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Simultaneous determination of hydrochlorothiazide, quinapril and quinaprilat in human plasma by liquid chromatography-tandem mass spectrometry

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ABSTRACT

A rapid and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the simultaneous estimation of hydrochlorothiazide, quinapril and its metabolite quinaprilat in human plasma. After solid phase extraction (SPE), the analytes and IS were chromatographed on a hypurity C8 (100 mm × 2.1 mm i.d., 5 μm particle size) column using 2 μL injection volume with a run time of 2.8 min. An isocratic mobile phase consisting of 0.5% (v/v) formic acid:acetonitrile (25:75, v/v) was used to separate all these drugs. The precursor and product ions of these drugs were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring mode (MRM) without polarity switch. The proposed method was validated over the range of 5–500 ng/mL for hydrochlorothiazide method and 5–1500 ng/mL for quinapril and quinaprilat. Inter-batch and intra-batch precision (coefficient of variation – % CV) across five validation runs lower limit of quantitation (LLOQ), lower quality control (LQC), middle quality control (MQC), higher quality control (HQC) and upper limit of quantitation (ULOQ) was less than 15. The accuracy determined at these levels was within ±13% in terms of relative percentage error.

1. Introduction

Hydrochlorothiazide (6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-doxide) is a diuretic, representative of the benzothiadiazine class sulfonamide derivatives, commonly known as thiazide [1]. Its action is to deplete the body's sodium stores. It also reduces the levels of chloride, bicarbonate, phosphate and magnesium. The drug also reduces the loss of calcium. The drug inhibits cell's reabsorption of NaCl. Thiazides are organic anions and bind to the site of the Cl^- on the sodium chloride transporter molecule preventing it from picking up NaCl. The depletion of NaCl stores in the body reduces blood pressure and cardiac output. After a few weeks, cardiac output returns to a normal level. Distal calcium reabsorption is also increased. Quinapril inhibits angiotensin converting enzyme, which catalyses formation of angiotensin II from its precursor, angiotensin I. Angiotensin II is a powerful vasoconstrictor and increases blood pressure through a variety of mechanisms. Due to reduced angiotensin production, plasma concentrations of aldosterone are also reduced, resulting in increased excretion of sodium in the urine and increased concentrations of potassium in the blood. Quinapril (3*S*)-2-[(2*S*)-2-[[(1*S*)-1-ethoxycarbonyl-3-phenyl-propyl] amino] propanoyl]-3,4-dihydro-1H-isoquinoline-3-carboxylic acid is a prodrug, which is hydrolyzed after absorption to the active diacid quinaprilat. Both quinapril and quinaprilat inhibit ACE activity, although the potency of quinapril is much lower than that of quinaprilat [1]. A simultaneous densitometric determination of hydrochlorothiazide and quinapril by TLC has been reported [2]. Several bioanalytical methods are reported to determine quinapril, quinaprilat and hydrochlorothiazide in different biological matrices like plasma [4,7–9,12–14,17,18], serum [10,11,16] and urine [4,5,8,13–15,19]. Sensitive and selective methods based on LC-MS/MS [12], HPLC [5–11,14–19], and GC [4,13] methodologies are reported. Most of the reported methods for the determination of hydrochlorothiazide, quinapril and quinaprilat are based on liquid chromatography. The proposed LC-MS/MS method utilizes a simple and less time consuming SPE procedure for sample

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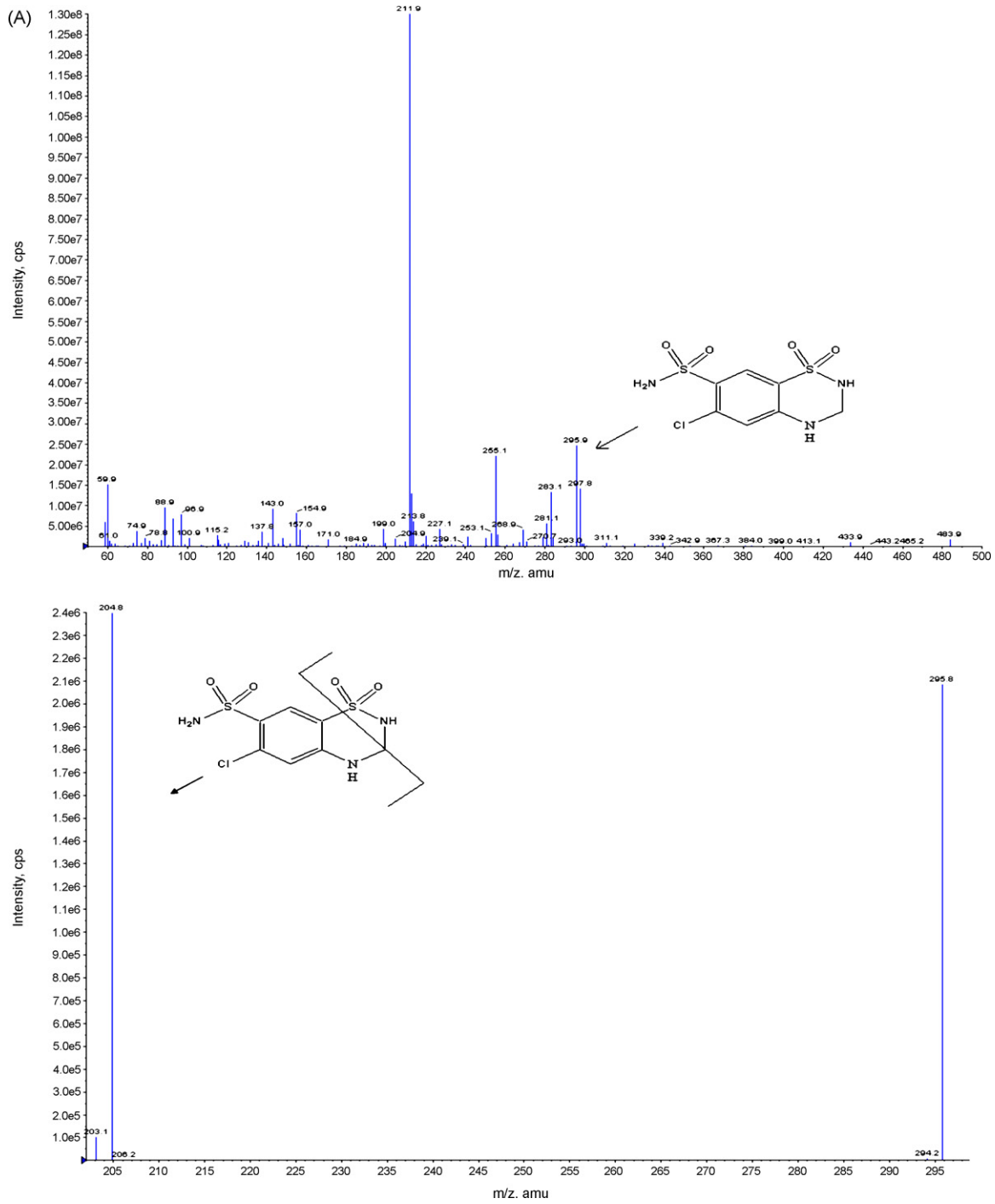

Fig. 1. Representative spectra of parent ion (A) and product ion (B) of hydrochlorothiazide.

extraction and allows the simultaneous determination of these drugs at low concentration levels.

The aim of this study was to develop a sensitive, selective and high throughput method for simultaneous estimation of hydrochlorothiazide, quinapril and quinaprilat in human plasma for therapeutic drug monitoring and pharmacokinetic studies. As a part of our ongoing research in this area, we have developed and validated a LC-MS/MS assay for this drug in human plasma. Special emphasis was given to optimize the extraction step in order to get quantitative and reproducible recovery for the analyte. The method presents a simple and clean SPE procedure without drying and reconstitution steps. The negative ion ESI mode selected for this study, gave high response for the analytes with mobile phase consisting of 0.5% (v/v) formic acid:acetonitrile (25:75, v/v). The analyte and IS were well separated with minimum matrix interference in a run time of 2.8 min under isocratic conditions. The lower limit of quantitation (LLOQ) of 5 ng/mL for all analytes was achieved.

2. Experimental

2.1. Chemicals and materials

Working standards of hydrochlorothiazide and quinapril hydrochloride were provided by Cadila healthcare Ltd. (Ahmed-

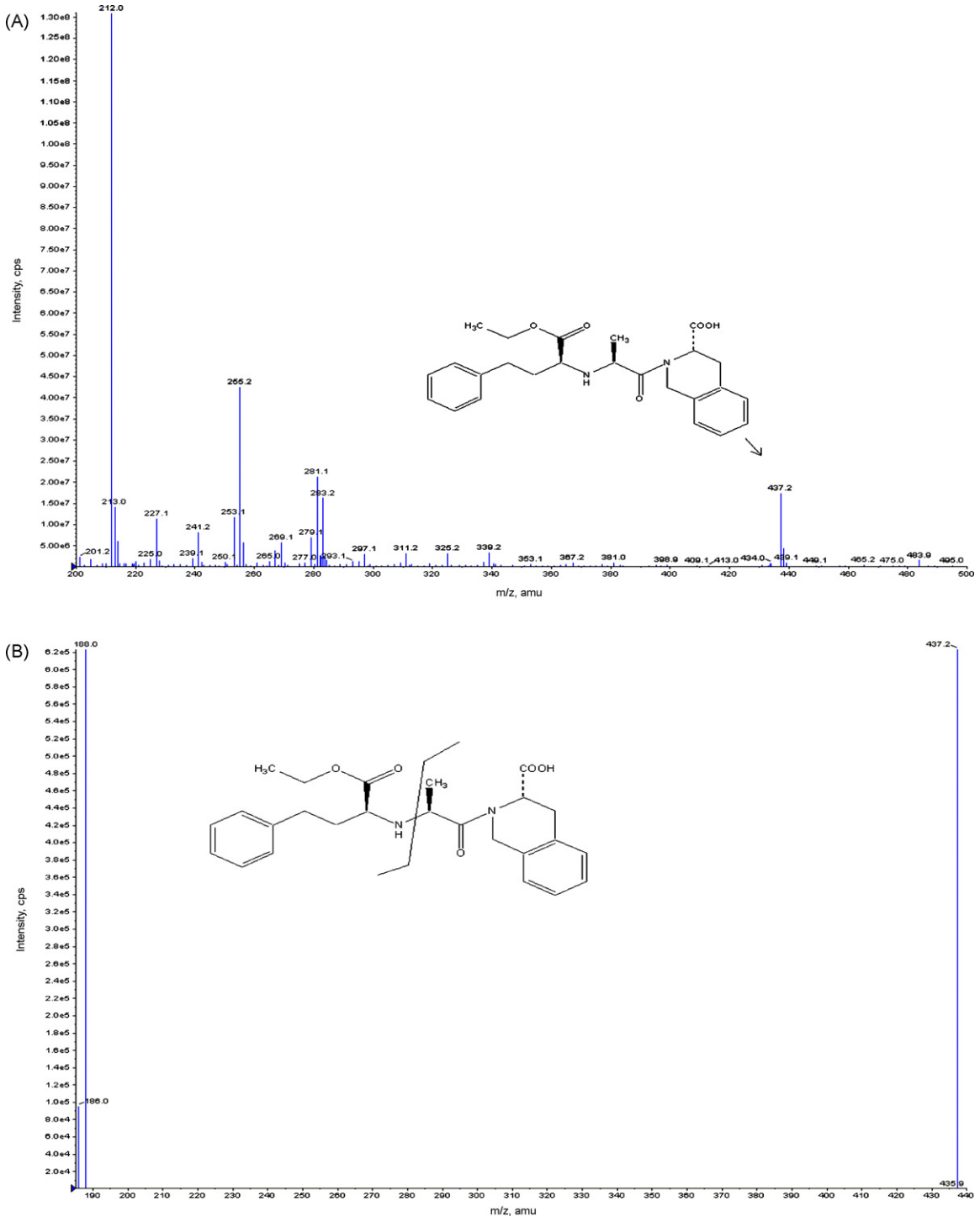

Fig. 2. Representative spectra of parent ion (A) and product ion (B) of quinapril.

abad, India) having purity greater than 99%. Quinaprilat ammonium salt was supplied by synfine research (Canada). Ramiprilat (IS) was provided by Cipla Ltd. (Mumbai, India). HPLC grade methanol and acetonitrile were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). AR grade ammonium acetate, glacial acetic acid and formic acid were procured from Merck (Mumbai, India). Purified water was obtained from Milli Q A10 gradient water purification system (Millipore, Banglore, India). Blank human plasma was collected in house with heparin as a coagulant and stored at −70 °C. Strata X, 30 mg; 1 mL solid phase extraction (SPE) cartridges were procured from Phenomenex (Torrance, CA, USA).

2.2. Liquid chromatography and mass spectrometric condition

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a binary LC-10AD prominence pump, autosampler (SIL-HTc) and solvent degasser (DGU-14) were used for all the analysis. The chromatographic system consisted of Hypurity C8 (100 mm × 2.1 mm i.d., 5 μm particle size) analytical column obtained from Thermo (India)

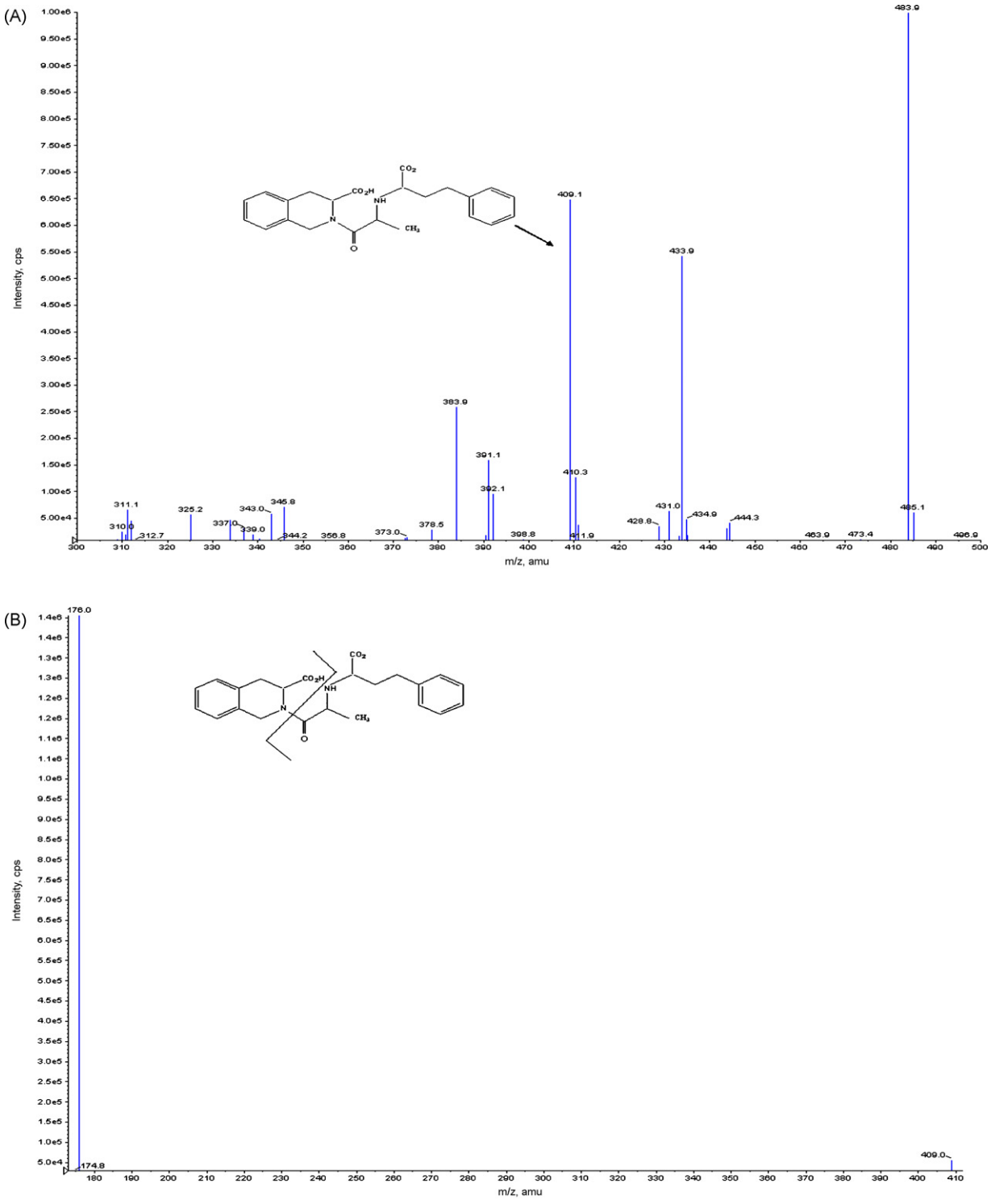

Fig. 3. Representative spectra of parent ion (A) and product ion (B) of quinaprilat.

Pvt. Ltd. The flow rate of the mobile phase under isocratic condition was kept at 0.20 mL/min. The auto sampler temperature was set at 10 °C and the injection volume was 2 μL. The mobile phase consisted of 0.5% (v/v) formic acid:acetonitrile (25:75, v/v). The total LC run time was 2.8 min. Detection of analyte and IS was performed on a triple quadrupole mass spectrometer, API-3000 (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray ionization source and operating in the negative ion mode. Analyst software version 1.4 was used to control all parameters of LC and MS. Quantitation was performed using multiple reaction monitoring (MRM) mode to study parent → product ion (m/z) transitions for hydrochlorothiazide (296.0 → 205.0), quinapril (437.2 → 187.8), quinaprilat (409.1 → 176.0) and IS (387.1 → 154.0), respectively. Figs. 1–4 show the mass spectra of parent and product ions for analyte and IS, respectively. Source dependent parameters optimized were gas 1 (Nebuliser gas): 12 psi; gas 2 (heater gas flow):

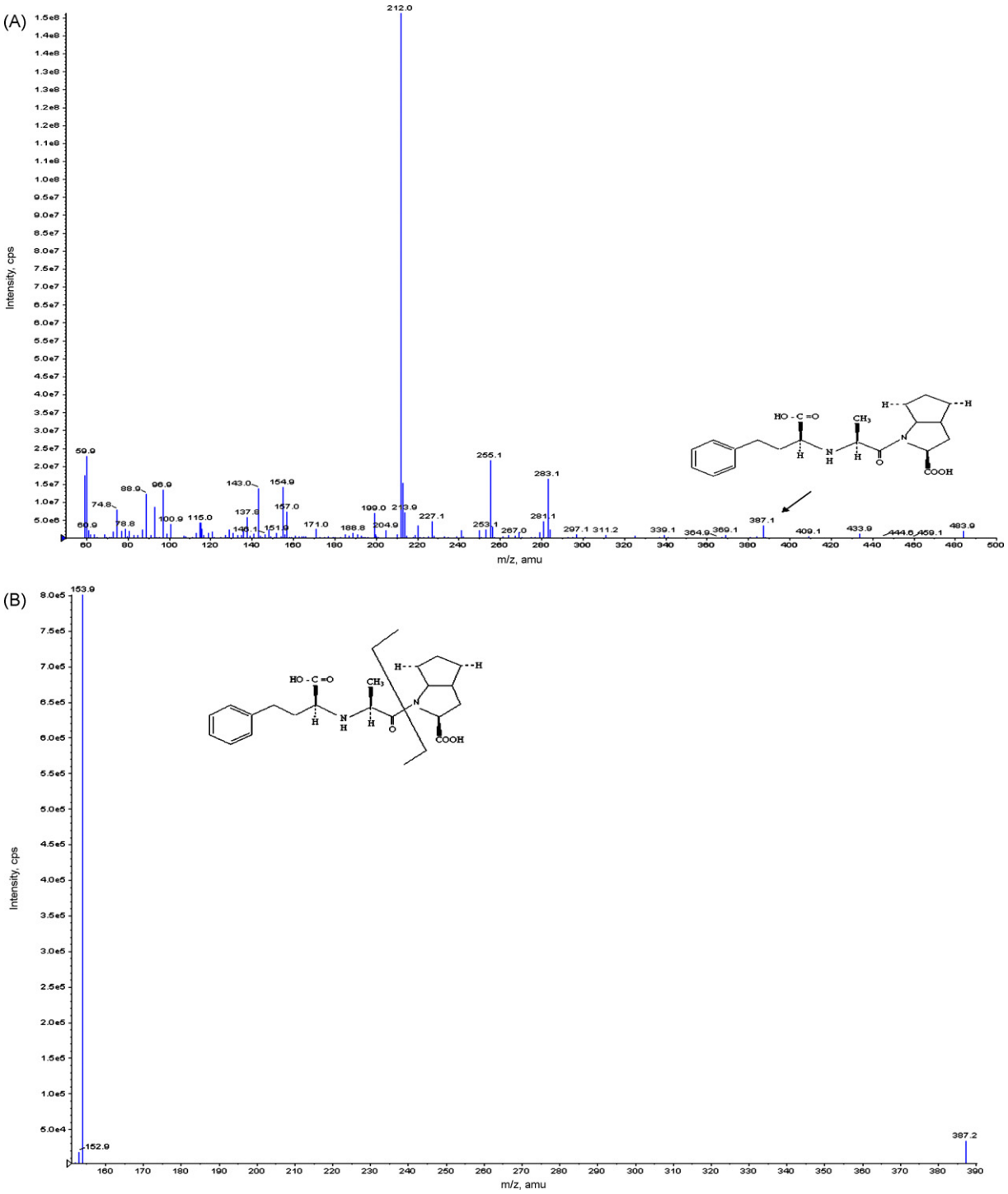

Fig. 4. Representative spectra of parent ion (A) and product ion (B) of ramiprilate (IS).

8000 cm^3/min; ion spray voltage (ISV): −4000 V; temperature (TEM): 450 °C. Compound dependent parameters like declustering potential (DP), entrance potential (EP), focusing potential (FP), collision energy (CE) and cell exit potential (CXP) were −40, −46, −26 V; −10, −10, −10 V; −90, −220, −120 V; −32, −28, −28 eV; −15, −13, −13 V; for hydrochlorothiazide, quinapril and quinaprilat, respectively. Nitrogen was used as collision activated dissociation (CAD) gas and was set at 12 psi. Both quadrupoles 1 and 3 were maintained at unit resolution and dwell time was set at 150, 150 and 250 ms for hydrochlorothiazide, quinapril and quinaprilat, respectively.

2.3. Analytical data processing

Chromatographic data were collected and integrated using Analyst software version 1.4. Peak area ratio of the analyte to IS was utilized for the construction of calibration curve. A weighing of $1/x^2$ (least-squares linear regression analysis, where x is the analyte concentration) was used for curve fitting. Concentration in unknown samples was calculated from the best-fit equation ($y = mx + c$), where y is the peak area ratio. The regression equation for the calibration curve was also

used to back-calculate the measured concentration at each QC level.

2.4. Standard and quality control preparation

Standard stock solution of hydrochlorothiazide (1 mg/mL), quinapril (1 mg/mL), quinaprilat (1 mg/mL) and the IS (1 mg/mL) were separately prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water/methanol (50:50, v/v; diluent). The IS working solution (1 μg/mL) was prepared by diluting its stock solution with diluent. Working solutions (0.5 mL) were added to drug-free human plasma (9.5 mL) as a bulk, to obtain concentration levels of 5, 10, 20, 40, 80, 160, 300, 400 and 500 ng/mL for hydrochlorothiazide; 5, 10, 30, 60, 200, 600, 900, 1200, and 1500 ng/mL for quinapril and quinaprilat as a single batch at each concentration. Quality control (QC) samples were prepared in human plasma at four concentration levels (5, 15, 150, 350 and 500 ng/mL^{-1}) for hydrochlorothiazide and (5, 15, 500, 1100 ng/mL^{-1}) for quinapril and quinaprilat. Separate analyte stock solutions were used for preparation of calibration standard solution and quality control samples. The calibration and control bulk samples were divided into aliquots and stored in the deep freezer at −70 °C until analysis.

2.5. Sample preparation

All frozen calibration standards and quality control samples were thawed at room temperature. The samples were adequately vortex mixed. 0.5 mL of plasma sample was dispensed into the eppendroff tubes and 50 μL of 1 μg/mL of internal standard and 500 μL of 50 mM ammonium acetate pH 4.3 ± 0.05 was added, followed by vortexing for 10 s. The samples were centrifuged at 15000 rpm at 10 °C for 5 min. The samples were then loaded on Strata X, 30 mg; 1mL cartridges preconditioned with 1 mL of methanol followed by 1 mL of 50 mM ammonium acetate pH 4.3 ± 0.05. Further, plasma was drained out under nitrogen pressure and cartridges were washed with 1 mL of water followed by 1 mL of 2% acetic acid in 10% (v/v) methanol in water to clean up the samples. After proper drying of the cartridge, elution was carried out using 0.5 mL acetonitrile: 4 mM ammonium acetate pH 3.0 ± 0.05. The eluate was transferred into vials, capped and placed in an autosampler rack for injection.

2.6. Method validation

A thorough and complete method validation of hydrochlorothiazide, quinapril and quinaprilat in human plasma was done following the USFDA guidelines [3]. The method was validated for selectivity, sensitivity, linearity, accuracy and precision, recovery, stability and matrix effect.

The selectivity towards endogenous and exogenous plasma matrix components was assessed in twelve different batches (six normal, three haemolysed and three lipemic) of human plasma samples by analyzing blank and spiked samples at LLOQ level. It was performed in two sets, in the first set, plasma samples were extracted and directly injected for LC-MS/MS detection and in the second set, blank plasma samples with least interference spiked with LLOQ working solution of hydrochlorothiazide, quinapril and quinaprilat were extracted and analyzed. The second set was used for sensitivity determination.

The linearity of the method was determined by the analysis of standard plots associated with a nine-point standard calibration curve. Five linearity curves containing nine non-zero concentrations were analyzed. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of the analytes was calculated from the simple linear equation using regression analysis of spiked plasma calibration standard with reciprocate of the drug concentration as a weighting factor (1/concentration × concentration, i.e. $1/x \times x$). The peak area ratio values of calibration standards were proportional to the concentration of the drug in plasma over the range tested.

Inter-batch and intra-batch accuracy and precision was evaluated at five different concentrations (LLOQ, LQC, MQC, HQC and ULOQ). Mean and standard deviation (S.D.) were obtained for calculated drug concentration at each level. Accuracy and precision were evaluated in terms of relative percentage error (%Er) and % CV, respectively.

Recovery presents the extraction efficiency of a method. It was performed at LQC, MQC and HQC levels. The relative recoveries were evaluated by comparing peak area of extracted samples (spiked before extraction) to that of unextracted samples (quality control working solutions spiked in extracted plasma).

Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions. Stock solution stability was performed by comparing area response of stability sample of analyte and internal standard with the area response of sample prepared from fresh stock solutions. Bench top stability, extracted sample stability (process stability), freeze thaw stability were performed at LQC and HQC level using six replicates at each level.

To study the effect of matrix on analyte quantification with respect to consistency in signal suppression, matrix effect was checked with six different lots of heparinised plasma. Two replicates each of LQC and HQC were prepared from different lots of plasma (total 24 QC samples).

3. Result and discussion

3.1. Method development

For optimum detection and simultaneous quantification of hydrochlorothiazide, quinapril and quinaprilat with IS in human plasma, it was necessary to adjust not only the chromatographic conditions and mass parameters but also to develop an efficient extraction method that gives consistent and reproducible recovery of analytes from plasma. Parent ions and product ions were optimized by infusing 500 ng/mL solutions into mass spectrometer in m/z 5–500 range, in positive and negative ion mode using electro spray ionization technique. Best intensity for $[M-H]^-$ ions was found in negative mode for all the three analytes including IS as they have an ability to donate protons. Most abundant product ions were obtained at m/z 205, 187.8 and 176.0 for hydrochlorothiazide, quinapril and quinaprilat, respectively, by applying sufficient CAD (collision activated dissociation) gas and CE (collision energy). Optimization of source temperature and heater gas flow is important as they play an important role in minimizing ion suppression and altering the sensitivity. Increase in source temperature and heater gas above 450 °C and 8000 cm^3/mL, respectively, augmented the intensity for the analyte. Minor changes in ion spray voltage and nebuliser gas did not have a marked effect on the signal intensity and were maintained at −4000 V and 12 psi, respectively. A dwell time of 0.15, 0.15 and 0.25 s for hydrochlorothiazide, quinapril and quinaprilat, respectively, was sufficient and no cross talk was found between all MRMs.

Chromatographic analysis of the analytes and IS was initiated under isocratic conditions with an aim to develop a simple separation process with a short run time. Separation was tried using various combinations of acetonitrile and buffer with varying contents of each component on variety of columns like C8 and C18

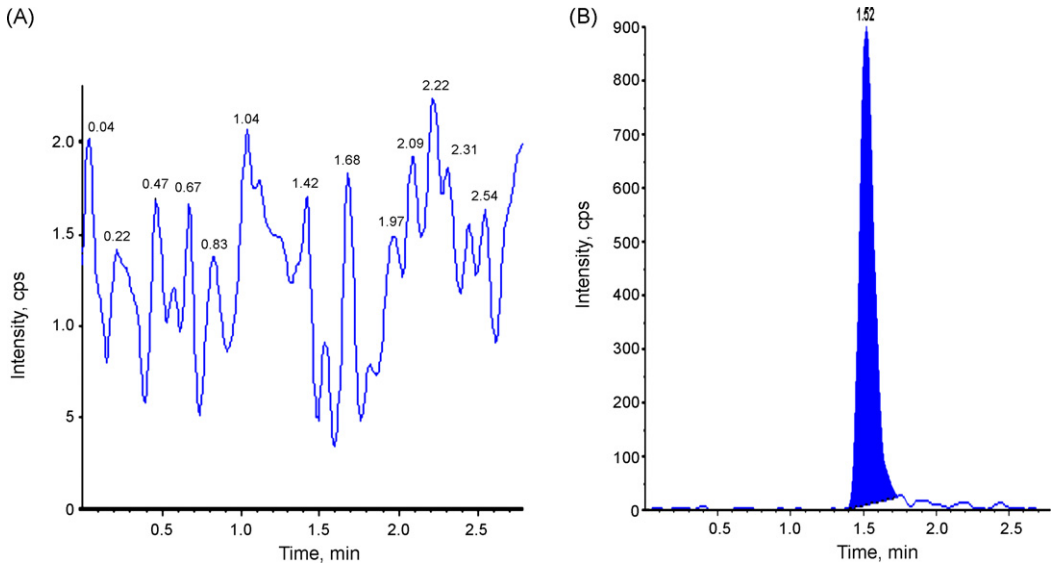

Fig. 5. Representative MS chromatogram of plasma blank (A) and LLOQ (B) for hydrochlorothiazide.

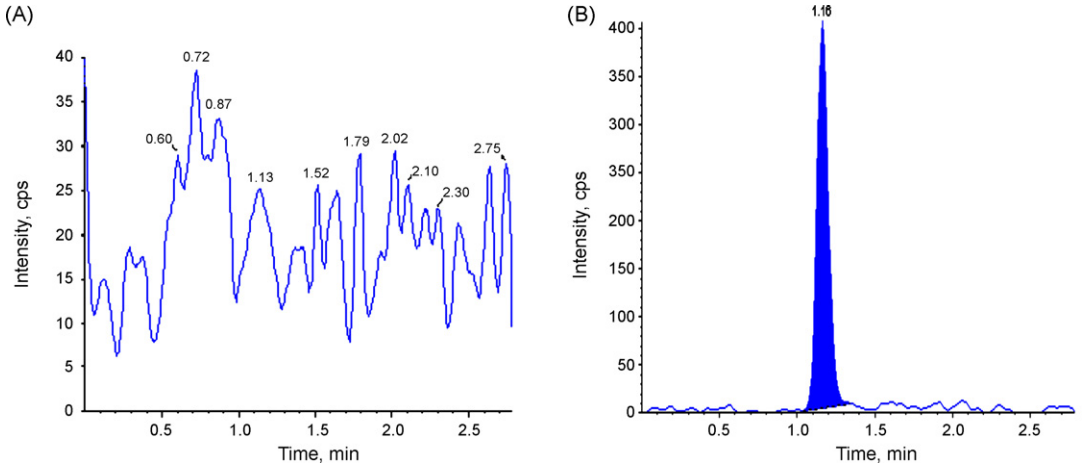

Fig. 6. Representative MS chromatogram of plasma blank (A) and LLOQ (B) for quinapril.

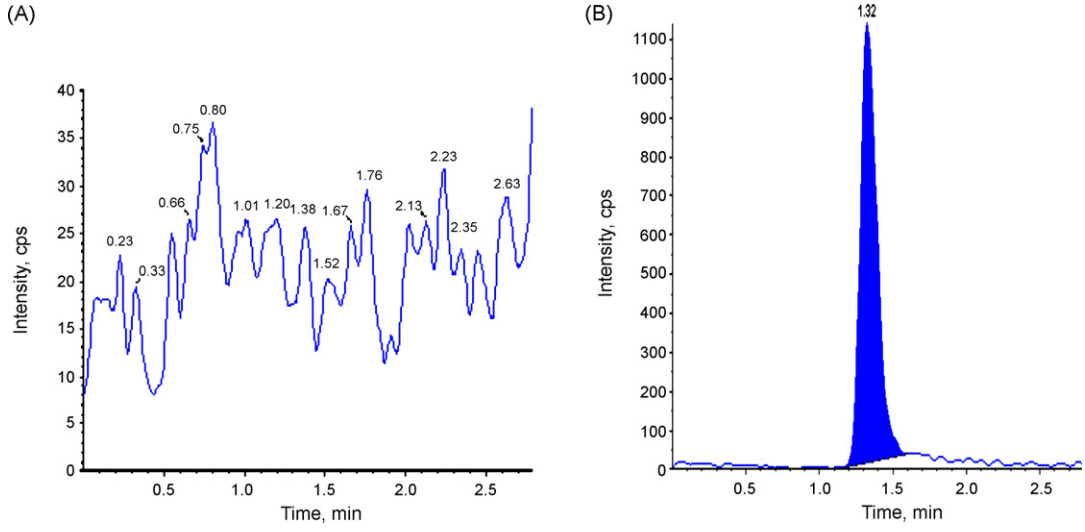

Fig. 7. Representative MS chromatogram of plasma blank (A) and LLOQ (B) for quinaprilat.

Table 1
Summary of calibration curve standards ($n = 5$)

Analyte	Nominal conc. (ng/mL)	Mean back calculated conc.(ng/mL)	% CV	% Er
	4.94	4.83	2.49	−2.29
	10.09	10.22	5.18	1.30
	20.18	21.07	6.44	4.43
	40.35	41.86	1.68	3.74
Hydrochlorothiazide	80.71	83.18	1.40	3.06
	161.41	165.63	2.71	2.61
	302.65	294.60	2.49	−2.66
	403.53	389.01	2.64	−3.60
	504.41	471.17	1.86	−6.59
	4.94	4.95	3.93	0.19
	10.09	9.14	16.23	−9.43
	30.26	32.55	14.77	7.56
	60.53	56.28	9.24	−7.03
Quinapril	201.76	203.85	6.34	1.04
	605.29	623.34	4.52	2.98
	907.93	950.85	13.03	4.73
	1210.57	1182.13	10.95	−2.35
	1513.22	1520.99	5.74	0.51
	4.93	4.86	3.52	−1.29
	10.06	10.20	7.01	1.45
	30.17	31.08	4.08	3.00
	60.34	61.16	1.24	1.37
Quinaprilat	201.13	201.09	1.61	−0.02
	603.38	601.53	1.01	−0.31
	905.08	892.91	1.94	−1.34
	1206.77	1185.13	2.03	−1.79
	1508.46	1492.36	1.08	−1.07

CV, coefficient of variation. %Er, relative percentage error.

Hypersil, hypurity; C18 advance high purity and Symmetry shield RP 18 to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape.

Use of buffer helped in achieving good response for MS detection operating in the negative mode. To get a good chromatographic separation with desired response it was observed that mobile phase as well as selection of column is an important criterion. Thus, a mobile phase consisting of 0.5% (v/v) formic acid:acetonitrile (25:75, v/v) was found suitable as the analyte were well separated by this phase. High content of acetonitrile (75%) in the mobile phase helped in eluting the analyte and IS within 2.8 min at a flow rate of 0.2 mL/min. Hypersil Hypurity C8 (100 mm × 2.1 mm, 5 μm particle size) column gave good peak shape and response even at LLOQ level for all the analyte including IS. Low injection volume of 2 μL reduced overloading of column with analytes, thereby ensuring more number of analyses on the same column.

For extraction of hydrochlorothiazide, quinapril, quinaprilat and IS with quantitative recovery and negligible matrix effect from plasma samples, an efficient extraction method was mandatory. A simple and rapid solid phase extraction method was developed using Strata-X33um Polymeric Reversed Phase (30 mg/1 mL) solid phase extraction (SPE) cartridges. Extraction of analytes was carried out with 0.5 mL of acetonitrile:4 mM ammonium acetate pH 3.0 ± 0.05 (80:20, v/v). The eluate was

Table 2
Intra-batch and inter-batch precision and accuracy

Analyte	Level	Conc. added (ng/mL)	Intra-batch			Inter-batch		
			Mean conc. found (ng/mL)[a]	%Er	% CV	Mean conc. found (ng/mL)[b]	%Er	% CV
	LLOQ	5.06	5.35	5.78	5.27	5.42	7.09	10.45
	LQC	15.18	14.83	−2.33	4.48	15.49	1.99	4.26
Hydrochlorothiazide	MQC	151.83	150.04	−1.18	1.35	152.61	0.51	5.15
	HQC	354.28	328.99	−7.14	1.72	341.78	−3.53	3.42
	ULOQ	503.58	475.71	−5.53	3.01	471.77	−6.32	2.44
	LLOQ	5.01	5.17	3.13	11.29	5.60	11.86	8.97
	LQC	15.03	13.14	−12.54	9.74	14.82	−1.34	8.78
Quinapril	MQC	500.86	450.85	−9.98	8.62	490.13	−2.14	13.19
	HQC	1101.88	969.19	−12.04	6.52	1043.74	−5.28	9.87
	ULOQ	1502.57	1412.94	−5.96	2.79	1474.55	−1.86	6.67
	LLOQ	5.00	4.82	−3.54	4.33	5.10	1.92	14.74
	LQC	15.00	14.53	−3.08	5.64	14.85	−0.98	5.88
Quinaprilat	MQC	499.88	492.65	−1.45	1.12	475.70	−4.84	3.58
	HQC	1099.74	1058.87	−3.72	1.22	1050.46	−4.48	2.91
	ULOQ	1499.64	1480.49	−1.28	2.99	1420.08	−5.31	1.29

%Er = relative percentage error, CV = coefficient of variance.
[a] Mean of six replicates observations at each concentration.
[b] Mean of eighteen replicates observations over three different analytical runs.

Table 3
Relative recovery of hydrochlorothiazide, quinapril, quinaprilat and IS

Analyte	Level	Relative recovery (mean ± S.D.%, $n = 6$)	% CV ($n = 6$)
Hydrochlorothiazide	LQC	91.0 ± 3.3	3.7
	MQC	91.2 ± 8.4	9.2
	HQC	102.8 ± 3.1	3.0
Quinapril	LQC	77.8 ± 6.9	8.9
	MQC	78.1 ± 6.3	8.0
	HQC	81.3 ± 4.7	5.7
Quinaprilat	LQC	86.2 ± 1.4	1.7
	MQC	88.7 ± 7.5	8.5
	HQC	95.5 ± 3.1	3.2
IS	LQC	105.7 ± 2.5	2.3
	MQC	105.4 ± 6.4	6.1
	HQC	108.0 ± 3.7	3.4

directly subjected into HPLC vials for injection. No interference was observed from any endogenous or exogenous plasma matrix.

It was difficult to find a compound, which could ideally mirror the analyte to serve as a good IS. Several compounds were investigated to find a suitable IS, and finally ramiprilate belonging to a same class of compounds was found most appropriate for the present purpose. There was no significant effect of IS on analyte recovery, sensitivity or ion suppression. The results of method validation using ramiprilate as the IS were acceptable in this study based on FDA guidelines.

3.2. Selectivity and sensitivity (LLOQ)

The selectivity of the method towards endogenous plasma matrix was evaluated in twelve different batches of human plasma by analyzing blanks and spiked samples at LLOQ levels. No significant direct interference in the blank plasma traces was observed from endogeneous substances in drug-free plasma at the retention time of the analytes. Figs. 5–7 demonstrates the selectivity results with the chromatograms of blank plasma and the peak response of analytes at LLOQ level. Excellent sensitivity was observed for a 2-μL injection volume.

3.3. Linearity, accuracy and precision, recovery

The nine-point calibration curve was linear over the concentration range 5–500 ng/mL for hydrochlorothiazide and 5–1500 ng/mL for quinapril and quinaprilat. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighing factors ($1/x$, $1/x^2$, none). The best linear fit and least-square residuals for the calibration curve were achieved with a $1/x \times x$ weighing factor giving a mean linear regression equation for the calibration curve of: $y = 0.0043\ (\pm 0.0006)x + 0.0024\ (\pm 0.0015)$; $y = 0.0011(\pm 0.001)x + 0.0002\ (\pm 0.0008)$ and $y = 0.0067\ (\pm 0.001)x + 0.0010\ (\pm 0.0032)$ for hydrochlorothiazide, quinapril and quinaprilat, respectively, where y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The observed mean back calculated concentration with accuracy (%) and precision (% CV) of five linearties are given in Table 1.

The precision and accuracy of the developed method was determined by analysis five quality control samples. Intra-day variation of the assay was assessed by injecting two batches containing these samples on the same day. Inter-day variation was assessed by injecting one batch on three different days. Each run consisted of six replicates at five concentration levels (LLOQ QC, LQC, MQC, HQC and ULOQ QC). Intra-batch precision was less than 12% and inter-batch precision was less than 15% for all three analytes. Accuracy was within 87–112% of their nominal concentration, respectively, as given in Table 2.

Six replicates at LQC, MQC and HQC level were prepared for recovery determination. Mean relative recovery found was 94.99%, 79.06% and 90.14% with a precision (% CV) of 7.12, 2.43 and 5.29 for hydrochlorothiazide, quinapril and quinaprilat, respectively. Recovery of IS was 106.38% with % CV of 1.35. The data of relative recovery are given in Table 3. This indicates that the extraction efficiency for the analyte as well as IS was consistent and reproducible.

3.4. Stability and matrix effect

For short-term stability determination, stored plasma were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples were extracted and analyzed as described above and the results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the autosampler

Table 4
Stability results

Stability		Level	Conc. added (ng/mL)	A ($n = 6$)	% CV	B ($n = 6$)	% CV	% Change
Bench top (24 h at room temp.)	Hydrochlororthiazide	LQC	15.18	15.431	2.17	15.445	4.02	0.09
		HQC	354.28	328.690	2.08	319.670	1.96	−2.74
	Quinapril	LQC	15.03	15.500	6.22	15.628	5.94	0.83
		HQC	1101.88	1069.096	10.75	1050.114	9.18	−1.78
	Quinaprilat	LQC	15.00	15.004	4.69	14.678	3.80	−2.18
		HQC	1099.74	1056.717	1.44	1057.943	0.96	0.12
Autosampler (24 h, 10 °C)	Hydrochlororthiazide	LQC	15.18	15.431	2.17	15.429	5.25	−0.02
		HQC	354.28	328.690	2.08	320.039	1.82	−2.63
	Quinapril	LQC	15.03	15.500	6.22	15.312	10.40	−1.21
		HQC	1101.88	1069.096	10.75	1059.721	8.65	−0.88
	Quinaprilat	LQC	15.00	15.004	4.69	14.612	2.02	−2.61
		HQC	1099.74	1056.717	1.44	1059.251	1.49	0.24
5th Freeze Thaw Cycle	Hydrochlororthiazide	LQC	15.18	15.431	2.17	15.270	1.64	−1.05
		HQC	354.28	328.690	2.08	328.129	1.75	−0.17
	Quinapril	LQC	15.03	15.500	6.22	15.053	3.56	−2.88
		HQC	1101.88	1069.096	10.75	1099.621	9.49	2.86
	Quinaprilat	LQC	15.00	14.931	3.09	14.805	2.17	−0.84
		HQC	1099.74	1044.281	4.24	1060.988	1.28	1.60

A, mean comparison sample conc. (ng/mL). *B*, mean stability sample conc. (ng/mL). % CV, coefficient of variation.

Table 5
Summary statistics for target parameters, test versus reference following a single dose of hydrochlorothiazide (25 mg) and quinapril (20 mg) tablet to twenty six volunteers

Analyte	Treat	T_{max}	C_{max} (ng/mL)	$T_{1/2}$ (h)	AUC0–∞ (ng/mL h)
Hydrochlorothiazide	Test	1.93	163.70	10.71	1305.32
	Reference	1.70	167.89	8.70	1113.52
Quinapril	Test	0.72	249.50	1.21	327.10
	Reference	0.58	290.30	1.04	332.90
Quinaprilat	Test	1.34	1014.16	3.29	3589.72
	Reference	1.17	1056.43	2.50	3321.18

for 24 h was also assessed. The result indicate that the solution of analytes and the IS can remain in the autosampler for at least 24 h without showing any significant loss in the quantified values, indicating that samples should be processed within this period of time.

Stock solution of analyte and IS were stable at room temperature for 18 h and at 2–8 °C for 9 days. Analytes in control human plasma was stable for minimum of five freeze and thaw cycles. Different stability experiments in plasma and the values for the precision and percent change are shown in Table 4. There was no significant degradation observed since the deviations in concentration was within 15% of their nominal values.

Matrix effect is due to co-elution of some components present in biological samples. These components may not give a signal in MRM of target analyte but can certainly decrease or increase the analyte response dramatically to affect the sensitivity, accuracy and precision of the method. Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC-MS/MS method for supporting pharmacokinetics studies. It was performed with the aim to see the matrix effect by processing six lots of different plasma samples in quadruplet ($n = 4$). LQC and HQC stock solutions were spiked post extraction in duplicate. Aqueous recovery solutions of LQC and HQC along with internal standard were also prepared. The results found were well within the acceptable limits as the % difference of post spiked samples and aqueous recovery samples at LQC and HQC levels were within 18% Moreover, the minor suppression of analyte signal due to endogenous matrix interferences does not affect the quantification of analytes and IS peak. Thus, the extraction method was rugged enough and gave accurate and consistent results when applied to subject sample analysis. % CV of peak area ratios of analyte to internal standard in post spiked 353 samples at Low, Medium and High quality control levels were within 15%.

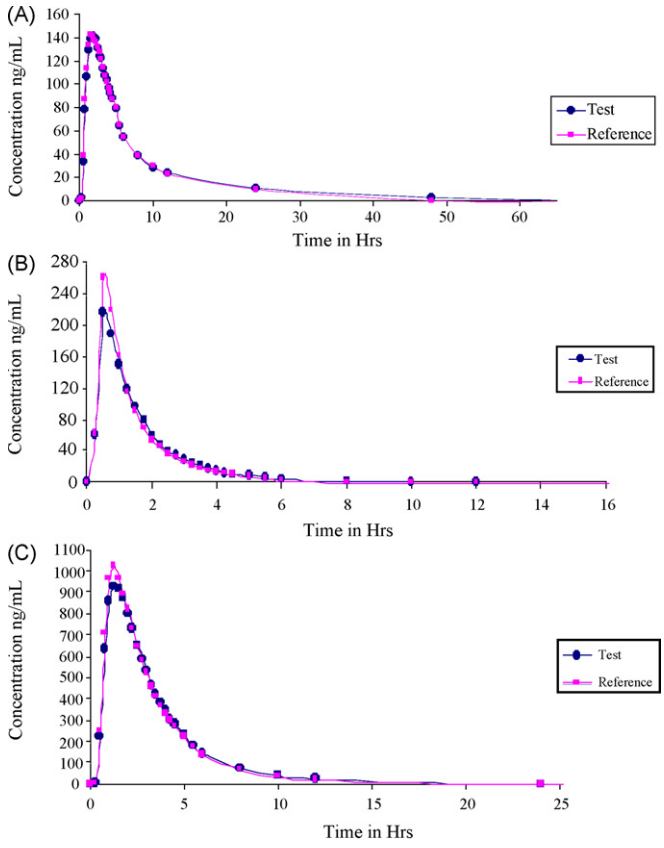

Fig. 8. Mean plasma concentration–time profile combined dosage of hydrochlorothiazide (25 mg) (A), quinapril (20 mg) (B) and quinaprilat (C).

3.5. Application of the method on healthy human volunteers

This analytical method was used in support of a single dose, open labelled, randomized, two-period, crossover bio-equivalence study to compare the rate and extent of absorption of test and reference formulations of hydrochlorothiazide (25 mg) and quinapril (20 mg) tablets in twenty-six healthy drug-free volunteers. From each of them 3 mL blood samples were withdrawn at pre-dose and at different time points post-dose. Following centrifugation at 4000 rpm for 10 min, the plasma samples were separated and stored at −70 °C, then were prepared and monitored according to the method we had built. The mean plasma concentration–time curves are shown in Fig. 8. Table 5 shows the t_{max} (time to peak plasma concentration), C_{max} (peak plasma concentration), $t_{1/2}$ (half-life) and $AUC_{0-\infty}$ (area under the plasma concentration time curve extrapolated to infinity).

4. Conclusion

The developed LC-MS/MS assay for hydrochlorothiazide, quinapril and quinaprilat is selective, rugged and suitable for routine measurement of subject samples. This method has significant advantages in terms of clean and reproducible SPE extraction procedure and a short chromatographic run time of 2.8 min. The extraction method gave consistent and reproducible recoveries for analytes and IS from plasma, with minimum matrix interference and ion suppression. The SPE eluate (2 μL) is directly submitted for LC-MS analysis without drying and reconstitution to give high throughput. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with test formulation of hydrochlorothiazide, quinapril and quinaprilat.

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