ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

High-sensitivity simultaneous liquid chromatography / tandem mass spectrometry (UPLC/MS/MS) assay of olmesartan medoxomil, hydrochlorothiazide and amlodipine besylate in human plasma

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Article History:

Received on: 04.02.2018
Revised on: 06.04.2018
Accepted on: 14.04.2018

Keywords:

Simultaneous estimation
Olmesartan medoxomil
Hydrochlorothiazide
Amlodipine Besylate
Telmisartan
UPLC-MS/MS
Human plasma

ABSTRACT

A simple and rapid UPLC-MS/MS method has been developed and validated for the analysis of Olmesartan medoxomil, Hydrochlorothiazide, Amlodipine besylate and Telmisartan as internal standard in plasma. Sample was prepared in LLE. LC separation was achieved in Acquity TQD. TQD detector and analytical column of RP-18 (50 mm* 2.1mm, 1.7 micron) at 40°C. The mobile phase consisted Acetonitrile and Ammonium acetate as a gradient elution up to 0.8 minutes/42% A, 5/10%, 6/10%, 6.1/42% and 10/42%. Total run time was 10 minutes operating with flow rate 0.3mL/ minutes. Mass spectroscopy detection was performed by negative and positive ion mode electro spray Olmesartan medoxomil, Hydrochlorothiazide and Amlodipine besylate, Telmisartan respectively. The method proved to be specific and linear over the range (40.34 to 8092.75) ng/mL for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine besylate. This technique also showed high sensitivity with a 2.14, 1.86 and 1.12 ng LOD and 6.28, 4.21 and 3.86 ng LOQ Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine besylate respectively. Percentage recovery ranged from 86 to 103% for Olmesartan medoxomil, Hydrochlorothiazide, Amlodipine Besylate and from 58 to 110 for Telmisartan. CV of inter and intra precision was found within 15%. The LC–MS/MS assay reported in this paper is rapid, simple, specific and sensitive for simultaneous quantification of Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate in human plasma and is fully validated according to commonly acceptable FDA guidelines. And the method can be useful for BA/BE studies and routine therapeutic drug monitoring with the desired precision and accuracy.

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ISSN: 0975-7538
DOI: https://doi.org/10.26452/ijrps.v9i2.1438

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INTRODUCTION

Calcium channel blockers have been widely used in the treatment of hypertension and angina pectoris, and combination therapy with an angiotensin II receptor blocker would enhance antihypertensive activity with greater efficacy and better tolerability, which maximize the blood pressure lowering effects and minimize the severity of their side effects of each component.

Olmesartan Medoxomil is an angiotensin II receptor antagonist which has been used for the treatment of high blood pressure. It is an ester pro drug,

it is completely and rapidly hydrolyzed to the active acid form. Angiotensin-II receptor antagonists should be used with caution in renal artery stenosis. Monitoring of plasma-potassium concentration is advised, particularly in the elderly and in patients with renal impairment; lower initial doses may be appropriate in these patients. Angiotensin-II receptor antagonists should be used with caution in aortic or mitral valve stenosis and in hypertrophic cardiomyopathy (W.C.Cushman., 2003). Hydrochlorothiazide (HCT) is a 6 - chloro - 3, 4 - dihydro - 7 -sulfamoyl - 2H - 1, 2, 4 - benzothiadiazine - 1, 1 - dioxide, is a thiazide diuretic. It increases sodium and chloride excretion in distilled convoluted tubule. Hydrochlorothiazide treats 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) (Borghi C *et al*., 2010). Amlodipine, (R, S)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1, 4-dihydropyridine) is a potent calcium channel blocker used for the treatment of hyper-tension and angina pectoris. It has high bioavailability, large volume of distribution and long elimination half-life (t1/2) ranging from 35 to 45 h (Philipp T C *et al*., 2007). A rapid and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) Method for the estimation of Amlodipine in human Plasma (Bhatt J *et al*., 2007) Determination of Amlodipine in human plasma by high-Performance liquid chromatography with Fluorescence Detection (Tatar S *et al*., 2001) Determination of Amlodipine in human Plasma by LC-MS/MS and its bioequivalence study in healthy chinese subjects (Chan-Mei Lv., *et al.,* 2013). Determination of Amlodipine in human plasma by electrospray ionization LCMS/MS method: validation and its stability studies (Anusak Sirikatitham *et al*., 2008). Determination of S- and R-Amlodipine in Rat Plasma using LC-MS/MS after oral administration of S-Amlodipine and racemic Amlodipine (Hye Hyun Yoo *et al.,* 2011). Spectrofluorimetric determination of Amlodipine in human plasma without derivatization (Yucel Kadioglu *et al*., 2012). Development and validation of a LC-MS/MS method for the simultaneous estimation of Amlodipine and Valsartan in human Plasma (Jangala *et al*., 2014). Simple RP-HPLC method for determination of triple drug combination of Valsartan, Amlodipine and hydrochlorothiazide in human plasma (Ritesh N. Sharma *et al.,* 2012). A novel, sensitive, bioanalytical method for estimation of Amlodipine besylate in rat plasma using fluorescence detection by RP-HPLC (Varghese, *et al.,* 2014). Spectrophotometric estimation of Olmesartan Medoxomil and hydrochlorothiazide in tablet (Rote AR *et al*., 2010). In vitro competitive metabolism study of Olmesartan Medoxomil in Rat Liver S9 Fractions using LC/MS, Pharmacology & Pharmacy (Muruganathan Gandhimathi *et. al*., 2011). Simultaneous Determination of Azelnidipine and Olmesartan Medoxomil by first derivative spectro photometric Method (Nilam Patel *et al*., 2012). Simultaneous determination of Telmisartan and Amlodipine in human plasma by LC–MS/MS and its application in a human pharmacokinetic study (Vasu Babu Ravi *et al*., 2012). Estimation of Telmisartan in human plasma by reversed phase liquid chromatography coupled with tandem mass Spectrometry - A Bioequivalence Study Application (James D Terish *et al*., 2011). The chemical structures of Olmesartan medoxomil, Hydrochlorohthiazide and Amlodipine besylate are shown in Fig.1 to Fig.3.

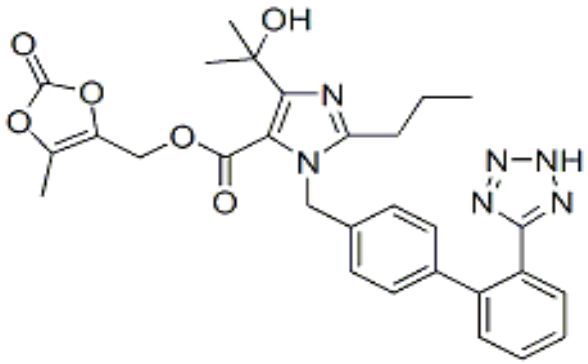

Figure 1: Chemical Structures of Olmesartan Hydrochloride

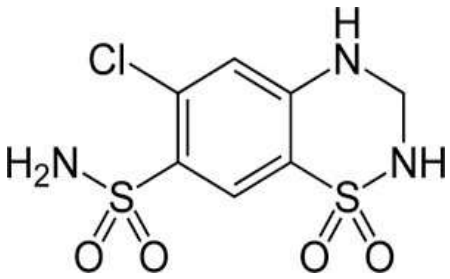

Figure 2: Chemical Structures of Hydrochlorothiazide

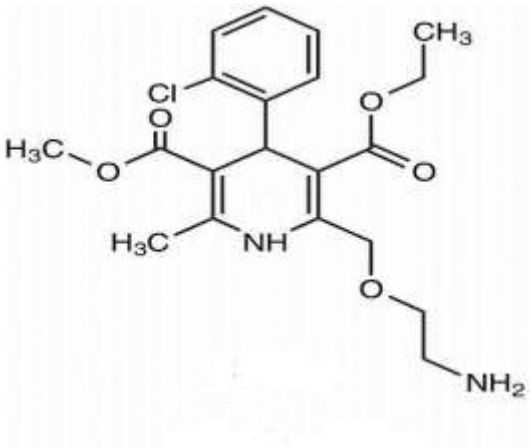

Figure 3: Chemical Structures of Amlodipine Besylate

MATERIALS AND METHODS

Reagents and Chemicals

Standards of Olmesartan medoxomil, Hydrochlorothiazide and Amlodipine Besylate were obtained from USP (Rockville, USA). Telmisartan was obtained from Clearsynth Labs Limited (Mumbai, India). Ammonium acetate was obtained from Sigma Aldrich (Bangalore, India). Trifluro acetic acid of GR grade was procured from Merck Private Limited. (Mumbai, India). HPLC grade methyl-tert-butyl ether and acetonitrile were procured from J.T. Baker Private Limited (Mumbai, India). Water used in the entire analysis was prepared from the Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma with disodium editate as an anticoagulant was obtained from clinical laboratory. Blank plasma was stored at –20 °C until use.

Instrumentation and Chromatographic conditions

UPLC/MS-MS ACQUITY TQD with binary pump, acuity column oven and TQD detector. Analytical column was used RP-C18 (50 mm* 2.1mm, 1.7 micron) at 40°C. The mobile phase consisted acetonitrile 5mM ammonium formate as a gradient elution up to 0.8 minutes/42% A, 5/10%, 6/10%, 6.1/42% and 10/42%. Total run time was 10 minutes operating with flow rate 0.3mL/ minutes. Mass spectroscopy detection was performed by negative and positive ion mode electro spray Olmesartan medoxomil, Hydrochlorothiazide and Amlodipine besylate, Telmisartan respectively.

Ionization and detection of analytes and IS were carried out on a triple quadrupole mass spectrometer, TQD (Waters), equipped with electrospray ionization (ESI) and operating in the positive ion mode and negative ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor parent→product ion (m/z) transitions 445.21→148.98, 167.07 for Olmesartan Medoxomil and 295.9→268.97, 204.54 for Hydrochlorothiazide, 409.12→238.11, 294.12 for Amlodipine besylate and 515.23→275.89, 210.84 Telmisartan as IS (Figure not shown). The source dependent parameters maintained for all analytes were Gas 1 (Nebulizer gas): 40.0 psig; Gas 2 (heater gas flow): 60.0 psig; ion spray voltage (ISV): 5000.0 V, turbo heater temperature (TEM): 550.0 °C; interface heater (Ihe): ON; entrance potential (EP): 10.0 V; collisional activated dissociation (CAD): 8 psig and curtain gas (CUR), nitrogen: 30 psig. Compound specific values of mass spectrometer parameters are listed in Table1 and product mass spectra of Olmesartan Medoxomil, Hydrochlorothiazide, Amlodipine Besylate and Telmisartan was shown in Figure 4. to Figure 7.

Sample preparation

The standard stock solution of Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate (1 mg/mL) and Telmisartan (1 mg/mL) were prepared by dissolving requisite amount in methanol. Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with serially diluted spiking solutions. Calibration curve standards were made at 40.34, 80.67, 403.36, 746.96, 1493.92, 2872.93, 5745.86 and 8092.75 ng/mL for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate. While QC samples were prepared at five concentration levels, viz. 5738 ng/mL (HQC, high quality control), 2869 ng/mL (MQC, medium quality control), 100 ng/mL (LQC, low quality control) and 40 ng/mL (LLOQ QC, lower limit of quantification quality control) for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate. The QC concentrations of 40.49, 100.71, 2869.22 and 5738.44 ng/mL were applied for all the three compounds. Stock solutions of Telmisartan (1.0 mg/mL) as IS were prepared by dissolving 1.0 mg each of them in appropriate volumes of acetonitrile. Mixed working IS, solutions containing 3000 ng/mL Telmisartan solution was prepared by appropriate dilution of the stock solution in acetonitrile. All the solutions (standard stock, calibration standards and quality control samples) were stored at 2–8°C until use.

Sample extraction protocols

Prior to analysis, all frozen subject samples, calibration standards and QC samples were thawed and allowed to equilibrate at room temperature. To 5mL of polypropylene centrifuge tube 500 micro liter of plasma sample was spiked with 50 micro liter internal standard solutions. 400 micro liter of 0.1% trifluoroacetic acid and ammonium acetate and 2.5 mL of tertiary butyl methyl ether were added. Sample were vortexed for 10 minutes from that 2ml supernenetent were extracted by LLE and dried the sample under N2 and reconstituted the sample with 300 micro liter of mobile phase. Vortex the sample and 10 micro liter was injected.

Method validation procedures

The bioanalytical method was fully validated following the USFDA guidelines. System suitability experiment was performed by six consecutive injections using the aqueous standard mixture of all the analytes and their IS at the start of each batch during method validation. System performance was studied by injecting one extracted blank (without analyte and IS) and one ULOQ (the upper limit of quantification) and LLOQ sample with IS at the beginning of each analytical batch and before

reinjection any sample during method validation. Carryover effect of autosampler was checked to verify any carryover of analyte at the start and at the end of each batch. The design of the experiment comprised the following sequence of injections viz., extracted blank sample→ULOQ sample→two extracted blank samples→LLOQ sample.

Selectivity of the method towards endogenous plasma matrix components was assessed in seven different batches of plasma, of which six were normal disodium edetate plasma and one each of lipidemic and haemolyzed plasma.

Linearity

Linearity of the method was determined by analysis of three linearity curves containing eight non-zero concentrations. Area ratio responses for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate and Telmisartan obtained from multiple reaction monitoring were used for regression analysis. Each calibration curve was analyzed individually by using least square weighted (1/x2) linear regression which was finalized during pre method validation. A correlation coefficient (r2) value of greater than 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least five times more than that of drug free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete and reproducible with a precision (%CV) less than 20% and accuracy within 80–120%. Deviation of the standards other than LLOQ from nominal concentration should not be more than ±15%.

Accuracy and Precision

For determining intra batch accuracy and precision, replicate analyses of plasma samples were performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ QC, LQC, MQC and HQC samples. Inter batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive validation days. Precision (%CV) at each concentration level from the nominal concentration should not be greater than 15%. Similarly, the mean accuracy should be within 85–115%, except for the LLOQ QC where it should be from 80% to 120% of the nominal concentration. Aliquots of 500 µL of extracted control plasma were then injected into the column by the autosampler.

Matrix effect

Relative recovery, absolute matrix effect and process efficiency were assessed. All three parameters were evaluated at HQC, MQC, LQC and LLQC levels in six replicates. Relative recovery (RE) was calculated by comparing the mean peak area response of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level. Recovery of IS was similarly estimated. Absolute matrix effect (ME) was assessed by comparing the mean peak area response of unextracted samples (spiked after extraction) with mean peak area of standard solutions.

Solution stability

All stability results were evaluated by measuring the area ratio response (drug/IS) of stability samples against freshly prepared comparison standards with identical concentration. Stock solutions of analytes and IS were checked for short term stability at room temperature and long term stability at 5 °C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Auto sampler stability (extract stability at 2–8 °C and at ambient temperature), bench top (at room temperature) and freeze thaw (four cycles) stability experiments were performed at LQC and HQC levels using six replicates. Freeze-thaw stability was evaluated by successive cycles of freezing (at -20 and -70°C) and thawing (without warming) at room temperature. Long term stability of spiked plasma samples stored at -20 and -70 °C was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15.0%.

Ruggedness

To authenticate ruggedness of the proposed method, it was performed with two precision and accuracy batches. The first batch was analyzed by different analysts while the second batch was studied on two different columns. Dilution integrity experiment was evaluated by spiking the QC sample at 1.7 times of ULOQ concentration for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate concentration in the screened plasma.

RESULTS AND DISCUSSION

Method development

To develop a selective, rugged and a reliable method for the simultaneous estimation of Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate in human plasma, the three commonly used extraction procedures were systematically investigated. The chromatographic and mass spectrometric conditions were suitably optimized to get the desired sensitivity, selectivity and linearity in regression curves.

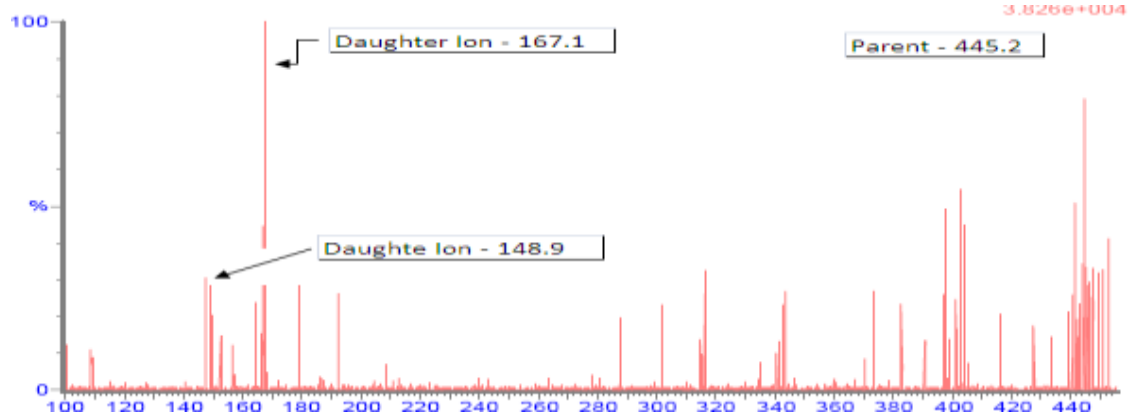

Figure 4: Product ion mass spectra of Olmesartan Medoxomil (m/z 445.2→167.1 and 148.9)

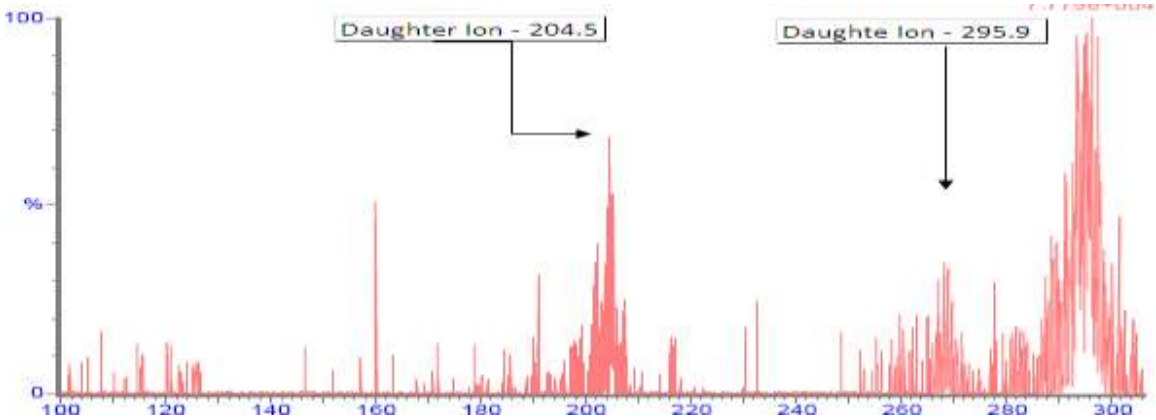

Figure 5: Product ion mass spectra of Hydrochlorothiazide (m/z 297→295.9 and 204.5)

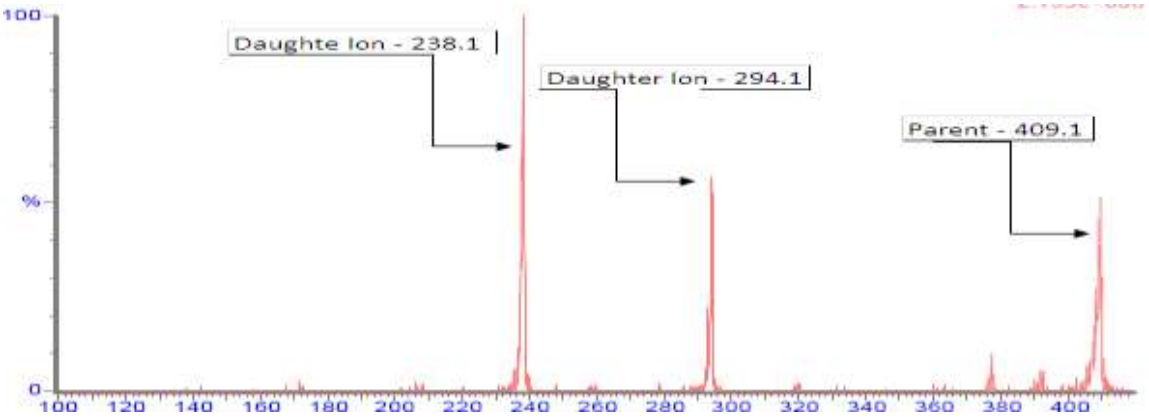

Figure 6: Product ion mass spectra of Amlodipine (m/z 409.1→294.1 and 238.1)

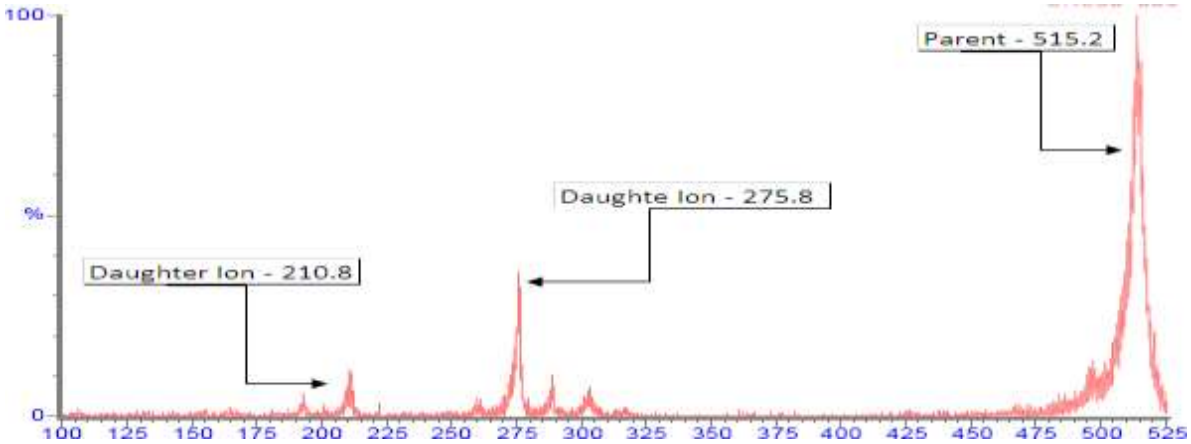

Figure 7: Product ion mass spectra of Telmisartan m/z 515.2→275.8 and 210.8)

Table 1: Values of compound specific mass spectrometer parameters

Compound	For-mula/Mass	Parent m/Z	Cone Voltage	Daughters	Collision Energy	Ion Mode
OLM	446.5	445.21	56	148.98	74	ES-
		445.21	56	167.07	42	ES-
HCTZ	297	295.97	46	268.97	38	ES-
		295.97	46	204.54	58	ES-
ALM	408.2	409.1	16	238.11	16	ES+
		409.1	16	294.12	20	ES+
TLM	514.2	515.23	72	275.89	78	ES+
		515.23	72	210.84	80	ES+

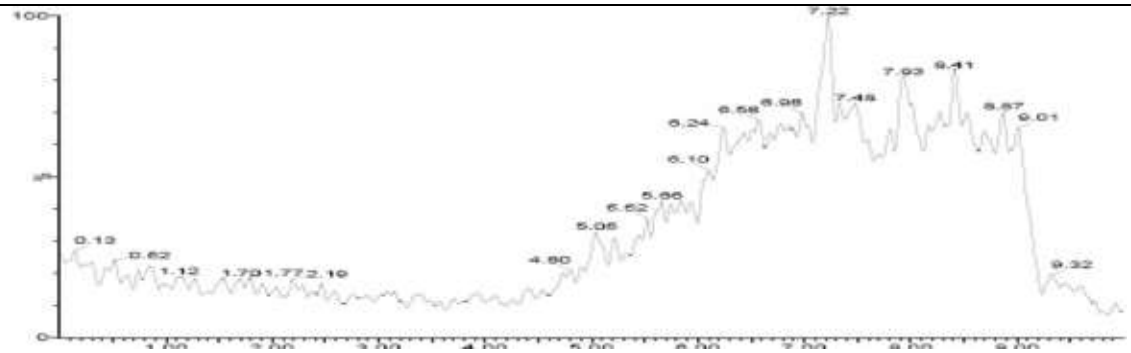

Figure 8: MRM ion-chromatograms of Double blank plasma (without IS) Positive ion mode

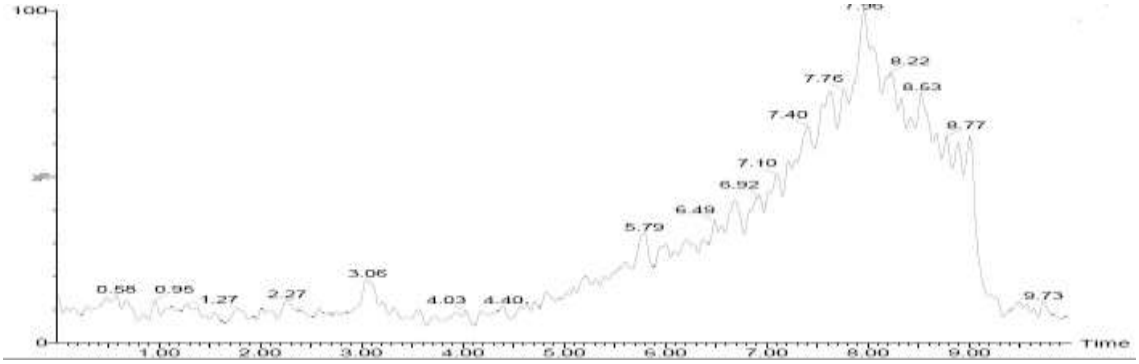

Figure 9: MRM ion-chromatograms of Double blank plasma (without IS) negative ion mode

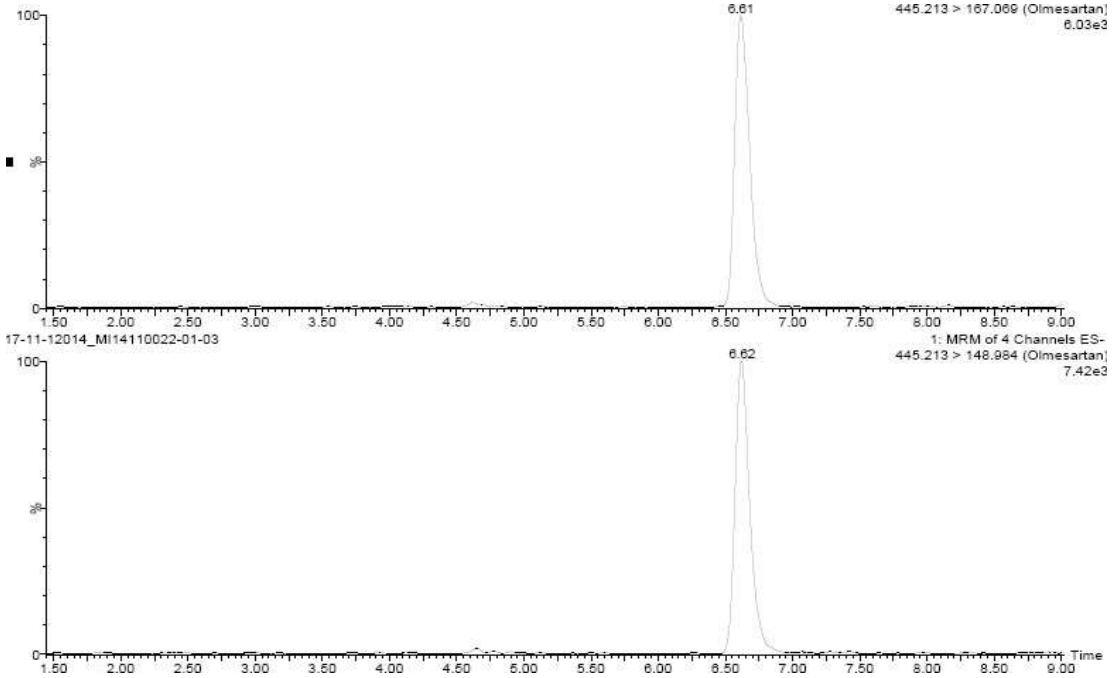

Figure 10: MRM ion-chromatograms of Olmesartan

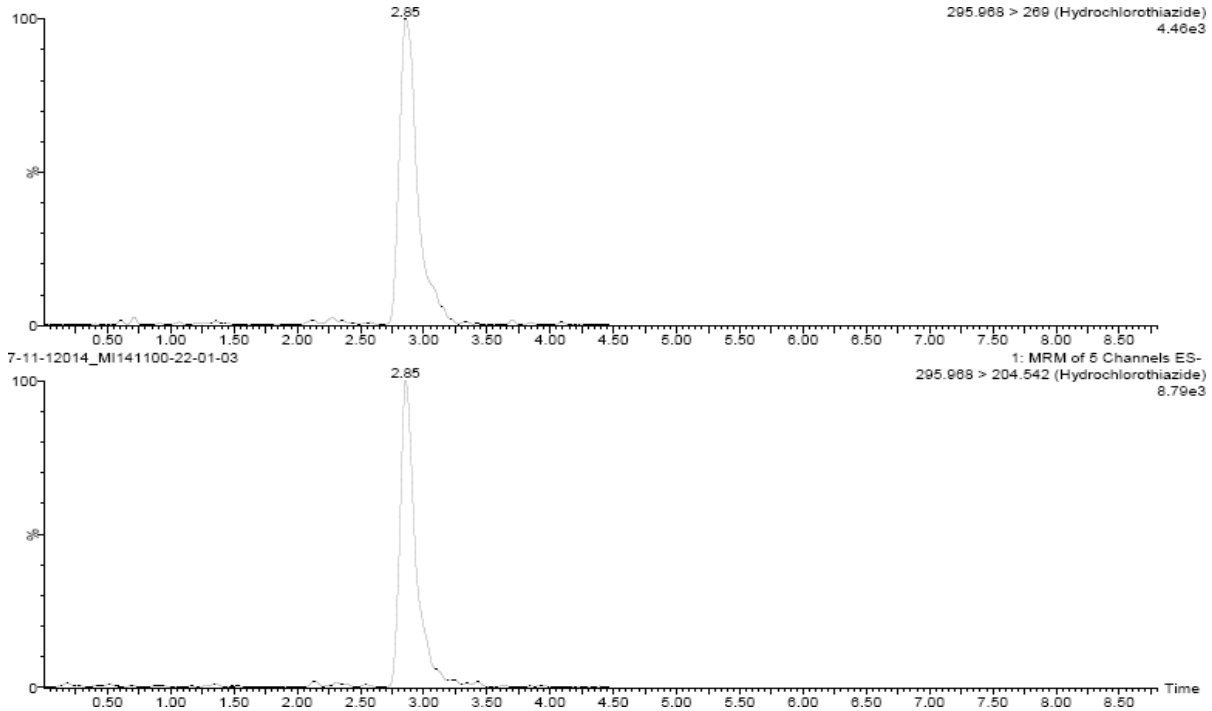

Figure 11: MRM ion-chromatograms of Hydrochlorothiazide

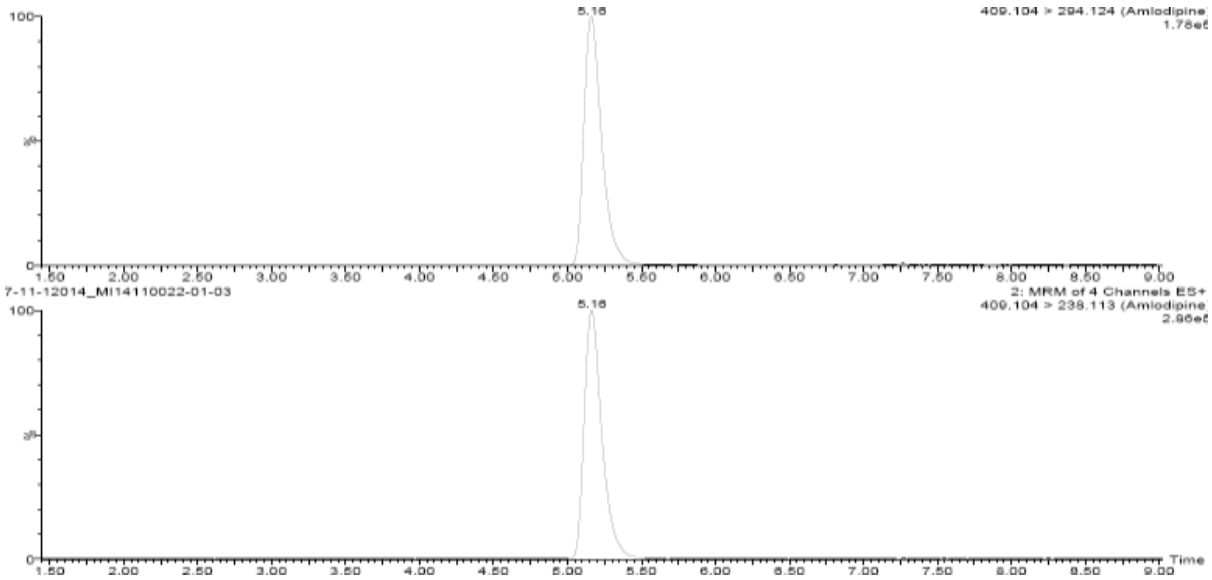

Figure 12: MRM ion-chromatograms Amlodipine

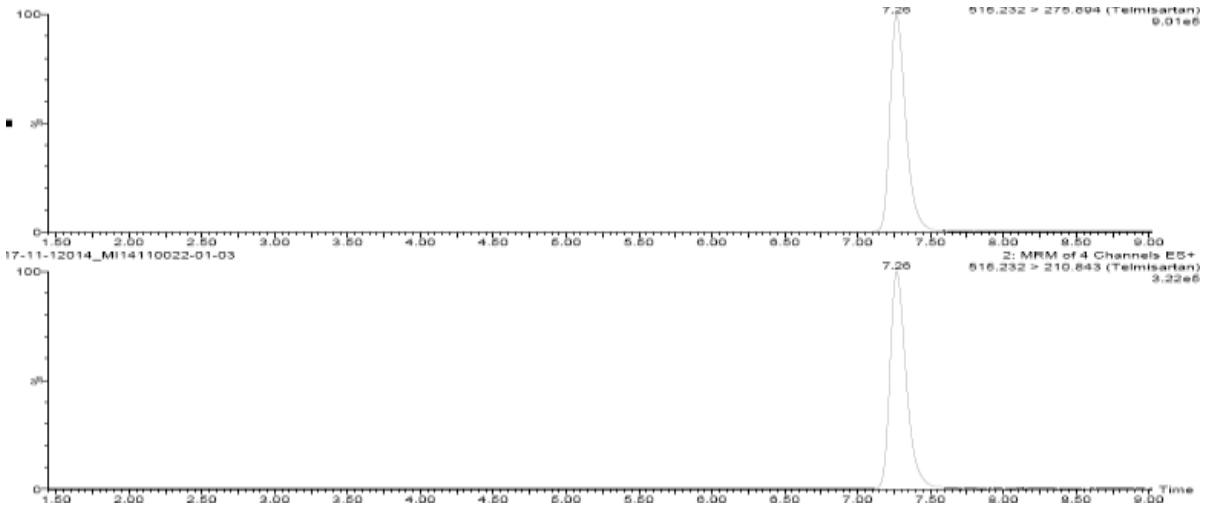

Figure 13: MRM ion-chromatograms of Telmisartan

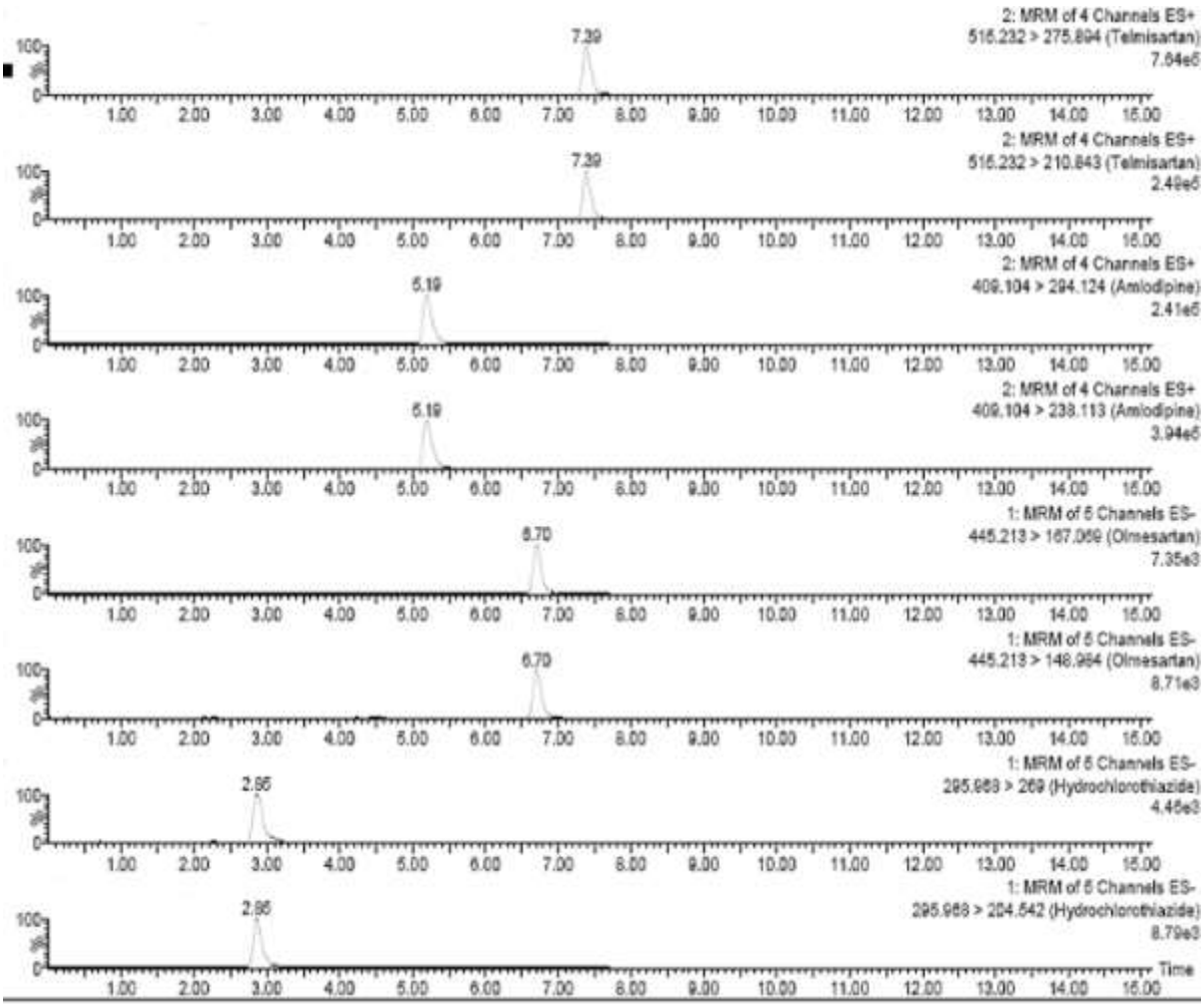

Figure 14: MRM ion-chromatograms of Olmesartan Medoxomil, Hydrochlorothiazide, Amlodipine Besylate and Telmisartan

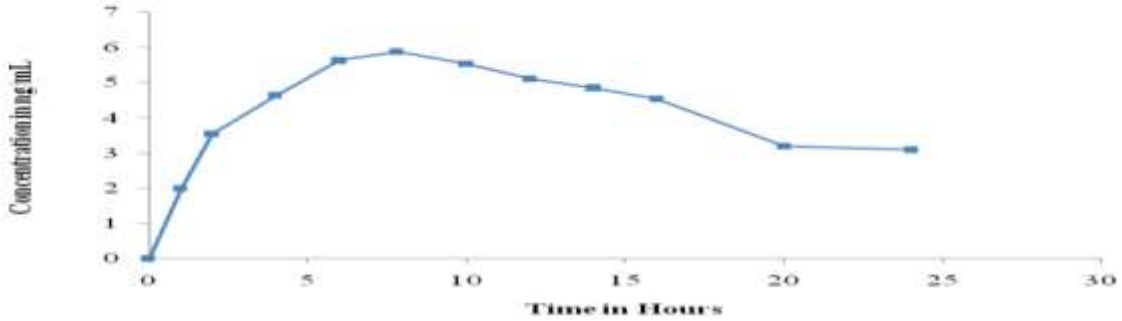

Figure 15: Mean plasma concentration time profile of Amlodipine in Human plasma

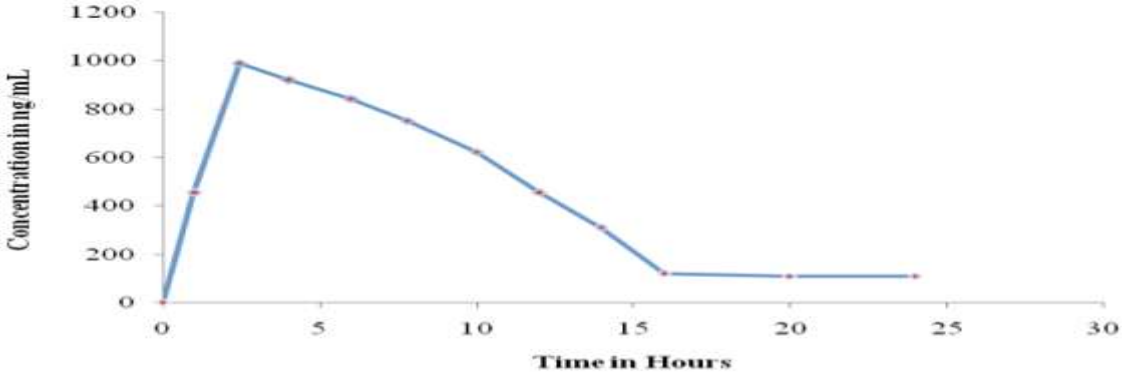

Figure 16: Mean plasma concentration time profile of Olmesartan Medoxomil in Human plasma

Mass spectrometry

Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate were tuned in positive ion mode electrospray on this adduct with two transitions for each analyte and one for the IS. Mass parameters were tuned in both positive and negative ionization modes for all three analytes. Good response was achieved in both mode for all the analytes and internal standard. Data from the MRM mode were considered to obtain better selectivity. Protonated form of each analyte and IS $[M+H]^+$ ion was the parent ion in the Q1 spectrum and was used as the precursor ion to obtain Q3 product ion spectra. The most sensitive mass transition was monitored from m/z 445.2 to 167.07 for Olmesartan Medoxomil, m/z 295.9 to 268.9 for Hydrochlorothiazide, m/z 409.1 to 294.12 for Amlodipine besylate and m/z 515.2 to 275.8 for Telmisartan respectively.

Optimization of extraction technique

Reported procedures for the estimation of Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate in human plasma have used either liquid-liquid extraction or solid phase extraction for sample preparation with little or no information on ion suppression or matrix interference. Considering the steroidal moiety in chemical structures of all the analytes by liquid-liquid extraction was tried by using the various combinations of organic solvents like diethyl ether, ethyl acetate, methyl tert-butyl ether, n-hexane and n-heptane. The samples were extracted in methyl tert-butyl ether gave good response and desired recovery through the extraction. After selective extraction of all three analytes, the organic supernatant layer was separated and evaporated to dryness. To reconstitute the final product, various combinations of Trifluro acetic acid, ammonium acetate, formic acid and ammonium formate solutions with acetonitrile were tried. The samples were reconstituted with mobile phase composition as ammonium acetate and acetonitrile 40:60% (v/v), which provided help to improve the sensitivity, compatibility and reproducible response.

Optimization of chromatographic conditions

To have a rugged and efficient chromatography, efforts were made to minimize matrix interference, achieve adequate run time in order to ensure high throughput and attain high sensitivity with good peak shapes. The analytical potential of four different reversed-phase columns was evaluated, namely, RP C18, (50 mm× 2.1 mm, 1.7 µm), Kinetex C18, (50 mm×4.6 mm, 2.6 µm), Thermohypersil BDS, (50 mm×4.6 mm, 2.6 µm) and HiQsil BDS 18, (50 mm×2.1 mm, 2.6 µm) analytical columns. Separation was tried using various combinations of methanol/acetonitrile in acidic buffer (2–20 mM ammonium formate, ammonium acetate) and additives like formic acid (0.01–0.1%) on these columns.

In the present work, the best chromatographic conditions as a function of analyte peak intensity, peak shape, adequate retention and analysis run time were achieved with RP C_{18}, (50 mm×2.6 mm, 1.7µm) using 5mM ammonium acetate and acetonitrile (up to 0.8 minutes/42% A, 5/10%, 6/10%, 6.1/42% and 10/42% (v/v) gradient programming) as the mobile phase. The total chromatographic run time was 10 min with a retention time of 2.85, 5.19, 6.70, and 7.39 minutes for Hydrochlorothiazide, Amlodipine besylate, Olmesartan medoxomil and Telmisartan respectively. The sensitivity achieved for Olmesartan medoxomil, Hydrochlorothiazide and Amlodipine besylate in the present work was 6.28, 4.21 and 3.86 ng/mL respectively. Based on the selectivity (unperturbed and stable base line) and signal-to-noise ratio (S/N≥22, 19 and 40 for all three analyte), it was possible to further lower the LLOQ by about two folds; however, it was not required based on the results of subject samples. Representative MRM ion chromatograms of extracted blank human plasma (double blank) and standard for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine besylate was shown in Fig. 8. to Fig. 14. demonstrate the selectivity of the method.

Telmisartan selected as internal standards in the present work. They had similar chromatographic behavior and were easily separated and eluted along with the analytes. There was no effect of IS on analyte recovery, sensitivity or ion suppression. The method was found successfully separating the interferences causing any ionization impact.

Assay performance and validation

Throughout the method validation, the precision (%CV) of the system suitability test was observed≤4.5 to 7.2% for all three analyte RT, IS RT and area ratio of analytes and respective IS, while the signal-to-noise ratio for system performance was≥ 22, 19 and 40 for Olmesartan medoxomil, Hydrochlorothiazide and Amlodipine besylate, respectively. Carryover evaluation was performed in each analytical run so as to ensure that it did not affect the accuracy and the precision of the proposed method. No enhancement in the response was observed in the double blank (without analyte and IS) after subsequent injection of the highest calibration standard (aqueous and extracted) at the retention time of the analyte and respective IS.

All three calibration curves were linear over the concentration ranges from 40.34 to 8092.75

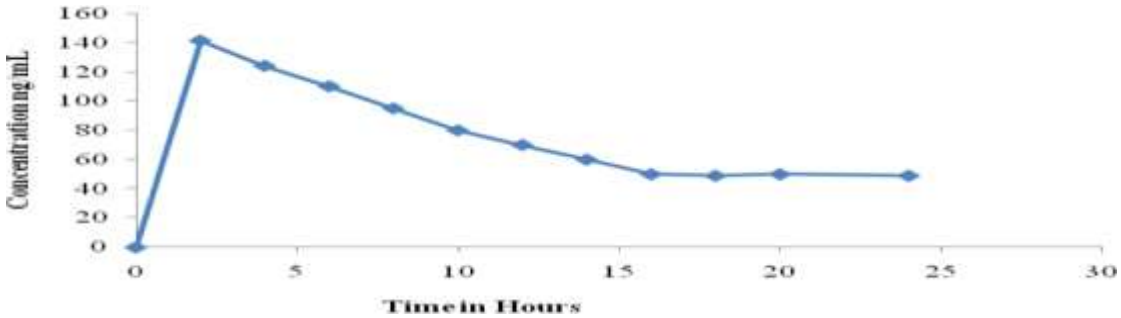

Figure 17: Mean plasma concentration time profile of Hydrochlorothiazide in Human plasma

Table 2: Accuracy and Precision for Olmesartan medoxomil

QC ID	LOQQC	LQC	MQC - 1	MQC - 2	HQC
Actual Concentration (ng/mL)	40.486	100.71	745.998	2869.22	5738.438
Calculated Concentration	42.3789	102.4149	810.5242	2900.94	6166.2686
(ng/mL)	45.7088	97.6026	805.4124	2852.068	5991.7606
	52.6088	112.7944	801.1589	2761.27	6033.1928
PA-01	49.6689	109.7168	812.4094	2776.305	6073.4568
	33.5102	104.7018	807.3623	2673.203	5861.9502
	43.1452	102.372	833.2822	2752.386	5819.9502
Mean	44.50347	104.9338	811.6916	2786.028	5819.8818
SD	6.648933	5.502256	11.28841	80.16829	130.563596
% CV	14.94	5.24	1.39	2.88	2.18
% Nominal	109.92	104.19	108.81	97.1	104.4
Calculated Concentration	26.191	88.6113	793.0336	2748.222	6139.8117

Table 3: Accuracy and Precision for Hydrochlorothiazide

QC ID	LOQQC	LQC	MQC - 1	MQC - 2	HQC
Actual Concentration (ng/mL)	40.486	100.71	745.998	2869.22	5738.438
Calculated Concentration	39.654	95.367	721.458	2654.124	5421.357
(ng/mL)	38.214	96.214	745.369	2665.514	5536.159
	39.321	99.687	768.369	2754.258	5741.258
PA-01	46.251	102.654	774.589	2965.258	5621.357
	45.213	103.587	745.369	2931.489	5782.369
	46.358	95.321	751.247	2865.147	5897.214
Mean	42.5018	98.805	751.0668	2805.965	5666.619
SD	3.81812	3.71838	18.94362	134.2303	173.99138
% CV	8.98	3.76	2.52	4.78	3.07
% Nominal	93.3	96.5	99.3	94.6	95.7

ng/mL for Olmesartan medoxomil, Hydrochlorothiazide and Amlodipine besylate respectively. A straight-line fit was made through the data points by the least square regression analysis and a constant proportionality was observed. The calibration curve (fitted by first order y=mx+b, where m is the slope, b is the intercept, x is the concentration and y is the peak area ratio of drug to IS) was plotted as the peak area ratio (drug to IS) on Y-axis vs. the nominal concentration of drug on X-axis.

The accuracy and precision (%CV) for the calibration curve standards were found within ±15.0% for all the drugs. The lowest concentration (LLOQ) in the standard curve that could be measured with acceptable accuracy and precision was found to be 6.28 ng/mL, 4.21 ng/mL and 3.86 ng/mL for Olmesartan medoxomil, Hydrochlorothiazide and Amlodipine besylate in plasma at a signal-to-noise ratio (S/N) of≥22, 19 and 40 respectively.

The intra-batch and inter-batch precision and accuracy were established from validation runs performed at HQC, MQC, LQC and LLOQ QC levels for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine was shown in Table 2 to Table 4.

The relative recovery and matrix factor data for all three analytes and IS are presented in Table 5 to Table 7. The relative recovery of the analyte was the 'true recovery', which was unaffected by the matrix as it was calculated by comparing the peak area ratio response (analyte/IS) of extracted (spiked before extraction) and unextracted (spiked after extraction) samples. The relative recovery was≥101.09% for Olmesartan Medoxomil and IS, ≥101.93% for Hydrochlorothiazide and IS,

Table 4: Accuracy and Precision for Hydrochlorothiazide

QC ID	LOQQC	LQC	MQC - 1	MQC - 2	HQC
Actual Concentration (ng/mL)	40.486	100.71	745.998	2869.22	5738.438
Calculated Concentration	35.264	92.357	698.325	2654.123	5321.459
(ng/mL)	38.214	102.388	741.258	2541.369	6214.325
	41.258	100.28	725.369	3121.247	5869.321
PA-01	40.123	102.358	732.258	2987.321	6001.258
	45.369	107.258	789.321	2654.213	5478.214
	47.258	95.236	741.256	2542.369	5832.265
Mean	41.2477	99.9795	737.9645	2750.1070	5786.1403
SD	4.45829	5.38755	29.73776	244.60196	331.48502
% CV	10.81	5.39	4.03	8.89	5.73
% Nominal	85.5	92.4	101.1	96.5	92.0

Table 5: Absolute matrix effect for Olmesartan Medoxomil

Lot No	Aqueous sample		Spiked sample		Area Ratio		Matrix factor
	Analyte area	IS Area	Analyte area	IS Area	Aqueous Sample	Spiked Sample	
1	18783	315412	19640	320752	0.0596	0.0612	1.03
2	19087	313876	19268	321175	0.0608	0.06	1.01
3	19458	318406	19558	317424	0.0611	0.0616	1.03
4	18744	322033	19040	317211	0.0582	0.06	1.01
5	18377	314887	19132	317001	0.0584	0.0604	1.01
6	18920	315559	18466	315517	0.06	0.0585	0.98
H			18908	312160		0.0606	1.02
				Mean	0.05968	0.0603	101.09
				SD			0.016776
				% CV			1.66

Matrix effect 101.09%

Table 6: Absolute matrix effect for Hydrochlorothiazide

Lot No	Aqueous sample		Spiked sample		Area Ratio		Matrix factor
	Analyte area	IS Area	Analyte area	IS Area	Aqueous Sample	Spiked Sample	
1	25510	315412	25417	320752	0.0809	0.0792	1.02
2	26514	313876	26651	321175	0.0845	0.083	1.02
3	26147	318406	25587	317424	0.0821	0.0806	1.02
4	26354	322033	25641	317211	0.0818	0.0808	1.01
5	25987	314887	25658	317001	0.0825	0.0809	1.02
6	26147	315559	25471	315517	0.0829	0.0807	1.03
H			24518	312160		0.0785	0
				Mean	0.0824	0.0806	101.93
				SD	0.0012	0.0014	0.0045
				% CV	1.46	1.75	0.44

Matrix effect 101.09%

≥103.27% for Amlodipine Besylate and its IS. Recovery was consistent across all QC levels. The matrix factor was given as the ratio of analysis of the analytical response obtained from analysis of six extracted blank matrix samples spiked after extraction with the analyte at four concentrations (LQC, MQC-2, MQC-1 and HQC) and IS (at the working concentrations) relative to the analytical response obtained from reference solutions (neat solution). CV (%) values for the samples were evaluated and matrix factor was calculated as the mean peak response in the presence of matrix ions divided by mean peak response in the absence of matrix ions.

Overall mean IS normalized matrix factor was observed 0.98 to 1.03, 1.01 to 1.03 and 0.99 to 1.05 for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate, respectively. % CV of matrix factor was observed 1.66, 0.44 and 2.53 for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate, respectively.

Table 7: Absolute matrix effect for Amlodipine Besylate

Lot No	Aqueous sample		Spiked sample		Area Ratio		Matrix factor
	Analyte area	IS Area	Analyte area	IS Area	Aqueous Sample	Spiked Sample	
1	41254	315412	41597	320752	0.1308	0.1297	1.01
2	41587	313876	40265	321175	0.1325	0.1254	1.06
3	42517	318406	40654	317424	0.1335	0.1281	1.04
4	40874	322033	40598	317211	0.1269	0.128	0.99
5	42598	314887	40874	317001	0.1353	0.1289	1.05
6	42157	315559	40258	315517	0.1336	0.1276	1.05
H			40258	312160		0.129	0
				Mean	0.1321	0.1281	103.27
				SD	0.0029	0.0014	0.0262
				% CV	2.22	1.09	2.53

Matrix effect 103.27%

Table 8: FT4 stability for Olmesartan medoxomil (at below -50°C and at below -15°C)

	LQC CS	HQC CS	At below -50°C		At below -50°C	
			LQC FT 4	HQC FT 4	LQC FT 4	HQC FT 4
Actual	100.71	5738.438	100.71	5738.438	100.71	5738.438
Concentration	106.1776	5900.922	98.6122	6217.067	104.2132	5898.211
	120.955	5905.805	103.4018	6357.143	102.0346	5679.941
	107.9245	5880.834	100.6013	6127.706	100.4351	5898.235
	109.1476	5695.979	113.7906	5855.83	104.3611	5877.605
	112.8357	5720.338	106.1627	5906.236	103.1181	5964.309
	121.3421	5814.001	103.5183	6137.871	95.4948	6043.5
Mean	113.0636	5819.647	104.3478	6100.309	101.6095	5893.634
SD	6.633358	92.69363	5.309928	189.3102	3.333944	121.1765
% CV	5.87	1.59	5.09	3.1	3.28	2.06
% Nominal	112.27	101.42	103.61	106.31	100.89	102.7
% Nominal			92.29	104.82	89.87	101.27

Table 9: FT4 stability for Hydrochlorothiazide (at below -50°C and at below -15°C)

	LQC CS	HQC CS	At below -50°C		At below -50°C	
			LQC FT 4	HQC FT 4	LQC FT 4	HQC FT 4
Actual concentration	100.71	5738.438	100.71	5738.438	100.71	5738.438
	93.251	5536.369	98.231	5321.258	95.369	5521.147
	101.258	5987.258	101.254	5641.236	99.258	5962.314
	106.321	5768.321	108.325	5897.325	106.354	5214.236
	105.369	5698.321	109.365	5789.369	92.147	5321.476
	106.547	5874.369	95.321	5532.365	90.231	5021.364
	99.325	5641.258	96.325	5987.251	99.367	5147.258
Mean	102.012	5750.9827	101.47017	5694.8007	97.121	5364.6325
SD	5.19513	162.61772	6.0686251	246.68811	5.83338416	338.019671
% CV	5.09267	2.8276511	5.9806989	4.3318129	6.0063057	6.30089147
% Nominal	98.7238	99.781869	99.250847	100.76627	103.69539	106.967961
% Nominal			99.47	99.02	95.21	93.28

The stability of Olmesartan Medoxomil, Hydrochlorothiazide, Amlodipine Besylate and respective IS in human plasma and stock solutions was examined under different storage conditions. Different stability experiments in plasma at two QC levels with the values for percent changes are shown in Table 8 to Table 10.

Method ruggedness was evaluated using reinjection of analyzed samples on different columns and mass spectrometer of the same make and with a different analyst. The precision (%CV) and accuracy values for different columns were found≤10.23% and 95.3–108.2% respectively, at all four QC levels for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate was found≤1.5% and 99.8–108.0% for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate respectively. The dilution integrity experi-

Table 10: FT4 stability for Amlodipine Besylate (at below -50°C and at below -15°C)

	LQC CS	HQC CS	At below -50°C		At below -50°C	
			LQC FT 4	HQC FT 4	LQC FT 4	HQC FT 4
Actual concentration	100.71	5738.438	100.71	5738.438	100.71	5738.438
	93.214	5896.324	95.214	5412.369	99.325	6210.211
	98.367	5987.258	97.258	5321.478	100.58	5987.325
	106.325	5641.258	91.587	5632.578	104.568	5214.265
	105.369	5536.258	96.357	5987.365	106.358	5532.149
	101.258	5983.214	94.578	5874.125	95.324	5821.369
	104.258	5641.236	95.147	5369.214	97.258	5932.147
Mean	101.4652	5780.925	95.0235	5599.5215	100.568833	5782.911
SD	4.987607	197.8347	1.9406575	280.10647	4.23142824	356.191137
% CV	4.915585	3.422199	2.0422922	5.0023288	4.20749461	6.15937435
% Nominal	99.25574	99.26505	105.98431	102.48086	100.140368	99.2309582
% Nominal			93.65	96.86	99.12	100.03

Table 11: Pharmacokinetic parameters of Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine (n=6, Mean ± SD)

Parameter	Olmesartan	Hydrochlorothiazide	Amlodipine
t m_{ax} (h)	2.5±0.8	2.00±0.34	7.8±0.23
C $_{max}$ (ng/mL)	980±230	141.23±28.21	5.89±0.86
AUC_{0-t} (ng h/mL)	7420±1830	768.25±421.31	268.23±83.02
AUC_{0-inf} (ng h/mL)	8514±830	821.54±78.35	341.21±111.30
$t_{1/2}$ (h)	10.4±1.2	5.87±0.35	30.31±16.17

ment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the ULOQ, which may be encountered during real subject sample analysis. The precision for dilution integrity of 1.7 times dilution was 3.24% and 2.72%, while the accuracy results were 106.0% , 107.2 and 111.2% for Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate respectively, which were well within the acceptance limit of 15% for precision (%CV) and 85–115% for accuracy.

Pharmacokinetic Study Result

In order to verify the sensitivity and selectivity of this method in real time situation, the present method was used to test the Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine in human plasma samples collected from healthy volunteers (n=6). The mean plasma concentration against time profile of Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine and pharmacokinetic data was shown in Table 11 and graphical illustration was shown in Fig. 15. to Fig. 17.

CONCLUSION

The LC–MS/MS assay reported in this paper is rapid, simple, specific and sensitive for simultaneous quantification of Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate in human plasma and is fully validated according to commonly acceptable FDA guidelines. The method showed suitability for pharmacokinetic studies in humans. The cost-effectiveness, simplicity of the assay and usage of liquid-liquid extraction, and sample turnover rate of less than 10.5 minutes per sample, make it an attractive procedure in high-throughput bioanalysis Olmesartan Medoxomil, Hydrochlorothiazide and Amlodipine Besylate. From the results of all the validation parameters, we can conclude that the developed method can be useful for BA/BE studies and routine therapeutic drug monitoring with the desired precision and accuracy.

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