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Liquid chromatography–mass spectrometry method for determination of ramipril and its active metabolite ramiprilat in human plasma

Zhimeng Zhu[a,*], Andre Vachareau[b], Len Neirinck[a]

[a]*Pharmascience, 6111 Royalmount Ave, Suite 100, Montreal, Quebec, H4P 2T4, Canada*
[b]*Celmed Bioscience, 2310 Alfred-Norbel Blvd., St-Laurent, Quebec, H4S 2A4, Canada*

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Abstract

A fast and robust liquid chromatography–mass spectrometry (LC–MS–MS) method has been developed for simultaneous quantitation of the angiotensin-converting enzyme (ACE) inhibitor, ramipril and its metabolite ramiprilat in human plasma. The method involves a solid-phase extraction from plasma, simple isocratic chromatography conditions and mass spectrometric detection that enables a detection limit at sub-nanogram levels. The proposed method has been validated with a linear range of 0.5–250 ng/ml for both ramipril and ramiprilat. The overall recoveries for ramipril and ramiprilat were 88.7 and 101.8%, respectively.

Keywords: Ramipril; Ramiprilat

1. Introduction

As an angiotensin-converting enzyme (ACE) inhibitor, ramipril, known as 2-{*N*-{(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl)}-L-alanyl}-(1*S*,3*S*,5*S*)-2-azabicyclo(3-3-0) octane carboxylic acid, is a prodrug used in regulation of hypertension and treatment of congestive heart failure [1–3]. Its active metabolite, ramiprilat is formed by hydrolysis of its ethyl ester through hepatic metabolism. The diacid metabolite competes with angiotensin-converting enzyme for substrate and blocks the conversion of angiotensin I to angiotensin II, resulting in a drop in blood pressure and a rise in plasma renin.

Several analytical methods for the determination of ramipril in pharmaceutical forms have been conducted by HPLC [4–9] and all these methods involve relatively high detection limits (i.e. in microgram level) due to poor absorption of ramipril in the UV region. A recent literature survey resulted in few publications available for analysis of ramipril and ramiprilat in biological fluid. Recently, an improved analytical method by GC–MS enabled the detectability to low nanogram levels (10 ng/ml) in human urine. However, the method requires laborious extraction, derivatization procedures and it has a low sensitivity [10]. Due to the relatively low dosing range of this ACE inhibitor, it was necessary to

*Corresponding author. Tel.: +1-514-340-5040; fax: +1-514-342-4118.
E-mail address: zzhu@pharmascience.com (Z. Zhu).

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develop a simple and sensitive analytical method for the quantitation of ramipril and its active metabolite, ramiprilat, in human plasma at sub-nanogram levels.

This paper describes the development and validation of the first LC–MS–MS method for the simultaneous determination of ramipril and ramiprilat in human plasma with an LOQ of 0.5 ng/ml.

2. Experimental

2.1. Materials and reagents

Ramipril and ramiprilat were from in-house supplies. The internal standards, enalapril maleate and enalaprilat were obtained from Sigma (Oakville, Ont., Canada) and Medicorp (Secunderabad, India), respectively. Acetonitrile, methanol and phosphoric acid were purchased from Fisher (Nepean, Ont., Canada). Sodium phosphate monobasic and formic acid were obtained from BDH (Toronto, Ont., Canada). The solid-phase extraction cartridges (Oasis HLB, 1 cc/30 mg) were purchased from Waters (Mississauga, Ont., Canada).

Drug-free human plasma samples were purchased from Biological Specialties (Colmar, PA, USA) and were stored at −20 °C prior to use.

2.2. Calibration curves

The stock solutions of ramipril, ramiprilat and their internal standards enalapril, enalaprilat were individually prepared in methanol at a concentration of 100 μg/ml. Dilutions of 10 and 1 μg/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 their internal standards. A seven-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 0.5 to 250 ng/ml for both ramipril and ramiprilat. Quality control samples were prepared at three concentration levels of 1.5, 50 and 180 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 $1/x^2$.

2.3. Sample preparation

A 0.5-ml aliquot of human plasma sample was mixed with 50 μl of internal standard working solution (2.00 μg/ml of enalapril and enalaprilat in methanol) and 0.5 ml of 0.05 *M* sodium phosphate pH 1.7 buffer. The sample mixture was loaded into an Oasis HLB extraction cartridge that was preconditioned with 1 ml methanol followed by 1 ml 0.05 *M* sodium phosphate buffer, pH 1.7. The extraction cartridge was washed with 1 ml of 0.1% phosphoric acid solution followed by 1 ml of deionized water. Ramipril, ramiprilat and their internal standards were eluted with 1 ml methanol and evaporated to dryness under a gentle stream of nitrogen. The extracted residues were dissolved in 100 μl of reconstitution solution; 10 μl of reconstituted sample was injected into the LC–MS–MS system.

2.4. Instrumentation

Chromatographic separation was carried out on an Agilent 1100 HPLC with an Inertsil Octyl column (5 μm, 50×2.1 mm) purchased from Chromatography Sciences (St-Laurent, Québec, Canada). A mobile phase consisting of acetonitrile, methanol and 0.1% formic acid (4:4:5, v/v) was used with a flow-rate of 0.210 ml/min. The total run time for each sample analysis was 2.5 min.

Mass spectra were obtained using a Sciex API 2000 mass spectrometer (Concord, Canada) equipped with a turbo ion-spray source. The data acquisition was ascertained by Analyst 1.1 software. The mass spectra of ramipril, ramiprilat and its internal standard enalapril and enalaprilat are presented in Fig. 1. Positive mode was used for the analysis. The strongest fragment of each compound, as indicated in Fig. 1 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, 377.2→234.1 for enalapril, 389.2→206.1 for ramiprilat and

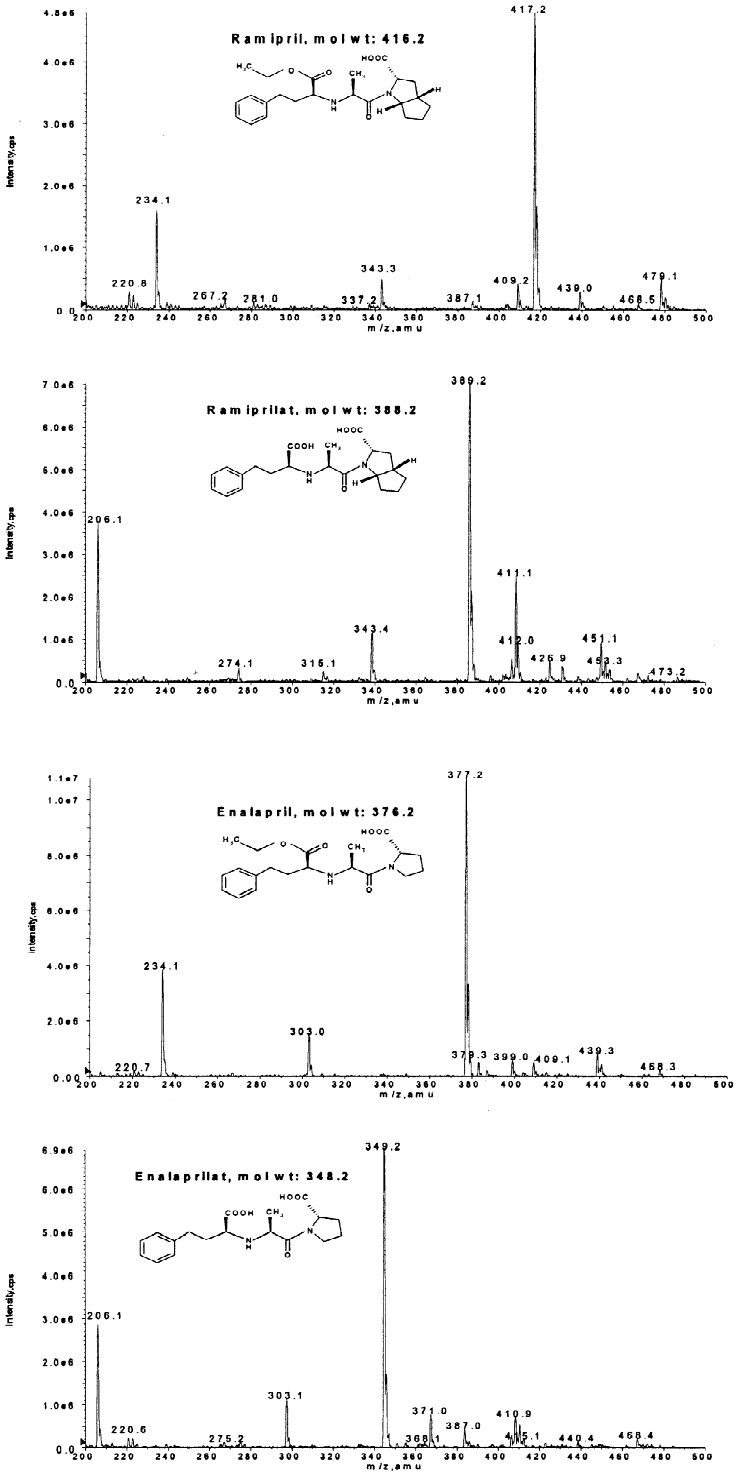

Fig. 1. Mass spectrum of ramipril, ramiprilat, enalapril and enalaprilat.

349.2→206.1 for enalaprilat. 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 enalaprilat.

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 LOQ and quality control samples 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 five 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 their internal standards.

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

As part of the method validation, stability was evaluated. 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 sitting in the auto-sampler at 4 °C for 72 h. The stability of spiked human plasma stored at −20 °C (long-term stability) was evaluated by analyzing low, mid and high quality control samples that were stored at −20 °C for 43 weeks together with freshly spiked standard curve and quality control samples. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen and thawed three times, with the plasma samples thawed once. Three aliquots of each low, mid and high concentration were used for the freeze–thaw stability evaluation. All stability evaluations were based on back-calculated concentrations.

3. Results

3.1. Limit of quantitation, linearity and precision

The limit of quantitation (LOQ) for ramipril and ramiprilat in human plasma is 0.5 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 0.5–250 ng/ml for both ramipril and ramiprilat. The results of the calibration samples are presented in Table 1. The average correlation coefficients were 0.9977 for ramipril and 0.9978 for ramiprilat. The results of inter-assay precision and accuracy are summarized in Table 2. The inter-assay precision was between 2.9 and 4.9% for ramipril and ramiprilat. The results for intra-assay precision and accuracy are listed in Table 3. For the quality control samples at a concentration of 1.5, 50 and 180 ng/ml, intra-assay precision was between 2.0 and 3.7% for ramipril and 2.4–3.2% for ramiprilat. The intra-assay precision for samples at LOQ level was 5.2% for ramipril and 10.8% for ramiprilat.

3.2. Selectivity

A representative chromatogram of extracted blank plasma is presented in Fig. 2. Representative chromatograms of extracted plasma samples containing 0.5 ng/ml (low standard) and 250 ng/ml (high standard) ramipril and ramiprilat are presented in Figs. 3 and 4. 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 their internal standards.

3.3. Recovery

Six replicates of quality control samples at 1.5, 50 and 180 ng/ml were prepared for recovery determi-

Table 1
Summary of ramipril and ramiprilat calibration standards

Analyte	Conc. added (ng/ml)	Conc. found (ng/ml)	RE (%)	CV (%)	*n*
Ramipril	0.5	0.499	−0.2	1.7	5
	1	0.996	−0.4	3.4	5
	5	5.18	3.6	2.3	5
	25	25.2	0.8	1.6	5
	100	104.2	4.2	1.1	5
	200	186.2	−6.9	1.8	5
	250	247.1	−1.2	2.2	5
Ramiprilat	0.5	0.502	0.4	2.8	5
	1	0.992	−0.8	5.4	5
	5	5.01	0.1	4.8	5
	25	24.8	−0.8	2.0	5
	100	102.2	2.2	3.0	5
	200	191.9	−4.0	1.9	5
	250	257.3	2.9	0.9	5

Table 2
Inter-assay precision of ramipril and ramiprilat in human plasma

Analyte	Conc. added (ng/ml)	Conc. found (ng/ml)	RE (%)	CV (%)	*n*
Ramipril	1.5	1.46	−2.7	3.2	18
	50	51.4	2.8	2.9	18
	180	179.1	−0.5	3.3	18
Ramiprilat	1.5	1.54	4.5	4.9	18
	50	53.5	7.1	3.8	18
	180	193.0	7.2	3.7	18

nation and the overall recovery for ramipril and enalapril was 88.7 and 87.8%, respectively while the recovery of ramiprilat and its internal standard enalaprilat was 101.8 and 87.3%, respectively.

3.4. Stability

Three replicates of low, mid and high quality control samples were used for the processed sample

Table 3
Intra-assay precision of ramipril and ramiprilat in human plasma

Analyte	Conc. added (ng/ml)	Conc. found (ng/ml)	RE (%)	CV (%)	*n*
Ramipril	0.5	0.46	−8.4	5.2	6
	1.5	1.46	−2.4	2.0	6
	50	51.6	3.2	2.1	6
	180	181.4	0.8	3.7	6
Ramiprilat	0.5	0.54	8.0	10.8	
	1.5	1.57	4.7	3.2	6
	50	54.8	9.7	2.4	6
	180	196.9	9.4	2.8	6

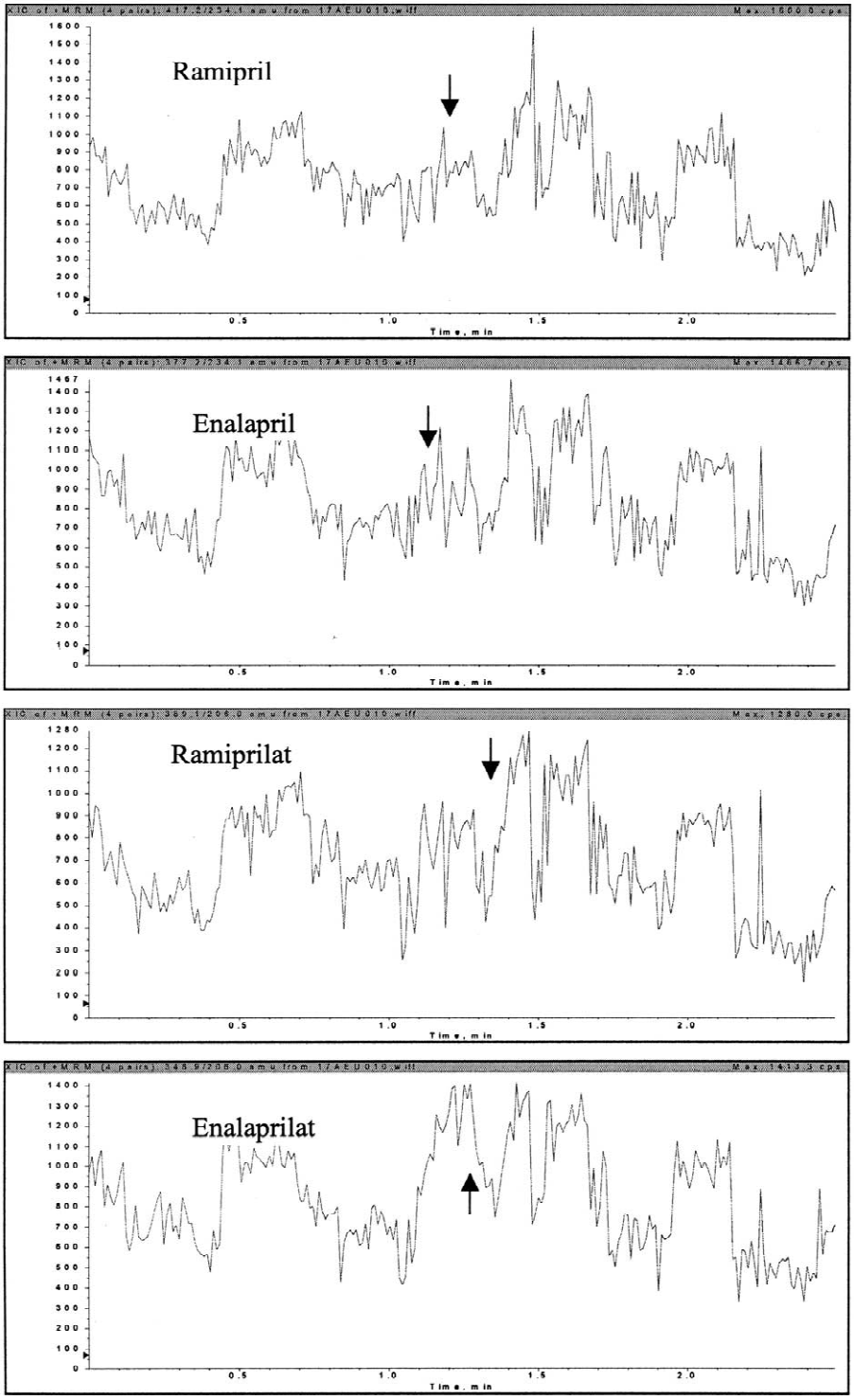

Fig. 2. Representative chromatograms of extracted blank plasma samples.

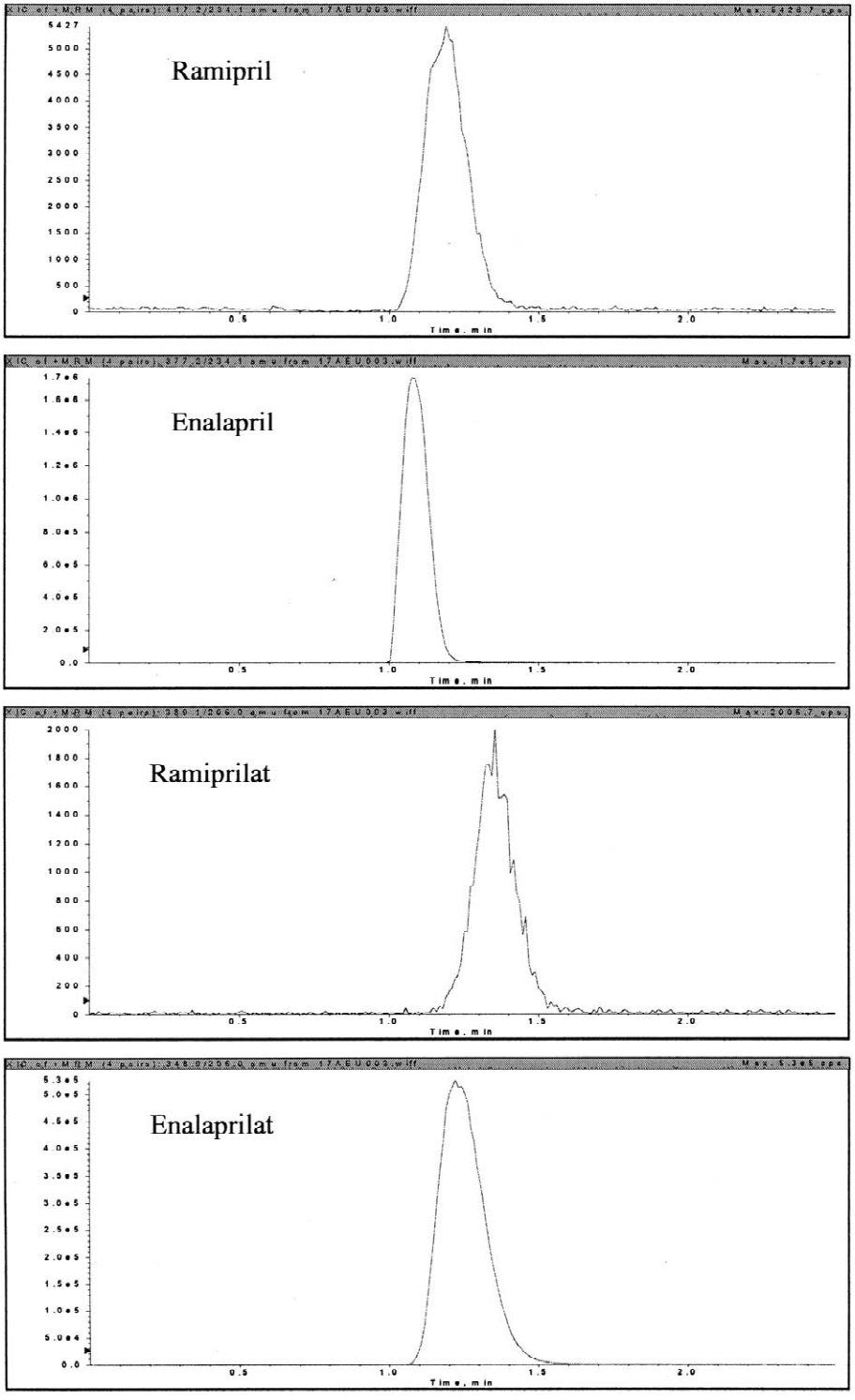

Fig. 3. Representative chromatograms of extracted plasma samples containing 0.5 ng/ml (LOQ).

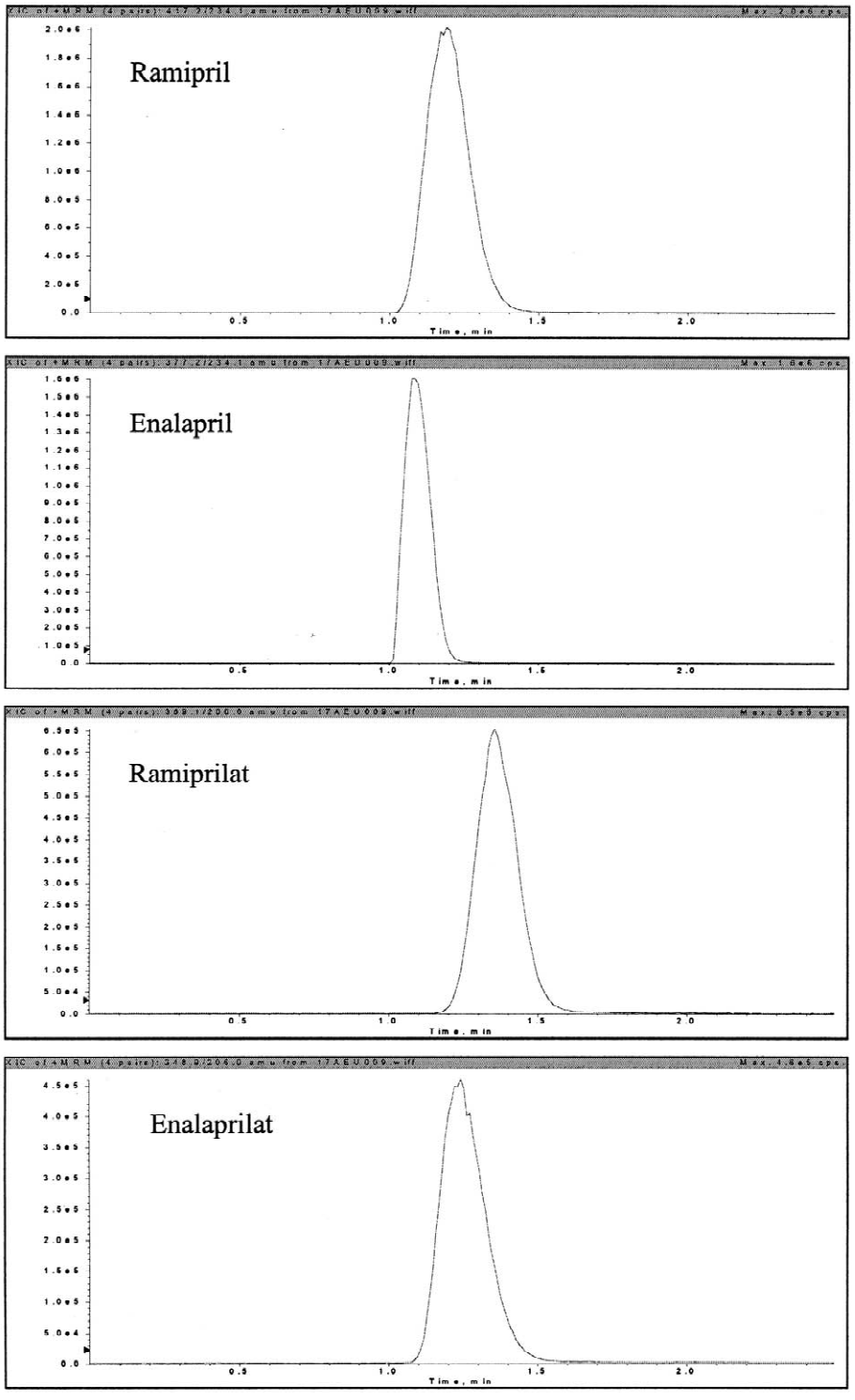

Fig. 4. Representative chromatograms of extracted plasma samples containing 250 ng/ml (high standard).

stability. Results indicated that the difference in the back-calculated concentration from time 0 to time 72 h is <3.2% for ramipril and <1% for ramiprilat which allowed us to conclude that processed samples are stable for at least 72 h at 4 °C in an autosampler. The stability results for both ramipril and ramiprilat show a difference between the fresh spiked sample and samples stored at −20 °C for 43 weeks: <5.2% for ramipril and <1.6% for ramiprilat. Therefore, it was concluded that the spiked ramipril and ramiprilat samples are stable for at least 43 weeks after storage at −20 °C in a freezer. The freeze–thaw stability results indicate that ramipril and ramiprilat are stable for at least three freeze–thaw cycles. The results of freeze–thaw stability indicated that the difference for ramipril is <5.9 and <1% for ramiprilat after three freeze–thaw cycles.

3.5. Application

The method has been applied to a study with more than 1200 plasma samples from human volunteers. The blood samples were collected from each volunteer over a period of 72 h after an oral administration of 10 mg ramipril. Samples were stored at −20 °C until analysis.

According to the *Compendium of Pharmaceuticals and Specialties*, after a single dose of 10 mg ramipril, the average C_{max} of ramipril is about 25 ng/ml ranging from 5 to 44 ng/ml. The average C_{max} of ramiprilat is around 20 ng/ml ranging from 5 to 59 ng/ml. Fig. 5 shows an average concentration versus sampling time profile of ramipril and ramiprilat in human plasma from male volunteers. The results show that after an oral dose of 10 mg of ramipril, the average C_{max} of ramipril is 26.2 ng/ml and ramiprilat is 16.8 ng/ml.

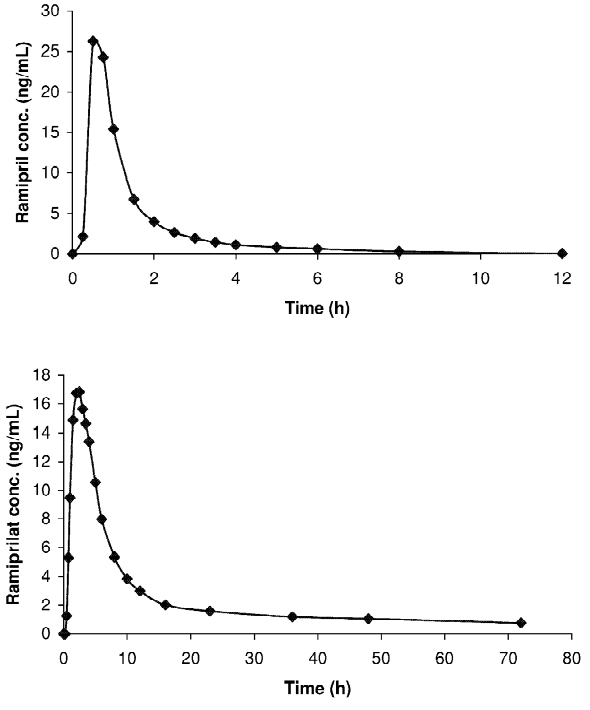

Fig. 5. Average plasma concentration of ramipril and ramiprilat following 10 mg ramipril oral dose to human volunteers.

4. Discussion

Ramipril and other ACE inhibitors such as perindopril, enalapril, captopril, and lisinopril have a structure similar to that of a proline-containing natural peptide, which contains structural diastereomers. Due to potential structural rotation of ACE inhibitors and their active metabolites, peak splitting and broadening are observed during reversed-phase liquid chromatography. In most of the previously reported HPLC methods, either low pH or an ion-pairing agent was used in the mobile phase to produce symmetric peaks for ACE inhibitors [4–7]. However, these modifications significantly limited their applications on LC–MS–MS. Moreover, it had been observed that the composition of an organic modifier in the mobile phase had a great effect on the chromatographic behavior of ACE inhibitors and their metabolites [11]. The method presented here overcomes these chromatographic problems with a simple mobile phase containing both organic modifiers, methanol and acetonitrile, and gives symmetric peaks for these ACE inhibitors. By using a column with an octyl stationary phase combined with a column temperature of 55 °C, peak splitting was avoided.

While various extraction methods had been tested for isolation of ramipril, ramiprilat and their internal standards from human plasma, the Oasis HLB extraction cartridge provided the best recovery due to its combined hydrophilic–lipophilic functional

groups. However, it could result in the blockage of the extraction cartridge. This can be resolved by a simple centrifugation before sample loading and during the sample extraction process. A high yield solid-phase extraction provided excellent specificity and ruggedness of the assay.

5. Conclusions

A rapid and sensitive analytical method for the determination of ramipril and ramiprilat in human plasma has been developed. The proposed method is the first LC–MS–MS method where precision and accuracy were demonstrated in the validation data. The method provided excellent specificity and linearity with a limit of quantitation of 0.5 ng/ml for both ramipril and ramiprilat.

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