



Trade Science Inc.

June 2008

Volume 7 Issue 7

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 7(7) 2008 [540-545]

Validated LC-MS/MS assay for the determination of ramipril levels in human plasma

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Received: 22nd May, 2008 ; Accepted: 27th May, 2008

ABSTRACT

A rapid, specific and sensitive liquid chromatographic-tandem mass spectrometric (LC-MS/MS) assay for the determination of ramipril levels in human plasma has been developed and validated. Enalapril was used as internal standard (IS). Protein precipitation using methanol in acidic medium yielded clean extracts with a consistent recovery of about 87 %. Analysis was performed on Waters Atlantis C18 column using a mobile phase of 0.1% formic acid and methanol (10:90, v:v). The eluent was monitored using tandem mass spectrometry electrospray ionization and multiple reaction monitoring (MRM) method. The peak height of the transition for ramipril m/z 417.3 \rightarrow 234.3 was measured against that of IS m/z 377.3 \rightarrow 234.2 to generate the standard curves. The relationship between ramipril concentration and peak height ratio (ramipril/IS) was linear over the range 0.5-80 ng/ml in human plasma. The intra-day and inter-day coefficients of variation were ≤ 7.0 and ≤ 10.2 , respectively. The method was applied to assess the stability of ramipril under various conditions generally encountered in the clinical laboratory. Ramipril in plasma was stable for at least 24 hr at RT, 3 months at -20°C , and after three freeze-thaw cycles. © 2008 Trade Science Inc. - INDIA

KEYWORDS

Ramipril;
Enalapril;
LC-MS/MS.

INTRODUCTION

Ramipril [CAS: 87333-19-5], 2-[N-{(S)-1-(ethoxy carbonyl)-3-phenylpropyl}-L-alanyl]-(1S, 3S, 5S)-2-azabicyclo(3-3-0)octane 3-carboxylic acid, is a prodrug inhibitor of angiotensin-converting enzyme (ACE) that is used in the treatment of hypertension and congestive heart failure^[1-3]. Ramipril is converted to ramiprilat by hepatic cleavage of the ester group. After the oral administration of a single 5 mg dose of ramipril, the bioavailability of ramipril and ramiprilat are 28% and 44% respectively^[4].

Several analytical methods have been reported in

the literature for the determination of ramipril in human plasma and pharmaceuticals formulations such as gas chromatography- mass spectrometry^[5-6], high performance liquid chromatography^[7-9], atomic absorption spectroscopy^[10], and voltammetry^[11]. However, these methods either required labor/time-consuming derivatization or had relatively high detection limits. To overcome these difficulties, LC-MS has been used as an alternative to HPLC in pharmacokinetic studies^[12]. Zhimeng Zhu et al.^[13] used an LC-MS/MS method for the determination of ramipril and ramiprilat following solid phase extraction. More recently, Xiao-yang Lu, et al.^[14] applied a simple method for the determination

of ramipril and ramiprilat levels, using peak areas ratios. Limited data on the stability of ramipril in dosage form are available in the literature^[9,15].

In this paper, we describe a simple, sensitive, and accurate method for determining ramipril levels in the therapeutic range in human plasma. The method employs a simple protein precipitation technique, separation by liquid chromatography, and detection by tandem mass spectrometry. The method was applied to determine ramipril stability under various conditions encountered in the clinical laboratory.

EXPERIMENTAL

Materials and reagents

All reagents were of analytical grade unless stated otherwise. Ramipril USP reference standard was kindly donated by Algorithm Sal Pharmaceutical, Lebanon. Enalapril was purchased from Merck Sharp and Dohme Research Lab., Rahway, NJ, USA. Methanol (HPLC grade) and phosphoric acid were obtained from Fisher Scientific, Fairlawn, NJ, USA. Formic acid was purchased from Riedel-de Haen Germany. Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital and Research Center (KFSH&RC). HPLC grade water was prepared by reverse osmosis and further purified by passing through a Milli-Q System (Millipore, Bedford, MA, USA).

Analytical system

The liquid chromatographic tandem mass spectrometric (LC-MS/MS) system included a Water Alliance 2795 separation module (Waters Associates, Inc Milford, MA, USA) used for solvent delivery and sample introduction system. Micromass Quattro micro API bench-top triple quadrupole mass spectrometer (Micromass, Manchester, UK) interfaced with a Z-spray ESI, was used as detector. Analysis was performed on a reversed phase Atlantis dC18 column (2.1×100 mm, 3µm) equipped with guard column symmetry C₁₈ (3.9 × 20 mm, 5µm) set at a temperature of 45°C. The mobile phase containing 0.1% formic acid (pH 2.7) and methanol (10:90, v:v) was filtered through a 0.22µm membrane filter (Millipore Corporation, Bedford, MA, USA), degassed before use, and delivered at a flow rate of 0.25 ml/min. Mass Lynx software (Ver4.0,

Micromass) working under Microsoft Window XP professional environment was used to control the instruments and for data acquisition, peak integration, peak smoothing, and signal-to-noise ratio measurements. The electrospray ionization source was operated in the positive-ion mode at a capillary voltage of 4.0 kV and a cone voltage of 30 V. Nitrogen was used as the nebulizing and desolvation gas at a flow rate of 10 and 600 L/hr, respectively. Argon was used as the collision gas at a pressure of 1.55×10⁻³ mbar. The optimum collision energy for ramipril and the IS was 25 eV. The ion source and the desolvation temperatures were maintained at 120 and 300 °C, respectively.

Characterization of the product ions using tandem mass spectrometry

One microgram solution of ramipril and IS were infused (10µl/min) into the mass spectrometer separately to characterize the product ions of each compound. The precursor ions [M+1]⁺ and fragmentation pattern were monitored using positive ion mode. The major peaks observed in the MS/MS scan were used to quantify ramipril and IS.

Preparation of standards and quality controls

Ramipril and enalapril (internal standard, IS) were separately dissolved in methanol at 1 mg/ml. The working solution of ramipril was prepared in drug-free plasma at a concentration of 100 ng/ml and the working solution of IS was prepared in methanol at a concentration of 500 ng/ml. The calibration standard solutions were prepared by mixing nine appropriate volumes of ramipril working solutions with 5 ml drug-free plasma to produce final concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, and 80.0ng/ml. Four quality controls: lower limit of quantification (LOQ), 3 × LOQ (3LOQ), 0.5 × upper limit of quantification (0.5UOQ), and 0.9 × UOQ (0.9UOQ) were prepared by mixing appropriate volumes of ramipril working solutions with 50 ml drug-free plasma to produce final concentrations of 0.5, 1.5, 40, and 72 ng/ml. The solutions were vortexed for one minute then 0.25 ml aliquots were transferred into 1.5ml eppendorf microcentrifuge tubes and stored at -20°C until used.

Sample preparation

50µl of the IS working solution(25ng of IS) and

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50 μ l of the phosphoric acid (1%) were added to two-hundred and fifty microliters of drug-free human plasma, calibration standards, or quality control samples placed in a 1.5ml eppendorf microcentrifuge tubes. After mild shaking, 375 μ l of methanol was added and the mixture was by vortexed for 1 minute. The mixture was then centrifuged at 13200 rpm for 10 minutes. Ten microliters of clear supernatant were injected onto the analytical column.

Validation

The method was validated for selectivity, linearity, precision, accuracy, recovery and stability. Selectivity was studied using the drug-free human plasma. Six different batches of plasma samples and eight commonly used drugs were assessed for potential interference with the assay. To evaluate linearity, the calibration curves were generated by plotting the peak height ratio (peak height of ramipril/peak height of IS) against the concentration of ramipril. A total of eight, 9 non-zero standard calibration curves were analyzed; the data was evaluated by comparing the correlation coefficient between theoretical and back-calculated concentrations of calibration standards. Precision and accuracy were determined by replicate analysis of samples containing known amounts of analytes. Recovery of ramipril was determined by comparing the peak height ratios of extracted standards in plasma to those of standards in mobile phase. Five replicates of 4 quality controls (LOQ, 3LOQ, 0.5ULQ, 0.9ULQ) were used to calculate the recovery.

Stability studies

Stability studies were performed as a part of method validation. A total of 40 aliquots of each 3LOQ and 0.9ULQ were prepared. Five aliquots of each sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed (counter stability, 24 hours at room temperature), five aliquots were stored at -20°C for three months before being processed and analyzed (long term freezer storage stability), and five aliquots were processed, and stored at room temperature for 5 hours or 24 hours at -20°C before analysis (autosampler stability). Finally, fifteen aliquots of each

sample were stored at -20°C for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to -20°C for another 24 hours. The cycle was repeated three times (freeze-thaw stability). Stock solution stability: Five aliquots of ramipril stock solution were diluted to (100ng/ml) in methanol and analyzed at baseline, after storage for 24 hours at room temperature, and after storage at -20°C for 3 months.

RESULTS AND DISCUSSION

Mass spectra

Precursor ions for ramipril, enalapril, and their corresponding product ions were determined from spectra obtained during the infusion of standard solutions into the mass spectrometer using an electrospray ionization source, operated at positive ionization mode with collision gas argon in Q2 of MS/MS system. Ramipril and IS mainly produced protonated molecules at m/z 417.3 and 377.3 respectively. Their product ions were scanned in Q3 after collision with argon in Q2 at m/z 234.3 for ramipril and at m/z 234.2 for IS. For quantification, peak heights of the transition m/z 417.3 \rightarrow 234.3 for ramipril against that of m/z 377.3 \rightarrow 234.2 for IS were measured in multiple reaction mode (Figure 1).

Sample preparation and LC conditions

A solid phase-liquid extraction using Oasis HLB

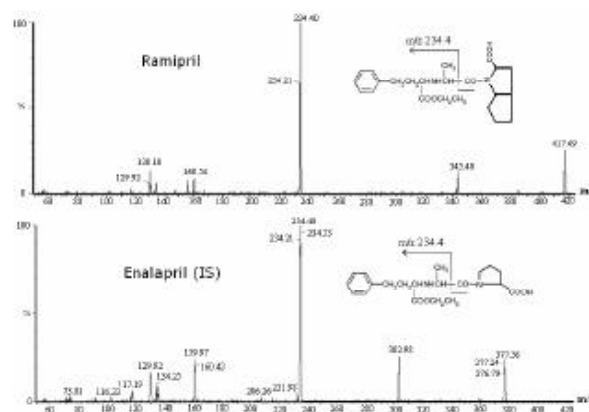


Figure 1: Chemical structures and MS-MS spectra of ramipril and enalapril obtained using electrospray ionization mode

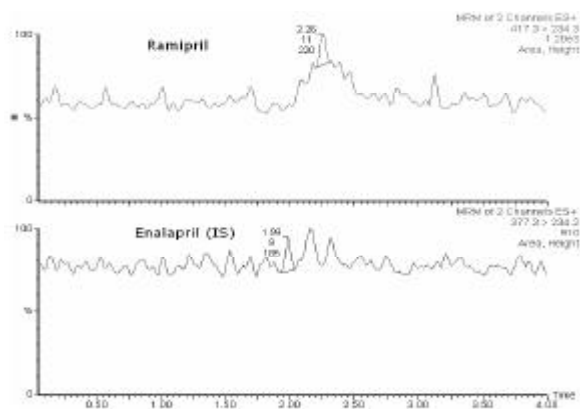


Figure 2 : A representative chromatogram of blank plasma

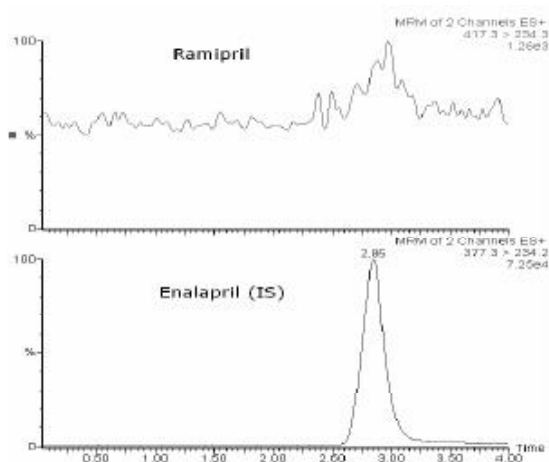


Figure 3: A representative chromatogram of blank plasma spiked with enalapril (25 ng)

extraction cartridge reported a ramipril recovery from plasma of 89 to 102%. However, the procedure was tedious and time consuming^[13]. An assay based on protein precipitation by methanol reported a recovery of 83 to 91% over the concentration range of 0.5 to 50ng/ml^[14]. We obtained an average recovery of 80% over the concentration range 0.5 to 72ng/ml using methanol alone. Therefore we used a mixture of methanol and phosphoric acid; the average recovery over the same range improved to 87%. For optimum detection and quantification of ramipril, we used a mobile phase composed of 0.1% formic acid and methanol (10: 90 v/v) and a flow rate 0.25ml/min. The high proportion of methanol in the mobile phase allowed completing the analysis in 3.5 minutes. Figures 2-5 depicts representative chromatograms: blank plasma (Figure 2), blank plasma with IS 25 ng (Figure 3), blank plasma spiked with ramipril 1.5 ng/ml and IS 25 ng (Figure 4), and

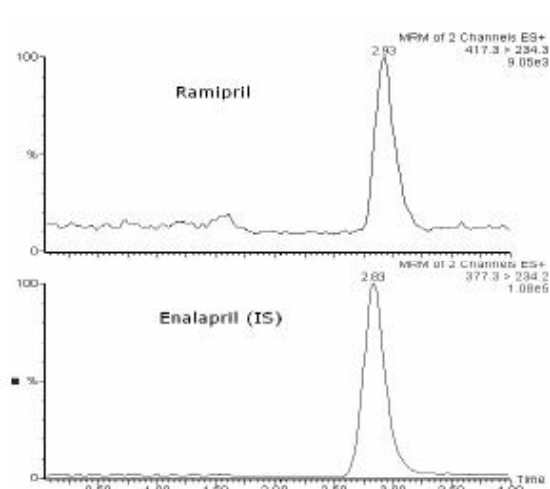


Figure 4: A representative chromatogram of blank plasma spiked with ramipril (1.5 ng/ml) and enalapril (25 ng)

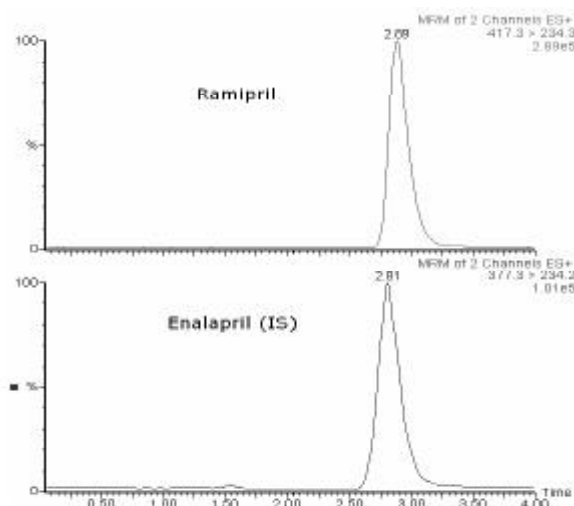


Figure 5: A representative chromatogram of blank plasma spiked with ramipril (72 ng/ml) and enalapril (25 ng)

blank plasma spiked with ramipril 72 ng/ml and IS 25 ng (Figure 5).

Method validation

The procedures used for validation are according to the US Food and Drug Administration (FDA) bioanalytical method validation guidance^[16].

1. Specificity

To evaluate assay specificity, we screened six different batches of human plasma and eight frequently used medications namely: acetaminophen, ibuprofen, aspirin, ranitidine, nicotinic acid, ascorbic acid, caffeine, and omeprazole for potential interference. None was found to interfere with the quantification of ramipril or

TABLE 1: Precision and accuracy of ramipril assay

Nominal Concentration (ng/ml)	Intra day (n=10)				Inter day (n=20)		
	Found Concentration (ng/ml)	*Precision (CV, %) (ng/ml)	**Accuracy (%)	Found Concentration	*Precision (CV, %)	**Accuracy (%)	
0.50	0.54 ± 0.03	7.0	108	0.53 ± 0.05	10.2	105	
1.5	1.58 ± 0.10	6.2	105	1.54 ± 0.12	8.0	102	
40	42.99 ± 2.80	6.5	107	39.84 ± 3.70	9.3	99	
72	77.74 ± 3.19	3.2	108	76.13 ± 4.56	6.0	106	

*Precision as coefficient of variation (CV, %) = Standard Deviation divided by mean measured concentration x 100, **Accuracy = Mean measured concentration/ Nominal concentration

TABLE 2 : Stability of ramipril under various conditions

Nominal Concentration (ng/ml)	Stability (%)								
	**Plasma						#Stock solution		
	Unprocessed		Processed		Freeze-Thaw Cycle				
	24 hrs RT	3 mons -20°C	5 hrs RT	24 hrs -20°C	1	2	3	24 hrs RT	3 mons -20°C
1.5	104	107	99	100	106	109	105	98	84
72	97	99	98	97	103	107	101		

the IS.

2. Linearity and limit of quantification

Linearity of ramipril was evaluated by analyzing a series of standards at nine different concentrations over the range of 0.50-80 ng/ml. The peak height ratio and concentration were subjected to regressive analysis. The mean equation obtained was $Y = 23.09 X - 0.3482$, $r^2 = 0.9970$ ($n = 8$). The limit of quantification (LOQ) was established as 0.5 ng/ml, and the detection limit as 0.2 ng/ml.

3. Accuracy and precision

Accuracy and precision were determined by measuring the levels of ramipril in spiked plasma samples. The spiked quantity of ramipril corresponded to LOQ (0.5 ng/ml), 3LOQ (1.5 ng/ml), 0.5ULQ (40ng/ml), and 0.9ULQ (72 ng/ml). The intra- and inter-day precision and accuracy were determined over three different days. The intra-day ($n=10$) precision and accuracy were $\leq 7.0\%$ and 105-108%, respectively. The inter-day ($n=20$) precision and accuracy were $\leq 10.2\%$ and 99-106%, respectively (TABLE 1).

4. Recovery

The recovery of ramipril was evaluated at four different concentrations, LOQ, 3LOQ, 0.5 ULQ, and 0.9ULQ in 5 replicates. The mean recovery of ramipril was found to be 87%. The recovery of the IS ($n=5$)

was 96%.

5. Robustness

The robustness of a method is a measure of its capacity to remain unaffected by small variations in method conditions. It provides an indication of the reliability of the method during normal applications. The robustness of the current assay was evaluated by altering the strength of formic acid ($\pm 0.02\%$), and the amount of methanol ($\pm 2.5\%$) in the mobile phase. No significant effects were observed.

6. Stability

The stability of the ramipril in plasma and processed samples was investigated. No decrease in the measured concentration or change in chromatographic behavior of the ramipril or the IS was observed. The stock/working solutions, plasma samples, or processed samples were stable after being maintained at room temperature for period of up to 24 hours. Plasma samples containing ramipril (3LOQ and 0.9UOQ) stored at -20°C were found to be stable for at least 3 months and at least after three freeze-thaw cycles. TABLE 2 summarizes the results of stability studies of ramipril. Similarly, the IS stock/working solutions were found to be stable for 24 hours at room temperature (108% and 104%, respectively) and two weeks at -20°C (95% and 100%, respectively).

CONCLUSION

In summary, the LC-MS/MS electrospray ionization method for the determination of ramipril described here is rapid, sensitive, reliable, and reproducible. It has been applied for studying ramipril stability under various clinical laboratory conditions. Potentially, it could be used for the determination of therapeutic levels of ramipril in small volume of human plasma.

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