Iranian Journal of Pharmaceutical Sciences
2016: 12 (1):2 1- 34
www.ijps.ir

Original Article

A quick and Sensitive Liquid Chromatography–Tandem Mass Spectrometry (LC-MS) Method for the Determination of Enalapril and Enalaprilat in Human Plasma: Application to a Bioequivalence Study

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Abstract

A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC-MS) method was developed for the estimation of enalapril and enalaprilat in human plasma. Detection of analytes was achieved by tandem mass spectrometry with electrospray ionization (ESI) interface in positive ion mode was operated under the multiple-reaction monitoring mode. Sample pretreatment involved in a one-step protein precipitation (PPT) with percholoric acid ($HClO_4$) of 0.15ml plasma. The reconstituted samples were chromatographed on C_{18} column by pumping methanol: water: acid formic74:24:2 (v/v) at a flow rate of 0.2 mL/min. Each plasma sample was chromatographed within 1.25 min. The standard curves were found to be linear in the range of 0.1–20 ng/mL for enalapril and enalaprilat with mean correlation coefficient of ≥0.999 for each analyte. The intra-day and inter-day precision and accuracy results were well within the acceptable limits. The limit of quantification (LOQ) was 0.1 ng/ml for enalapril and enalaprilat. The mean (SD) C_{max}, T_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values of enalapril versus enalaprilat after administration of the 10 mg enalapril are 141.33(3.51) versus73.33 (5.03) ng/mL, 1.15(1.45) versus 4.12 (1.74) hours, 142.57 (34.34) versus 425.94(13.09) ng/mL/h, and 150.74 (16.69) versus 455.80 (65.11) ng/mL/h respectively. The mean (SD) $t_{1/2}$ was 2.72 (2.01) hours for the enalapril and 6.34 (2.13) hours for the enalaprilat. The developed assay method was successfully applied to a pharmacokinetic study in human male volunteers.

Keywords: Enalapril, Enalaprilate, Human plasma, LC-MS.

1. Introduction

Enalapril, N-[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl]-l-proline (Figure 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 (Figure 1B), is the major metabolite of enalapril and has been shown

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Cite this article as: Danafar H, Hamidi M, A quick and sensitive Liquid Chromatography–Tandem Mass Spectrometry (LC-MS) Method for the Determination of Enalapril and Enalaprilat in Human Plasma: Application to a Bioequivalence Study. Iranian Journal of Pharmaceutical Sciences, 2016, 12 (1): 21-34.

to be effective in the treatment of hypertension and congestive heart failure without causing significant side effects [1–4]. Enalapril and enalaprilat are often determined simultaneously in biological fluids. Therefore, the simultaneous detection of enalapril and enalaprilat in human plasma is of prime importance for pharmacokinetic studies. 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 (>3.5min), large volume of plasma sample (>0.5ml), or low extraction recovery may not meet the requirement for high throughput, speed and sensitivity in bio sample analysis for quantitative analysis. Although these problems could be solved by using both low pH and high column temperature, [12, 13] these reported HPLC methods are not adequate for pharmacokinetic studies due to relatively high detection limits [14-16]. As a result, a simple method that can simultaneously determine enalapril and enalaprilat in human plasma was required. The pervious our work was determination of ezetimibe, amlodipine and atorvastatin by LC–MS method in human plasma [17-18]. Our aim was to develop and validate a simple and rapid LC–MS method for the quantification of enalaprilat and enalapril in human plasma. The method has been used effectively during a bioequivalence revise on a generic product of the drug with the representative results being accessible in the final part of the article. The developed assay method was successfully applied to a pharmacokinetic study in human male volunteers after oral administration of 10 mg enalapril.

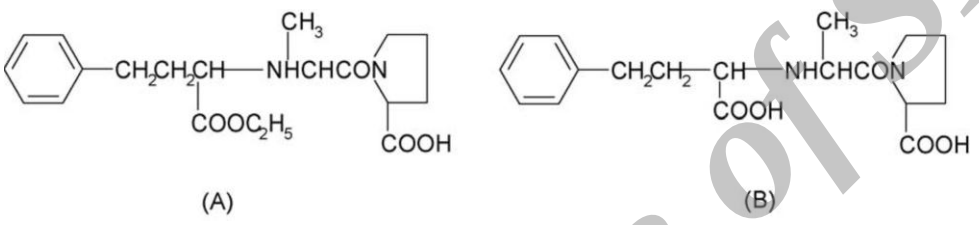


Figure 1. Structural formulae for (A) enalapril) and (B) enalaprilat.

2. Materials and Methods

2.1. Materials

Enalaprilat and enalapril maleate USP Reference standards (USPC Inc., Rockville, MD) were kindly donated by Dr Abidi Pharmaceutical Co. (Tehran, Iran).Other chemicals and solvents were from chemical lab or HPLC purity grades, whenever needed, and were purchased locally. Drug-free human plasma was provided by Iranian

Blood Transfusion Organization after routine safety evaluations.

2.2. Instrumentation and Operating Conditions

2.2.1. Liquid Chromatography

Liquid chromatography was performed using an Agilent LC-1200 HPLC system consisting of an auto sampler (agilent). The column was a Zorbax XDB-ODS C18 column (2.1mm×30mm, 3.5 micron) and was operated at 25◦C.The mobile phase including methanol: water: acid formic 74:24:2 (v/v)was used at a flow rate of 0.2 ml/min.

2.2.2. Mass Spectrometry

Mass spectrometric detection was performed using an Agilent LCMS-6410 quadrupole mass spectrometer with an electrospray ionization (ESI) interface. The ESI source was set at positive ionization mode. The mass selective detector was used in the multiple reaction monitoring (MRM) mode for the highest possible selectivity and sensitivity. The MS operating conditions were optimized as follows: Ion spray voltage was set to 4000V, temperature of the ion transfer capillary was 250 ◦C, Nebulizer gas (NEB) was 30psi, Dwell time per transition (ms) 200, Gas flow 8 l/min, Collision gas for enalapril and enalaprilat 20. Quantitative determinations were performed in multiple reactions monitoring scan mode using the following transitions: m/z 377→234forenalapril, m/z 349→206 for enalaprilat. The quantification was performed via peak-area. Data acquisition and processing were accomplished using Agilent LC-MS solution Software forLCMS-6410 system.

2.3. Standard Preparation

A stock solution of 0.2 mg/ml enalapril and enalaprilat in methanol were prepared, from which the concentrations of 0.1, 0.5, 0.1, 2.5, 5 and 10ng/ml for enalapril and enalaprilat were prepared by serially diluting this solution with the proper amount of mobile phase and plasma.

2.4. Sample Preparation and Extraction Procedure

To 150µL calibration standards, QC samples, or plasma samples, 50µL perchloric acid was added. The mixtures were vortex mixed for 20 s. After centrifugation at 15 000×g in microcentrifuge tubes for 20 min, aliquot of 10 µl was injected into the LC–MS system.

2.5. Analysis Validation Tests

2.5.1. Standard Curve (Linear Range)

The plasma samples with a series of known concentrations, prepared as described, were analyzed in three separate runs and, in each case, the linear regression analysis was carried out on known concentrations of enalapril and enalaprilat against the corresponding peak heights and, then, the regression coefficient (r), slope, and y-intercept of the resulting calibration curves were determined.

2.5.2. Within-run Variations

In one run, three samples with concentrations of 0.1, 10, and 20ng/ml (from high, middle, and low regions of the standard curve) for enalapril and enalaprilat were prepared in triplicate and analyzed by developed LC-Mass method. Then, the coefficient of variations (CV %) of the corresponding determined concentrations were calculated in each case.

2.5.3. Between-run Variations

On three different runs, samples from upper, intermediate, and lower concentration regions used for construction of standard curve (the same as within-run variations test) were prepared and analyzed by LC-Mass method. Then, the corresponding CV% values were calculated.

2.5.4. Absolute Recovery (Accuracy)

For each sample tested for within- and between-run variations, the absolute recovery of the method was determined as the percent ratio of the measured concentration (determined using standard curve) to the corresponding nominal added concentration.

2.5.5. Relative Recovery (Matrix Effect)

Three samples with concentrations 0.1, 10, and 20ng/ml (from high, middle, and low regions of the standard curve) for enalapril and enalaprilat were prepared in triplicate and analyzed by developed LC-Mass method. Then, the ratio of the recorded peak heights to the peak heights resulted from the direct injection of the aqueous solutions of enalapril and enalaprilat with the same concentrations were determined as percentage in each case.

2.5.6. *Limits of Detection and Quantitation*

Limit of detection (LOD) of the method was determined as the lowest enalapril and enalaprilat concentration producing a signal-to-noise (S/N) ratio of about 3, 4 respectively. Limit of quantitation (LOQ) was determined as the lowest enalapril and enalaprilat concentration capable of being quantitated with enough accuracy and precision.

2.5.7. Stability

2.5.7.1. Freeze and Thaw Stability

Three concentration levels of QC plasma samples were stored at the storage temperature (−20 ◦C) for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-thaw cycle were repeated twice, then the samples were tested after three freeze (−20 ◦C)-thaw (room temperature) cycles.

2.5.7.2. Short-term temperature stability. Three concentration levels of QC plasma samples were kept at room temperaturefor a period that exceeded the routine preparation time of samples (around 6 h).

2.5.7.3. Long-term Stability.

Three concentration levels of QC plasma samples kept at low temperature (−20 ◦C) were studied for a period of 4 weeks.

2.5.7.4. Post-preparative Stability.

The autosampler stability was conducted reanalyzing extracted QC samples kept under the autosampler conditions (4 ◦C) for 12 h.

2.6. Clinical Study Design

Twelve male subjects were enrolled in a randomized, two-treatment, two-period, single-dose crossover study with a week washout between the first dosing in period I and the first dosing of period II. Single dose study subjects fasted from the night before dosing until 2 h after dosing for each session. 10mgenalapril was administered and blood samples were obtained prior to dose administration (time 0) and at 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 24.0and

48.0 h after the dose. The blood samples were immediately centrifuged at 1600×g for 10 min. The plasma was removed and stored at −20^{0}C until analysis was done.

2.6.1. Pharmacokinetic Study

The pharmacokinetic parameters for enalapril were designed using standard non-compartmental methods. The peak serum concentration (Cmax) and the time to reach it (Tmax) were evaluated from visual examination of the data and used as criteria of the rate of absorption. The apparent elimination rate constant (β) was determinate by linear regression of log-transformed data in the terminal phase of the serum concentration-time profile [19]. The elimination half-life ($t_{1/2}$) was considered by the quotient of 0.693/β. In addition the area under plasma concentration time (AUC0-t) curve was determined by the linear trapezoidal rule from the measured serum concentrations from zero to time of the last quantifiable concentration (Ct). The $AUC_{0-\infty}$, the area under the serum concentration-time curve extrapolated to perpetuity, was designed according to the following equation [19]: $AUC_{0-\infty}= AUC_{0-t} +$ Ct/Kel.

3. Results and Discussion

3.1. Sample Preparation

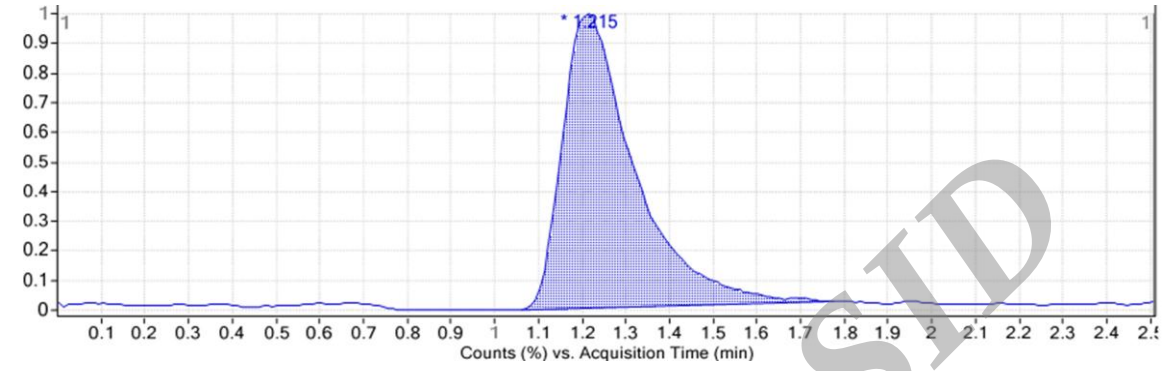
Liquid–liquid extraction (LLE) and solid-phase extraction (SPE (techniques often used in the preparation of biological samples for their ability to improve the sensitivity and robustness of assay. SPE was employed in the extract of enalapril and enalaprilat from plasma samples [9] in which the recoveries 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 protein precipitation [10] were increased but the sensitivity was not satisfactory without a concentrate procedure. In the present method, a protein precipitation method was adopted which provided high recovery for both analytes. Under the optimal LC–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 was simplified. Both methanol and HClO4 could be taken as the protein precipitant for they provided equivalent extraction recovery. HClO4 was chosen as the precipitant for its better compatibility with mobile phase.

3.2. LC–MS Condition Optimization

LC–MS operation parameters were carefully optimized for determination of enalapril and enalaprilat. Themass spectrometerwas 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. 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. The product ion scan spectra showed high abundance fragment ions at m/z 234 and 206 for enalapril and enalaprilat,

respectively. The collision gas pressure and collision energy of collision-induced decomposition (CI) were optimized for maximum response of the fragmentation ofthe two compounds. Multiple reaction monitoring (MRM) usingthe precursor→product ion transition of m/z 377→m/z 234, m/z 349→m/z 206 was employed for quantification of enalapril and enalaprilat, respectively. The multiple-reaction monitoring mode (MRM) (+) chromatograms extracted from

(A)



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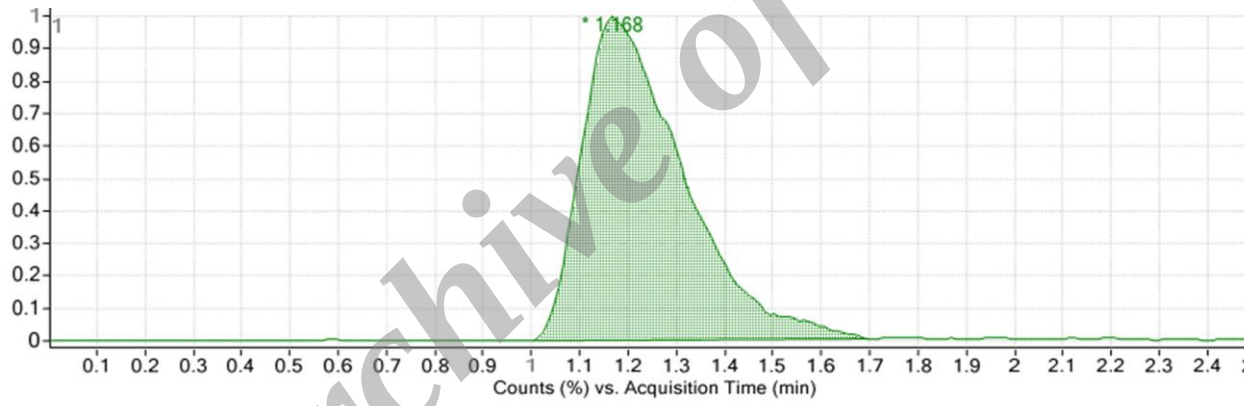

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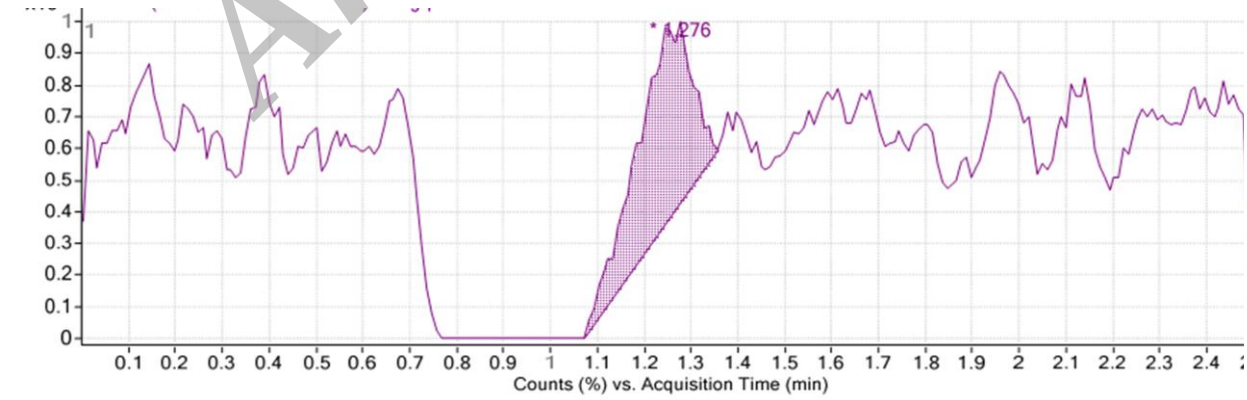

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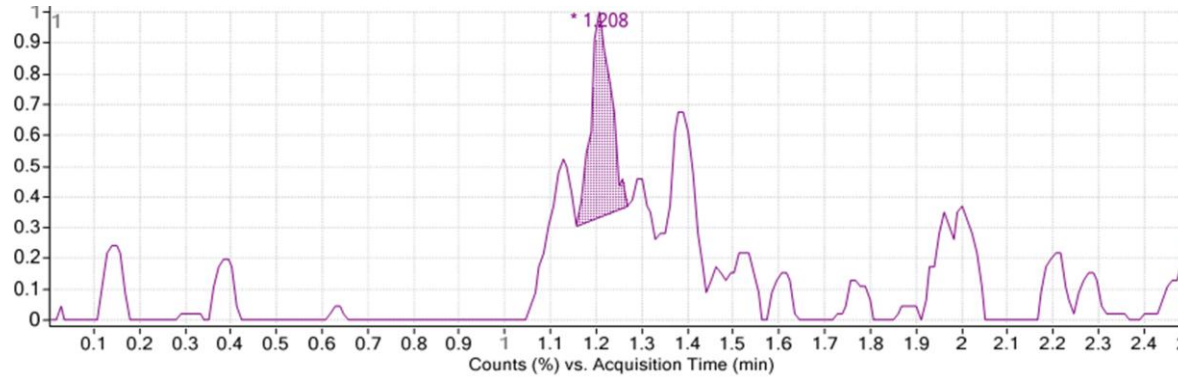

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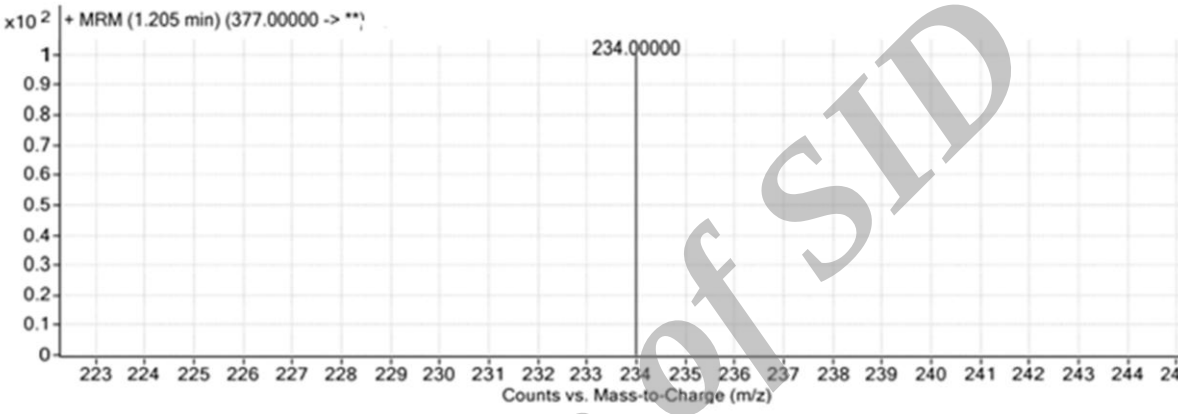

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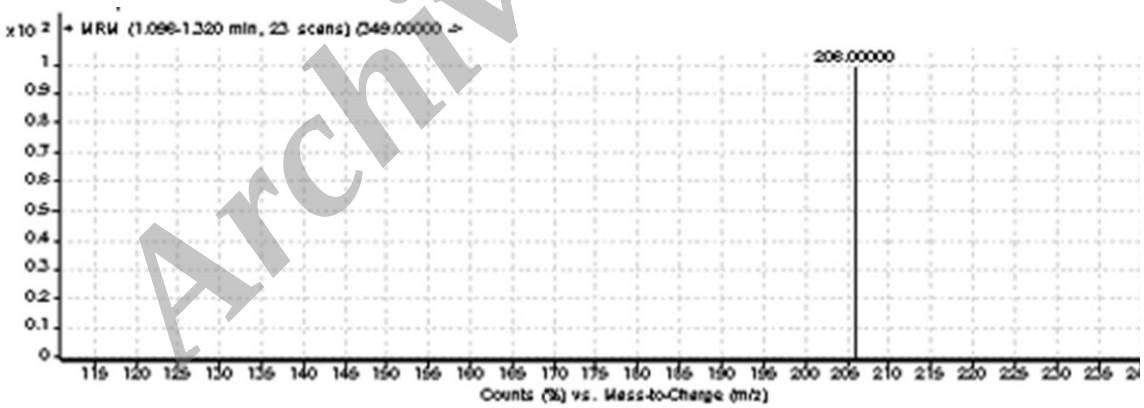

Figure 2. The MRM (+) chromatograms of enalapril and enalaprilat (A) Supplemented plasma (concentration of enalapril = 5 ng/ml). (B) Supplemented plasma (concentration of enalaprilat = 5 ng/ml). (C) LOQ (concentration of enalapril = 0.1 ng/ml).(D) LOQ (concentration of enalaprilat = 0.1 ng/ml). (E) The mass specrum MRM of enalapril (F) The mass specrum MRM of enalaprilat.

supplemented plasma are depicted in figure 2. The retention times of enalapril and enalaprilat were1.23 min. The total HPLC–MS analysis time was 1.25 min per sample.

3.3. Method Validation

3.3.1. Assay Specificity

No interferences of the analytes were observed. The HPLC chromatogram for a blank

Table 1. Within–run variations and accuracy of the LC-Mass method for quantitation of enalapril(n=3) .

Nominal Added Concentration (ng/ml)	Sample Number	Measured Concentration (ng/ml)	Mean (SD)	CV%	Accuracy (%)
0.1	1	0.099	0.098	3.55	98
	2	0.095	(0.0035)		
	3	0.102			
10	1	9.65	9.92	2.4	99.2
	2	10.12	(0.24)		
	3	10.01			
20	1	20.11	19.98	0.6	99.9
	2	19.87	(0.12)		
	3	19.96			

Table 2. Within–run variations and accuracy of the LC-Mass method for quantitation of enalaprilat(n=3) .

Nominal Added Concentration (ng/ml)	Sample Number	Measured Concentration (ng/ml)	Mean (SD)	CV%	Accuracy (%)
0.1	1	0.098	0.1 (0.012)	11.47	100
	2	0.12			
	3	0.10			
10	1	10.25	10.04 (0.18)	1.86	100.4
	2	9.89			
	3	9.98			
20	1	19.51	20.38(0.76)	3.73	101.9
	2	21.02			
	3	20.58			

Table 3. Between–run variations and accuracy of the LC-Mass method for quantitation of enalapril (n=3).

Nominal Added Concentration (ng/ml)	Run Number	Measured Concentration (ng/ml)	Mean (SD)	CV%	Accuracy (%)
0.1	1	0.098	0.097	2.67	**97**
	2	0.102	(0.002)		
	3	0.097			
10	1	10.57	10.18	3.32	**101.18**
	2	9.96	(0.33)		
	3	10.01			
20	**1**	**20.1**	**19.99**	**1.92**	**99.95**
	2	**19.57**	**(0.38)**		
	3	**20.32**			

plasma sampleindicating no endogenous peaks at the retention positions of enalapril and enalaprilat.

3.3.2. Linearity and LOQ

The method produced linear responses throughout the enalapril and enalaprilat.

Table 4. Between–run variations and accuracy of the LC-Mass method for quantitation of enalaprilat(n=3

Nominal Added Concentration (ng/ml)	Run Number	Measured Concentration (ng/ml)	Mean (SD)	CV %	Accuracy (%)
0.1	1	0.098	0.10	10.98	100
	2	0.102	(0.011)		
	3	0.12			
10	1	10.31	10.01 (0.26)	2.56	100.1
	2	9.87			
	3	9.86			
20	1	20.38	20.07 (0.26)	1.29	100.35
	2	19.98			
	3	19.89			

Table 5. Relative recovery of enalapril by the LC-Mass method (N=3).

Nominal Added Concentration (ng/ml)	Sample Number	Recovery (%)	Mean (SD)	CV%
0.1	1	90.11	95.17 (4.40)	4.62
	2	97.32		
	3	98.09		
10	1	97.20	94.08 (4.33)	4.59
	2	89.14		
	3	95.89		
20	1	101.00	96.47 (6.46)	6.69
	2	99.34		
	3	89.08		

Table 6. Relative recovery of enalaprilat by the LC-Mass method (N=3).

Nominal Added Concentration (ng/ml)	Sample Number	Recovery (%)	Mean (SD)	CV%
0.1	1	93.19	93.62	3.4
	2	90.67	(3.18)	
	3	97.00		
10	1	89.09	95.34	7.27
	2	94.14	(6.93)	
	3	102.81		
20	1	91.00	94.71	7.95
	2	103.39	(7.53)	
	3	89.76		

concentration range of 0.1-10 ng/ml for enalapril and enalaprilat, which is suitable for intended purposes. A typical linear regression equation of the method was: y = 2867 x +1132,forenalaprilandy = 456.2 x +121,for enalaprilat, with x and y representing concentration (in ng/ml) and peak height (in arabitiary units), respectively, and the regression coefficient (r) of 0.999.The LLOQ for the two compounds was 0.1 ng/ml in plasm corresponded to an on-column sensitivity of 1.06 pg, which was lower than those reported in literature [5–9,11].The lower limit of detection for enalapril and enalaprilat were 0.08 ng/ml. Figures. 2. C , D

Table 7. Data showing stability of enalapril in human plasma at different QC levels (n=5).

	% Accuracy for 0.1(ng/ml)	% change(bias)	% Accuracy for 10 (ng/ml)	% change(bias)	% Accuracy for 20(ng/ml)	% change(bias)
Short-term stability	91.18	2.21	91.2	1.13	90.18	2.34
Freeze and thaw stability	92.3	1.15	94.01	3.21	95.21	2.54
Long-term stability	96.15	1.27	93.65	1.21	95.58	-1.15
Post-preparative stability	97.14	-1.1	91.87	3.30	91.14	1.12

Table 8. Data showing stability of enalaprilat in human plasma at different QC levels (n=5).

	% Accuracy for 0.1(ng/ml)	% change(bias)	% Accuracy for 10 (ng/ml)	% change(bias)	% Accuracy for 20(ng/ml)	% change(bias)
Short-term stability	95.57	-1.32	90.65	2.13	95.65	3.02
Freeze and thaw stability	96.56	2.43	93.25	-2.12	94.73	2.14
Long-term stability	93.61	2.12	94.52	2.21	95.94	2.31
Post-preparative stability	91.65	3.01	92.31	1.31	91.57	1.13

Table 9. Pharmacokinetic parameters of enalapril and enalaprilat (Mean ± SD).

Pharmacokinetics Parameters	Enalapril	Enalaprilat
C_{max} (ng/ml)	141.33 ±3.51	73.33±5.03
t_{max}	1.15±1.45	4.12±1.74
AUC_{0-t} (ng/L.hr)	142.57 ±34.34	425.94±13.09
$AUC_{0-\infty}$ (ng/L.hr)	150.74±16.69	455.80 ±65.11
$T_{½}$ (h)	2.72 ±2.01	6.34 ±2.13

show the chromatogram of an extracted sample that contained (LOQ) of enalapril and enalaprilat. Figures 2. A, B show the chromatogram of an extracted sample that contained of enalapril and enalaprilat with concentrations of 5ng/ml.Figure 2. E shows the precursor→product ion transition of m/z 377→m/z 234 of enalapril. Figure 2.F shows the precursor→product ion transition of m/z 349→m/z 206 of enalaprilat.

3.3.3. Within-run Variations and Accuracy

The within-run variations of the developed LC-Mass method as well as the corresponding absolute recoveries are shown in Table1, 2.

3.3.4. Between-run Variations and Accuracy

The between-run variations of the developed LC-Mass method as well as the corresponding absolute recoveries are shown in Table 3, 4.

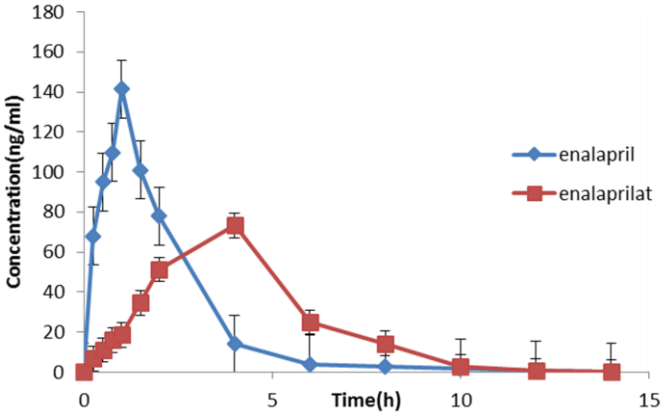

Figure 3. Mean plasma concentration–time profile of enalapril and enalaprilat after oral administration of 10 mg enalapril maleate to 12 healthy male.

3.3.5. Extraction Recovery

The extraction recovery determined for enalapril and enalaprilat were shown to be consistent, precise and reproducible. Datawere shown below in Table 5, 6.

3.3.6. Stability

Table 7,8summarizes the freeze and thaw stability, short term stability, long-term stability and post-preparative stability data enalapril and enalaprilat. All the results showed the stability behavior during these tests and there were no stability related problems during the samples routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies. The stability of working solutions was tested at room temperature for 6 h. based on the results obtained, these working solutions were stable within 6 h.

3.4. Bioequivalence Study

The mean serum concentration-time profiles after single oral dose administration of enalapril and enalaprilat are showed in Figure 3. As it is shown, the mean serum concentration-time curves from both of the test and reference products are about super imposable. Furthermore, there was no important distinction between enalapril serum concentrations at each time point subsequent oral administration of the enalapril. At the first case time (0.5 h), the drug was computable in all subjects following the administration of both arrangements. The resulting pharmacokinetic parameters of enalapril and enalaprilat are shown in Table 9. Mean maximum serum concentrations of 141.33± 3.51ng/ml and 73.33 ± 5.03ng/ml were obtained for the enalapril and enalaprilat, respectively. Tmax, the time required to reach the maximum serum concentration, was 1.02 ± 1.45 h and 4.21 ± 1.74 h, respectively. The parameters used as procedures of the amount of absorption are AUC0-t, $AUC_{0-\infty}$. The $AUC_{0-t}$and $AUC_{0-\infty}$for the enalapril were 142.57 ± 34.34ng·h/ml and 150.74 ± 16.69 ng·h/ml, respectively. The considered values for the enalaprilat were 425.94 ± 13.09ng·h/ml and 455.80 ± 65.11ng· h/ml in the order mentioned.

4. Conclusion

A sensitive, selective, accurate and precise HPLC methodwith selected ion monitoring by single quadrupole mass spectrometer with ESI

interface in positive ion mode with multiple-reaction monitoring mode was developed and validated for determination of enalapril and enalaprilat in human plasma. The reported method offers several advantages such as a rapid and simple extraction scheme, and a short chromatographic run time, which makes the method suitable for the analysis of large sample batches resulting from the pharmacokinetic, bioavailability or bioequivalent study of enalapril and enalaprilat.

Acknowledgement

The authors would like to thank the authority of the Faculty of Pharmacy, Zanjan University of Medical Sciences, for their support.

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