RESEARCH ARTICLE

SIMULTANEOUS ESTIMATION OF RAMIPRIL AND ITS ACTIVE METABOLITE RAMIPRILAT IN HUMAN PLASMA BY ESI-LC-MS/MS

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ABSTRACT

A rapid and sensitive liquid chromatography tandem mass spectrometry method has been developed and validated for the simultaneous determination of ramipril and ramiprilat in human plasma. The solid-phase extraction technique was used for the extraction of ramipril and ramiprilat from human plasma. Enalapril was used as the internal standard (IS).Chromatography was performed on a Aquasil C18, 100mm×2.1mm, 5µ column, with the mobile phase consisting of acetonitrile-deionsed water (in a 65:35 ratio) and 1.0mLL^{-1} ammonium trifluoroacetate solution (1.0M), followed by detection using mass spectrometry. The method involves a simple reversed isocratic chromatography condition and mass spectrometry detection, which enables detection at sub-nanogram levels. The method was validated and the lower limit of quantification for ramipril and ramiprilat was found to be 0.1 ngmL^{-1}and 0.1 ngmL^{-1}, respectively. The mean recovery for ramipril and ramiprilat ranged from 63.5 to 74.3%. This method increased the sensitivity and selectivity; resulting in high-throughput analysis of ramipril and ramiprilat using single IS in a single experiment for bioequivalence studies, with a chromatographic run time of 3.0 min only.

Key words: Ramipril, Human Plasma, Solid-phase extraction technique, ESI-LC-MS/MS

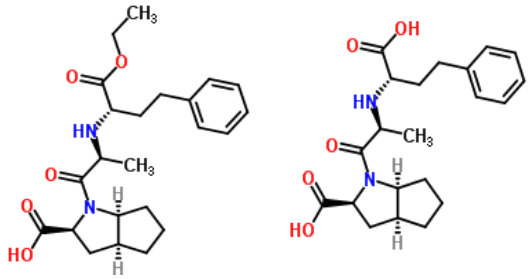
1. INTRODUCTION

The rennin–angiotensin system acts through two factors, i.e. angiotensin-converting enzyme, which converts angiotensin I to angiotensin II, and angiotensin receptors I and II to maintain volume homeostasis, control blood pressure and prevent ischemia.Therefore, controlling both the factors simultaneously provides effective blood pressure control and reduces the risk of cardiovascular events.

Ramipril and ramiprilat compete with angiotensin I and block the conversion of angiotensin I to angiotensin II. Angiotensin II contracts the muscles of most arteries in the body, including the heart, thereby narrowing the arteries and elevating the blood pressure. Ramipril is chemically designated as (2S,3aS,6aS)-1-[(2S)-2-[[(2S)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]-3,3a,4,5,6,6a-hexahydro-2H-cyclopenta[d]pyrrole-2-carboxylic acid. Ramipril and ramiprilat structure showed in Fig.1.

A number of methods have been reported for the simultaneous determination of ramipril and ramiprilat, including liquid chromatography with tandem mass spectrometric detection (LC–MS/MS) using liquid–liquid extraction, GC–MS using derivatisation technique and high-performance liquid chromatography (HPLC). Although the above methods are fast and robust, they require a large number of complicated steps for sample pretreatment.

LC–MS/MS was demonstrated to be superior to all the above mentioned techniques in terms of sensitivity, selectivity, simplicity and analysis throughput. This paper describes the LC technology coupled with triple quadruple tandem mass spectrometry that has been applied to the analysis for the simultaneous determination of ramipril and ramiprilat using enalapril as the internal standard (IS). The use of solid phase extraction technique (SPE) using plexus cartridges 30mg 1cc from Analchem Inc. (IL, USA) reduced the background noise produced by electrospray ionization (ESI), enabling us to develop a single and more sensitive method for ramipril and ramiprilat with a high sample throughput due to the short chromatographic condition and simple sample preparation.



Ramipril **Ramiprilat**

Figure 1: Chemical structure of Ramipril and Ramiprilat

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Ramipril, ramiprilat and enalapril standards (purity > 99.8%) were obtained from Varda Biotech (India). Tri-potassium salts of ethylenediaminetetraacetic acid (K3EDTA) plasma of healthy volunteers were obtained from In-house Clinic Cliantha Research, Ahmedabad (India). Acetonitrile (HPLC grade) and methanol were obtained from Qualigens, Germany. Milli-Q water (Millipore Co., MA, and USA) purification system was used to obtain purified water for the HPLC analysis.

2.2. Instrumentation

Chromatography was performed at ambient temperature, with the mobile phase consisting of acetonitrile and deionised water (65:35, V/V) plus 1.0mLL-1 ammonium

trifluaroacetate solution (1.0M). An Aquasil C18 (100mm×2.1mm, 5µ) column obtained from Thermo Hypersil, FL, USA, was used for the chromatographic separation at a flow rate of 0.3 mL/min. The mobile phase was delivered by a high performance liquid chromatography (HPLC) pump and the sample was injected by a HPLC autosampler (Schimadzu, JAPAN). Detection was performed by an API-4000 LC-MS/MS tandem quadrupole mass spectrometer (AB SCIEX, USA) fitted with an ESI source operating in a positive ion mode. A Plexus 30mg/1cc solid-phase extraction (SPE) cartridge for sample preparation was obtained from Analchem.

Mass spectra were obtained using a Sciex API 4000 mass spectrometer equipped with a turbo ion-spray source. The data acquisition was ascertained by Analyst 1.4.2 software. The mass spectra of ramipril, ramiprilat and its internal standard enalapril are presented in Fig. 2. The strongest fragment of each compound, as indicated in Fig. 2 was selected and used as Q3 ion to be monitored. The mass transition ion-pair was selected as follows: 417.2→234.1 for ramipril, 389.2→206.1 for ramiprilat and 377.2→234.2 for enalapril.

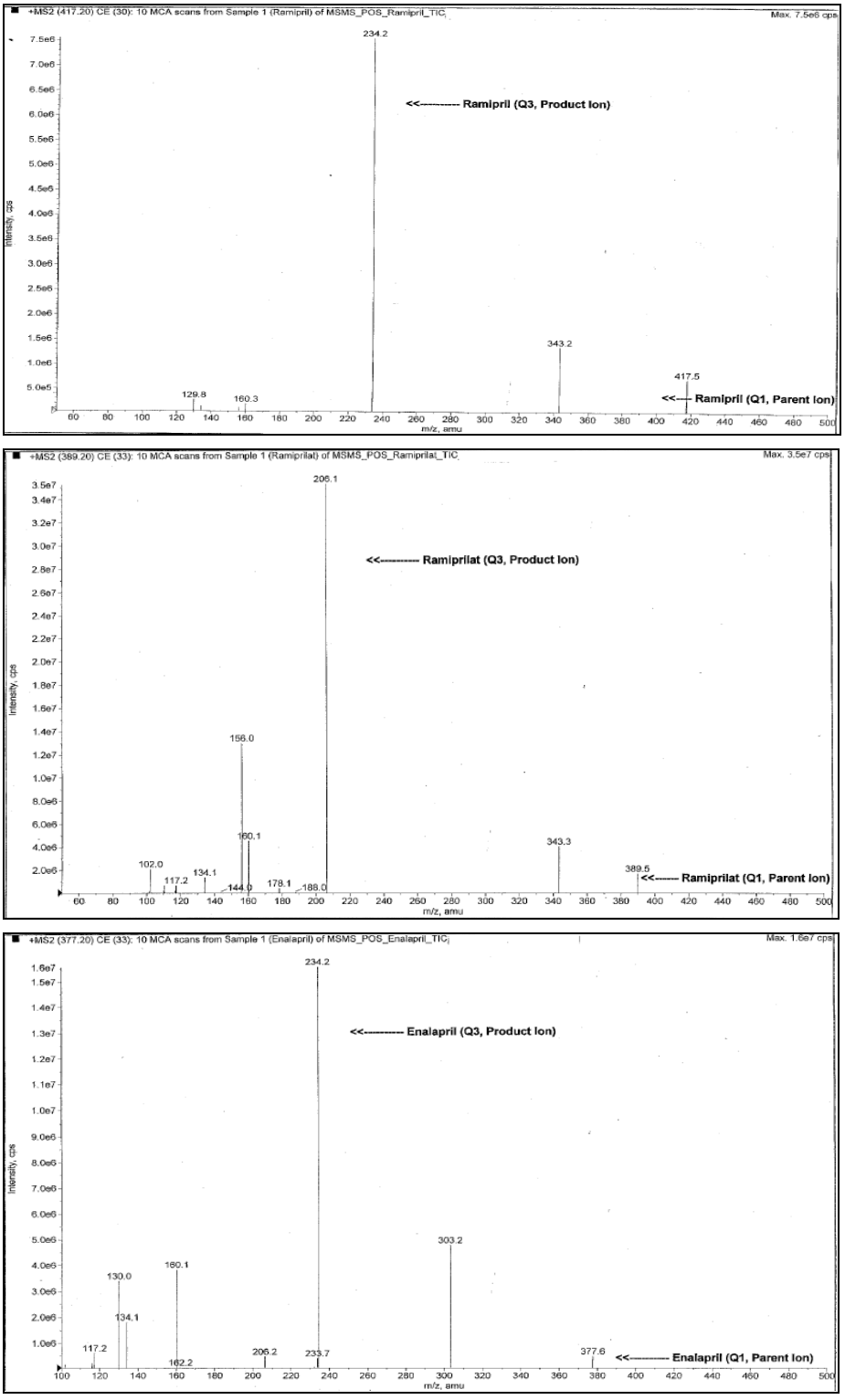


Figure 2: Representative mass spectra of ramipril, ramiprilat, enalapril and fragment ion

Mass detection was obtained at a unit-mass resolution for all channels Quantitation of ramipril and ramiprilat in human plasma was based on the peak area ratios of ramipril versus its internal standard enalapril and ramiprilat versus enalapril.

2.3. Preparation of standards and quality control samples

The stock solutions of ramipril, ramiprilat and internal standard enalapril were individually prepared in methanol at a concentration of 100µg/ml. Dilutions of 2.000/2.000 ng/mL to 500.0/500.0 ng /mL were made from the stock solutions of ramipril and ramiprilat. These diluted solutions were used to prepare the calibration curve and quality control samples.

Blank human plasma was screened prior to spiking to ensure it was free of endogenous interference at the retention times for ramipril, ramiprilat and internal standard. A ten-point standard curve of ramipril and ramiprilat was prepared by spiking the blank plasma with appropriate amounts of ramipril and ramiprilat. The calibration curve ranged from 0.1 to 25.00 ng / ml for both ramipril and ramiprilat. Quality control samples were prepared at four concentration levels of 0.3, 2.5, 10.0 and 18.75 ng / ml for both ramipril and ramiprilat and in a manner similar to the standard from the stock solution. A weighted least-squares linear regression was used for quantitation of ramipril and ramiprilat in this study and the weighting factor was $1/x^2$.

2.4. Extraction procedure

A 0.5mL aliquot of human plasma sample was mixed with 100 µL of internal standard working solution (50.00 ng/ml of enalapril in deionized water) and then added 25µL of 25% orthophosphoric acid solution vortex to mix. The sample mixture was loaded into a Plexus extraction cartridge that was pre-conditioned with 1 ml methanol followed by 1 ml deionized water. Centrifuge for 1.0 minute at 3000 rpm for each step. The extraction cartridge was washed with 1 mL of deionized water. Ramipril, ramiprilat and internal standard were eluted with 1 mL methanol by centrifugation for 1.0 minute at 3000 rpm and evaporated to dryness under a gentle stream of nitrogen (40ºC). The extracted residues were dissolved in 0.3 ml of reconstitution solution; 10 µL of reconstituted sample was injected into the LC/MS/MS system.

2.5. Validation

The method has been validated for selectivity, linearity, precision, accuracy, recovery and stability. The accuracy was determined by replicate analysis of samples containing known amounts of analytes. The intra-assay precision and accuracy was determined with six replicates of LLOQ, ULOQ and quality control samples (HQC, MQC-1, MQC-2 and LQC) at each level that were extracted from the sample batch. The inter-assay precision and accuracy was determined by analyzing the quality control samples that were tested on three different occasions. Inter-assay and intra-assay precision and accuracy evaluations were based on back-calculated concentrations.

The selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. This test was performed by analyzing the blank plasma samples from different sources (or donors) to test for interference at the retention time of ramipril, ramiprilat and internal standard.

The relative recovery of ramipril and ramiprilat was evaluated by comparing the peak area response of extracted analytes and internal standard with that of reference quality control solutions at the same concentration level and reconstituted into blank plasma extracts.

The stability of drugs in human plasma was studied by subjecting into different storage conditions at two different concentration (LQC and HQC) levels. The plasma samples were kept at room temperature for 25 h for evaluation of bench top stability. And −20º ± 10ºC as well as -70º ± 20ºC for long term freezer stability. Freeze/thawed stability was also evaluated after subjecting into six cycles of freezing and thawing. The stability was evaluated by comparing with a freshly prepared calibration standard and QC samples.

All stability evaluations were based on back-calculated concentrations.

Table 1: Back Calculated concentration of ramipril and ramiprilat (n=4).

	Concentration ($ngmL^{-1}$)									
	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8	STD-9	STD-10
	0.1	0.2	0.5	1.0	2.0	5.0	9.0	15.0	20.0	25.0
Ramipril	**Slope** = 0.232436 **Intercept** =-0.000731827 **r^2**=0.9994613									
Mean	0.09966	0.2011	0.4988	1.008	2.018	4.981	9.102	14.78	20.39	24.29
S.D.	0.00065	0.0015	0.0134	0.0283	0.0371	0.244	0.2957	0.5446	0.4975	0.6763
% CV	0.7	0.8	2.7	2.8	1.8	4.9	3.2	3.7	2.4	2.8
Accuracy (%)	99.7	101	99.8	101	101	99.6	101	98.5	102	97.2
Ramiprilat	**Slope =0.0273276 Intercept =0.000071752 r^2= 0.9989181**									
Mean	0.09911	0.2033	0.5051	1.003	1.916	5.175	9.102	14.42	20.09	25.26
S.D.	0.00073	0.0037	0.0219	0.0522	0.0410	0.154	0.3551	0.9846	0.5490	0.7687
% CV	0.7	1.8	4.3	5.2	2.1	3.0	3.9	6.8	2.7	3.0
Accuracy (%)	99.1	102	101	100	95.8	104	101	96.1	100	101

SD: standard deviation; n: total number of observation; STD: standard

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3. RESULTS

3 .1 Limit of quantitation, linearity and precision

The limit of quantitation (LOQ) for ramipril and ramiprilat in human plasma is 0.1 ng/ mL. The calculation was based on the peak area ratio of analyte versus its internal standard. The calibration curves are linear in the concentration range 0.1000–25.00 ng/mL for both ramipril and ramiprilat. The results of the calibration samples are presented in Table 1. The average correlation co-efficient were 0.9994 for ramipril and 0.9989 for ramiprilat. The inter-batch precision and accuracy were determined from three analytical batches by analyzing spiked QC samples. The intra-batch precision and accuracy of the assay were measured by analyzing six spiked samples of ramipril and ramiprilat at each QC level (0.3, 2.5, 10.0 and 18.75 ngmL−1 of ramipril and ramiprilat). Intraday and inter-day precision ranged from 1.4 to 3.1% and 2.5 to 5.3% for ramipril, 2.8 to 13.0% and 4.1 to 8.3% for ramiprilat while accuracy was within 97.9 to 104% and 99.7 to 102% for ramipril, 94.7 to 104% and 96.2 to 103% for ramiprilat respectively, as given in Table 2.

Table 2: Intraday and inter day accuracy of method for ramipril and ramiprilat

Levels	Conc Added ($ngmL^{-1}$)	Intra day				Inter day			
		n	Mean conc ($ngmL^{-1}$)[a]	Accuracy(%)	% CV found	n	Mean Conc ($ngmL^{-1}$)[b]	Accuracy(%)	% CV found
Ramipril									
LLOQ	0.1000	6	0.1043	104	2.1	18	0.1024	102	2.9
LQC	0.3000	6	0.3062	102	2.6	18	0.3074	102	2.5
MQC-2	2.500	6	2.553	102	3.1	18	2.493	99.7	5.3
MQC-1	10.00	6	10.11	101	1.4	18	10.19	102	3.0
HQC	18.75	6	18.36	97.9	1.4	18	19.07	102	4.7
Ramiprilat									
LLOQ	0.1000	6	0.09472	94.7	13.0	18	0.09623	96.2	8.3
LQC	0.3000	6	0.3186	104	7.3	18	0.3076	103	6.1
MQC-2	2.500	6	2.467	98.7	4.2	18	2.491	99.6	5.4
MQC-1	10.00	6	9.723	97.2	7.2	18	9.928	99.3	6.1
HQC	18.75	6	18.44	98.3	2.8	18	18.48	98.6	4.1

CV, coefficient of variance; n, total number of observation,
a Mean of 6 replicates observation at each concentration, b Mean of 18 replicates observations over three different analytical batch.

3 .2 Selectivity

A representative chromatogram of extracted blank plasma is presented in Fig. 3. Representative chromatograms of extracted plasma samples containing 0.1 ng/mL (low standard) and 25.00 ng/mL (high standard) ramipril and ramiprilat are presented in Figs. 4 and 5. Six different sources of drug free human plasma samples were screened and no endogenous interference was observed at the retention times of ramipril, ramiprilat and internal standard.

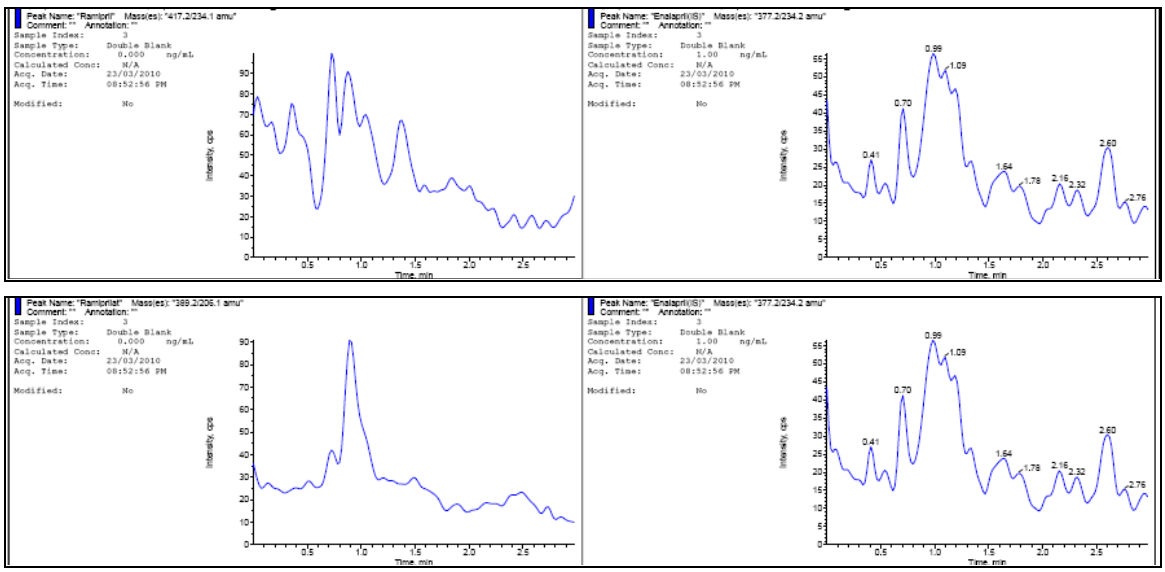

Figure 3: Representative chromatograms of extracted blank plasma samples.

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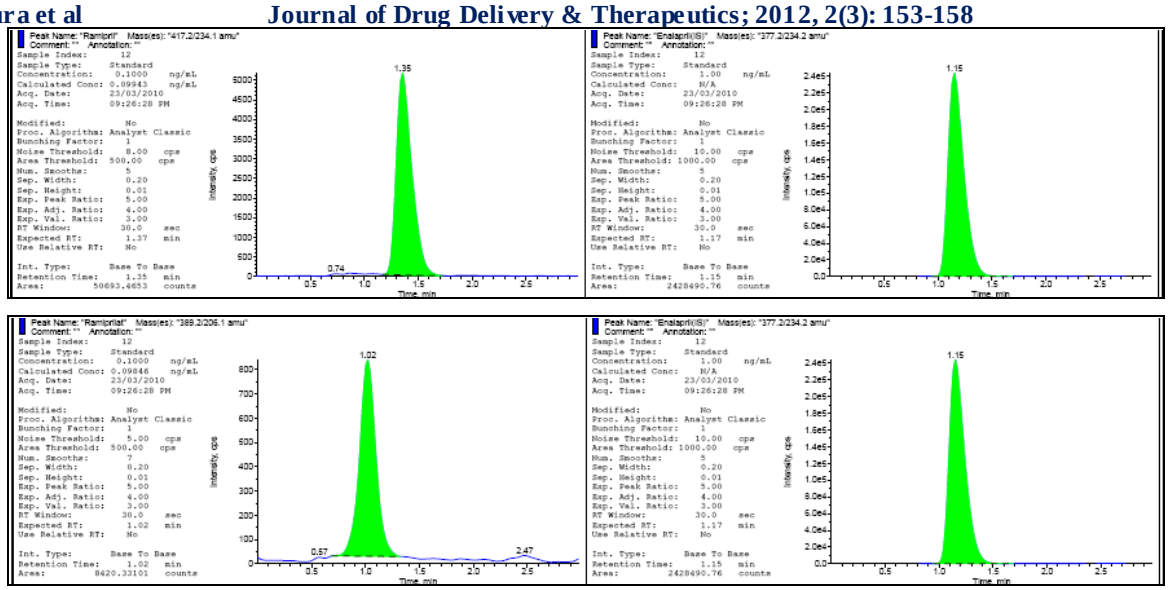

Figure 4: Representative chromatograms of extracted plasma sample containing 0.1000 ngmL^{-1} both ramipril and ramiprilat (LLOQ).

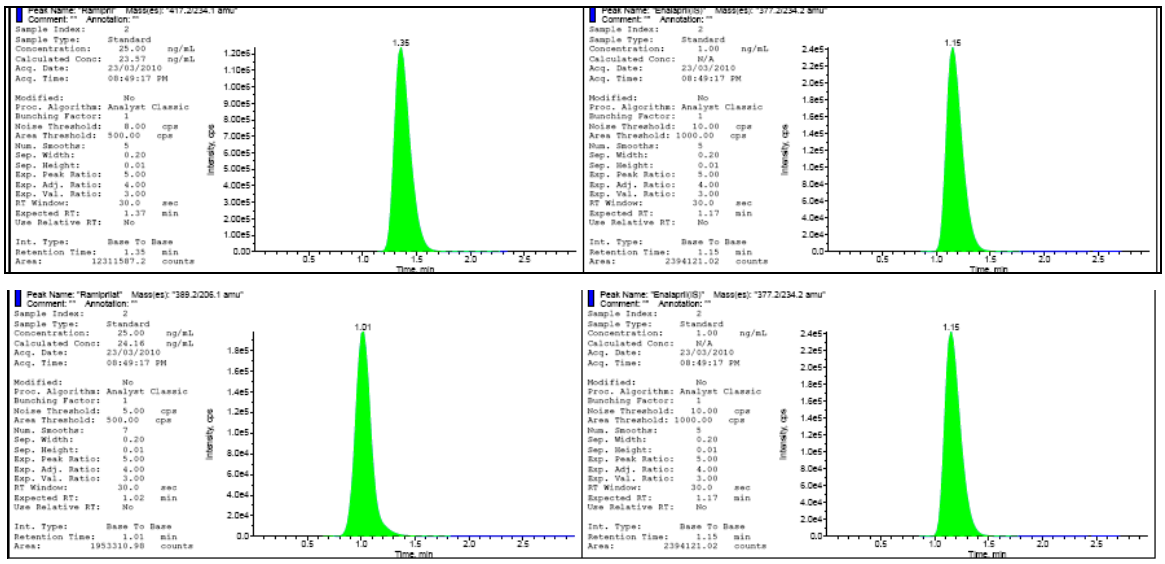

Figure 5: Representative chromatograms of extracted plasma sample containing 25 ngmL^{-1} both ramipril and ramiprilat (ULOQ)

3 .3 Recovery

The recovery was based on the comparison of the peak areas of extracted plasma QC samples at low 0.5000/0.5000 ngmL^{-1} (LQC), Medium 2.500/2.500 ngmL^{-1} (MQC-2) and 10.00/10.00 ngmL^{-1} (MQC-2) and high 18.75/18.75 ngmL^{-1} concentration with unextracted QC samples for each concentration, the peaks areas of six (6) replicates of extracted and unextracted samples were compared. The mean recovery and precision (%CV) of ramipril/ramiprilat from plasma was 63.5%/74.3% and 1.4%/2.2% respectively. The mean recovery and precision (%CV) of internal standard from plasma was 76.1% and 3.2% respectively.

Table 3: Matrix effect evaluation for ramipril and ramiprilat (n=4)

	Concentration ngmL^{-1}											
	HQC LOT-1	HQC LOT-2	HQC LOT-3	HQC LOT-4	HQC LOT-5	HQC LOT-6	LQC LOT-1	LQC LOT-2	LQC LOT-3	LQC LOT-4	LQC LOT-5	LQC LOT-6
Ramipril												
Mean	18.11	18.23	17.13	18.12	18.30	18.08	0.2998	0.2917	0.2928	0.2919	0.2938	0.2997
S.D.	0.118	0.486	0.206	0.302	0.588	0.342	0.0103	0.0158	0.0177	0.0106	0.0050	0.0125
%CV	0.7	2.7	1.2	1.7	3.2	1.9	3.4	5.4	6.0	3.6	1.7	4.2
Ramiprilat												
Mean	17.98	18.53	18.32	18.08	17.26	18.16	0.2970	0.2938	0.2965	0.2936	0.2966	0.3021
S.D.	0.282	0.2193	0.460	0.246	0.256	0.419	0.0117	0.0133	0.0087	0.0078	0.0054	0.0259
%CV	1.6	1.2	2.5	1.4	1.5	2.3	3.9	4.5	2.9	2.7	1.8	8.6

3. 4 Matrix Effect

There were four sets of QC samples for ramipril/ramiprilat at high (18.75/18.75) (HQC) and low (0.3000/0.3000) (LQC) concentration injected using six different plasma matrices to study the matrix effect. The precision (%CV) for the plasma matrices for HQC and LQC concentration QC samples were in the range of 0.7%/1.2% to 3.2%/2.5% and 1.7%/1.8% to 6.0%/8.6% respectively for

ramipril/ramiprilat. These results indicate that there is no significqant matrix effect. Table 3 shows the statistical data of the results.

3 .5 Stability

3.5a Freeze thaw Stability

QC samples (20 sets) prepared at high (HQC) and low (LQC) concentration were divided into 5 cycles consisting of 4 sets of QC samples per cycle. Initial 4 sets of QC samples were stored at -20ºC±10ºC for at least 24 hours and were thawed at room temperature in a water bath for minimum of 2 hours; subsequent QC samples were refrozen for at least 12 hours under the same conditions. Samples from all six freeze and thaw cycles were extracted and compared against a fresh calibration standard curve. The observed values were compared against the nominal values. The results indicated that the samples were stable for at least six freeze thaw cycles.

3.5b Bench Top Stability

Six sets of plasma QC samples at high (HQC) and low (LQC) for ramipril and ramiprilat were kept at room temperature for 25 hours. After 25 hours, the samples were extracted and injected using fresh calibration standard curve. The results indicated that the plasma samples were stable at room temperature for at least 25 hours.

3.5c Processed sample stability at room temperature

Concentration of extracted replicates was compared to the nominal concentration. The results showed that ramipril and ramiprilat was stable for at least 96 hours at room temperature after extraction and sample preparation and prior to sample analysis.

3.5c Processed sample stability at refrigerator temperature

The concentration of extracted replicates to the nominal concentration are Compared. The result showed that ramipril and ramiprilat samples were stable during storage in the refrigerator (4ºC±6ºC) for at least 96 hours.

4 APPLICATION OF THE METHOD

The method was applied to the analysis of plasma samples obtained from pharmacokinetic study. The study was conducted as a randomized, single-dose, two treatments, two-sequence, two-period, crossover study with at least 21 days washout period between each administration, in 48 healthy adult male human subjects under fasting condition. Each subject received ramipril 2.5 mg tablet of test or reference. Blood samples were collected using K3EDTA vaccutainers at the following times: pre-dose, 0.0, 0.167, 0.333, 0.50, 0.667, 0.833, 1.0, 1.25, 1.50, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 12.0, 16.0, 24.0 Ambulatory sample (48.0, 72.0, 96.0 & 120.0) hours post dose after administration.

Pharmacokinetic parameters were calculated from the subjects who had successfully completed period I and Period II.

5. CONCLUSION

A highly sensitive and selective method for the simultaneous determination of ramipril and ramiprilat was developed using HPLC-MS/MS with turbo-ESI. This developed assay method was used in a pharmacokinetic study in which 48 healthy male volunteers were given a 2.5mg of ramipril.This method allows for a much higher sample throughput due to short chromatographic time (3.0 min) and simple sample preparation. This validated method is an excellent analytical option for simultaneous, rapid quantification of ramipril and ramiprilat in human plasma.

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