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Short communication

Simultaneous determination of enalapril and enalaprilat in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A rapid, sensitive, and highly selective liquid chromatography–tandem mass spectrometry method was developed and validated for simultaneous determination of enalapril and its major active metabolite enalaprilat in human plasma. The analytes were extracted from plasma samples by liquid–liquid extraction, separated on a Zorbax Extend-C_{18} column, and detected by tandem mass spectrometry with a Turbo IonSpray ionization interface. The method has a lower limit of quantification (LLOQ) of 0.1 ng/ml for both enalapril and enalaprilat. The chromatographic run time was approximately 3.5 min. The standard calibration curves for both enalapril and enalaprilat were linear in the concentration ranges of 0.10–100.0 ng/ml in human plasma. The intra- and inter-run precisions, expressed as the relative standard deviation (R.S.D.), were less than 7.7 and 7.8%, determined from QC samples for enalapril and enalaprilat, and accuracy was within ±3.9 and ±2.7% in terms of relative error, respectively. The method was successfully applied for the evaluation of the pharmacokinetics of enalapril and enalaprilat in 20 volunteers after an oral dose of 10 mg enalapril maleate.

Keywords: Enalapril; Enalaprilat

1. Introduction

Enalapril, *N*-[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl-L-proline, is a prodrug that requires de-esterification to an active diacid metabolite enalaprilat [1]. Enalaprilat is an active angiotensin converting enzyme (ACE) inhibitor that has been shown to be effective in the treatment of hypertension and congestive heart failure by dilating peripheral vascular resistance without causing significant changes in heart rate or cardiac output [1–4]. Following oral administration of enalapril in healthy subjects, absorption is rapid. The terminal half-life of enalapril is approximately 2 h after a single oral dose of 10 mg, maximum plasma concentrations of enalapril are reached in approximately 1 h and it is not detected above 10 ng after 4 h. However, enalaprilat is detectable for up to 72 h and has a half-life of approximately 30–35 h [1,3]. Therefore, an analytical method with sufficient sensitivity and selectivity is needed to monitor the plasma concentrations after an oral administration for enalapril for 12 and 72 h for enalaprilat.

A wide variety of analytical techniques have been applied to the quantification of enalapril and enalaprilat in biological fluids, including gas chromatography–mass spectrometry (GC–MS) [5], radioimmunoassay (RIA) [6,7], enzyme-linked immunosorbent assay (ELISA) [8], and time-resolved-fluoroimmunoassay (TR-FIA) [9]. However, these methods all have their own disadvantages. RIA, ELISA, and TR-FIA were sensitive, but preparations of antibodies were needed and the cross-reactions with structurally similar compounds were often observed. GC–MS has been developed for the simultaneous quantification of enalapril and enalaprilat in

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human plasma down to 0.2 ng/ml. Although, the sensitivity of this method is adequate for the pharmacokinetic studies of enalapril and enalaprilat, the derivatization step makes it a complicated and time-consuming procedure. Recently, Lee et al. [10] described an LC–MS–MS method for simultaneous determination of enalapril and enalaprilat in plasma. Plasma samples were extracted by solid-phase extraction and analyzed by gradient HPLC. The chromatographic run time was approximately 5.5 min, and the lower limit of quantification (LLOQ) for enalapril and enalaprilat were 0.2 and 1.0 ng/ml, respectively. It permitted plasma drug monitoring for only 12 h after an oral administration. Although, this could be sufficient for pharmacokinetic study of enalapril, as the terminal half-life of enalaprilat is about 30–35 h after a single oral dose of enalapril 10 mg, it is not sufficient for pharmacokinetic study of enalaprilat.

In this study, we describe a rapid, sensitive, and selective high-performance liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS) method for the simultaneous quantification of enalapril and enalaprilat in human plasma using daidzein as the internal standard (I.S.). The chromatographic run time is approximately 3.5 min. The method has a lower LLOQ of 0.1 ng/ml for both enalapril and enalaprilat. It allows plasma drug monitoring for at least 72 h after oral administration. The method was successfully applied for the evaluation of the pharmacokinetics of enalapril and enalaprilat in 20 volunteers after an oral dose of 10 mg enalapril maleate.

2. Experimental

2.1. Chemicals and reagents

Enalapril (99.5% purity) and enalaprilat (99.3% purity) were obtained from the Changhang Drug Research Ltd. Co. (Huhehot, Inner Mongolia, China), and daidzein (internal standard, 99.1% purity) was purchased from Huike Botanical (Xi'an, Shaanxi, China). Methanol (Yuwang Chemical, Shandong, China) was of HPLC-grade, and other chemicals used were of analytical grade. Blank (drug free) human plasma was obtained from Shenyang Blood Donor Service (Liaoning, China). Distilled water, prepared from demineralized water, was used throughout the study. Plasma was obtained by centrifugation of blood treated with the anticoagulant sodium heparin and stored at −20 °C until analysis.

2.2. Instrumentation

An API 4000 triple quadrupole mass spectrometer (Applied Biosystems-SCIEX, Concord, Ontario, Canada) equipped with a Turbo IonSpray source and an HP-1100 pump, an HP-1100 autosampler (Aglient, Wilminton, DE, USA) were used for LC–MS–MS analyses. The data processing was carried out using Analyst 1.3 software (Applied Biosystems-SCIEX).

2.3. Standards and quality control samples

Stock solutions of enalapril and enalaprilat were prepared by dissolving the accurately weighed reference compounds in methanol to give a final concentration of 400 μg/ml of both. The solution was then serially diluted with mobile phase to achieve standard working solutions at concentrations of 0.5, 1.2, 4.0, 15, 50, 100, 250, and 500 ng/ml for both enalapril and enalaprilat. Stock solution of I.S. was prepared in methanol at the concentration of 400 μg/ml and diluted to 4 μg/ml with water. Structural formulae of enalapril, enalaprilat, and daidzein are shown in Fig. 1. All solutions were stored at 4 °C and were brought to room temperature before use.

For the preparation of standard curves or quality control samples, the standard working solutions (100 μl) were used to spike blank plasma samples (500 μl), both in prestudy validation and during the analysis of samples from the pharmacokinetic study.

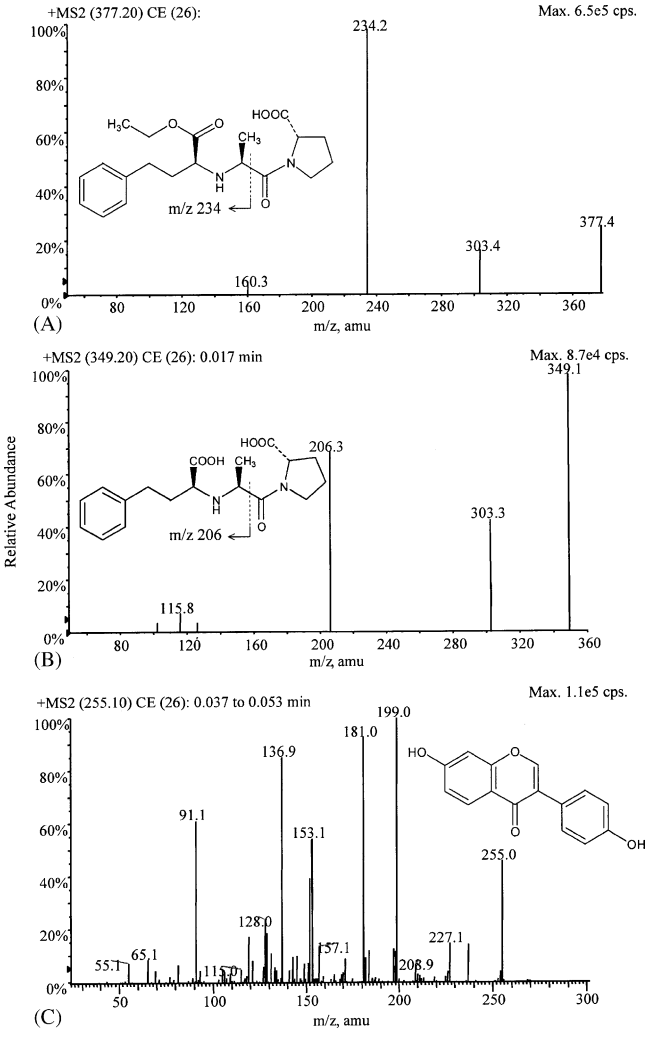

Fig. 1. Product ion spectra of $[M+H]^+$ of enalapril (A), enalaprilat (B) and daidzein (C).

2.4. Sample preparation

To a 0.5-ml aliquot of plasma sample, 100 μl of internal standard (4 μg/ml daidzein in water), 100 μl of phosphoric acid (0.5 M), and 100 μl of mobile phase were added. The samples were briefly mixed and 3 ml of ethyl acetate were added. The mixture was vortex-mixed for approximately 1 min and placed on a mechanical shaker for 10 min. After centrifugation at 2000 × *g* for 5 min, the upper organic layer was removed and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dry residues were reconstituted with 100 μl of the mobile phase and vortex-mixed. A 20-μl aliquot of the solution was injected onto the LC–MS–MS system for analysis.

2.5. Chromatographic conditions

Chromatography was performed on a Zorbax Extend-C_{18} analytical column (150 mm × 4.6 mm i.d., 5 μm, Agilent, Wilminton, DE, USA) a SecurityGuard C_{18} guard column (4 mm × 3.0 mm i.d., Phenomenex, Torrance, CA, USA). The column was maintained at 30°. The compounds were eluted isocratically at a flow rate of 0.6 ml/min. The mobile phase consisted of methanol–water–formic acid (70:30:1, v/v/v).

2.6. Mass spectrometric conditions

The mass spectrometer was operated in the positive ion detection mode. Nitrogen was used as the nebulizing, turbo spray, and curtain gas, with the optimum values set at 55, 35, and 10 psi, respectively. The temperature of the vaporizer was set at 400 °C and ESI needle voltage was adjusted to 5000 V. The declustering potential were set at 65, 60, and 50 V for enalapril, enalaprilat, and daidzein, respectively. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of *m/z* 377 → *m/z* 234 for enalapril, *m/z* 349 → *m/z* 206 for enalaprilat and *m/z* 255 → *m/z* 199 for daidzein (I.S.), respectively, with a dwell time of 200 ms per transition. Fig. 1 shows the product ion spectra of $[M+H]^+$ of enalapril, enalaprilat and daidzein. For collision-induced dissociation (CID), nitrogen was used as the collision gas at a pressure of 4 Pa. The optimized collision energy was 26 eV. The mass spectrometer was operated at unit mass resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3.

2.7. Method validation

Plasma samples were quantified using the peak area ratios of enalapril and enalaprilat to that of the I.S. Peak area ratios were plotted against concentrations of enalapril and enalaprilat, respectively. The sample concentrations were calculated using weighted ($1/x^2$) least squares linear regression.

To evaluate linearity, plasma calibration curves were prepared and were assayed in duplicate on three separate days. Accuracy and precision were also assessed by determining QC samples at three concentration levels on three different validation days. The accuracy was expressed by (mean observed concentration)/(spiked concentration) × 100% and the precision by relative standard deviation (R.S.D.).

The extraction recoveries of enalapril and enalaprilat at three QC levels were evaluated by comparing peak areas of analytes obtained from plasma samples with the analytes spiked before extraction to those spiked after the extraction. The matrix effect experiments were carried out by extracting blank plasma from six different sources, reconstituting the final extract in mobile phase containing a known amount of the analytes, analyzing the reconstituted extracts and then comparing the peak areas of the analytes with those of standard solutions in the mobile phase.

The stability of enalapril and enalaprilat in the reconstituted solution was assessed by placing QC samples under ambient conditions for 24 h. The freeze–thaw stability of enalapril and enalaprilat was also assessed by analyzing QC samples undergoing three freeze (−20 °C)–thaw (room temperature) cycles. Subsequently, the enalapril and enalaprilat concentrations were measured and compared with freshly prepared samples, respectively.

2.8. Application

To demonstrate the reliability of this method for the study of pharmacokinetics of enalapril and enalaprilat, it was used to determine concentrations of both in plasma samples 0–72 h after administration of 10 mg enalapril maleate to 20 healthy volunteers. Blood samples were drawn in heparinized tubes at 0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 13, 24, 36, 48, and 72 h after oral administration. The obtained plasma samples were immediately separated by centrifugation at 2000 × g for 10 min and stored frozen at −20 °C until analysis.

3. Results and discussion

3.1. Sample preparation

Lee et al. [10] used a 96-well solid-phase extraction cartridge to extract enalapril and its metabolite from plasma samples, in which the recovery were not reported. In the present experiment, we attempted to use another method. Precipitating plasma protein by organic solvent (methanol, acetonitrile) or trichloroacetic acid can be accompanied by interference of large amounts of endogenous substances during detection, especially in long-term use. Moreover, samples are also diluted by this method. Therefore, a liquid–liquid extraction method was evaluated. Due to the differences of hydrophobic character and pK_a value between enalapril and enalaprilate, it is difficult to obtain the identical extraction recoveries for them. Under the present LC–MS–MS conditions, MS response of enalapril was about five times more sensitive than that of enalaprilat, therefore, we optimized the extraction condition for a higher recovery of enalaprilat. Since, enalapril and enalaprilat (diacid metabolite) are both acidic

compounds, pH value of the plasma samples is a key factor for extraction. The effect of pH (pH 2, 3, 4, and 5 in plasma) was tested during development of the extraction procedure. Enalapril is easily extracted from plasma samples adjusted to pH 4–7. The best yield of extraction (75–80%) was obtained at pH 5. Whereas, enalaprilat could be extracted from plasma at lower pH levels. And the optimal pH value to extract enalaprilat is 3, which could provide not only high recovery for the metabolite, but also reproducible recovery for parent drug. Four different solvents were also investigated: diethyl ether, ethyl acetate, diethyl ether–dichloromethane (60:40, v/v), and *n*-hexane–dichloromethane–isopropanol (20:10:1, v/v/v) with ethyl acetate generating the best result. In addition, it was found that neither increase of extraction solvent volume (>3 ml) and/or extraction time (>10 min) nor repeating the extraction increase the recovery of enalapril and enlaprilate.

3.2. LC–MS–MS analysis

Although, enalapril and enalaprilat are both acidic compounds, the signal intensities obtained in the positive mode was much higher than that obtained in the negative mode. Initially, enalapril and enalaprilat responses to APCI and ESI were evaluated by recording direct inlet full-scan mass spectra in the positive ionization mode, respectively. ESI spectra revealed higher signals for *m*/*z* 377 (enalapril) and *m*/*z* 349 (enalaprilat) compared to the APCI source. Further assay development was, therefore, limited to the ESI source. The full scan spectra were dominated by protonated molecules $[M+H]^+$, and no significant fragments ions were observed. The collision-induced dissociation (CID) of protonated enalapril (*m*/*z* 377; $[M+H]^+$) resulted in the loss of the proline moiety, leading to the main fragment ion at *m*/*z* 234 (Fig. 1A). Other fragmentation showed relative intensities below 25% and were not likely to improve the sensitivity when used in MRM mode. A similar fragment pathway for enalaprilat was also observed: the major fragment ion was *m*/*z* 206. Fig. 1B shows the MS–MS CID full scan spectrum of enalaprilat. The protonated molecules (*m*/*z* 349; $[M+H]^+$) was still the base peak and the collision energy had been enhanced, but it only increased the response of other fragment ions. Additional tuning of the ESI source and CID parameters onto the transition *m*/*z* 377 → *m*/*z* 234 (enalapril) and *m*/*z* 349 → *m*/*z* 206 (enalaprilat) further improved the sensitivity.

Although, the stable isotope labeled compounds of the analytes would be the ideal I.S., structurally related compounds were commercially available with a high chemical similarity to the analytes and were expected to achieve similar chromatographic behaviors, mass spectrometric behaviors and extraction characteristics. Our first attempt used ramipril and ramiprilat as the I.S. of enalapril and enalaprilat, respectively. However, it was found that, in our method, the recovery of ramiprilat was low and variable. On the other hand, ramipril had a good and stable recovery, but the peak broadening and tailing presented a new problem. Although, it could be solved by using a high column temperature (60 °C) [11], the bonded stationary phases tend to deteriorate rapidly at high column temperatures. Next, we attempted to use daidzein as the I.S., which is also an acid. It was found that daidzein had an adequate and stable recovery, gave a symmetric and sharp peak, and had a similar retention time to the analytes. In the positive ESI mode, daidzein formed predominately the protonated molecule $[M+H]^+$ in full scan spectrum. To determine daidzein using the MRM mode, full scan and product ion spectra of daidzein were investigated under the present HPLC conditions. Fig. 1C shows product ion spectrum of $[M+H]^+$ ions of daidzein. Several fragment ions were observed in the product ion spectrum. The major fragment ion at *m*/*z* 199 was chosen in the MRM acquisition for daidzein.

3.3. Chromatographic conditions

The chromatographic conditions were investigated to optimize sensitivity, speed, and peak shape. Methanol was chosen as the organic solvent because it provided a higher sensitivity and lower background noise than acetonitrile. The compositions of mobile phase were optimized by flow injection analyses with varying percentages of methanol. It was found that high organic solvent content (about 70%) in HPLC system decreased the background noise and provided rapid separation and stable MS signal throughout the analytical run, allowing an enhancement of sensitivity. But when the percentage of methanol was increased to 80%, the reproducibility decreased. It was also found that the presence of a low amount of formic acid in the mobile phase could improve the sensitivity by promoting the ionization of the analytes. To achieve symmetrical peak shapes, a short chromatographic analysis time, and to eliminate the matrix effect, a mobile phase consisting of methanol–water–formic acid (70:30:1, v/v/v) was used in the experiment.

Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with enalapril and enalaprilat at the LLOQ and daidzein, and a plasma sample from a healthy volunteer 6 h after an oral administration. There is no interference from endogenous substances with the analytes and I.S. Typical retention times for enalapril, enalaprilat and daidzein were 2.5, 2.5, and 3.2 min, respectively. The total run time was about 3.5 min.

No matrix effect for enalapril (R.S.D. = 3.8%) and enalaprilat (R.S.D. = 7.8%) was observed for the six different plasma pools indicating that no undetected co-eluting compounds that could influence the ionization of the analytes.

3.4. Linearity, precision, and accuracy

Visual inspection of the plotted duplicate calibration curves and correlation coefficients >0.99 confirmed that the calibration curves of enalapril and enalaprilat were linear over the concentration range of 0.1–100 ng/ml, respectively. The relative calibration graphs are given, respectively, by the equations: $y = (5.652 \pm 0.794) \times 10^{-3} + (1.427 \pm 0.238) \times$

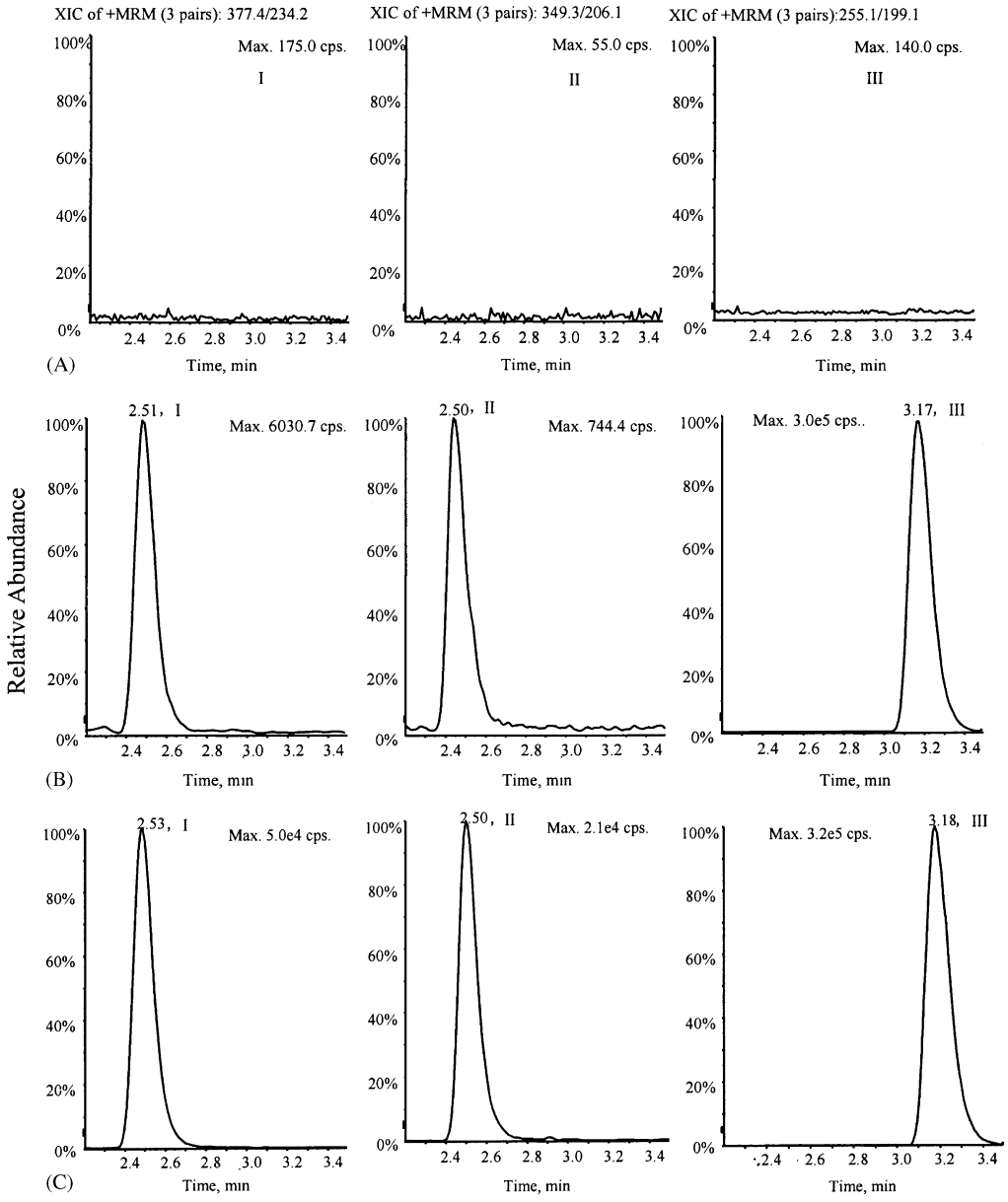

Fig. 2. MRM chromatograms of enalapril (I), enalaprilat (II), and daidzein (III) in human plasma samples. (A) Blank plasma sample; (B) plasma sample spiked with enalapril (0.1 ng/ml), enalaprilat (0.1 ng/ml) and I.S. (800.0 ng/ml); (C) volunteer plasma sample 6 h after oral dose of 10 mg enalapril maleate.

$10^{-1}x$ for enalapril and $y = (1.199 \pm 0.289) \times 10^{-3} + (3.961 \pm 0.398) \times 10^{-3}x$ for enalaprilat. The method showed very good precision and accuracy. Tables 1 and 2 summarize the intra- and inter-day precisions and accuracies for enalapril and enalaprilat from QC samples, respectively.

In this assay, the intra- and inter-run precisions were less than 7.7 and 7.8%, and accuracies were within ±3.9 and ±2.7%, as determined from QC samples for enalapril and enalaprilat, respectively. The results, calculated using one-way ANOVA, indicated that the values were within the

Table 1
Precision and accuracy results for enalapril in human plasma ($n = 3$ days, six replicates per day)

Added C (ng/ml)	Found C (ng/ml)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Relative error (%)
0.24	0.24 ± 0.02	7.7	5.0	1.0
10.0	10.4 ± 0.50	5.0	1.8	3.9
90.0	87.5 ± 3.10	3.5	3.8	−2.8

Table 2
Precision and accuracy results for enalaprilat in human plasma ($n = 3$ days, six replicates per day)

Added C (ng/ml)	Found C (ng/ml)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Relative error (%)
0.24	0.24 ± 0.01	4.7	5.3	0.5
10.0	10.0 ± 0.44	3.8	7.8	−0.5
90.0	92.4 ± 4.51	5.2	1.3	2.7

acceptable range and the method was accurate and precise [12].

The LLOQ was defined as the lowest concentration on the calibration curve, which was established using six replicate preparations of plasma samples. The precision and accuracy from LLOQ samples were 8.7 and −4.4% for enalapril and 9.8 and 12.1% for enalaprilat, respectively. The results showed that the LLOQ offered by the present LC–MS–MS was 0.1 ng/ml for both enalapril and enalaprilat, which was more sensitive than the reported methods [5–10].

3.5. Extraction recovery and storage stability

The recoveries observed (value ± S.D., $n = 6$) were 62.4 ± 3.3, 62.4 ± 3.5 and 67.8 ± 2.3% (0.24, 10, and 90 ng/ml, respectively) for enalapril, 22.3 ± 0.3, 24.3 ± 1.0, and 26.3 ± 1.1% (0.24, 10, and 90 ng/ml, respectively) for enalaprilat, and 86.4 ± 5.6% for I.S. (800 ng/ml). Enalaprilat gave by far the lowest recovery, but since, reproducibility was evaluated positively, the relative standard deviation of different concentrations were all below 2.0%, this result was considered acceptable, especially taking into account the adequate LLOQ.

Stability analysis was carried out with plasma quality control samples (0.24, 10, and 90 ng/ml, respectively). All samples showed no significant degradation under the conditions previously described in Section 2.

3.6. Application

The method was applied to determine the plasma concentration of enalapril and enalaprilat after an oral administration of enalapril maleate (10 mg) to 20 healthy volunteers. Mean plasma concentration–time profiles of enalapril and enalaprilat are presented in Fig. 3. The main pharmacokinetic parameters of enalapril and enalaprilat in 20 volunteers were calculated. After oral administration of 10 mg enalapril, the mean C_{max} values for enalapril maleate and enalaprilat were 113.9 ± 42.3 and 34.5 ± 14.8 ng/ml, respectively. Corresponding mean T_{max} values were 0.88 ± 0.36 h for enalapril and 3.95 ± 0.95 h for enalaprilat, respectively. The mean plasma elimination half-life of enalapril was 1.83 ± 0.73 h and for enalaprilat was 29.2 ± 12.8 h. These results are consistent with previous determinations of enalaprilat pharmacokinetics after oral enalapril administration [1,3,13]. Due to lack of sensitive analytical techniques, enalapril was undetectable 4 h postdose in previous reports [1,3,13]. In the present study, the plasma concentration of enalapril could be detected up to 12 h.

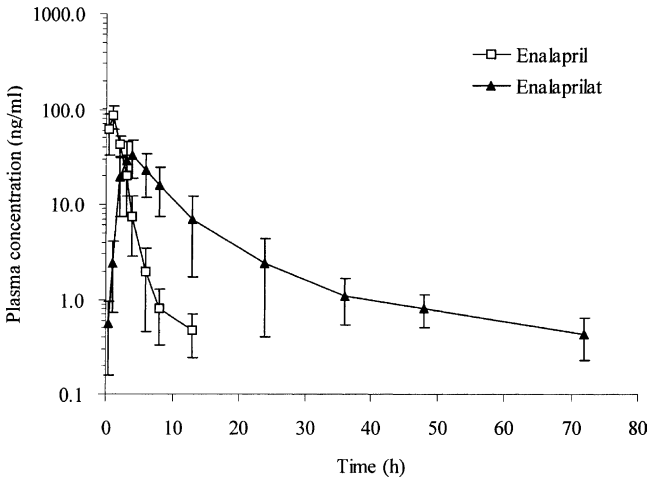

Fig. 3. Mean plasma concentration–time profile of enalapril and enalaprilat after an oral administration of 10 mg enalapril maleate to 20 healthy volunteers.

4. Conclusions

An LC–MS–MS method was developed and validated for the simultaneous determination of enalapril and its major active metabolite enalaprilat in human plasma. The method is rapid, sensitive, and selective with an LLOQ of 0.1 ng/ml for both enalapril and enalaprilat using 0.5 ml of human plasma. It allows the determination of enalaprilat up to 72 h after an oral administration. The short chromatographic cycle time (3.5 min) allowed rapid analysis with minimal matrix interference. The method proved to be superior with respect to sensitivity, selectivity and speed of analysis, compared with the analytical methods reported previously. The method was successfully applied for the evaluation of the pharmacokinetics of enalapril and enalaprilat in 20 volunteers after an oral dose of 10 mg enalapril maleate.

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