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Short communication

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

Xiao-yang Lu *, Jian-zhong Shen-Tu, Jian Liu

The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, PR China

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Abstract

This study presents a rapid, specific and sensitive LC–MS/MS assay for the determination of ramipril and ramiprilat in human serum using enalapril as an internal standard (IS). A Waters Atlantis C18 column (2.1 mm × 100 mm, 3 μm) and a mobile phase consisting of 0.1% formic acid–methanol (25:75, v/v) were used for separation. The analysis was performed by the selected reaction monitoring (SRM) method, and the peak areas of the *m/z* 417.3 → 234.3 and *m/z* 389.3 → 206.2 transition for ramipril and ramiprilat, respectively, were measured versus that of the *m/z* 377.3 → 234.2 for IS to generate the standard curves. The assay linearities of ramipril and ramiprilat were confirmed over the range 0.10–100 ng ml^{-1} and 0.25–100 ng ml^{-1}, respectively, and limits of quantitation for them were 0.10 and 0.25 ng ml^{-1}, respectively. The linear ranges correspond well with the serum concentrations of the analytes obtained in clinical pharmacokinetic studies. Intraday and interday relative standard deviations of ramipril and ramiprilat were 2.8–6.4% and 4.3–4.6%, 4.4–6.7% and 3.5–4.7%, respectively. The recoveries of ramipril and ramiprilat from serum were in the range of 81.0–98.2%. The developed LC–MS procedures were applied for the determination of the pharmacokinetic parameters of ramipril and ramiprilat following a single oral administration of 10 mg ramipril tablets in 18 Chinese healthy male volunteers.

Keywords: Ramipril; Ramiprilat; High-performance liquid chromatography–mass spectrometric; Pharmacokinetics

1. Introduction

Ramipril(2-[*N*-[(S)-l-ethoxycarbonyl-3-phenylpropyl-L-alanyl]-(1S,3S,5S)-2-azabicyclo[3-3-0]-octane-3-carboxylic acid, see Fig. 1) is an orally active inhibitor of angiotensin converting enzyme (ACE), which is a prodrug used in the treatment of all forms of hypertension, heart failure and following myocardial infarction to improve survival in patients with clinical evidence of heart failure [1,2]. The active diacid metabolite, ramiprilat (see Fig. 1), is formed by hydrolysis of its ethyl ester from ramipril [3].

For clinical studies, it is necessary to establish an accurate and specific analytical technique, which permits measurements of ramipril and its active metabolite in biological specimens at different therapeutic levels. Previous studies have reported several different methods for the qualitative and quantitative detections of ramipril in human plasma and pharmaceutical formulations, such as gas chromatography–mass spectrometics (GC–MS) [4,5], high-performance liquid chromatography (HPLC) [6,7], atomic absorption spectroscopy [8], and voltammetric study [9], etc. However, these published methods either required time-consuming derivatization or had relatively high detection limits (i.e. in microgram level). In addition, ramipril is characterized by its low ability to absorb light in the UV region. Recently, LC–MS has been used as an alternative of HPLC in many clinical investigations such as metabolic and pharmacokinetic studies [10]. The method provides more specific, selective and sensitive quantitative results with reduced sample preparation and analysis time relative to other commonly employed techniques, particularly when the analytes have poor UV absorption properties and when the analytes are contaminated with endogenous plasma constituents. Further, LC–MS has been reported for the determinations of ramipril and ramiprilat in human plasma with solid-phase extraction [11].

In this paper, we describe the simultaneous determinations of ramipril and ramiprilat (enalapril (see Fig. 1) as an internal stan-

* Corresponding author. Tel.: +86 571 8723 6531; fax: +86 571 8723 6531.
E-mail address: lxy00711@yahoo.com.cn (X.-y. Lu).

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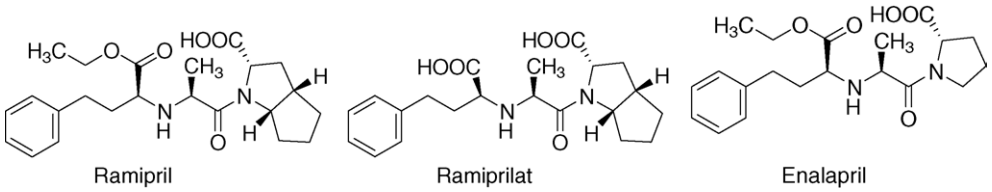

Fig. 1. The chemical formula of the studied compounds.

dard (IS)) in human serum using modified LC–MS/MS method, and this method has been successfully applied to a clinical study of post marketing surveillance in Chinese volunteers.

2. Experimental

2.1. Chemicals and reagents

Ramipril and ramiprilat pure drug sample (purity 99.8%) were kindly provided by Huahai Pharmaceutical Co. Ltd., Zhejiang, China. Tritace® tablets (batch no. L420) containing 5 mg ramipril per tablet were from commercial sources. The internal standard, enalapril pure drug sample (purity 99.8%) was obtained from National Institute for the Control of Pharmaceuticals and Biological Products, Beijing, China. Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Water was purified by Milli-Q system from Millipore Co. (Milford, MA, USA). Other chemicals were of analytical grade.

2.2. Instrumentation

Chromatographic separations were carried out on a Surveyor HPLC with a Waters Atlantis C18 column (2.1 mm × 100 mm, 3 μm). A mobile phase consisting of 0.1% formic acid–methanol (25:75, v/v) was used with a flow rate of 0.2 ml min^{-1}. The mobile phase was filtered by passing through a 0.22 μm membrane filter. Measurements were made at a temperature of 45 °C using 10 μl loop.

Mass spectra were obtained using a Finnigan TSQ Quantum discovery mass spectrometer (USA) and operating fitted with the electrospray ionization (ESI) condition. The positive-ion selected reaction monitoring (SRM) mode was chosen for the quantification. The electrospray voltage was fixed at 3500 eV and the HPLC fluid was nebulized using N_2 and capillary temperature was 300 °C. The sheath and auxiliary gas pressure were 35 and 10 eV, respectively. The peak areas of the LC–MS/MS transition from m/z 417.3 $[M+H]^+$ ion, to a product ion at m/z 234.3 by SRM with collision energy of 28 eV was measured for ramipril. Ramiprilat and the internal standard were monitored using the transitions from m/z 389.3 → m/z 206.2, and m/z 377.3 → m/z 234.2, respectively. The data acquisition was ascertained by Xcalibur software.

2.3. Standard solution

Ramipril and ramiprilat stock solutions were prepared in methanol (0.10 mg ml^{-1}) and stored at 4 °C. Working standards from the concentrated stock solutions were prepared with methanol to yield the final concentrations of 1.0, 2.5, 5.0, 25.0, 50.0, 100.0, 250.0, 500.0, 1000.0 ng ml^{-1}. Enalapril, the internal standard, stock solution 0.1 mg ml^{-1} also stored at 4 °C, was diluted with methanol to give a final concentration of 1.0 ng ml^{-1}.

2.4. Sample preparation

Serum samples for quantitation were drawn from 18 healthy volunteers after a single oral administration of 10 mg ramipril. The samples were stored frozen at −20 °C until analysis. This study was approved by the Ethical Committee on Clinical Investigation, the First Affiliated Hospital, College of Medicine, Zhejiang University and was performed in accordance with the declaration of Helsinki and its amendments.

Serum samples for development and validation of this analytical method were obtained from healthy volunteers in our laboratory. The obtained serum samples were pooled and stored frozen at −20 °C until use.

The working standards 30 μl were mixed with 300 μl blank serum to make a series of serum samples, which were added with 0.6 ml enalapril (1.0 ng ml^{-1}, internal standard). Then the samples were mixed up vigorously for 5 min and centrifuged at 14,000 rpm (2 °C) for 10 min with a CEMTRAL/7R refrigerated centrifuge (The Great British). Its supernatants were poured into another tube to be centrifuged at 14,000 rpm (2 °C) for 5 min, 10 μl was injected onto the LC–MS system.

2.5. Validation

2.5.1. Selectivity

The selectivity was performed on the human serum from 18 individual healthy donors receiving no medication for the assessment of potential interferences with endogenous substances at the retention time of ramipril, ramiprilat, and their internal standard.

2.5.2. Linearity

Serum samples spiked with ramipril, ramiprilat and IS working solutions were processed according to the procedure described above for the construction of calibration curves. The nine-point (0.1, 0.25, 0.50, 2.50, 5.00, 10.00, 25.00, 50.00, 100.00 ng ml^{-1}) calibration curves were obtained by plotting the peak area ratio (y) of ramipril or ramiprilat to the IS against the concentration (x) of ramipril or ramiprilat. The concentrations of calibration standards were analyzed in triplicate and the linearity was evaluated by comparing the correlation coefficient (r) between theoretical and back-calculated concentrations of calibration standard samples.

2.5.3. Precision

The intraday precision of LC–MS was evaluated by replicate ($n = 5$) analysis of serum samples containing ramipril and ramiprilat, IS added, at three different concentrations of 0.50, 5.0, and 50.00 ng ml^{-1}. The interday precision was evaluated at the above concentration levels for 5 days. The precision was estimated by the relative standard deviation (R.S.D.%).

2.5.4. Recovery

The recoveries of ramipril and ramiprilat were evaluated in quintuplicate at three concentration levels of 0.50, 5.0, and 50.00 ng ml^{-1} from peak area ratios of assayed samples comparison to the one of reference standards prepared in methanol. The recoveries were calculated as the areas of ramipril or ramiprilat using the formula:

$$\%\text{Recovery} = \frac{\text{peak}_{\text{serum}}}{\text{peak}_{\text{methanol}}} \times 100$$

2.5.5. Stability

The processed serum samples (0.50, 5.0, and 50.00 ng ml^{-1}) treated as sample preparation were kept at room temperature for 24 h and then the stability was determined. The freeze–thaw stability was determined after three repeated freezing and thawing cycles on day 0, 10 and 20.

2.6. Pharmacokinetic studies

Eighteen healthy male Chinese volunteers were enrolled in this study. After a 12 h fasting, each volunteer was given an oral dose of 10 mg Titrace® tablet (2 × 5 mg). Three mililiter blood samples were collected at predose, and at 0.17, 0.33, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 15.0, 24.0, 36.0, 48.0, 60.0, 72.0 h postdose. Serum samples were prepared for the determination of ramipril and ramiprilat concentrations as described in Section 2.4. Noncompartmental pharmacokinetic analyses were performed on ramipril and ramiprilat by Pharmaceutical Kinetics Software (PKS) 1.0.2 software package (Shanghai Hongneng Software Co. Ltd., China). The maximum concentration (C_{max}) and maximum time (t_{max}) were observed values. The elimination rate constant (K_e) was estimated from the terminal linear segment of the log serum concentration/time data. The elimination half-life ($T_{1/2}$) was calculated from ln 2/K_e. The area under the curve ($AUC_{0\rightarrow t}$)was estimated by trapezoidal rule with extrapolation.

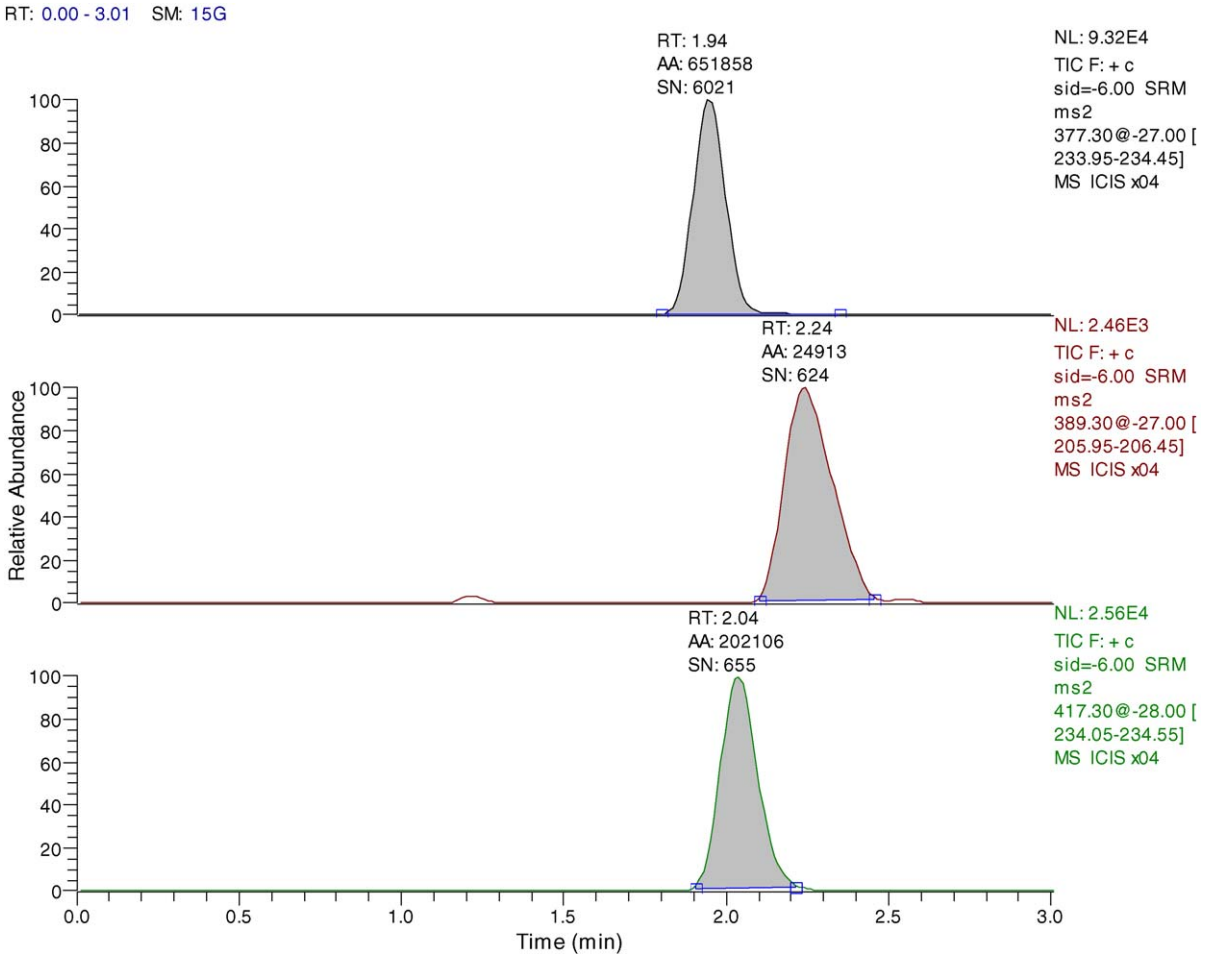

Fig. 2. Representative LC–MS chromatograms from human serum spiked with 2.0 ng ml^{-1} ramipril and ramiprilat, 1.0 ng ml^{-1} enalapril and monitored at m/z 417.3, 389.3, and 377.3, respectively.

3. Results and discussion

3.1. Development of LC–MS method

For optimum detection and quantitation of ramipril and ramiprilat in serum by mass spectrometry, it was necessary to adjust the chromatographic and mass spectrometric conditions. A mobile phase composed of 0.1% formic acid–methanol in a ratio 25:75 (v/v) and flow rate 0.2 ml min^{-1} was used to ensure complete ionization and detection of the drugs and IS by a MS detector at relatively low concentrations without interference of sample matrixes. The high percentage of methanol in the mobile phase allows rapid detection of the examined compounds with retention times less than 2.5 min.

3.2. Validation of LC–MS method

3.2.1. Selectivity

Observed retention times were about 2.04, 2.24, and 1.94 min for ramipril, ramiprilat and IS, respectively (Fig. 2). Serum samples from different sources were found to be free from interfering molecular ions at the retention times of ramipril, ramiprilat, and IS (Fig. 3).

3.2.2. Linearity

The calibration curves of ramipril and ramiprilat in serum were linear in the range from 0.10 to 100.00 ng ml^{-1}, and 0.25 to 100.00 ng ml^{-1}, respectively. The regression equations of calibration curves were $y = 0.1838x - 0.0149$ ($r = 0.9997$), and $y = 0.0322x - 0.0048$ ($r = 0.9999$) for ramipril and ramiprilat, respectively. Their correlation coefficients showed good linearities. This result showed the usefulness of the present LC–MS method in the assays of ramipril and ramiprilat from low to high serum levels. The limits of quantitation (LOQ) of ramipril and ramiprilat in serum were determined to be approximately 0.10 and 0.25 ng ml^{-1}, respectively. The limits of detection (LOD) of ramipril and ramiprilat were determined to be approximately 0.05 and 0.08 ng ml^{-1}, respectively.

Table 1
Intraday precision of LC–MS for determination of ramipril and ramiprilat in serum

	Nominal concentration (ng ml^{-1})					
	Ramiprilat			Ramipril		
	0.50	5.0	50.0	0.50	5.0	50.0
	Calculated concentration (ng ml^{-1})					
	0.49	5.09	48.80	0.53	5.28	50.97
	0.49	5.11	49.09	0.54	5.35	51.45
	0.50	5.24	48.09	0.50	4.52	50.02
	0.54	4.85	50.99	0.53	5.10	55.86
	0.57	4.70	55.34	0.51	5.04	54.30
Mean	0.52	5.00	50.46	0.52	5.06	52.52
S.D.	0.03	0.22	2.93	0.01	0.33	2.46
R.S.D.%	6.7	4.4	5.8	2.8	6.4	4.7

Table 2
Interday precision of LC–MS for determination of ramipril and ramiprilat in serum

	Day	Nominal concentration (ng ml^{-1})					
		Ramiprilat			Ramipril		
		0.50	5.0	50.0	0.50	5.0	50.0
		Calculated concentration (ng ml^{-1})					
	1	0.52	5.00	50.35	0.52	5.05	52.35
	2	0.50	4.62	46.36	0.53	4.95	47.71
	3	0.47	4.71	49.29	0.51	4.66	48.13
	4	0.50	4.71	48.45	0.50	4.49	47.33
	5	0.46	5.00	50.90	0.48	4.81	50.90
Mean		0.49	4.81	49.07	0.51	4.79	49.28
S.D.		4.64	3.39	3.52	4.34	4.45	4.45
R.S.D.%		4.7	3.5	3.6	4.3	4.6	4.5

Table 3
Recovery of ramipril and ramiprilat from human serum ($n = 5$)

	Recovery (%) ± S.D.		
	0.50 (ng ml^{-1})	5.0 (ng ml^{-1})	50.0 (ng ml^{-1})
Ramipril	83.4 ± 3.9	88.0 ± 1.8	90.7 ± 2.6
Ramiprilat	81.0 ± 2.6	98.2 ± 2.8	93.2 ± 1.3

Table 4
The stability of ramipril and ramiprilat in human serum ($n = 5$)

Drug	Nominal concentration (ng ml^{-1})	Found concentration mean ± S.D. (ng ml^{-1})		R.S.D.%
		0 h	24 h	
Ramiprilat	0.50	0.52	0.51	0.7
	5.00	5.00	4.77	2.3
	50.00	50.46	48.64	1.8
Ramipril	0.50	0.52	0.50	1.8
	5.00	5.06	4.90	1.6
	50.00	2.52	47.12	5.4

3.2.3. Precision

The intraday precision showed a relative standard deviation (R.S.D.%) of 2.8–6.4% for ramipril and 4.4–6.7% for ramiprilat (Table 1). The interday R.S.D.% were 4.3–4.6% and 3.5–4.7% for ramipril and ramiprilat, respectively (Table 2).

Table 5
Freeze–thaw stability of ramipril and ramiprilat in human serum ($n = 5$)

Drug	Nominal concentration (ng ml^{-1})	Found concentration mean ± S.D. (ng ml^{-1})			R.S.D.%
		Day 0	Day 10	Day 20	
Ramiprilat	0.50	0.52	0.46	0.49	6.0
	5.00	5.00	5.24	5.19	2.5
	50.00	50.46	55.91	49.53	6.6
Ramipril	0.50	0.52	0.56	0.53	3.9
	5.00	5.06	4.49	4.53	6.7
	50.00	52.52	48.97	45.92	6.7

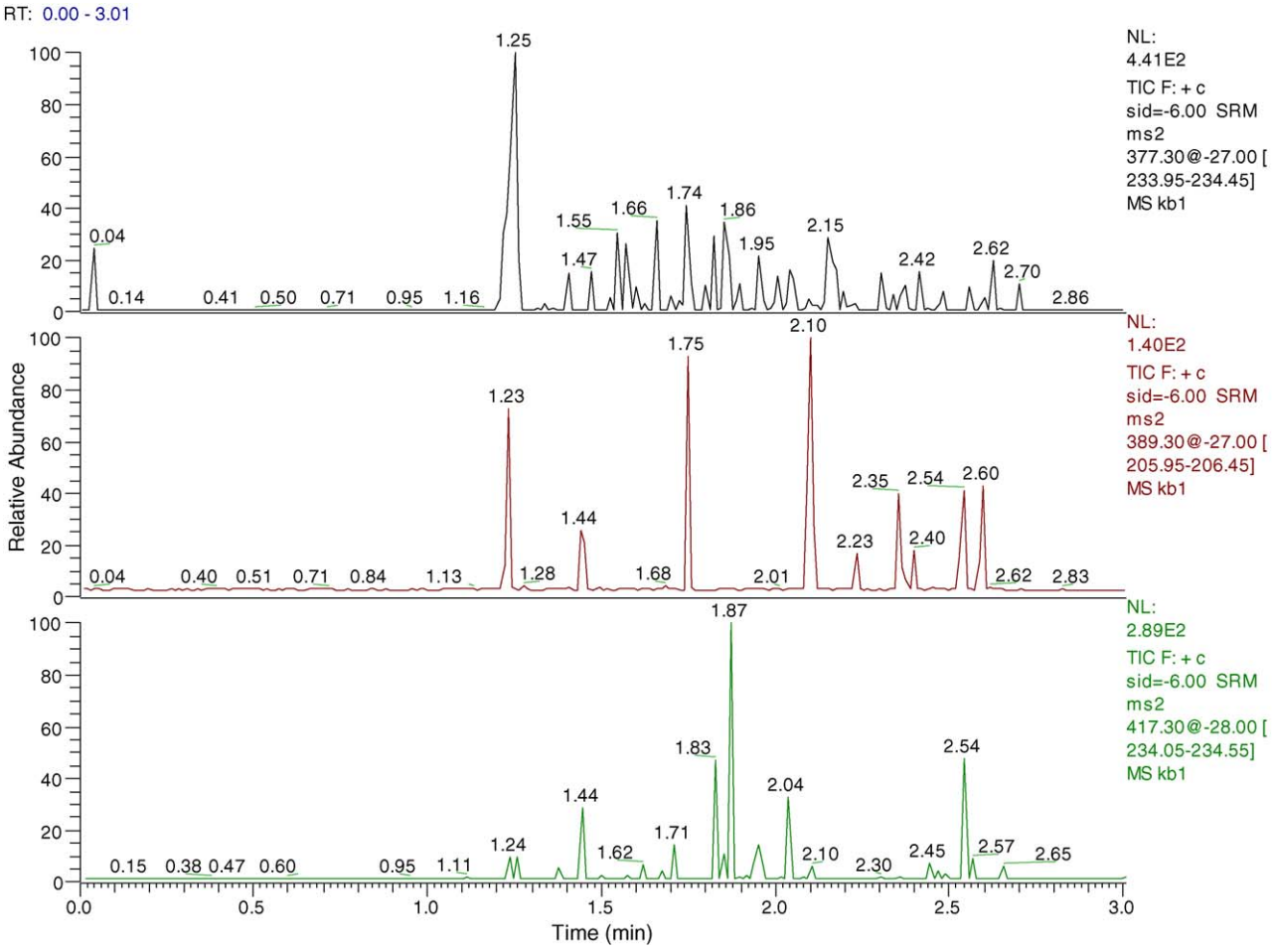

Fig. 3. Representative LC–MS/MS chromatograms from blank serum sample monitored at *m/z* 377.30 → 342.3, *m/z* 389.30 → 206.2, and *m/z* 417.30 → 234.3, respectively.

The data proved good precision of the developed LC–MS method.

3.2.4. Recovery

The mean recoveries of serum samples from low to high concentrations after treatment as Section 2.4 was 81.0–98.2% (Table 3) for ramipril and ramiprilat. These results suggested that there were no relevant differences in serum treatment recoveries at different concentration levels for both ramipril and ramiprilat.

3.2.5. Stability

No significant loss of ramipril and ramiprilat (≤5.4%, R.S.D.) was observed after storage of serum samples at room temperature for at least 24 h (Table 4). Serum samples were stable over at least three freeze–thaw cycles (Table 5), indicating that the

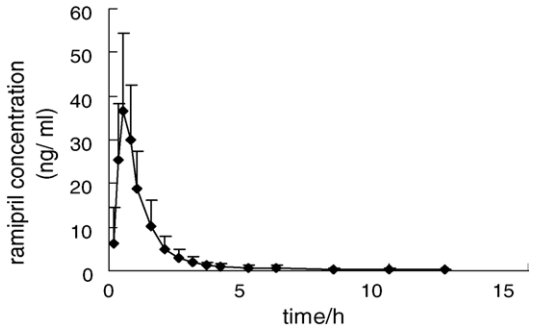

Fig. 4. Serum concentration of ramipril–time curve after a single dose oral administration of 10 mg ramipril to the Chinese healthy volunteers ($n = 18$).

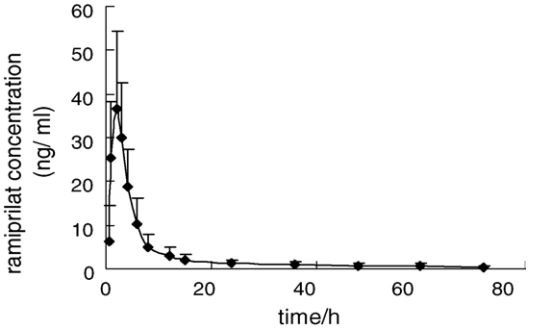

Fig. 5. Serum concentration of ramiprilat–time curve after a single dose oral administration of 10 mg ramipril to the Chinese healthy volunteers ($n = 18$).

Table 6
Pharmacokinetic parameters of ramipril and ramiprilat after administration of 10 mg ramipril to the Chinese healthy volunteers ($n = 18$)

Pharmacokinetic parameters	$\bar{x} \pm$ S.D.	
	Ramipril	Ramiprilat
t_{max} (h)	0.53 ± 0.14	2.44 ± 0.62
C_{max} (ng ml^{-1})	42.34 ± 13.27	43.28 ± 13.83
$AUC_{0\rightarrow t}$ (ng h ml^{-1})	41.70 ± 13.62	311.34 ± 99.34
$AUC_{0\rightarrow\infty}$ (ng h ml^{-1})	42.59 ± 13.97	323.10 ± 100.80
$t_{1/2}$ (k_e) (h)	2.55 ± 1.55	18.81 ± 6.86
K_e (h^{-1})	0.4088 ± 0.2860	0.04297 ± 0.01862

serum samples can be frozen and thawed at least three times prior to analysis.

3.3. Clinical application

The developed method was applied to the determination of ramipril and ramiprilat in human serum for a clinical study of post marketing surveillance in Chinese people. Figs. 4 and 5 shows mean serum concentration–time profiles of ramipril and ramiprilat after an oral administration of Tritace® tablet (10 mg) to 18 healthy male volunteers. Mean values of the different pharmacokinetic parameters were listed in Table 6.

4. Conclusions

The LC–MS/MS method for quantifying ramipril and ramiprilat in human serum has been developed. Method validation has been demonstrated by a variety of tests for selectivity, linearity, sensitivity, precision, recovery, and stability. The developed LC–MS/MS has several advantages compared to the previously reported LC–MS/MS method [11], as it provides lower levels of quantitation of ramipril and ramiprilat, and simpler sample pretreatment. The LC–MS/MS method has been successfully applied in pharmacokinetic analyses of ramipril and ramiprilat in Chinese volunteers.

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