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Development and validation of UPLC tandem mass spectrometry assay for separation of a phase II metabolite of ramipril using actual study samples and its application to a bioequivalence study

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ABSTRACT: In this paper, we present a validated UPLC-MS/MS assay for determination of ramipril and ramiprilat from human plasma samples. The assay is capable of isolating phase II metabolites (acylglucornides) of ramipril from *in vivo* study samples which is otherwise not possible using conventional HPLC conditions. Both analytes were extracted from human plasma using solid-phase extraction technique. Chromatographic separation of analytes and their respective internal standards was carried out using an Acquity UPLC BEH C_{18} (2.1 × 100 mm), 1.7 μm column followed by mass spectrometric detection using an Waters Quattro Premier XE. The method was validated over the range 0.35–70.0 ng/mL for ramipril and 1.0–40.0 ng/mL for ramiprilat.

Keywords: ramipril; ramiprilat; UPLC-MS/MS; multiple reaction monitoring

Introduction

Ramipril (Fig. 1a) 2-[N-[(S)-1-ethoxycarbonyl-3-phenylproyl-L-alanyl]-(1S,3S,5S)-2-azabicyclo[3-3-0]-ocatne-3-carboxylic acid is a prodrug used in all forms of hypertensions, heart failure and following myocardial infraction to improve survival in patients with clinical evidence of heart failure. The active diacid metabolite, ramiprilat (Fig. 1b) is formed by hydrolysis of the ethyl ester group from ramipril.

Previous studies have reported several different methods for qualitative and quantitative determination of ramipril in human plasma, serum and some pharmaceutical formulations, such as voltametry (Al-Majed *et al*., 2000), high-performance liquid chromatography (Hogan *et al*., 2000 and Belal *et al*., 2001), atomic absorption spectroscopy (Abdellatef *et al*., 1999), gas chromatography tandem mss spectrometry (Maurer *et al*., 1998; Nordstrom *et al*., 1993). Few LC-MS/MS methods have also been reported with solid-phase extraction for analysis in human serum and plasma (Lu *et al*., 2006; Zhu *et al*., 2002). However, none of the articles mentioned above, especially the ones employing LC-MS/MS, highlighted the issue of interference from the phase II metabolite of ramipril. There has also been reported a case of interference from an unknown metabolite using a GC-MS assay procedure for ramipril and ramiprilat from human urine and plasma. The interference from the unknown metabolite was reduced by including a washing step during SPE after extraction/alkylation, prior to acylation (Persson *et al*., 2006).

This phenomenon and many others like it have been listed in an excellent review article (Jemal *et al*., 1999). It was noted that acylglucornides are some of the most problematic metabolites in bioanalysis as they have a tendency to be unstable and hydrolyze to release the original drug under neutral and alkaline conditions as well as at elevated temperatures. It was also suggested that mildly acidic conditions at pH 3–5 would be very helpful in reducing the hydrolysis of acylglucornides in biological samples. Storing the plasma samples at reduced temperatures (by keeping them on ice) and immediate buffering of the aliquotted plasma samples to lower the pH to 3–5 was also suggested as an option to stop the hydrolysis process of acylglucornides in plasma.

Contribution to the main drug area due to in-source fragmentation of its conjugated metabolite has also been discussed (Yan *et al*., 2003).

Very recently, a new method development and validation approach has been reported which helps to develop a reliable method for the simultaneous quantitation of ramipril and ramiprilat in the presence of numerous labile metabolites in human EDTA plasma using LC-MS/MS. In this new approach, the use of a synthesized labile acyl glucuronide of ramipril has been employed. Individual and pooled incurred (study) samples in the development and validation process have also been incorporated (Tan *et al*., 2009).

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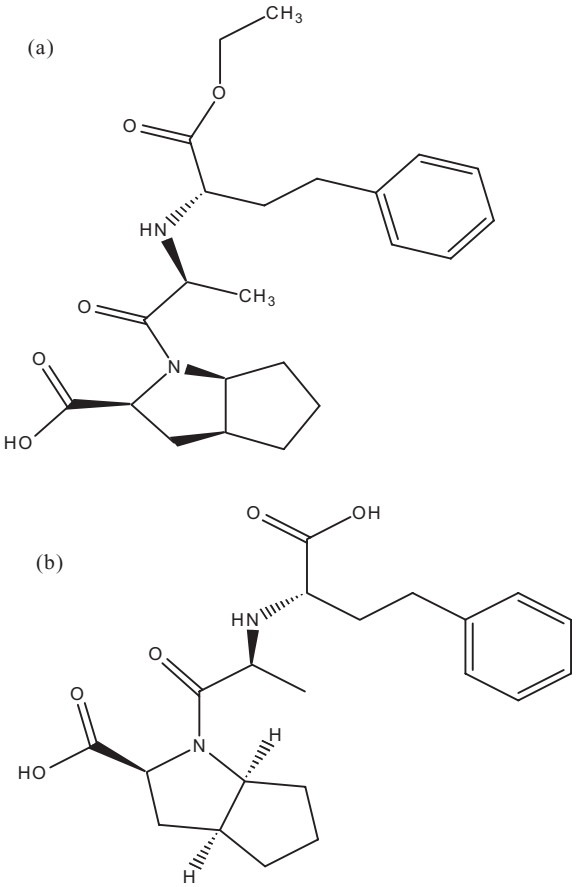

Figure 1. Chemical structure of (a) ramipril and (b) ramiprilat.

In this regard, we would like to present a case study of development and validation of UPLC-MS/MS assay in our laboratory for ramipril and ramiprilat which is free from any potential interference from its phase II metabolite. The internal standards used were enalapril (Fig. 2a) and enalaprilat (Fig. 2b) for ramipril and ramiprilat, respectively. Actual study samples were used to achieve the separation between the analyte and its phase II metabolite (acylglucornides). Special emphasis was given to optimize the extraction step in order to obtain quantitative and reproducible recovery for the analyte as well as metabolite. The use of UPLC conditions and a 1.7 µm particle size column further enhanced this much-needed separation.

Experimental

Materials and Chemicals

Working standard of both Ramipril and Ramiprilate were obtained from Varda Biotech Ltd.(Mumbai, India)both having purity greater than 93%. The internal standards, enalapril and enalaprilat, were both procured as USP Reference standards (Rockville, MD, USA). HPLC-grade methanol and acetonitrile were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Fluka grade formic acid, ammonium acetate and glacial acetic acid were procured. Purified water was obtained from Milli Q A10 gradient water purification system (Millipore, Bangalore, India). Blank human blood was collected with heparin used as the anticoagulant from healthy and drug-free volunteers after obtaining their signed consent. After centrifugation at 4000 rpm at room temperature, plasma was collected and stored at

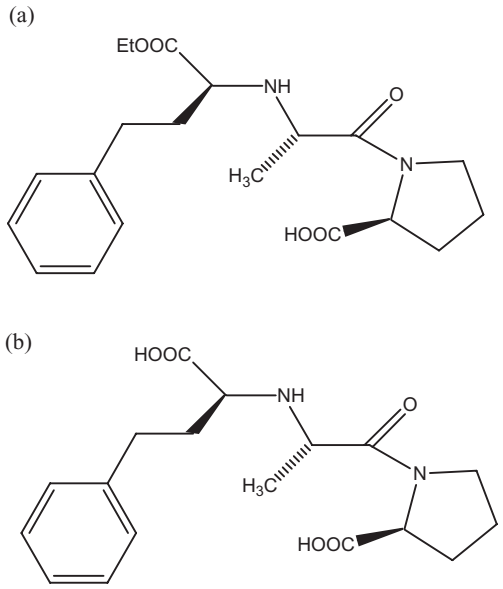

Figure 2. Chemical structure of (a) enalapril and (b) enalaprilat.

–20°C. Strata-X (30 mg/1 mL), solid-phase extraction (SPE) cartridges were procured from Phenomenex (USA).

Ultra Performance Liquid Chromatography and Mass Spectrometric Condition

Chromatographic separation was carried out using a Waters Quattro Premier XE mass spectrometer with an Acquity UPLC BEH C18 (2.1 × 100 mm), 1.7 µm column purchased from Waters, India (Mumbai, India). A mobile phase consisting of 2 mM ammonium formate buffer of pH 2.50 ± 0.05–acetonitrile (40:60, v/v) was delivered with a flow rate of 0.3 mL/min. The total run time for each sample analysis was 3.5 min. Mass spectra were obtained using an electrospray ionization source operated in the multiple reaction monitoring (MRM) mode. Sample introduction and ionization were in the positive ion mode. The cone voltage was set as 30, 33, 25 and 20 V for ramipril, ramiprilat, enalapril and enalaprilat, respectively. The capillary and extractor voltages were optimized at 3.5 kV and 3.0 V, respectively. Argon was used as collision gas. The collision energy was set as 24, 22, 20 and 18 V for ramipril, ramiprilat, enalapril and enalaprilat, respectively The desolvation and cone gas pressures were optimized during tuning as 800 and 45 L/h, respectively. The source and desolvation temperatures were 150 and 450°C respectively. The mass transition ion-pair selected were m/z 417.2 → 234.2 for ramipril, m/z 389.4 → 206.1 for ramiprilat, m/z 377.2 → 234.2 for enalapril and m/z 349.2 → 206.2 for enalaprilat. The data acquisition software used was Mass Lynx version 4.1. For quantification, the peak area ratios of the target ions of the drugs to those of the internal standard were compared with weighted (1/concentration^2) least squares calibration curves in which the peak area ratios of the calibration standards were plotted vs their concentrations.

Preparation of Standards and Quality Control Samples

Two separate stock solutions each of ramipril and ramiprilat were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as the subject sample analysis. For bulk spiking, screened blank plasma samples from six different lots with least interference at the retention time of the analytes and the internal standards were pooled together and used.

The stock solutions of ramipril, ramiprilat and the internal standards were prepared in methanol at a free base concentration of 1000 ppm. Aliquots of these stock solutions were kept stored under refrigeration at 2–4°C for determination of stock solution stability.

Primary dilutions were prepared from stock solutions by dilution with 50 mM ammonium acetate pH 4.30 ± 0.05 buffer–methanol (50:50, v/v). The secondary dilutions and subsequent working solutions were prepared as and when required using the same diluent as for the primary dilutions. These working standard solutions thus prepared were used to prepare the calibration curve and quality control samples.

An eight-point standard curve was prepared by spiking the previously screened blank plasma with appropriate amount of both ramipril and ramiprilat stock dilutions. The calibration curve ranged from 0.35 to 70.0 ng/mL for ramipril and from 1.0 to 40.0 ng/mL for ramiprilat. Quality control samples were prepared at three concentration levels of 1.0, 25.0 and 50.0 ng/mL for ramipril and 3.0, 13.0 and 28.0 ng/mL for ramiprilat in a manner similar to the preparation of calibration curve samples from the stock dilutions.

Extraction Procedure

Plasma samples to be processed were thawed at room temperature. The thawed samples were vortexed to ensure complete mixing of contents. A 500 µL aliquot of plasma was taken in a polypropylene tube and 50 µL of IS dilution mixture (about 700.000 ng/mL of enalapril and 450.000 ng/mL of enalaprilat) was added. A 500 µL aliquot of 50 mM ammonium acetate buffer of pH 4.30 ± 0.05 was added to it and the sample was vortexed for about 30 s followed by centrifugation for 5 min at 15000 rpm. An SPE cartridge (Strata-X, 30 mg/1 mL) was conditioned with 1 mL of methanol followed by 1 mL of 50 mM ammonium acetate buffer of pH 4.30 ± 0.05. The samples were loaded on cartridges and then drained out by applying positive pressure. Each cartridge was washed twice with 1 mL of water and then dried at 30 psi for about 30 s. The sample was eluted by passing 750 µL of mobile phase and transferred into an autosampler vial for injection. A 5 µL aliquot of the eluant was injected into the UPLC-MS/MS system.

Method Validation

Selectivity. Selectivity was performed by analyzing the blank plasma samples from 10 different sources (or donors) to test for interference at the retention time of ramipril, ramiprilat and the internal standards. These 10 sources comprised six normal controlled plasma lots and two controlled plasma lots each of hemolyzed and lipemic plasma containing the same anticoagulant as the study samples.

Linearity and lower limit of quantification. The linearity of the method was determined by analysis of five standard plots associated with an eight-point standard calibration curve. The ratio of area response for analyte to IS was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression. The calculation was based on the peak area ratio of analyte vs the area of internal standard. The concentration of the analyte were calculated from the calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/x^2$). Several regression types were tested and the linear regression (weighted with 1/concentration^2) was found to be the simplest regression, giving the best results. The correlation coefficients were ≥0.9912 for ramipril and ≥0.9952 for ramiprilat. Across the eight points taken as calibration standards, the RSD obtained over five batches was ≤10.5 and ≤8.0 for ramipril and ramiprilat, respectively. 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. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0% whereas for LLOQ it should not be more than ±20.0%.

Accuracy and precision. The intra-batch and inter-batch accuracy and precision were determined by replicate analysis of the four quality control levels on three different days. In each of the precision and accuracy batches, six replicates at each quality control level were analyzed. Mean and standard deviation (SD) were obtained for calculated drug concentration over these batches. Accuracy and precision were calculated in terms of relative error (%RE) and coefficient of variation (%CV) respectively.

Matrix effect. The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) constitutes an important and integral part of validation for quantitative LC-MS-MS method for supporting pharmacokinetics studies. It was performed by processing six lots of different normal controlled plasma samples in quadruplet ($n = 4$). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The RSD for six values at each level was calculated by taking the mean value obtained by injecting the post extracted samples prepared in duplicate from each plasma lot.

Recovery. Absolute recoveries of the analyte were determined at the three different quality control levels, viz. low- (LQC), medium- (MQC) and high-quality control (HQC), by comparing the peak areas of the extracted plasma samples with those of the unextracted standard mixtures (prepared in the elution solution at the same concentrations as the extracted samples) representing 100% recovery. The extraction efficiencies of ramipril, ramiprilat and the internal standards were determined by analysis of six replicates at low, medium and high quality control concentrations for ramipril, ramiprilat and at one concentration for the internal standards. The percentage recovery was evaluated by comparing the peak areas of extracted analytes to the peak areas of non-extracted standards.

Dilution integrity. The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was performed at 1.6 times the ULOQ concentration. Six replicates samples of half and quarter concentration were prepared and their concentrations were calculated by applying the dilution factor of 2 and 4 respectively against the freshly prepared calibration curve.

Stability. All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against comparison samples of identical concentration. Stock solutions of ramipril, ramiprilat and their respective internal standards were checked for short-term stability at room temperature and long term stability at 2–8°C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Bench-top stability, autosampler stability (process stability), freeze–thaw stability and long-term stability in plasma were performed at LQC and HQC level using six replicates at each level. Freeze–thaw stability was evaluated by successive cycles of freezing (at −20°C) and thawing (without warming) at room temperature. To meet the acceptance criteria, the difference between the stability and fresh samples was set at to be within ±15%.

Results and Discussion

Six replicates at low, medium and high quality control concentration for ramipril and ramiprilat were prepared for recovery determination. The mean recoveries for ramipril and ramiprilat were 93.4 and 84.5% with RSD values of 5.6 and 13.0%, respectively. The mean recoveries for enalapril and enalaprilat were 85.4 and 97.7% with RSD values of 5.5 and 3.4%, respectively.

A minimal matrix effect for ramipril and ramiprilat was observed from the six different plasma lots tested. The RSD of the area ratios of post-spiked recovery samples at LQC and HQC levels were within 6.3 and 2.0%, respectively for ramipril and within 5.1 and 2.2%, respectively for ramiprilat. For the internal standards, the RSD of the area over both LQC and HQC levels was 10.0% for enalapril and 10.6% for enalaprilat. These results obtained were well within the acceptable limits, i.e. the RSD of

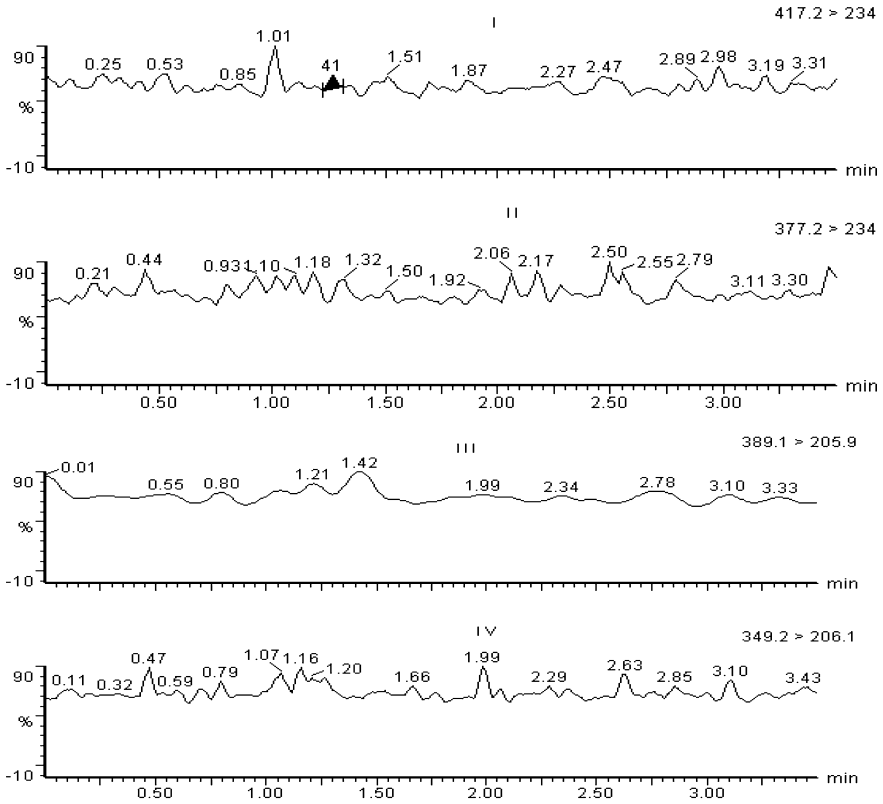

Figure 3. Chromatogram of blank plasma sample for ramipril (I)and ramiprilat (II) with respective internal standard enalapril (III) and enalaprilat (IV).

the area ratio was within ±15% at each level tested for the two analytes and within ±20% over both the levels tested for the internal standard.

The high selectivity of MS-MS detection allowed the development of a very specific and rapid method for the determination of ramipril and ramiprilat in plasma. Representative chromatograms obtained from blank plasma and blank plasma spiked with LLOQ standard for ramipril and ramiprilat are presented in Figs 3 and 4. No significant interfering peak of endogenous compounds was observed at the retention time of analyte in blank human plasma containing heparin as the anti-coagulant in 10 different plasma lots, which were compared with six replicates of extracted samples at the LLOQ level.

The LLOQ, defined as that concentration of ramipril and ramiprilat which can still be determined with acceptable precision (%RSD < 20) and accuracy (bias within ± 20%) was found to be 0.35 and 1.0 ng/mL, respectively, for ramipril and ramiprilat.

Accuracy is defined as the percentage relative error (%RE) and was calculated using the formula $\%RE = (E - T)(100/T)$ where E is the experimentally determined concentration and T is the theoretical concentration. Assay precision was calculated by using the formula $\%RSD = (SD/M)(100)$ where M is the mean of the experimentally determined concentrations and SD is the standard deviation of M. Refer to Tables 1 and 2 for the intrarun and interrun accuracy and precision summary for ramipril and ramiprilat.

As a part of the method validation, various stabilities were evaluated. Analytes were considered stable if the recoveries of the mean test responses were within 15% of appropriate controls. The stability of spiked human plasma kept at room temperature of about 25°C (bench-top stability) was evaluated for 24 h. The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0) with the samples that were re-injected after keeping in the autosampler at 4°C for 58 h. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen and thawed five times with freshly spiked quality control samples. Six aliquots of each low and high concentration were used for all the stability evaluations and compared with freshly spiked quality control samples at the two quality control levels tested.

The goal of this work was to develop and validate a simple, rapid and sensitive assay method for the simultaneous extraction and quantification of ramipril and its metabolite, ramiprilat, suitable for determining the pharmacokinetics of this compound in clinical studies. To achieve the goal, during method development different options were evaluated to optimize sample extraction, detection parameters and chromatography. Since ramipril was found to exist in unionized form in the acidic pH, in this assay, the plasma samples were treated with 50 mM ammonium acetate buffer of pH 4.30 ± 0.05 and loaded on to the Phenomenex Strata-X 30 mg/1 cm^3 SPE cartridges. These cartridges were selected for the current assay as they were found to be the most reproducible and gave less batch-to-batch variation when compared with other cartridges of the same make. In addition, these cartridges have both hydrophilic as well as lipophilic properties which further support their use in the current assay. In the state of nonionic forms, the strong binding of analytes to the copolymer of SPE cartridge enables sufficient cleanup. Electrospray ionization (ESI) was evaluated to get a better response from analytes. It was found that the best signal was achieved with ESI positive ion

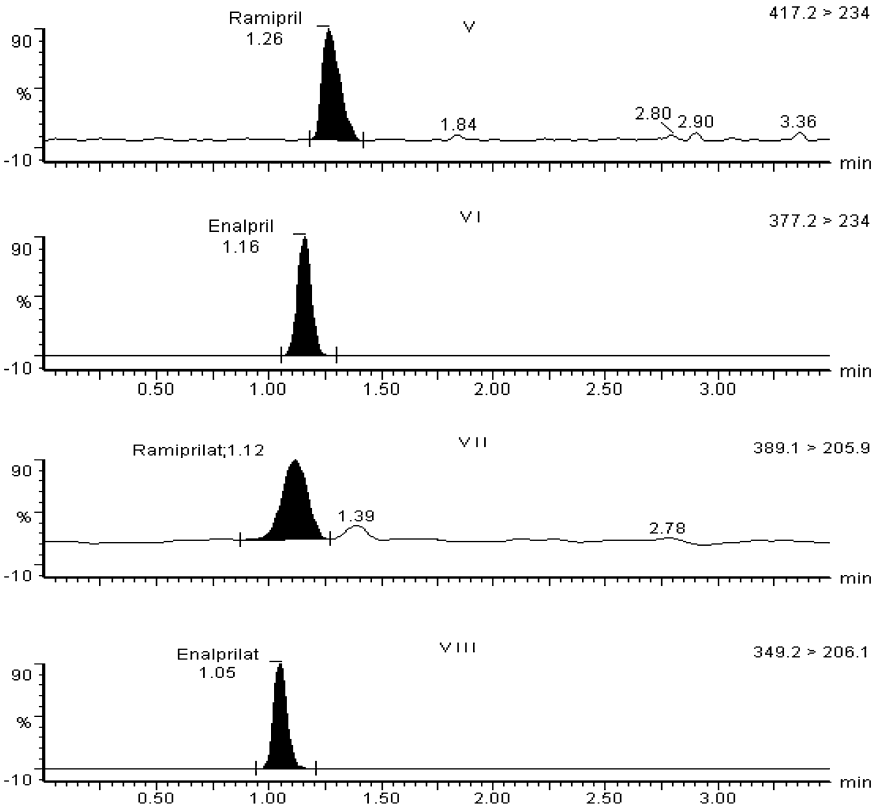

Figure 4. Blank plasma spiked at LLOQ level (CS-1, 0.3 ng/mL) for ramipril (V) and ramiprilat (1.04 ng/mL; VI) with respective internal standards enalapril (VII) and enalaprilat (VIII).

Table 1. Intra-run and Inter-run precision and accuracy for ramipril

Level	Concentration added (ng/mL)	Intra-batch			Inter-batch		
		Mean concentration found (ng/mL)[a]	RE (%)	%CV	Mean concentration found (ng/mL)[b]	RE (%)	%CV
LLOQ	0.362	0.334	−7.7	8.78	0.329	−9.12	14.76
LQC	1.05	1.011	−3.7	8.36	0.978	−6.86	12.36
MQC	25.083	25.027	−0.2	4.9	25.273	0.76	5.57
HQC	50.166	50.422	0.5	8.56	49.215	−1.90	12.18

RE = relative error; CV = coefficient of variance.
[a] Mean of 12 replicates observations at each concentration.
[b] Mean of 18 replicates observations over three different analytical runs.

mode. Further optimization in chromatographic conditions increased the signal of analytes. Using 2 mM ammonium formate buffer of pH 2.50 ± 0.05:acetonitrile (40: 60, v/v) as the mobile phase delivered at a flow rate of 0.3 mL/min resulted in improved signal when compared with other combinations using the same reagents.

Initially, analysis of study samples was started using an already existing HPLC-MS/MS assay validated in our laboratory. Briefly, this assay consisted of extraction of ramipril and ramiprilat from human plasma using solid-phase extraction, which was similar to the modified method. Chromatographic separation of analytes and their respective internal standards was carried out using a Hypersil Hypurity C_{18} (50 × 4.6 mm i.d., 5 µm particle size) column followed by detection using an Applied Biosystems API 4000 mass spectrometer in conjunction with a Shimadzu HPLC as the front end.

A chromatogram obtained from initial analysis of real samples is presented in Fig. 5 for ramipril. On close inspection of the chromatograms of study samples, especially those in the onset period, it was observed that the peak shape of ramipril was different when compared with those of the calibrants and the quality control samples. The visual inference one could logically deduce was that there were two closely eluting peaks at almost the same retention time as ramipril. We deduced that this could have been due to co eluting peaks from the study sample matrix or some coeluting metabolite.

At this moment, the subject sample analysis was stopped and an attempt was made to resolve the two peaks. The first simple

Table 2. Intra-run and Inter-run precision and accuracy for ramiprilat

Level	Concentration added (ng/mL)	Intra-batch			Inter-batch		
		Mean concentration found (ng/mL)[a]	RE (%)	%CV	Mean concentration found (ng/mL)[b]	RE (%)	%CV
LLOQ	1.047	1.049	0.2	6.53	1.049	0.19	6.22
LQC	2.754	2.761	0.3	6.48	2.761	0.25	5.56
MQC	13.295	13.049	−1.9	5.67	13.049	−1.85	5.59
HQC	28.859	28.836	−0.1	3.13	28.836	−0.08	3.29

RE = relative error; CV = coefficient of variance.
[a] Mean of 12 replicates observations at each concentration.
[b] Mean of 18 replicates observations over three different analytical runs.

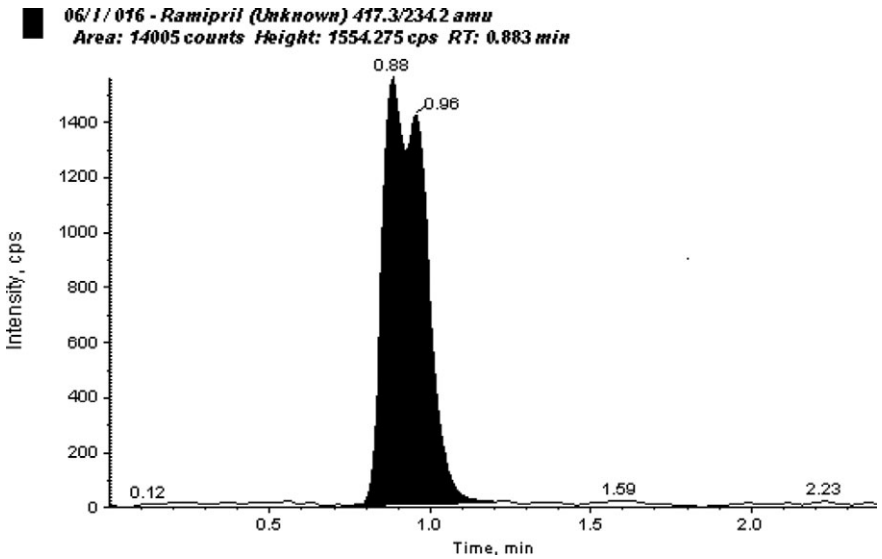

Figure 5. Real subject sample chromatogram from HPLC-MS/MS assay showing the merging of metabolite peak.

approach taken was to use a column of the same make as that used in validation but with a longer length for better resolution. Although this helped in partially resolving the merging peaks, the run time per sample was almost three times of original sample analysis time of 2.5 min, thus affecting the throughput.

Hence, it was decided to shift the assay from normal HPLC to a faster UPLC separation. Consequently, the assay was shifted to on to a UPLC-MS/MS system and an Acquity UPLC BEH C18 (2.1 × 50 mm), 1.7 µm column was employed for separation. The mobile phase was modified to 2 mM ammonium formate buffer of pH 2.50 ± 0.05 as the aqueous phase and acetonitrile in the volume ratio of 40:60 v/v. Decreasing the amount of organic modifier in the mobile phase led to better separation between ramipril and the interfering peak. The use of a column with low particle size i.e. 1.7 µm also played a major role in the chromatographic separation and also helped to keep the run time per sample to an agreeable 3.5 min. A representative chromatogram of a subject sample and a calibration standard using these new UPLC conditions is given in Figs 6 and 7, respectively. From the chromatogram, it was now evident that this interfering peak was indeed leading to overestimation of the main ramipril peak, thus confirming our earlier assumption.

The next part of our investigation was to identify this interfering peak. This drug being a mono carboxylic acid, there was a very good chance of formation of the phase II metabolite in study samples. To verify this, a real sample was used and its full-scan, product ion scan and neutral loss scan were run to confirm the molecular weight of this interfering peak.

It was found that the molecular weight (MW) of the merging peak was 593. On subjecting it to collision-induced dissociation, the fragments had molecular weights of 417, 234 and 343 (Figs 8 and 9), which were the same as the molecular weights of the parent compound, ramipril. The product ion spectrum also showed identical fragments at 234 and 343 *m/z* (Fig. 10). Neutral loss scan of 176 (which is used for confirmation of formation of glucuronide), showed that the molecular weight of the unknown peak was indeed 593.

Once the merging peak was resolved, the assay was then revalidated as discussed above using the UPLC-MS/MS conditions and then used for sample analysis. The initially analyzed subject samples analyzed as per the previously validated HPLC-MS/MS method were reanalyzed using the revised conditions. The back-calculated concentrations from the initial subjects and the repeated subjects were then compared. It was observed that, due to interference from the phase II

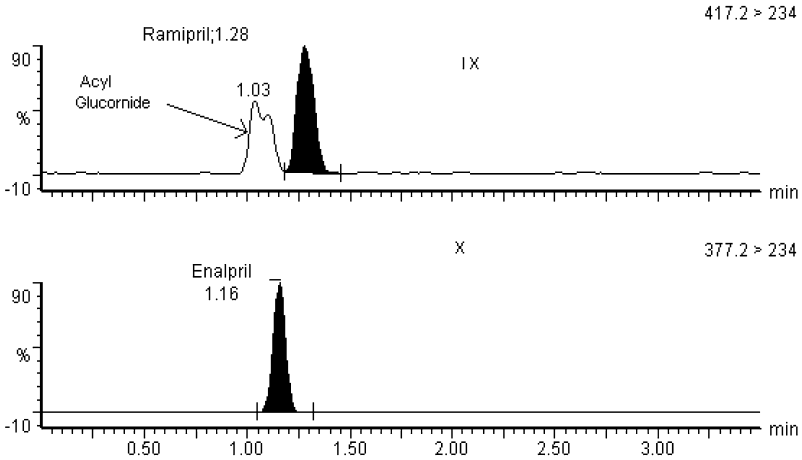

Figure 6. Chromatogram of real subject sample using UPLC-MS/MS assay.

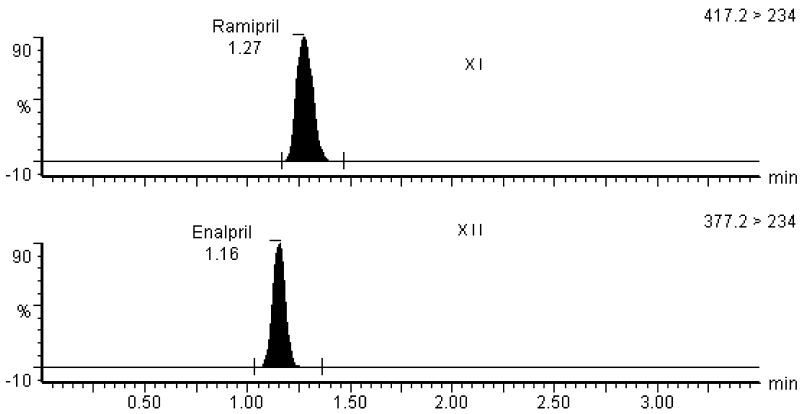

Figure 7. Chromatogram of calibration standard using UPLC-MS/MS assay.

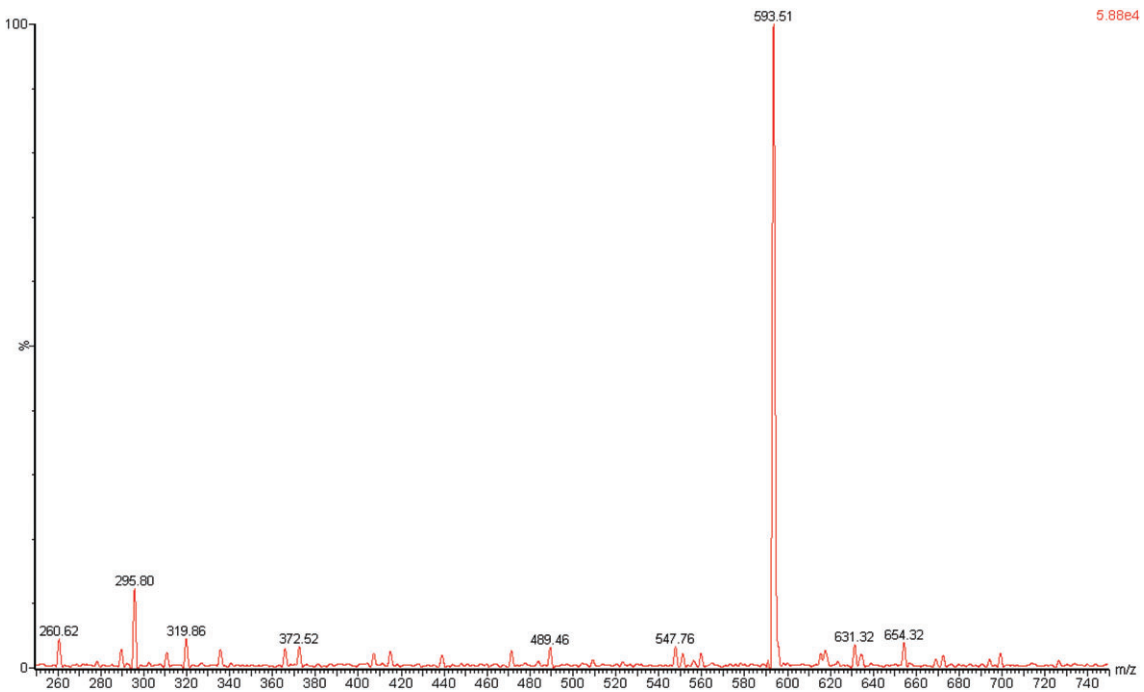

Figure 8. ESI positive full-scan spectrum of unknown metabolite peak with base peak at 593 *m/z*.

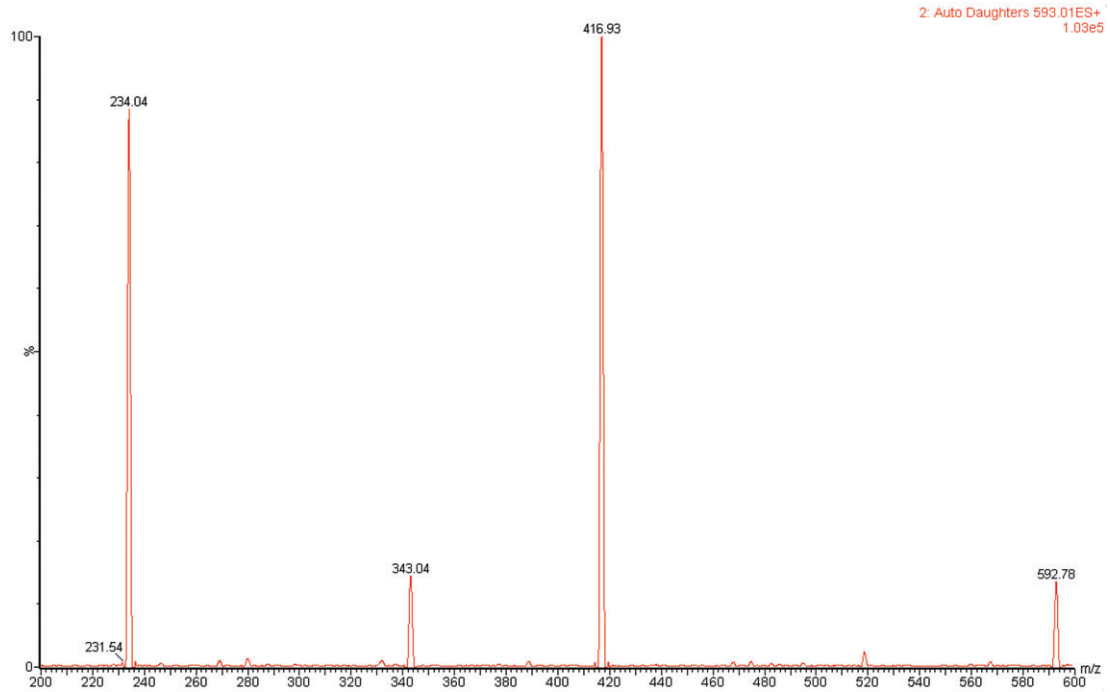

Figure 9. ESI positive full MS/MS scan spectrum of unknown metabolite peak with base peak at 593 *m/z* showing fragments at 417, 234 and 343 *m/z*.

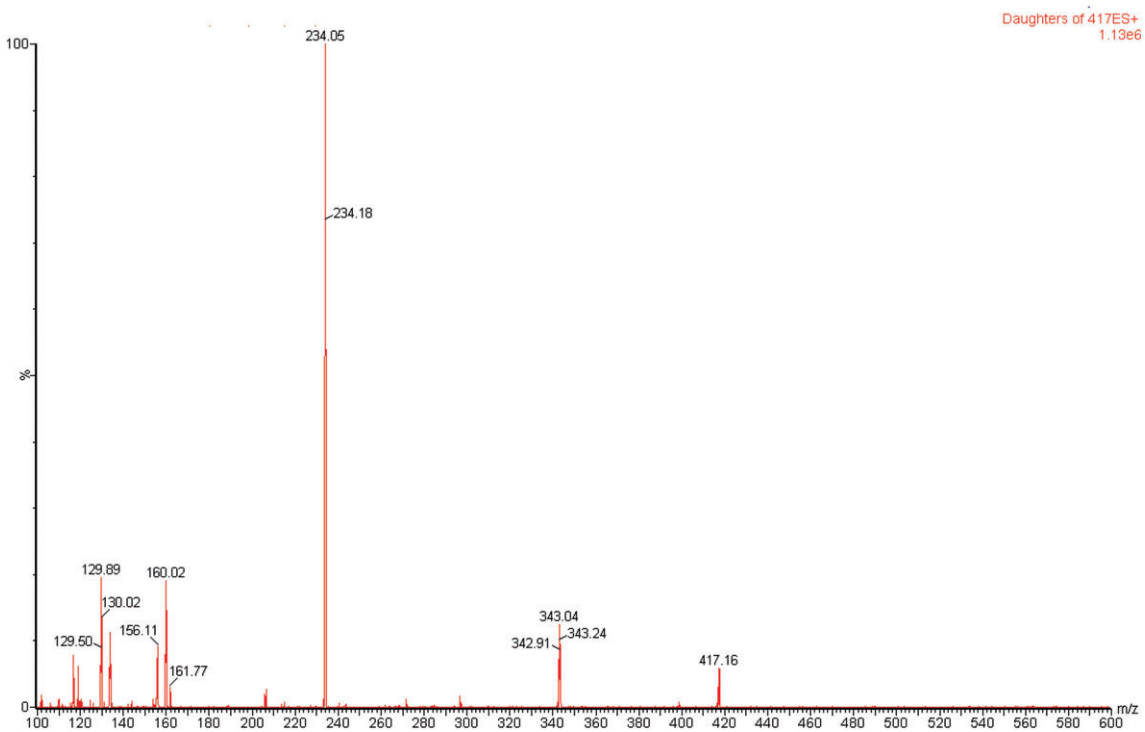

Figure 10. ESI-positive full MS/MS scan spectrum of ramipril showing major fragments at 234 and 343 *m/z*.

metabolite, ramipril levels were indeed being overestimated (Table 3).

Application of Method

This validated method was used to quantitate both ramipril and ramiprilat concentrations in 55 healthy human volunteers under fasting conditions after administration of a single capsule containing 10 mg of ramipril as an oral dose (test and reference). Post analysis the pharmacokinetic parameters were computed using SAS 9.1.3 software. The statistical data which were evaluated were C_{max} (maximum observed drug concentration during the study), AUC_{0-t} (area under the plasma concentration–time curve measured to the last quantifiable concentration, using the trapezoidal

Table 3. Comparison of concentration of mean test and reference formulation using UPLC-MS/MS and HPLC-MS/MS assay

Sampling time (h)	Mean test concentration using UPLC-MS/MS assay (ng/mL)	Mean test concentration using HPLC-MS/MS assay (ng/mL)	Mean reference concentration using UPLC-MS/MS assay (ng/mL)	Mean reference concentration using HPLC-MS/MS assay (ng/mL)
0	0	0	0	0
0.17	0	0	0	0
0.25	0.026	0.031	0.078	0.107
0.33	0.246	0.288	0.378	0.407
0.5	1.782	2.619	1.253	1.621
0.67	3.747	4.811	2.525	3.164
0.83	5.12	7.834	3.63	4.857
1	5.414	8.178	4.438	6.281
1.25	5.693	9.264	5.796	8.185
1.5	7.091	10.989	8.043	11.505
2	8.405	12.465	8.85	13.495
2.5	6.961	10.92	6.648	11.021
3	5.26	8.189	4.952	8.793
3.5	3.724	6.713	3.578	6.93
4	2.336	4.241	2.323	4.929
5	0.683	1.674	0.535	2.003
6	0.174	0.873	0.173	1.028
8	0.051	0.33	0.042	0.411
12	0.021	0.045	0	0.055
24	0	0	0	0
36	0	0	0	0
48	0	0	0	0
72	0	0	0	0
96	0	0	0	0
120	0	0	0	0
144	0	0	0	0
168	0	0	0	0

Table 4. Summary of statistics for target parameters, test vs reference following a single dose of ramipril (10 mg) to 55 volunteers under fasting conditions

Analyte	Treatment	C_{max} (ng/mL)	T_{max} (h)	$AUC_{0\text{-}inf}$ (ng h/mL)	$T_{1/2}$ (h)
Ramipril	Test	10.19	1.73	20.443	0.6968
	Reference	10.32	1.50	21.312	0.7508
Ramiprilat	Test	17.08	4.29	295.964	73.4948
	Reference	17.59	4.39	287.792	69.2616

rule), $AUC_{0\text{–}inf}$ ($AUC_{0\text{–}t}$ plus additional area extrapolated to infinity, calculated using the formula $AUC_{0\text{–}t} + C_t/K_{el}$, where C_t is the last measurable drug concentration, T_{max} is the time to observe maximum drug concentration, K_{el} is the apparent first-order terminal rate constant calculated from a semi-log plot of the plasma concentration vs time curve, using the method of least square regression and $T_{1/2}$ is the terminal half-life as determined by quotient $0.693/K_{el}$. All the statistical parameters computed using SAS 9.1.3 software post-study are tabulated in Table 4.

Conclusion

ACE inhibitors such as ramipril need very selective assay for pharmacokinetics and bioequivalence studies. As these drugs are bound to form phase II metabolites, during method development, due emphasis should be given to separation of these from parent which is generally used for bioequivalence estimation. There are two ways one can assure this separation either by using a presynthesized metabolite and use it for spiking purpose during development of the assay or by using actual study samples to establish the assay resolution. Here we have used study samples to set up the assay after encountering the interference issue. Also this assay demonstrates how UPLC with lower particle size column can help in isolating metabolites which elute very closely with the parent.

References

Abdellatef HE, Ayad MM and Taha EA. Spectrophotometeric and atomic absorption spectrometric determination of ramipril and perindopril

through ternary complex formation with eosin and Cu(II). *Journal of Pharmaceutical and Biomedical Analysis* 1999; **18**(6): 1021–1027.

Al-Majed AA, Belal F, Abadi A and Al-obaid AM. The voltametric study and determination of ramipril in dosage forms and biological fluids. *Il Farmaco* 2000; **55** 3): 233–238.

Belal F, Al-Zaagi IA, Gadkariem EA and Abounassif MA. A stability-indicating LC method for the simultaneous determination of ramipril and hydrochlorothiazide in dosage forms. *Journal of Pharmaceutical and Biomedical Analysis* 2001; **24**(3): 335–342.

Hogan BL, Williams M, Idiculla A, Veysoglu T and Parente E. Development and validation of a liquid chromatographic method for the determination of the related substances of ramipril in Altace capsules. *Journal of Pharmaceutical and Biomedical Analysis* 2000; **23**(4): 637–651.

Jemal M, Ouyang Z, Chen B-C and Teitz D. Quantitation of the acid and lactone forms of atorvastatin and its biotransformation products in human serum by high-performance liquid chromatography with electrospray tandem mass spectrometry. *Rapid Communication in Mass Spectrometry* 1999; **13**(11): 1003–1015.

Lu X-Y, Shen-Tu J-Z and Liu J. High-performance liquid chromatography–mass spectrometric analysis of ramipril and its active metabolite ramiprilat in human serum: Application to a pharmacokinetic study in the Chinese volunteers. *Journal of Pharmaceutical and Biomedical Analysis* 2006; **40**(2): 478–483.

Maurer HH, Kraemer T and Arlt JW. Screening for the detection of angiotensin-converting enzyme inhibitors, their metabolites, and AT II receptor antagonists. *Therapeutic Drug Monitoring* 1998; **20**(6): 706–713.

Nordstrom M, Abrahamsson T, Ervik M, Forshult E and Regardh CG. Central nervous and systemic kinetics of ramipril and ramiprilat in the conscious dog. *Journal of Pharmacology and Experimental Therapeutics* 1993; **266**: 147–152.

Persson BA, Fakt C, Ervik M, Ahnoff M. Interference from a glucuronide metabolite in the determination of ramipril and ramiprilat in human plasma and urine by gas chromatography–mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 2006; **40**(3): 794–798.

Tan A, Jin W, Deng F, Hussain SA, Musuku A and Massé R. Bioanalytical method development and validation using incurred samples—simultaneous quantitation of ramipril and ramiprilat in human EDTA plasma by LC-MS/MS. *Journal of Chromatography B: Biomedical Sciences and Applications* 2009; **877**(29): 3673–3680.

Yan Z, Caldwell GW, Jones W and Masucci J. Cone voltage induced in-source dissociation of glucuronides in electrospray and implications in biological analyses. *Rapid Communications in Mass Spectrometry* 2003; **17**(13):1433–1442.

Zhu Z, Vachreau A and Neirinck L. Liquid chromatography–mass spectrometry method for ramipril and its active metabolite ramiprilat in human plasma. *Journal of Chromatography B: Biomedical Sciences and Applications* 2002; **779**(2): 297–306.

