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Short communication

Simultaneous quantification of enalapril and enalaprilat in human plasma by high-performance liquid chromatography–tandem mass spectrometry and its application in a pharmacokinetic study

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ABSTRACT

A rapid, selective and sensitive high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method was developed to simultaneously determine enalapril and enalaprilat in human plasma. With benazepril as internal standard, sample pretreatment involved in a one-step protein precipitation (PPT) with methanol of 0.2 ml plasma. Analysis was performed on an Ultimate™ XB-C_{18} column (50 mm × 2.1 mm, i.d., 3 μm) with mobile phase consisting of methanol–water–formic acid (62:38:0.2, v/v/v). The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction-monitoring (MRM) mode via electrospray ionization (ESI) source. Each plasma sample was chromatographed within 2.5 min. The linear calibration curves for enalapril and enalaprilat were both obtained in the concentration range of 0.638–255 ng/ml ($r^2 \geq 0.99$) with the lower limit of quantification (LLOQ) of 0.638 ng/ml. The intra-day precision (R.S.D.) was below 7.2% and inter-day R.S.D. was less than 14%, while accuracy (relative error R.E.) was within ±8.7 and ±5.5%, determined from QC samples for enalapril and enalaprilat which corresponded to requirement of the guidance of FDA. The HPLC–MS/MS method herein described was fully validated and successfully applied to the pharmacokinetic study of enalapril maleate capsules in 20 healthy male volunteers after oral administration.

1. Introduction

Enalapril, *N*-[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]-L-proline (Fig. 1A), belongs to the series of substituted *N*-carboxymethyl dipeptides. Enalapril is a prodrug which is hydrolyzed after absorption forming the active angiotensin converting enzyme (ACE) inhibitor. The active form, enalaprilat (Fig. 1B), is the major metabolite of enalapril and has been shown to be effective in the treatment of hypertension and congestive heart failure without causing significant side effects [1–4]. Therefore, enalapril and enalaprilat are often determined simultaneously in biological fluids.

Several analytical methods for enalapril and enalaprilat in biological samples have been reported, including gas chromatography–mass spectrometry (GC–MS) [5], radioimmunoassay (RIA) [6] and enzyme kinetics [7]. Recently, liquid chromatography–mass spectrometry, LC–MS/MS [8,9] and LC–MS [10,11], was used in the determination of enalapril and enalaprilat. But the long analysis time (>3.5 min), large volume of plasma sample (>0.5 ml), or low extraction recovery may not meet the requirement for high throughput, speed and sensitivity in biosample analysis.

This paper describes an improved, rapid, selective and sensitive HPLC–MS/MS method, which enables simultaneous determination of enalapril and enalaprilat with good accuracy at low drug concentrations in human plasma. The lower limit of quantification (LLOQ) corresponding to an on-column sensitivity was 1.06 pg, which was lower than that reported in the literatures [5–9,11]. The total run time of the method per sample was 2.5 min which was shorter than reported ones [8,9,10]. The sample preparation was simple which involved only one-step protein precipitation (PPT) with methanol. This method was fully validated and applied to the pharmacokinetic study in healthy volunteers after oral administration of 10 mg enalapril in capsules.

2. Experimental

2.1. Chemicals and reagents

Reference standards of enalapril (99.2% of purity) and enalaprilat (99.2% of purity) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR

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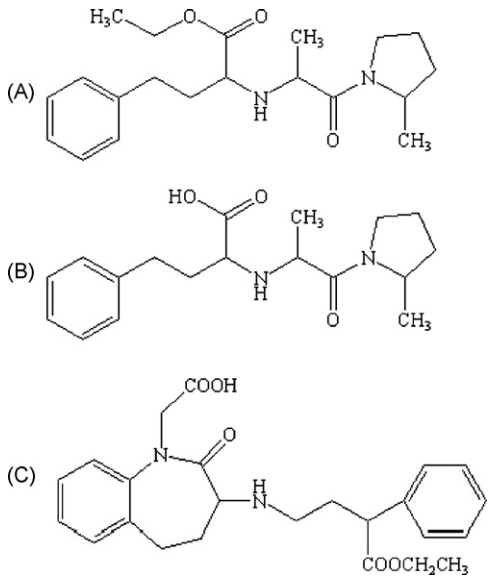

Fig. 1. Chemical structures of enalapril (A), enalaprilat (B) and benazepril (C).

China). Benazepril (IS, 99.4% of purity) was kindly provided by Medicinal Chemistry Department of Shenyang Pharmaceutical University. Methanol of HPLC grade was obtained from Tedia (Fairfield, OH, USA). Formic acid (HPLC grade) was purchased from Dikma (Richmond Hill, NY, USA). Water was purified by redistillation and filtered through a 0.22 μm membrane filter before use.

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

The separation was performed on an ACQUITY UPLC™ system (Waters Corp., Milford, MA, USA) with cooling autosampler. An Ultimate™ XB-C_{18} column (50 mm × 2.1 mm, 3 μm, Welch Materials Inc., Chatham Road Ellicott City, USA) was employed for separation at ambient temperature. The mobile phase was composed of methanol–water–formic acid (62:38:0.2, v/v/v). The flow rate was set at 0.20 ml/min. The autosampler temperature was kept at 4 °C and 5 μl of sample solution was injected with partial loop mode. After each injection, the sample manager experienced a needle wash process, including strong wash (methanol:water = 80:20) and weak wash (methanol:water = 20:80).

2.2.2. Mass spectrometric conditions

A triple quadrupole tandem mass spectrometer (Micromass® Quattro micro™ API mass spectrometer, Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) interface was used for analytic detection. The ESI source was set in positive ionization mode. Quantification was performed using MRM of the transitions of m/z 377 → 234 for enalapril, m/z 349 → 206 for enalaprilat and m/z 425 → 351 for IS, respectively, with scan time of 0.10 s per transition. The optimal MS parameters were as follows: capillary voltage 3.5 kV, cone voltage 25 kV, source temperature 110 °C and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 500 and 30 L/h, respectively. Argon was used as the collision gas at a pressure of approximately 0.261 Pa. The optimized collision energy for the two analytes and IS was 18 eV. All data collected in centroid mode were acquired and processed using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standards and quality control samples

Stock standard solutions of enalapril and enalaprilat were prepared by dissolving approximate 10 mg of accurately weighted substance in 100 ml of methanol. And the solutions were then serially diluted with methanol to provide working standard solutions of desired concentrations. In addition, appropriate amounts of the two compounds were dissolved in methanol to give a final concentration of 102 μg/ml each for preparation of QC samples. The IS (10.5 mg) was dissolved and diluted with methanol to yield a stock solution with a concentration of 1.05 μg/ml, which was further diluted with methanol yielding an IS working solution at concentration of 31.5 ng/ml. All the solutions were stored at 4 °C and brought to room temperature before use.

Calibration standards were prepared daily by spiking appropriate working standard solutions (50 μl of enalapril and 50 μl of enalaprilat) to 200 μl of blank plasma giving concentrations of 0.638, 1.28, 6.38, 12.8, 25.5, 63.8, 127 and 255 ng/ml. The quality control (QC) samples were prepared with blank plasma at LLOQ, low, middle and high concentrations of 0.638, 1.53, 51.0 and 204 ng/ml and stored aliquot at −20 °C after preparation. One set of standards and quality controls were analyzed on each analysis day with the same procedure for plasma samples as described below.

2.4. Plasma sample preparation

Methanol (100 μl) and 200 μl of plasma were pipetted to the 1.5 ml polypropylene micro-centrifuge tube. To each tube, 300 μl of IS solution was added. The mixture was vertex-mixed for 60 s and centrifuged at 13,000 rpm for 10 min. The supernatant (300 μl) was transferred to an autosampler vial, and an aliquot of 5 μl was injected into the HPLC–MS/MS system for analysis.

2.5. Method validation

The method was validated for selectivity, linearity, precision, accuracy, extract recovery and stability according to the FDA guideline for validation of bioanalytical methods [12]. Validation runs were conducted on 3 consecutive days. The peak area ratios of enalapril and enalaprilat to the IS of QC samples were interpolated from the calibration curve on the same day to give concentrations of the two analytes. The results from QC samples in three runs were used to evaluate the precision and accuracy of the method developed.

2.5.1. Selectivity

The selectivity was evaluated by comparing the chromatograms of six different batches of blank plasma obtained from six subjects with those of corresponding standard plasma samples spiked with enalapril, enalaprilat, IS (31.5 ng/ml) and plasma sample after oral dose of enalapril maleate capsules.

2.5.2. Linearity and LLOQ

The calibration curves of enalapril and enalaprilat were both constructed using standard plasma samples at eight concentrations in the range of 0.638–255 ng/ml with weighted ($1/x^2$) least squares linear regression. The LLOQ is defined as the lowest concentration on the calibration curve at which an acceptable accuracy (R.E.) within ±20% and a precision (R.S.D.) below 20% can be obtained.

2.5.3. Precision and accuracy

The intra-day precision and accuracy were evaluated by determining a replicate analysis of QC samples of enalapril and enalaprilat on the same day. The run consisted of a calibration curve and six replicates of each LLOQ, low, mid, and high concentration

quality control samples. For determining the inter-day accuracy and precision, analysis of three batches of QC samples was performed on different days.

2.5.4. Extraction recovery and matrix effect

The extraction efficiency of enalapril and enalaprilat was determined by analyzing six replicates of plasma samples at three QC concentration levels of 1.53, 51.0 and 204 ng/ml for each of enalapril and enalaprilat. The recovery was calculated by comparing the peak areas of the enalapril and enalaprilat added into blank plasma and extracted using the PPT procedure with those obtained from the two compounds spiked into post-extraction supernatant at three QC concentration levels. The matrix effect was measured by comparing the peak response of sample spiked post-extraction (A) with that of pure standard solution containing equivalent amounts of the two compounds (B). The ratio $(A/B \times 100)\%$ was used to evaluate the matrix effect. The extraction recovery and matrix effect of IS were also evaluated using the same method.

2.5.5. Stability

The stability of enalapril and enalaprilat in human plasma was assessed by analyzing replicates ($n = 6$) of low and high QC samples during the sample storage and processing procedures. The freeze–thaw stability was determined after three freeze–thaw cycles. Post-preparation stability was estimated by analyzing QC samples at 0 and 8 h in the autosampler at 4 °C. Six aliquots of QC samples were stored at −20 °C for 50 days and at ambient temperature for 4 h to determine long-term and short-term stability, respectively. All stability testing QC samples were determined by using calibration curve of freshly prepared standards.

2.6. Pharmacokinetic study

The pharmacokinetic study was approved by the local Ethics Committee and carried out in the hospital. All volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. Two enalapril maleate capsules (containing 5 mg enalapril each) were administered to each healthy male volunteer after 12 h fasting. Blood samples were collected before and at 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 12.0, 24.0 and 36.0 h post-dosing. The plasma was immediately separated by centrifugation and stored frozen at −20 °C until analysis.

The maximum plasma concentrations (C_{max}) and their times (T_{max}) were noted directly from the measured data. The elimination rate constant (k_e) was calculated by linear regression of the terminal points in semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve (AUC_{0-t}) to the last measurable plasma concentration (C_t) was calculated by using the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as

$$AUC_{0-\infty} = AUC_{0-t} + \frac{C_t}{k_e}$$

3. Results and discussion

3.1. HPLC–MS/MS condition optimization

HPLC–MS/MS operation parameters were carefully optimized for determination of enalapril and enalaprilat. The mass spectrometer was tuned in both positive and negative ionization modes with ESI for both enalapril and enalaprilat containing secondary amino and carboxy groups. Both signal intensity and ratio of signal to noise obtained in positive ionization mode were much greater than those in negative ionization mode. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[M+H]^+ m/z$ 377, 349 and 425 for enalapril, enalaprilat and IS, respectively. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain highest intensity of protonated molecules of the two compounds and IS. The product ion scan spectra showed high abundance fragment ions at m/z 234, 206 and 351 for enalapril, enalaprilat and IS, respectively. The collision gas pressure and collision energy of collision-induced decomposition (CID) were optimized for maximum response of the fragmentation of the two compounds. Multiple reaction monitoring (MRM) using the precursor → product ion transition of m/z 377 → m/z 234, m/z 349 → m/z 206 and m/z 425 → m/z 351 was employed for quantification of enalapril, enalaprilat and IS, respectively.

It is reported that the liquid chromatographic behavior of enalapril and enalaprilat are poor because of interconversion between *cis*- and *trans*-rotamers, such as a peak splitting and broadening [13]. Therefore, optimization of mobile phase is important for improving peak shape, detection sensitivity and shortening run time of enalapril and enalaprilat. Methanol and acetonitrile were both attempted as the organic modifier of mobile phase. It was found that the peaks were more symmetric when acetonitrile was adopted, however, much lower detection response was presented. For a sample of 5.1 ng/ml for each analyte, the peak area (mean ± S.D., $n = 4$) of enalapril was 3249 ± 69.0 with acetonitrile as mobile phase compared to 4457 ± 90.8 with methanol, and that of enalaprilat was 623 ± 24.0 versus 1264 ± 28.5. Response of analytes was crucial for quantification, therefore methanol was chosen as the organic phase. The proportion of methanol in mobile phase affected the peak shape, with 62% generating the best result. The ionization of enalapril, enalaprilat and benazepril was increased by adding formic acid in the mobile phase. In the tested concentration range of formic acid, 0.1–0.5%, both analytes and IS were found to have highest response in the mobile phase with 0.2% formic acid. Finally, a mobile phase consisting of methanol–water–formic acid (62:38:0.2, v/v/v) was used. Although the chromatography was performed on an HPLC column (not under UPLC conditions), the small dead volume of Acquity system benefits the separation efficiency and run time. Under the optimal conditions, the total run time for each sample was only 2.5 min, with symmetric peak shape and high sensitivity.

3.2. Sample preparation procedure

Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are techniques often used in the preparation of biological samples for their ability to improve the sensitivity and robustness of assay. SPE was employed in the extract of enalapril and enalaprilat from plasma samples [9] in which the recoveries were not reported. LLE was also reported in the literature [8] for the sample pretreatment of enalapril and enalaprilat in human plasma, the recoveries were only around 65% and 24% for the two compounds, respectively. The significantly different extraction recoveries for enalapril and enalaprilat are due to the difference in hydrophobic character between them. Compare with LLE, the recoveries of enalapril and enalaprilat with PPT [10] were increased but the sensitivity was not satisfactory without a concentrate procedure.

In the present method, a PPT method was adopted which provided high recovery for both analytes and IS. Under the optimal HPLC–MS/MS conditions, the obtained sensitivity was higher than that reported in the literature [10]. Therefore no further concentration procedure was needed, the sample preparation procedure

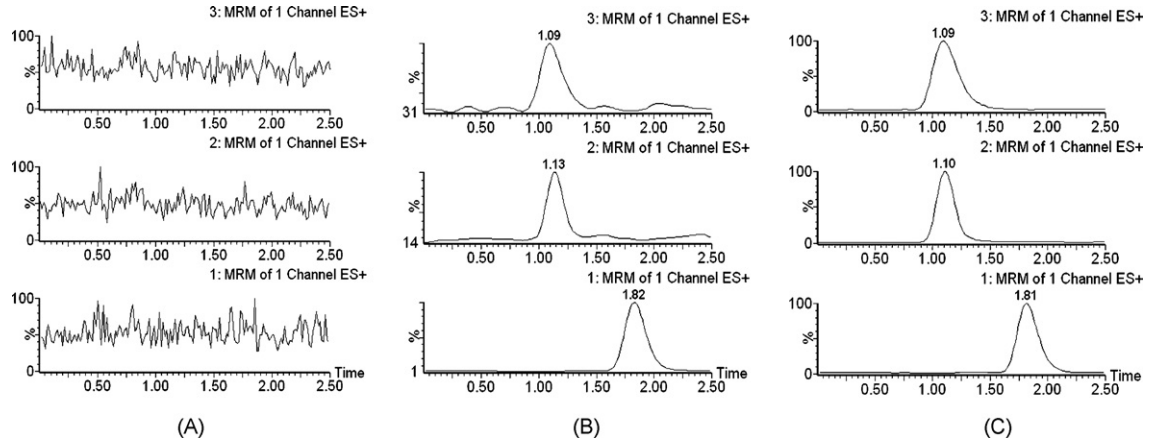

Fig. 2. Representative MRM chromatograms of enalapril (channel 3), enalaprilat (channel 2) and benazepril (channel 1) in human plasma samples. (A) Blank plasma sample; (B) blank plasma sample spiked with enalapril and enalaprilat at the LLOQ of 0.638 ng/ml and IS (31.5 ng/ml); (C) plasma sample from a volunteer 2.0 h after oral administration of enalapril. The retention times for enalapril, enalaprilat and IS were 1.09, 1.10 and 1.81 min, respectively.

was simplified. Both methanol and acetonitrile could be taken as the protein precipitant for they provided equivalent extraction recovery. Methanol was chosen as the precipitant for its better compatibility with mobile phase. IS working solution in methanol was used as protein precipitant, which further simplified the sample pretreatment procedure. In this process, however, the IS may not fully interact with the sample. For comparison another set of samples were prepared by evaporating IS solution to dryness, fully mixing with 200 μl of plasma and then protein-precipitated with 400 μl of methanol. It was found that the recovery of IS (85.0%) was almost the same as that (84.1%) using IS solution as precipitant.

3.3. Method validation

3.3.1. Selectivity

Selectivity was determined by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. As shown in Fig. 2, no interference from endogenous substance was observed at the retention time of enalapril, enlaprilat and benazepril. Carry-over was eliminated by rinsing system. This was demonstrated by analyzing blank samples immediately following the samples at highest concentration.

3.3.2. Linearity and LLOQ

The peak area ratios of analytes to IS in human plasma varied linearly over the concentration range tested (0.638–255 ng/ml). Typical equations for the calibration curves were: $y = 4.50 \times 10^{-2} x + 5.26 \times 10^{-3}$, $r = 0.996$ for enalapril and $y = 2.48 \times 10^{-2} x - 1.07 \times 10^{-4}$, $r = 0.997$ for enalaprilat, respectively. The LLOQ for the two compounds was 0.638 ng/ml in plasma corresponded to an on-column sensitivity of 1.06 pg, which was lower than those reported in literature [5–9,11].

3.3.3. Precision and accuracy

The data of intra- and inter-day precision and accuracy for enalapril and enalaprilat from QC samples are summarized in Tables 1 and 2, respectively. The precision and accuracy of the present method conform to the criteria for the analysis of biological samples according to the guidance of FDA where the R.S.D. determined at each concentration level is required not exceeding 15% (20% for LLOQ) and R.E. within ±15% (±20% for LLOQ) of the actual value [12].

Table 1
Precision and accuracy for determination of enalapril in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days).

Concentration (ng/ml)		R.S.D. (%)		R.E. (%)
Added	Found (mean ± S.D.)	Intra-day	Inter-day	
0.638	0.661 ± 0.070	8.7	17	3.5
1.53	1.40 ± 0.06	4.5	2.3	−8.7
51.0	48.8 ± 2.1	3.4	8.2	−4.4
204	194 ± 9.5	4.6	6.8	−4.7

3.3.4. Extraction recovery and matrix effect

The extraction recoveries from QC samples at low, middle and high concentrations were 94.9 ± 1.2%, 91.6 ± 0.6%, 82.9 ± 0.9% for enalapril and 100 ± 8.7%, 87.4 ± 4.6%, 83.9 ± 3.8% for enalaprilat, respectively, whereas 84.1 ± 1.5% for IS. The recoveries were much higher than those reported in the literature [8,10] for the two compounds.

In terms of matrix effect, all the ratios defined as in Section 2 were between 85% and 115%. No significant matrix effect for enalapril and enalaprilat was observed indicating that no co-eluting substance could influence the ionization of the analytes.

3.3.5. Stability study

The results from all stability tests are presented in Table 3, which demonstrate a good stability of enalapril and enalaprilat over all steps of the determination. The method is therefore proved to be applicable for routine analysis.

3.4. Pharmacokinetic application

This validated HPLC–MS/MS method was successfully applied to a pharmacokinetic study of enalapril capsule in 20 healthy male volunteers following oral administration of 10 mg enalapril. Mean

Table 2
Precision and accuracy for determination of enalaprilat in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days).

Concentration (ng/ml)		R.S.D. (%)		R.E. (%)
Added	Found (mean ± S.D.)	Intra-day	Inter-day	
0.638	0.637 ± 0.070	9.0	18	−0.2
1.53	1.51 ± 0.13	7.2	14	−1.1
51.0	49.6 ± 2.1	4.2	9.7	−2.8
204	193 ± 12	6.4	5.6	−5.5

Table 3
Stability of enalapril and enalaprilat in plasma samples ($n = 6$).

Stability	Concentration found (mean ± S.D.)	
	1.53 (ng/ml)	204 (ng/ml)
Enalapril		
Three freeze–thaw cycles	1.48 ± 0.14	214 ± 6.6
Long-term (−20 °C for 50 days)	1.46 ± 0.12	213 ± 7.8
Short-term (room temperature for 4 h)	1.45 ± 0.10	210 ± 9.7
Post-preparative (4 °C for 8 h)	1.46 ± 0.13	197 ± 6.4
Enalaprilat		
Three freeze–thaw cycles	1.48 ± 0.13	215 ± 6.5
Long-term (−20 °C for 50 days)	1.54 ± 0.08	221 ± 5.9
Short-term (room temperature for 4 h)	1.58 ± 0.11	214 ± 11
Post-preparative (4 °C for 8 h)	1.65 ± 0.14	206 ± 0.14

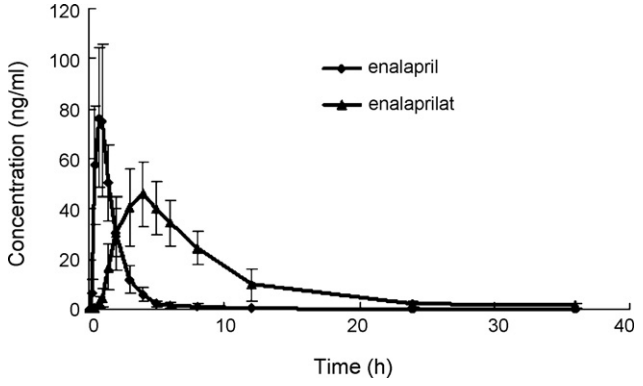

Fig. 3. Mean plasma concentration–time profile of enalapril and enalaprilat after oral administration of two enalapril maleate capsules (containing 50 mg each) to 20 healthy male Chinese volunteers (each point represents mean ± S.D.).

plasma concentration–time curve of enalapril and enalaprilat in single dose study is shown in Fig. 3.

The maximum plasma concentration (C_{max}) was 90.5 ± 28.4 and 47.5 ± 12.4 ng/ml, the time of maximum plasma concentration (T_{max}) was 0.860 ± 0.310 and 4.20 ± 1.06 h, the area under the plasma concentration–time curve from 0 h to the time of last measurable concentration (AUC_{0-t}) was 136 ± 36 and 401 ± 89 ng/ml h, area under the plasma concentration–time curve from 0 h to infinity ($AUC_{0-\infty}$) was 138 ± 36 and 420 ± 91 ng/ml h, the half-life of drug elimination at the terminal phase ($t_{1/2}$) was 1.35 ± 0.61 and 6.71 ± 2.22 h for enalapril and enalaprilat, respectively. These parameters were in accordance with those reported in the literatures [5–11].

4. Conclusion

A sensitive, selective and rapid HPLC–MS/MS method for simultaneous determination of enalapril and its major active metabolite enalaprilat in human plasma is described. Comparing with the analytical methods reported previously, the method proved to be superior with respect to the on-column sensitivity, chromatographic analysis time and extraction recovery. The method has been successfully applied to the pharmacokinetic study of enalapril given in capsule form to healthy volunteers.

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