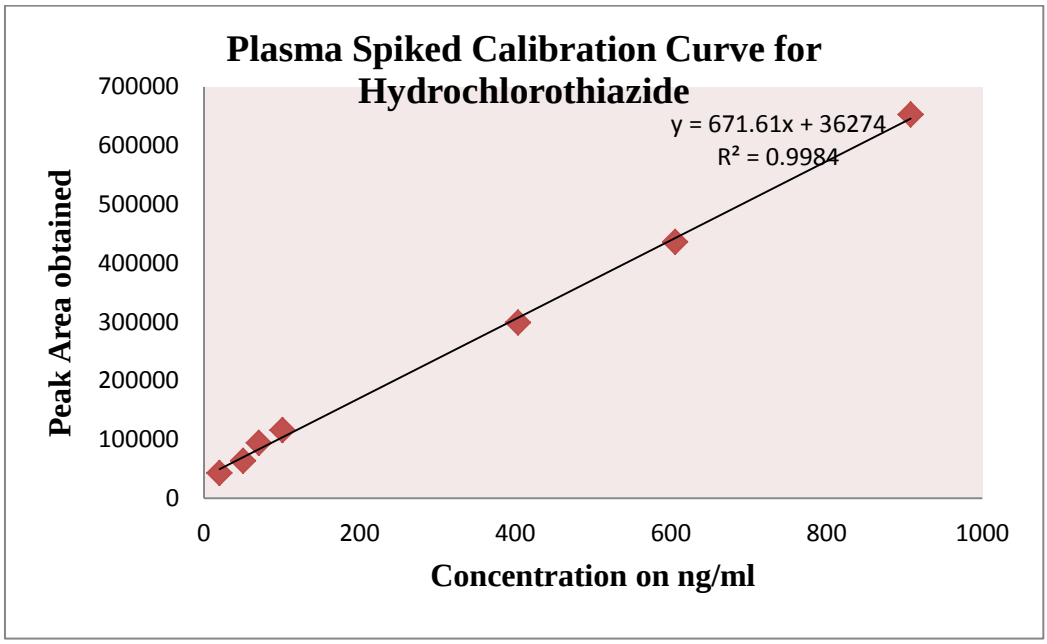


Figure 3.J: Linearity graph for Hydrochlorothiazide



3.5.3 Precision and accuracy

Intra-day assay precision and accuracy were determined by analyzing six replicates at four different QC levels in the same day on two runs. Precision was carried out at HQC, MQC, LQC and LLOQC for both the drugs in calibration curve range. Detector response at the retention time of both the drugs in each level was determined and the %CV of the response was calculated. The acceptance criteria included accuracy within ±15% deviation (SD) from the nominal values, except LLOQ QC, where it

should be ±20% and a precision of ≤15% relative standard deviation (RSD), except for LLOQ QC, where it should be ±20%. Whereas batch acceptance criteria included 67% for overall quality control samples and 50% at each level respectively. The results confirmed that the method was found to be precise and accurate. Results were given in table 3.12.

Table 3.12: Precision at HQC and LQC levels

Precision at HQC					
S.NO	**Sample ID**	**Telmisartan**		**Hydrochlorothiazide**	
		Area obtained	**Observed Concentration**	**Area obtained**	**Observed Concentration**
P and A at HQC	PA001	651758	906.55	651758	906.55
	PA002	651963	906.835	651963	906.835
	PA003	653911	909.545	653911	909.545
	PA004	652459	907.525	652459	907.525
	PA005	652988	908.261	652988	908.261
	PA006	651790	906.595	651790	906.595
Nominal Conc.		**801.272ng/ml**		**908.019ng/ml**	
N		**6**		**6**	
Average		**712193**	**1.46381**	**845.094**	**1.17547**
SD		**1203.06**	**800.956**	**652478**	**907.552**
%CV		**0.169**	**0.18276**	**0.12952**	**0.12952**
Accuracy (%)		**99.961**		**99.949**	
Precision at MQC					
S.NO	**Sample ID**	**Telmisartan**		**Hydrochlorothiazide**	
		Area obtained	**Observed Concentration**	**Area obtained**	**Observed Concentration**
P and A at MQC	PA007	184722	98.2898	115028	99.3102
	PA008	185936	98.9358	114693	99.021
	PA009	182299	97.0006	114239	98.629
	PA010	185258	98.575	114069	98.4822
	PA011	185563	98.7373	116025	100.171
	PA012	186936	99.4679	115025	99.3076
Nominal Conc.		**170.27ng/ml**		**100.891ng/ml**	
N		**6**		**6**	
Average		**1568.15**	**0.83441**	700.113	0.60445
SD		**185119**	**98.5011**	114847	99.1535
%CV		**0.8471**	**0.8471**	0.60961	0.60961
Accuracy (%)		**98.5011**		**99.1535**	

Table 3.13: Precision at LQC and LLOQC levels

Precision at LQC					
S.NO	Sample ID	Telmisartan		Telmisartan	
		Area obtained	Observed Concentration	Area obtained	Observed Concentration
P and A at LQC	PA013	97021	99.895	63021	98.7527
	PA014	96578	99.439	63229	99.0786
	PA015	97367	100.251	63182	99.005
	PA016	97442	100.328	63589	99.6427
	PA017	96987	99.860	62950	98.6414
	PA018	96875	99.745	62597	98.0883
Nominal Conc.		**70.111ng/ml**		**50.445ng/ml**	
N		**6**		**6**	
Average		**320.069**	**0.32931**	**329.868**	**0.5169**
SD		**97045**	**99.9197**	**63094.7**	**98.8681**
%CV		**0.330**	**0.330**	**0.52281**	**0.52281**
Accuracy (%)		**99.9197**		**98.8681**	
Precision at LQC					
S.NO	Sample ID	Telmisartan		Telmisartan	
		Area obtained	Observed Concentration	Area obtained	Observed Concentration
P and A at LLOQC	PA019	88012	99.729	42142	98.4304
	PA020	87896	99.598	42969	100.362
	PA021	87936	99.643	42551	99.3857
	PA022	88693	100.501	42367	98.9559
	PA023	88581	100.374	42914	100.234
	PA024	87158	98.761	42636	99.5842
Nominal Conc.		**40.064ng/ml**		**20.178ng/ml**	
N		**6**		**6**	
Average		**553.016**	**0.6268**	**316.996**	**0.7404**
SD		**88046**	**99.768**	**42596.5**	**99.492**
%CV		**0.628**	**0.628**	**0.744**	**0.744**
Accuracy (%)		**99.768**		**99.492**	

3.5. Recovery:

Recovery of the analytes from the extraction procedure was determined by comparing the peak areas of the analytes inspiked plasma samples (six each of HQC, MQC, and HQC samples) with those of the analytes in samples prepared by spiking the extracted drug-free plasma samples with the same amounts of the analytes at the step immediately prior to chromatography.

Table 3.14: Recovery of Telmisartan

S. NO	Recovery at HQC level				Recovery at MQC level				Recovery at LQC level			
	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery
1	736925	908211	650.154	81.1403	382863	441893	303.728	86.642	90176	105863	59.722	85.182
2	729963	909917	642.805	80.223	381190	459631	290.731	82.934	91589	106397	60.353	86.082
3	731580	905583	647.312	80.7855	380326	432816	308.043	87.872	93691	118580	55.395	79.011
4	739281	914792	647.541	80.8141	385601	445021	303.750	86.648	93902	107951	60.987	86.986
5	728284	909882	641.351	80.0416	376920	446179	296.141	84.477	93698	105821	62.079	88.544
6	731417	904861	647.684	80.832	382047	446377	300.036	85.588	90581	107486	59.084	84.272
SD	**4261**	**3591**	**3.3435**	**0.417**	**2878.38**	**8658**	**6.204**	**1.770**	**1698.42**	**4925**	**2.306**	**3.290**
Mean	**732908**	**908874**	**646.141**	**80.64**	**381491**	**445319**	**300.405**	**85.693**	**92272.8**	**108683**	**59.603**	**85.013**
CV	**0.581**	**0.395**	**0.517**	**0.517**	**0.75451**	**1.9443**	**2.065**	**2.065**	**1.841**	**4.533**	**3.870**	**3.869**
Standard Deviation					**2.74313**							
Average recovery of three levels					**83.782**							
% Recovery					**3.27413**							

Table 3.15: Recovery of Hydrochlorothiazide

S. NO	Recovery at HQC level				Recovery at MQC level				Recovery at LQC level			
	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery
1	682239	849631	729.124	80.2983	275819	315896	352.365	87.3132	64281	85861	37.7663	74.8664
2	679228	845822	729.175	80.3039	286932	319671	362.233	89.7585	61820	82661	37.7265	74.7874
3	671852	841028	725.368	79.8846	265102	328476	325.703	80.7067	60528	86394	35.342	70.0604
4	642811	842836	692.525	76.2676	295017	339178	351.02	86.98	65936	84105	39.5475	78.3972
5	637492	849928	681.063	75.0054	259670	329541	317.998	78.7975	62820	80281	39.4733	78.2501
6	637028	842014	686.964	75.6553	279954	330208	342.146	84.7811	64559	84028	38.7571	76.8303
SD	**21540.5**	**3886**	**22.81**	**2.512**	**13230.5**	**8295**	**16.970**	**4.205**	**1979.76**	**2225.1**	**1.567**	**3.105**
Mean	**658442**	**845210**	**707.37**	**77.902**	**277082**	**327162**	**341.911**	**84.723**	**63324**	**83888**	**38.102**	**75.532**
CV	**3.271**	**0.460**	**3.224**	**3.224**	**4.77493**	**2.536**	**4.963**	**4.963**	**3.126**	**2.652**	**4.111**	**4.111**
Standard Deviation					**4.771**							
Average recovery of three levels					**79.386**							
% Recovery					**6.011**							

The results of the recovery conforms that the % recovery was found to be 3.27 for Telmisartan and 6.011 for Hydrochlorothiazide in three levels. The results of the recovery were given in table 3.14 and 3.15 for Telmisartan and Hydrochlorothiazide respectively

3.5.5 Stability of the drug in solution:

Stability tests were conducted to evaluate the analytestability in stock solutions and in plasma samples underdifferent experimental conditions.

3.5.5.1Short term stability:

The stock solution stability at room temperature for 8 hours was per-formed by comparing the area response of the analytes(stability samples) with the response of the sample prepared from fresh stock solution. Freshly prepared and stability stored solution at 801.272ng/ml for Telmisartan and 908.019ng/ml Hydrochlorothiazide were analysed and area response of the two conditions were compared and % stability was calculated. % stability was found to be within the range of more than 98% for both the drugs.

Table 3.16. Short term stability results for Telmisartan and Hydrochlorothiazide

S. NO	Telmisartan			Hydrochlorothiazide		
	Area obtained for		% Stability	Area obtained for		% Stability
	Fresh Stock	Stability stock		Fresh Stock	Stability stock	
1	912581	901526	98.789	846628	841028	99.339
2	911286	904789	99.287	851573	840856	98.741
3	913952	902851	98.785	846692	843942	99.675
4	910179	896925	98.544	859015	844103	98.264
5	913631	897530	98.238	848715	836925	98.611
6	909425	904744	99.485	845593	838128	99.117
SD	**1850.15**	**3458.07**	**0.463**	**5031.9**	**2932.12**	**0.516**
Mean	**911842**	**901394.17**	**98.855**	**849703**	**840830**	**98.96**
CV	**0.2029**	**0.384**	**0.468**	**0.592**	**0.349**	**0.522**
% Stability	**98.855**			**98.96**		
% Change	**1.145**			**1.04**		

3.5.5.2 Long term stability:

Long term stability of the sample solution was studied by incubating the solution at a below 10°C (stability samples) in a refrigerator for 11 days. Solution at 801.272ng/ml for Telmisartan and 908.019ng/ml Hydrochlorothiazide was used for stability study. The solutions were analyzed in after the stability period. % stability and % assay were calculated. % stability was found to be 98.457 for Telmisartan with a % change of 1.543. % stability for hydrochlorothiazide was found to be 98.333 and % change was 1.667. Results were given in table 3.17.

Table 3.17: Long term stability results for Telmisartan and Hydrochlorothiazide

S. NO	Telmisartan			Hydrochlorothiazide		
	Area obtained for		% Stability	Area obtained for		% Stability
	Fresh Stock	Stability stock		Fresh Stock	Stability stock	
1	942891	929671	98.598	866917	856921	98.847
2	951273	931157	97.885	861801	844281	97.967
3	955107	930179	97.390	865528	859362	99.288
4	946931	936901	98.941	869018	851029	97.930
5	952037	945143	99.276	860581	849361	98.696
6	953975	941128	98.653	869286	845536	97.268
SD	**4621.51**	**6442.746**	**0.697**	**3649.52**	**6043.65**	**0.740**
Mean	**950369**	**935696.5**	**98.457**	**865522**	**851082**	**98.333**
CV	**0.486**	**0.689**	**0.7078**	**0.422**	**0.710**	**0.753**
% Stability	**98.457**			**98.333**		
% Change	**1.543**			**1.667**		

3.5.5.3 Freeze Thaw Stability:

The stability studies of plasma samples spiked with Telmisartan and Hydrochlorothiazide were subjected to three Freeze thaw cycles. The mean concentrations of the stability samples were compared to the theoretical concentrations. Freeze Thaw Stability was studied at HQC and MQC levels. Response at the retention time of the each drug was noted and the % stability was calculated. % stability was found to be 100.293 and 100.482 for Telmisartan; 100.380 and 95.305 for Hydrochlorothiazide in HQC and MQC levels respectively. High stability was observed for the proposed method. Results were given in table…3.18

Table 3.18: Freeze Thaw Stability results for Telmisartan and Hydrochlorothiazide

S.NO	Telmisartan				Hydrochlorothiazide			
	At HQC (ng/ml)		At LQC (ng/ml)		At HQC (ng/ml)		At LQC (ng/ml)	
	Fresh	Stability	Fresh	stability	Fresh	stability	Fresh	stability
1	802.141	802.128	40.154	41.221	908.123	908.589	20.361	19.152
2	801.361	803.631	40.914	41.014	909.632	910.281	20.498	19.365
3	800.943	802.063	41.225	39.651	907.581	911.476	19.936	18.637
4	799.75	804.586	39.879	39.481	906.636	911.693	19.881	18.992
5	801.22	804.125	39.425	40.691	908.036	913.571	20.781	19.705
6	802.58	805.636	39.661	40.361	908.921	914.286	19.632	19.553
N	6	6	6	6	6	6	6	6
SD	0.988	1.405	0.715	0.713	1.041	2.093	0.435	0.3905
Mean	801.333	803.695	40.209	40.403	908.155	911.64	20.1815	19.234
% CV	0.123	0.175	1.778	1.765	0.115	0.230	2.153	2.030
Accuracy	100.008	100.302	100.564	100.846	100.015	100.40	100.017	95.322
Stability	100.293		100.482		100.380		95.305	

3.5.5.4 Bench-top stability:

Bench top stability, using six sets each of LQC and HQC was determined at six hours. The quality control samples were quantified against the freshly spiked calibration curve standards of concentration range equivalent to that used for calculation of precision and accuracy. Results were found to be stable up to 6 hours as per the acceptance criteria. The percent nominal ranged from 98.716 at HQC and 93.874 at LQC for Telmisartan and 100.777 at HQC and 92.032 at LQC for Hydrochlorothiazide. Results were given in table 3.19

Table 3.19: Bench-top stability results for Telmisartan and Hydrochlorothiazide

S. NO	Telmisartan				Hydrochlorothiazide			
	At HQC (ng/ml)		At LQC (ng/ml)		At HQC (ng/ml)		At LQC (ng/ml)	
	Fresh	stability	Fresh	stability	Fresh	stability	Fresh	stability
1	795.653	795.514	39.636	38.157	905.693	916.635	21.119	18.874
2	799.154	794.825	40.185	39.636	907.759	911.125	19.898	19.896
3	798.582	796.621	41.128	38.571	905.596	910.141	21.556	19.254
4	799.125	797.103	42.225	38.253	906.674	914.471	20.693	18.631
5	796.251	796.634	42.174	37.556	907.581	911.392	21.175	19.204
6	799.936	794.415	39.996	38.142	906.058	917.856	20.225	18.874
N	6	6	6	6	6	6	6	6
SD	1.74182	1.09561	1.1275	0.69502	0.94085	3.19527	0.62715	0.44437
Mean	798.117	795.852	40.891	38.386	906.56	913.603	20.7777	19.1222
% CV	0.21824	0.13767	2.75736	1.81063	0.10378	0.34974	3.0184	2.32384
Accuracy	99.6062	99.3236	102.063	95.8113	99.8393	100.615	102.972	94.7674
Stability	98.716		93.874		100.777		92.032	

3.5.5.5Auto-sampler stability:

The auto-sampler stability was evaluated by keeping the QC samples at 40^0C for 24 h in auto-sampler before analysis. Auto sampler stability was studied at HQC and LQC level. % stability was found to be 99.16129 and 93.036 for Telmisartan and 100.796 and 107.519 for Hydrochlorothiazide at HQC and LQC levels. Stability results were found to be accepted. Results were given in table.. 3.20

Table 3.20: Auto-sampler stability results for Telmisartan and Hydrochlorothiazide

S. NO	Telmisartan				Hydrochlorothiazide			
	At HQC (ng/ml)		At LQC (ng/ml)		At HQC (ng/ml)		At LQC (ng/ml)	
	Fresh	stability	Fresh	stability	Fresh	stability	Fresh	stability
1	796.585	799.025	38.174	36.691	902.258	909.25	18.896	20.221
2	793.674	799.471	39.158	35.871	905.563	916.654	19.874	21.235
3	799.251	795.582	39.271	37.582	907.586	911.281	19.579	20.825
4	794.48	800.25	38.517	37.108	901.251	905.593	18.367	20.634
5	796.637	769.582	40.143	36.253	908.887	916.98	20.124	21.128
6	794.583	771.25	40.218	35.58	905.659	914.691	19.827	21.391
N	6	6	6	6	6	6	6	6
SD	2.047	14.642	0.829	0.759	2.962	4.5129	0.674	0.434
Mean	795.868	789.193	39.247	36.514	905.201	93.408	19.444	20.906
% CV	0.257	1.855	2.113	2.078	0.327	0.495	3.467	2.077
Accuracy	99.326	98.493	97.960	91.139	99.690	100.483	96.365	103.606
Stability	99.16129		93.036		100.796		107.519	

3.6 Discussion of the Results

Bioanalytical methods employed for the quantitative determination of drugs and their metabolites in biological matrix (plasma, urine, saliva, serum etc) play a significant role in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. The main advantages of LCMS/MS include low detection limits, the ability to generate structural information, the requirement of minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities. Hence a simple, rapid and sensitive liquid chromatography–tandem mass spectrometric (LC–MS/MS) assay method has been developed and fully validated for the simultaneous quantification of Telmisartan and Hydrochlorothiazide in human plasma.

Analytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a matrix. A compound can often be measured by several methods and the choice of analytical method involves many considerations, such as: chemical properties of the analyte, concentrations levels, sample matrix, cost of the analysis, and speed of the analysis, quantitative or qualitative measurement, and precision required and necessary equipment. The analytical chain describes the process of method development and includes sampling, sample preparation, separation, detection and evaluation of the results.

The isocratic mobile phase, a mixture of Acetate buffer (pH 4.4), Methanol and Acetonitrile in the ratio of 60:20:20 (v/v) acetonitrile and 5 mM ammonium acetate (pH-4.0) (50:50, v/v) was delivered at 0.5mL/min into the electrospray ionization chamber of the mass spectrometer. Quantitation was achieved with MS–MS detection in positive ion mode for both the analytes using a MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray™ interface at 500 °C. The ion spray voltage was set at 5500 V. The source parameters, viz. the nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 45, 20, 45 and 10 psi, respectively. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM).

At the optimized conditions, the standard drug Telmisartan elute at a retention time of 4.39min and Hydrochlorothiazide elutes at 5.73min. The separation was found to be accurate and symmetric peaks with high resolution was observed. The optimized chromatographic and mass parameters were given in table 3.10. LC chromatogram of standard was given at figure 3.C; mass spectra were given in figure 3.G for Telmisartan and 3.H for Hydrochlorothiazide.

A thorough validation of the method was carried out as per the US FDA guidelines. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability. Selectivity of the method was assessed by analyzing six blank human plasma matrix samples. The responses of the interfering substances or background noises at the retention time of the Telmisartan and Hydrochlorothiazide are acceptable if they are less than 20% of the response of the lowest standard curve point or LLOQ.

Matrix effect is investigated to ensure that selectivity and precision are not compromised within the matrix screened. Three blank samples from each of at least six batches of matrix under screening are extracted. For matrix effect LQC (lower quality control), MQC (middle quality control) and HQC (higher quality control) spiking dilutions and internal standard dilution are spiked in the above extracted blank samples. Recovery comparison sample at LQC, MQC and HQC concentration level along are prepared and screened. The results confirmed that no significant matrix effect was observed for the method developed in the study.

The calibration curves were plotted between response factor and concentration of the standard solutions against concentration of the analyte. The linearity ranges were found to be 40.064ng/ml - 801.272ng/ml for Telmisartan and 20.178ng/ml - 908.019ng/ml for Hydrochlorothiazide. The calibration curves were constructed on 11 different days over a period of four weeks to determine the variability of the slopes and intercepts. The results indicated no significant interday variability of slopes and intercepts over the optimized concentration range. A high correlation of 0.997 ($y = 831.2x + 54897$) and 0.998 ($y = 671.6x + 36274$) for was observed for Telmisartan and Hydrochlorothiazide respectively. Figure 3.I and 3.J shows the plasma spiked calibration

curves obtained in the developed method. Table 3.11 shows the results the plasma spiked calibration curve results for Telmisartan and Hydrochlorothiazide.

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

Evaluation of the stability of samples was based on the comparison of various samples against freshly prepared samples of the same concentration. Percentage difference between the back calculated concentrations obtained for the sample under investigation and freshly prepared sample was evaluated. Six aliquots, each of LQC and HQC concentrations were used for stability study. High % stabilities were obtained for different stability studies like short term, long term, bench top, freeze thaw and auto-injector stabilities. This confirms that the method was stable.

3.7 Conclusion

A method using LC-MS/MS for the determination of Telmisartan and Hydrochlorothiazide in plasma employing simple liquid-liquid extraction was developed. The method is rapid, simple, specific and sensitive, and additionally demonstrates good accuracy and precision. Compared with the available methods, the present method features high selectivity and sensitivity. We believe that this high through put method could provide a useful tool for the determination of Telmisartan and Hydrochlorothiazide in plasma. The established method was successfully applied to a pharmacokinetic study and to assess the plasma concentration.

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