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Rapid and sensitive liquid chromatography/tandem mass spectrometry method for simultaneous determination of enalapril and its major metabolite enalaprilat, in human plasma: Application to a bioequivalence study

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A rapid and most sensitive method for simultaneous determination of enalapril (ENP) and its metabolite, enalaprilat (ENPT), in human plasma using ESI-LC-MS/MS (electrospray ionization liquid chromatography tandem mass spectrometry) positive ion multiple reactions monitoring (MRM) mode, was developed and validated. The procedure involves a simple solid-phase extraction (SPE) followed by evaporation of the sample. Chromatographic separation was carried out on a Hypurity C_{18} column (50 mm × 4.6 mm, 5 μm) with an isocratic mobile phase and a total run time of 2.0 min only. The MRM of ENP and ENPT is 377.10 → 234.20 and 349.20 → 206.10 respectively. The standard calibration curves showed excellent linearity within the range of 0.064 to 431.806 ng/mL for ENA and 0.064 to 431.720 ng/mL for ENPT ($r \geq 0.990$). This is the only method which can quantitate upto 0.064 ng/mL for both ENP and ENPT in a single run with the shortest analysis time. In matrix effect experiment, this method shows a % CV (% coefficients of variation) of less than 5, which means that the proposed method is free from any kind of irregular ionization process.

This method was successfully applied to a pharmacokinetic study after oral administration of enalapril maleate 20 mg tablet in Indian healthy male volunteers.

Keywords: enalapril; enalaprilat; LC-MS\MS; human plasma; matrix effect; pharmacokinetic.

Introduction

Enalapril maleate (Figure 1A) N-[1-(S)-carboxy-3-phenylpropyl]-L-alanyl-L-proline (MK-422) is a potent angiotensin I-converting enzyme (ACE) inhibitor.[1] Renin, synthesized by the kidneys, is released into the circulation where it acts on a plasma precursor to produce angiotensin I, which is converted by angiotensin-converting enzyme to angiotensin II, a potent vasoconstrictor that also causes release of aldosterone from the adrenals; both of these actions increase blood pressure. Enalapril (ENP) blocks the conversion of angiotensin I to angiotensin II, decreasing blood pressure, decreasing aldosterone secretion, slightly increasing serum potassium levels, and causing sodium and fluid loss; increased prostaglandin synthesis also may be involved in the antihypertensive action.[2]

Enalapril maleate is a pro-drug without direct biological activity which is rapidly absorbed after oral administration and de-esterified *in vivo* to its active metabolite enalaprilat (ENPT), a potent angiotensin converting enzyme inhibitor.

Following oral administration of ENP, peak serum concentrations of ENP occur within about 1 h. Based on urinary recovery, the extent of absorption of ENP is approximately 60%. ENP absorption is not influenced by the presence of food in the gastrointestinal tract. Following absorption, ENP is hydrolyzed to ENPT, which is a more potent angiotensin converting enzyme inhibitor than ENP; ENPT is poorly absorbed when administered orally. Peak serum concentrations of ENPT occur three to four hours after an oral dose of enalapril maleate.[3,4] Excretion of ENP is primarily renal. Approximately 94% of the dose is recovered in the urine and faeces as ENPT or ENP. The principal components in urine are ENPT, accounting for about 40% of the dose, and intact ENP. There is no evidence of metabolites of ENP, other than ENPT (Figure 1B).[5]

A wide variety of analytical techniques have been applied to the quantification of ENP and ENPT in biological fluids includes, gas chromatography/mass spectrometry (GC/MS),[6] liquid chromatography coupled with UV detection,[7–12] capillary

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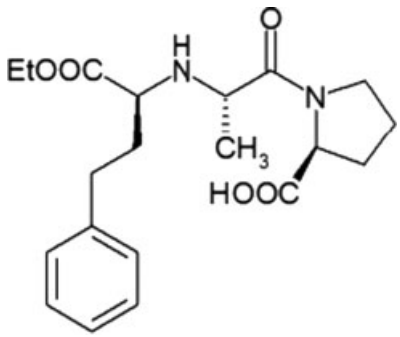

Figure 1A. Chemical structure of ENP.

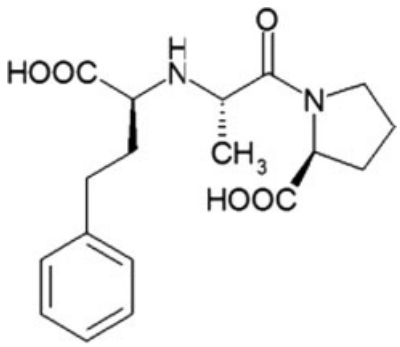

Figure 1B. Chemical structure of ENPT.

electrophoresis (CE),[13,14] flow injection analysis based on the formation of a copper Complex,[15] radioimmunoassay (RIA),[16,17] enzyme linked immunosorbent assay (ELISA)[18] and time-resolved fluoroimmunoassay (TR-FIA).[19] ENP is a dipeptide with a proline peptide bond and can exist as rotamers. The inter-conversion between cis and trans-configurations leads to poor chromatographic properties and prevents generation of a single sharp peak for quantitative analysis. Although these problems could be solved by using low pH and high column temperature,[9,10] these reported HPLC methods are not adequate for pharmacokinetic studies due to relatively high detection limits.[7,8,11] However, these methods 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 ENP and ENPT in human plasma down to 0.2 ng/ml. Although, the sensitivity of this method is adequate for the pharmacokinetic studies of ENP and ENPT, the derivatization step makes it a complicated and time-consuming procedure. Lee *et al*.[20] described an LC-MS/MS method for simultaneous determination of ENP and ENPT in plasma. Plasma samples were extracted by solid-phase extraction (SPE) and analyzed by gradient HPLC. The chromatographic run time was approximately 5.5 min, and the lower limit of quantification (LLOQ) for ENP and ENPT 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 ENP, as the terminal half-life of ENPT is about 30–35 h after a single oral dose of ENP 20 mg, it is not sufficient for pharmacokinetic study of ENPT. Moreover, among all the published manuscripts which descried the quantitation of ENP and ENPT by LC-MS/MS[20–28] no methods can detect up to

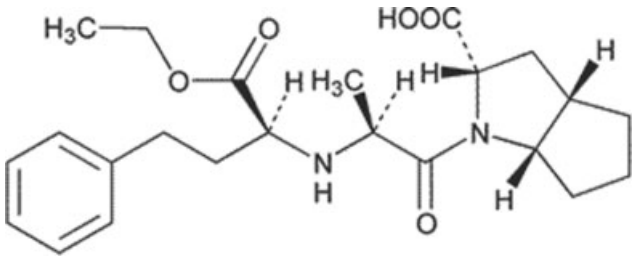

Figure 1C. Chemical structure of ramipril.

0.064 ng/mL (LLOQ) with acceptable accuracy and precision and shortest 2.0 min run time.

In this study, we describe a rapid, most sensitive, and selective HPLC coupled with tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of ENP and ENPT in human plasma using ramipril (Figure 1C) as the internal standard (IS). The chromatographic run time is approximately 2.0 min. The method has a lower LLOQ of 0.064 ng/ml for both ENP and ENPT. It allows plasma drug monitoring for at least 72 h after oral administration. The method was successfully applied for the evaluation of the pharmacokinetics and bioequivalence study of ENP and ENPT in 36 healthy, male, Indian volunteers after an oral dose of 20 mg enalapril maleate tablet. The method had been validated by evaluating the precision, accuracy and other validation parameters for human plasma samples, as mentioned in regulatory guidelines.[29]

Experimental

Apparatus and software

The HPLC system with an auto sampler was a Shimadzu LC-20AD (Shimadzu, Tokyo Japan) coupled with Applied Biosystem Sciex (MDS Sciex, ON, Canada) API 4000 Tandem mass spectrometry. The auto sampler was SIL-HTC from Shimadzu, Tokyo Japan. The solvent delivery module was LC-20AD from Shimadzu, Tokyo Japan. The chromatographic integration was performed by Analyst software (version: 1.4.2; Applied Biosystems ON, Canada). Positive pressure unit used for SPE was from Orochem technologies Inc. (Lombard, IL, USA). The Caliper turbovap LV concentration workstation used to evaporate the samples was purchased from Caliper Life Sciences (Hopkinton, MA, USA).

Chemicals and reagents

ENA (99.7%), ENPT (99.29%) and IS, were procured from the Quality Control Department of Cadila Pharmaceuticals Limited (Ahmedabad, Gujarat, India) and Varda Biotech (p) Limited (Mumbai, India), respectively. All reagents were of analytical reagent grade unless stated otherwise. Water used for the preparation of mobile Phase and other solutions was collected from Milli Q_{PS} (Billerica, Massachusetts Millipore, USA) installed in the Laboratory. HPLC-grade Methanol, acetonitrile and glacial acetic acid were supplied by J. T. Baker, Massachusetts USA and Finar Chemicals Limited (Ahmedabad, India), respectively. Lichrosep sequence SPE cartridge (30 mg, 1 mL) from Merck used for solid phase extraction. Drug free Human K_2EDTA Plasma was used during validation and study sample analysis was supplied by the Clinical Unit of Cadila Pharmaceuticals Limited (Ahmedabad, India). Plasma was stored at $-30 \pm 5\ ^{\circ}C$ before sample preparation and analysis.

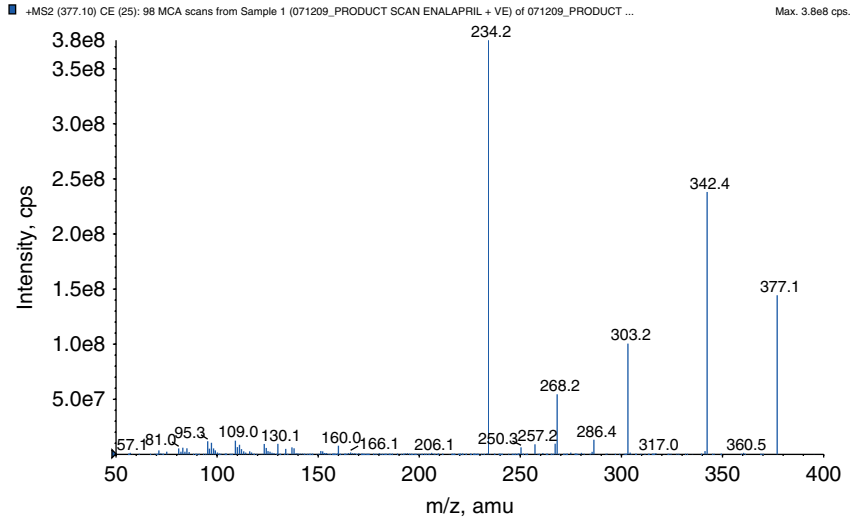

Figure 2A. Product ions scan of ENP.

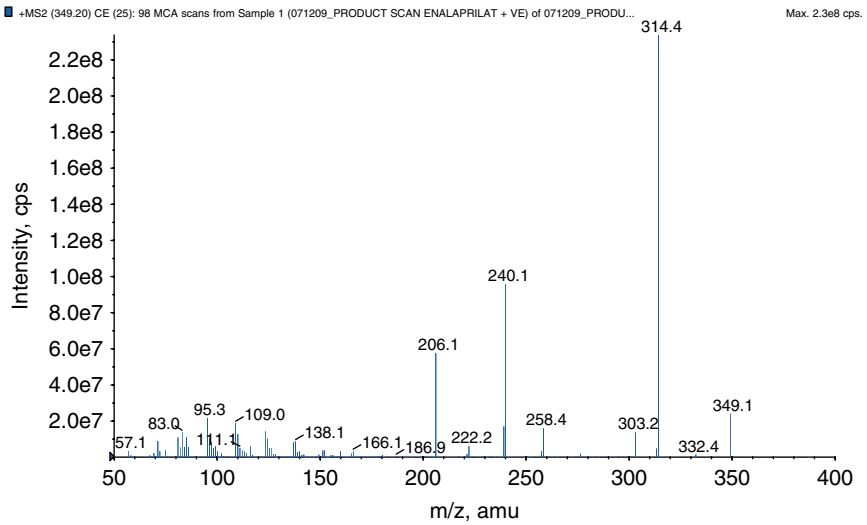

Figure 2B. Product ions scan of ENPT.

Standards and working solutions

An individual stock standard solution of ENA, ENPT, and IS containing 1 mg/mL was prepared by dissolving pure compound in methanol. Intermediate and working solutions were prepared from corresponding stock solutions by diluting with a mixture of water: methanol 50:50 *v/v*. Calibration standards were prepared in the range of 0.064 to 431.806 ng/mL for ENP and 0.064 to 431.720 ng/mL for ENPT using nine concentration levels each. Quality control samples of three different levels low (0.189 ng/mL), medium (17.000 ng/mL) and high (351.470 ng/mL) for ENP and low (0.189 ng/mL), medium (20.080 ng/mL) and high (351.400 ng/mL) for ENPT were also prepared. All these stock solutions, calibration standards and Quality control samples were stored at 6 ± 2 °C.

Characterization of the product ions using tandem mass spectrometry

One micro molar ENP, ENPT, and IS solutions were separately infused into the mass spectrometer at a flow rate of 10 µL/min, to characterize the product ions of each compound. The precursor ions $[M+H]^+$ and the pattern of fragmentation were monitored using the positive ion mode. The major and stable peaks observed in the MS/MS scan were used to quantify ENP, ENPT, and IS. Figure 2A represents the fragmentation pattern of ENP; Figure 2B represents the fragmentation pattern of ENPT; and Figure 2C represents the fragmentation pattern of IS.

Analytical system

The plasma ENP and ENPT concentrations were quantified using SCIEX API 4000 LC-MS/MS system (MDS Sciex, ON Canada), equipped with an ESI interface used to generate positive ions $[M+H]^+$. The compounds were separated on a reversed Phase column (Hypurity C18, 50 × 4.6 mm ID, particle size 5 µ, Thermo Electron Corporation, London UK), with an isocratic mobile phase consisting of 0.1% *v/v* ammonia solution (pH 2.88; adjusted with acetic acid) in water-methanol (15 : 85, *v/v*). The mobile phase was eluted at 0.30 mL/min. Total analysis time of single injection was 2.0 min. Injection volume was 15 µL. Auto sampler rinsing volume was 500 µL. The auto sampler temperature was maintained at 5 °C.

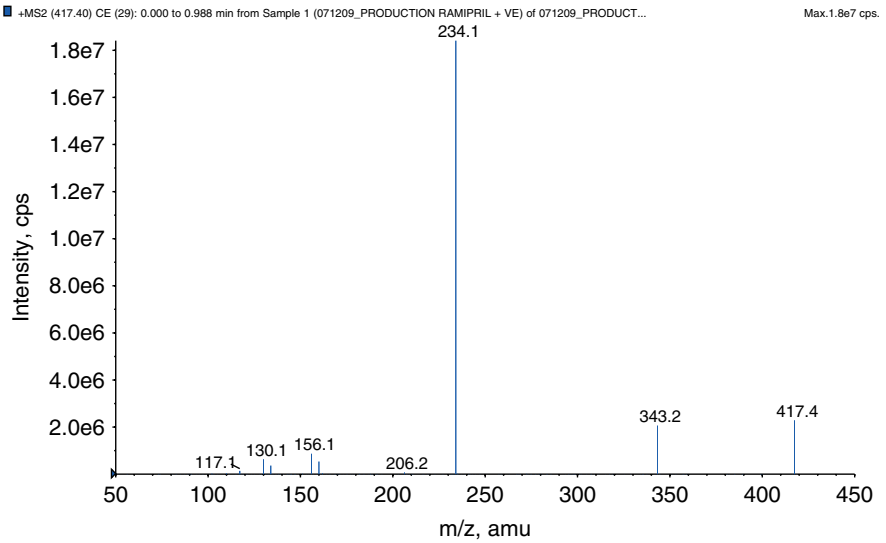

Figure 2C. Product ions scan of ramipril.

The optimized ion spray voltage and temperature were set at 5500 V and 400 °C. The typical ion source parameters, viz., declustering potential, collision energy, entrance potential and collision cell exit potential were 60, 25, 10, and 12 V for ENP; 78, 29, 12 and 15 V for ENPT; 78, 29, 12 and 15 V for IS, respectively. Nitrogen gas was used for the gas 1, gas 2, curtain gas, and collision-activated dissociation gas, which were set at 30, 40, 10, and 12 psi, respectively. Quantification was performed by multiple reaction monitoring of the protonated precursor ion and the related product ion for ENP and ENPT using the IS method with a peak area ratios and a linear least-squares regression curve with weighting factor of $1/x^2$. The mass transitions used for ENP, ENPT, and IS were *m/z* 277.10 → 234.20, *m/z* 349.20 → 206.10 and *m/z* 417.40 → 234.10, respectively, with a dwell time of 200 ms per transition. Quadrupoles Q1 and Q3 were set on a unit resolution. The analytical data were processed by Analyst software (Version 1.4.2; Applied Biosystems ON).

Sample treatment

SPE technique was used to extract ENP and ENPT. 400 µL plasm sample was transferred to a ria vial for analysis. 25 µL of IS (1 µg/mL) and 300 µL 2.0% *v/v* ortho phosphoric acid to water was added into it, the sample was vortexed for 15 s. In SPE, cartridge was conditioned with 1 mL of methanol followed by 1 mL of water, then the plasma samples were loaded on the cartridges, cartridges were washed with 1 mL of water, followed by air drying and elution with 1 ml of 0.2% *v/v* acetic acid in methanol. Methanol was collected and evaporated to dryness at 50 °C under the stream of nitrogen. The residue was then reconstituted with 200 µL of mobile phase and injected (15 µL) to LC-MS/MS.

Method validation

The validation parameters were specifically, linearity, sensitivity, accuracy, precision, and matrix effects of the assay and the recovery and stability in human plasma, according to the US Food and Drug Administration (FDA) guidance for the validation of bioanalytical methods.[29]

Selectivity was studied by comparing the chromatograms of six different lots of plasma obtained from six subjects, with the plasma samples having been spiked with ENP, ENPT, and IS. Calibration curves were prepared by assaying standard plasma samples at ENP and ENPT concentrations, ranging from 0.064 to 431.806 ng/mL for ENP and 0.064 to 431.720 ng/mL for ENPT.

The linearity of each method matched calibration curve was determined by plotting the peak area ratio (y) of ENP or ENPT to IS versus the nominal concentration (x) of ENP or ENPT, respectively. The calibration curves were constructed by weighing ($1/x^2$) least-squares linear regression.

The LLOQ for ENP or ENPT in human plasma was defined as the lowest concentration giving at least 10-fold, acceptable accuracy (80–120%), and sufficient precision (within 20%).

Intra- and inter-day accuracy and precision for this method were determined at three different concentration levels on at least two different days, six replicates were analyzed with independently prepared calibration curves. The percentage accuracy was expressed as (mean observed concentration)/(nominal concentration) ×100, and the precision was the relative standard deviation (RSD, %).

To evaluate the matrix effects of ENP, ENPT, and IS on the ionization of the analyte, low quality control (LQC) samples from six different plasma batches in triplicate were processed. For recovery calculation, the peak areas obtained by direct injection of solvent (or neat) standard solutions spiked after extraction into plasma extracts as A and the peak areas for solvent (or neat) standard solutions spiked before plasma extraction as B, the extraction recovery value can be calculated as follows:

$$\text{Extraction recovery (\%)} = B/A \times 100 \quad (1)$$

The stability of ENP or ENPT in human plasma was assessed by analyzing six replicate samples spiked with LQC and HQC (high quality control) samples, respectively, under five different conditions: after short-term storage for 8.30 h at room temperature; after long-term storage of 6 months at −30 °C; after four freeze-thaw cycles; after 7 h on bench top, and after 48.30 h within the auto sampler. The concentrations obtained were compared with the nominal values of the QC samples.

Clinical protocol

The bioequivalence study protocol presented in this paper was approved by the Independent Ethics Committee. This was a randomized, two-treatment, two-period, two-sequence, single-dose, two-way crossover bioequivalence study on ENP Maleate BP 20 mg tablet, subjects were administered a single dose of ENP maleate 20 mg tablet along with 200 mL of drinking water after an overnight fasting of at least 10 h in each period with at least a 10-day washout period between each administration. All subjects were healthy, adult, male, human Indian volunteers of Indian origin. In each period, a total of 20 blood samples (5 mL each) will be collected, prior to drug administration (0.0) and at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 9.0, 12.0, 16.0, 24.0, 36.0, 48.0 and 72.0 h thereafter.

The blood samples were immediately centrifuged at 2000 × g for 10 min at 4 °C, and the plasma samples were stored at −30 °C until LC-MS/MS analysis.

Results and discussions

Optimization of chromatographic condition and sample clean-up

In order to have an optimal selectivity and sensitivity different types of column and mobile phase were used.[30,31] Length of the columns varied from 50 cm to 150 cm, and the particle size varied from 3.5 µ to 5.0 µ. Columns of different types of stationary phase like C_8, C_{18} and Cyano were used which showed considerable matrix effects on peak shape and intensity. Finally, Hypurity C_{18} column of 50 × 4.6 mm, 5 µ was selected for analysis based on good peak shape and even matrix effects.

The influence of buffer molarity, pH, and types of organic modifier on the signal intensities was also studied at the optimized declustering potential. Based on the peak intensity of the ENP, ENPT, and IS, an isocratic mobile Phase consisting of 0.1% v/v ammonia solution (pH: 2.88, adjusted with acetic acid) in water and methanol at a ratio of 15:85 *v/v*. The mobile Phase was eluted at 0.30 mL/min for further studies. Higher proportions of organic modifier in the mobile Phase were found to improve the signal intensity. Initially, 95 : 5 (*v/v*) acetonitrile: buffer at a flow rate of 0.30 mL/min was tried. On the other hand, very high proportions of organic phase led to improper elution leading to peak deformation. Therefore, the 85 : 15 (*v/v*) organic phases to buffer were selected as optimum at a flow rate of 0.30 mL/min. Moreover, reconstitution of the extracted dry residue in mobile phase improved the peak symmetry.

The sample clean-up technique was also optimized in order to get minimum interference of endogenous compounds or matrix effects and good analyte recovery. Different techniques like protein precipitation, liquid-liquid extraction (LLE) and SPE were used for sample extraction. Based on non-interference and optimal recovery, SPE was found to be the best suitable for sample preparation.

Full scan spectra of ENP, ENPT, and IS showed the presence of parent ions only. Significant and stable daughter ion was also scanned in infusion mode. So the detection was made in multiple ions monitoring mode with Q1 and Q3 set at *m/z* 377.10 → 234.20 for ENP, 249.20 → 206.10 for ENPT and for IS, the transition monitored was *m/z* 417.40 → 234.10.

To the best of our knowledge, there are no reported LC-MS/MS methods were available with this LLOQ value and shortest analysis time.

Method validation

Linearity

Linearity of calibration standards was assessed by subjecting the spiked concentrations and the respective peak areas using $1/X^2$ linear least-squares regression analysis. Linearity ranged from 0.064 to 431.806 ng/mL for ENP ($r>0.990$), 0.064 to 431.72 ng/mL for ENPT ($r>0.990$). In aqueous solution, accuracy of all calibration standards was within 85–115%, except LLOQ where it was 80–120%.

Specificity and selectivity

Six different lots of blank plasma, one each of haemolyzed and lipemic effects were analyzed to ensure that no endogenous interference took place with the mass transitions chosen for ENP, ENPT, and IS. Six LLOQ level samples along with plasma blank from the respective plasma lot were prepared from six different lots of plasma and analyzed. In all six plasma blanks, the response at the retention time of ENP and ENPT was < 20% of LLOQ response and at the retention time of IS, the response was < 5% of mean IS response in LLOQ. Figure 3 represents the chromatogram of plasma blank.

Limit of quantitation

To check the reproducibility of the method at the highest and lowest concentration level, six injections at each level was injected. For ENP the % accuracy at LLOQ and ULOQ (upper limit of quantification) level was 99.90 and 106.19, and the % CV (% coefficients of variation) was 4.15 and 0.37. Whereas for ENPT the % accuracy at LLOQ and ULOQ level was 108.79 and 102.54, and the % CV was 11.26 and 0.46. Figure 4A represents the LLOQ of ENP and Figure 4B represents the LLOQ of ENPT.

Accuracy and precision

For the validation of the assay, QC samples were prepared at three concentration levels of low, medium, and high. Six replicates of each QC samples were analyzed together with a set of calibration standard. The accuracy of each sample preparation was determined by injection of calibration samples and three QC samples in six replicate for at least two days. Obtained accuracy and precision (inter- and intra-day) are presented in Table 1A for ENP and Table 1B for ENPT. The results show that the analytical method is accurate, as the accuracy is within the acceptance limits of 100 ± 15% at all concentration levels. The precision around the mean value was never greater than 15% at any of the concentration studied. Summary of QC samples of all precision and accuracy batches were presented in Table 2.

Recovery study

A recovery study was performed by comparing processed QC samples of three different levels in six replicate with aqueous samples of same level. The recovery of ENP at LQC level was 87.87%, medium quality control (MQC) level was 83.80% and for high quality control (HQC) level was 84.54%. The mean recovery of ENP was 85.40%; % CV of mean recovery of all three QCs were 2.54.

The recovery of ENPT at LQC level was 90.94%, medium quality control (MQC) level was 89.94%, and for high quality control (HQC) level was 92.17%. The mean recovery of ENPT was 91.02%; % CV of mean recovery of all three QCs were 1.23. Recovery of IS was 91.73%.

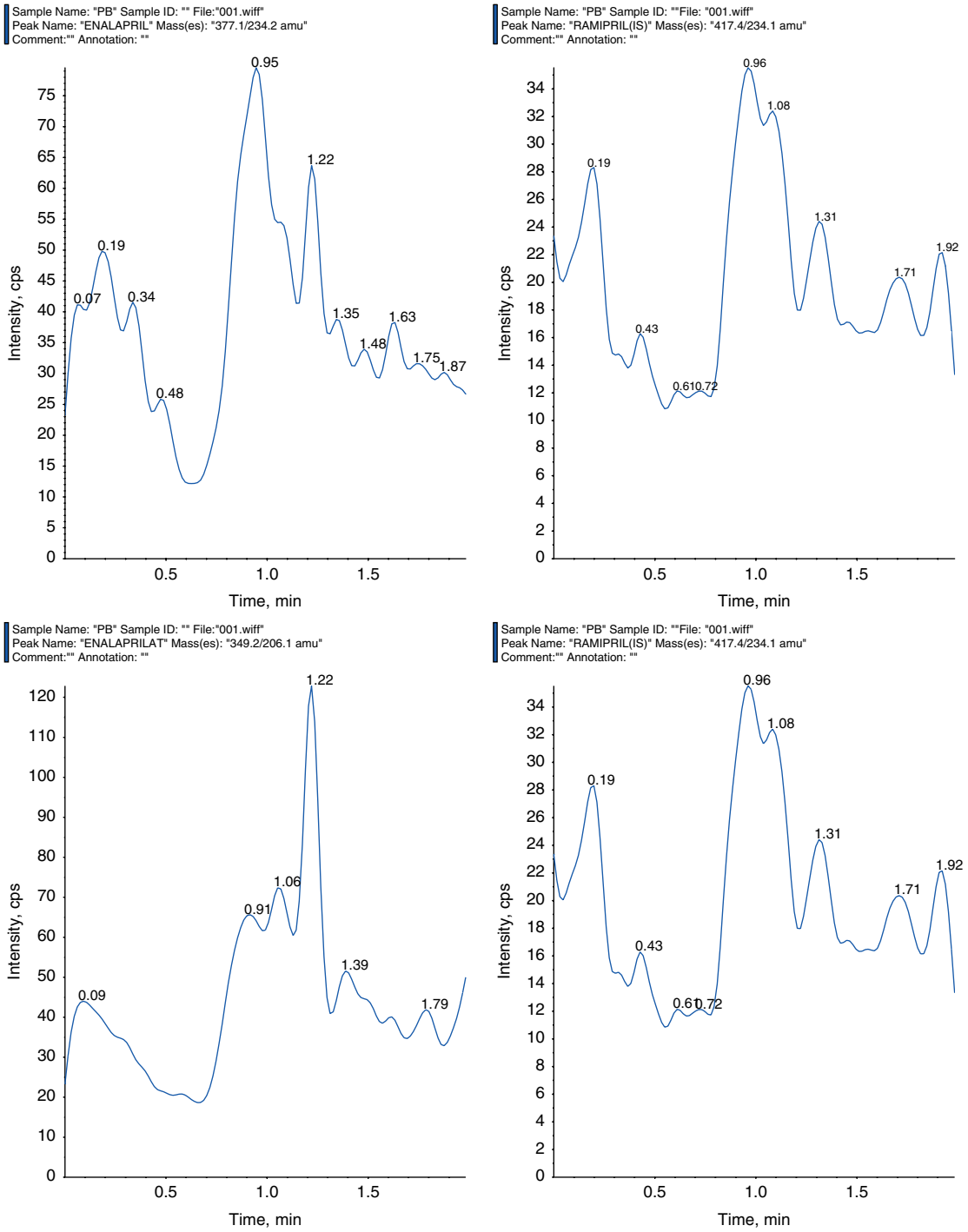

Figure 3. Representative chromatogram of plasma blank.

Matrix effects

Matrix effect was determined to examine whether there was co-elution of any phospholipids, analyte or IS with any matrix or its components which can cause irregular ionisation. To evaluate the matrix effects, eighteen LQC and HQC samples, three each from six different plasma lots were processed and analyzed. For ENP, the average % accuracy for all LQC level was 90.67 and all HQC level was 102.15 and %CV of all LQC samples was 2.32 and all HQC samples was 3.23.

Whereas, for ENPT the average % accuracy for all LQC level was 92.17 and all HQC level was 100.96 and % CV of all LQC samples was 4.32 and all HQC samples was 4.49.

The % CV value for both ENP and ENPT was within 5, it means that the proposed method is free from any kinds of irregular ionization process.

Haemolysis effects

To determine haemolysis effects six QC samples were prepared in haemolyzed plasma with all three concentration levels of low,

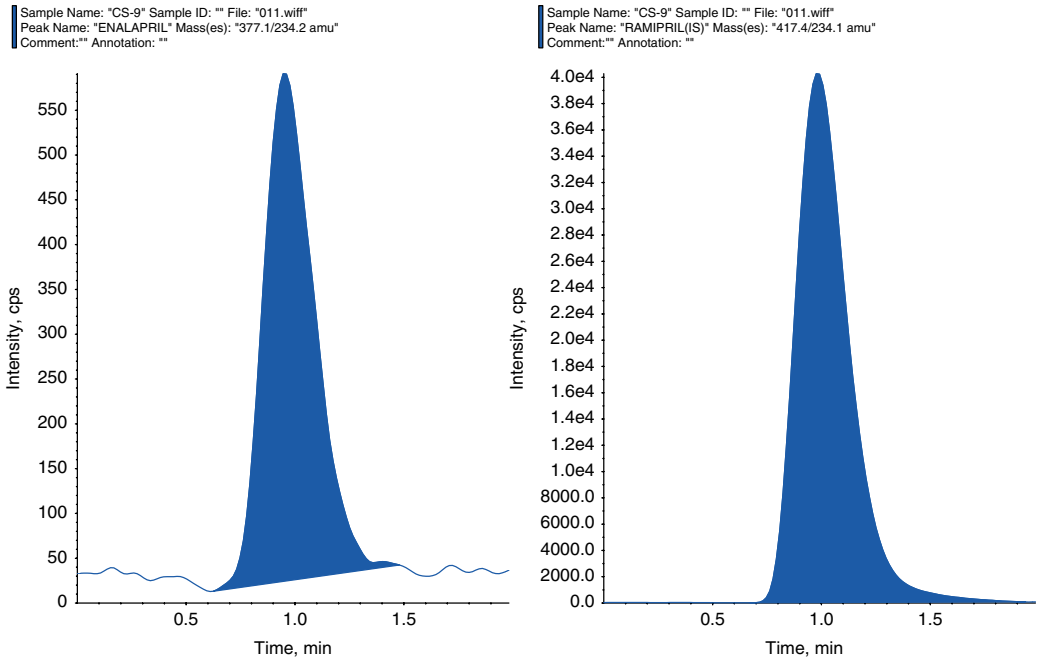

Figure 4A. Representative chromatogram of lowest standard of ENP (0.064 ng/mL).

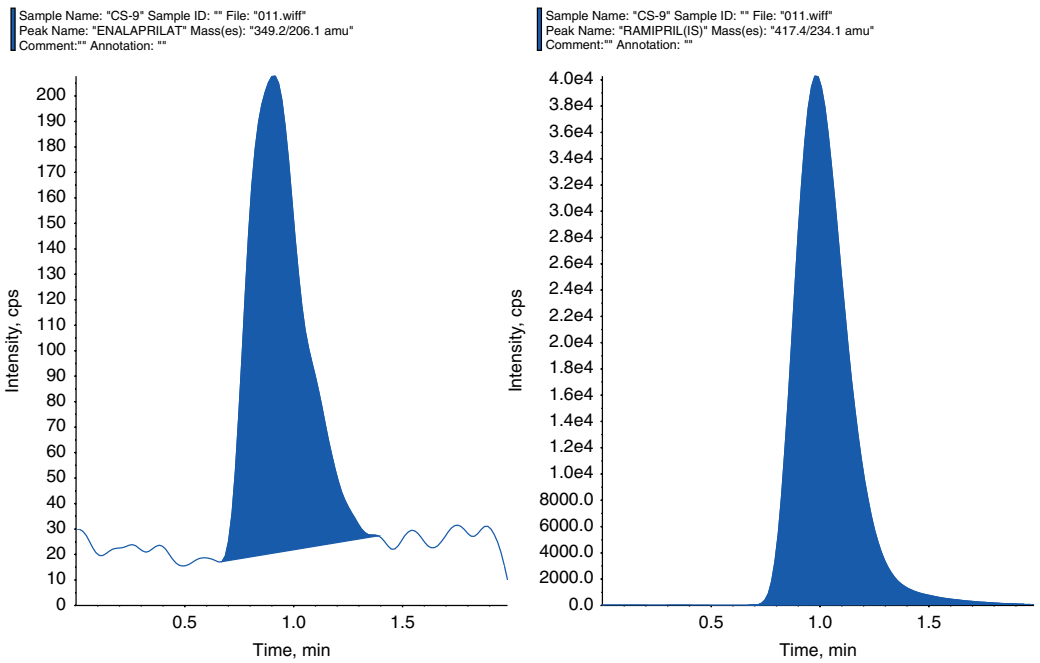

Figure 4B. Representative chromatogram of lowest standard of ENPT (0.064 ng/mL).

medium, and high. Six replicates of each QC sample were analyzed together with a set of calibration standard prepared in normal plasma. The accuracy of each sample preparation was determined by injection of calibration samples and two QC samples in six replicate. For ENP the average % accuracy for LQC level was 90.23, for MQC level was 100.07, and for HQC level was 102.04. The % CV of LQC was 3.04; for MQC was 2.47; and for HQC was 3.11.

For ENPT, the average % accuracy for LQC level was 91.00; for MQC level was 100.18; and for HQC level was 101.50. The % CV of LQC was 6.80; for MQC was 3.74; and for HQC was 4.69.

Lipemic effects

To determine lipemic effects, six QC samples were prepared in lipemic plasma with all three concentration levels of low, medium, and high. Six replicates of each QC samples were analyzed together with a set of calibration standard prepared in normal plasma. The accuracy of each sample preparation was determined by injection of calibration samples and two QC samples in six replicates. For ENP, the average % accuracy for LQC level it was 90.87; for MQC level it was 99.99; and for HQC level was 101.99. The % CV of LQC was 1.55; for MQC it was 2.27; and for HQC it was 2.99.

Table 1 A. Inter- and Intra-day accuracy and precision of ENP. Each mean and % CV of intra-day accuracy and precision represent six observations (n=6). The inter-day accuracy and precision are averages and % CV of three intra-day observations

	QC Levels	Mean accuracy	Mean Precision (% CV)
Day 1	LQC	100.53	11.58
	MQC	97.59	2.97
	HQC	100.34	4.77
Day 2	LQC	100.53	5.79
	MQC	106.88	3.59
	HQC	104.76	4.53
Day 3	LQC	105.82	11.50
	MQC	107.65	3.11
	HQC	105.12	4.38

Table 1 B. Inter- and intra-day accuracy and precision of ENPT. Each mean and % CV of intra-day accuracy and precision represent six observations (n=6). The inter-day accuracy and precision are averages and % CV of three intra-day observations

	QC Levels	Mean accuracy	Mean Precision (% CV)
Day 1	LQC	105.82	8.50
	MQC	111.82	4.53
	HQC	99.19	5.50
Day 2	LQC	95.24	10.00
	MQC	112.35	5.47
	HQC	99.33	3.29
Day 3	LQC	105.82	6.50
	MQC	113.35	4.14
	HQC	98.01	5.97

Table 2. Overall statistics of QC samples of precision and accuracy batches during validation

Analyte name	QC Levels	Nominal conc. (ng mL^{-1})	N	Mean conc. (ng mL^{-1})	SD(±)	%RSD	% Accuracy
Enalapril	LQC	0.189	18	0.190	0.018	9.470	100.530
	MQC	17.000	18	17.680	0.965	5.460	104.000
	HQC	351.470	18	363.440	17.431	4.800	103.410
Enalaprilat	LQC	0.189	18	0.190	0.016	8.420	100.530
	MQC	20.080	18	19.130	0.860	4.490	112.530
	HQC	351.400	18	347.400	16.610	4.780	98.840

For ENPT the average % accuracy for LQC level was 93.67; for MQC level it was 100.13; and for HQC level it was 101.25. The % CV of LQC was 5.68; for MQC it was 3.01; and for HQC it was 4.75.

Stability studies

The stability of ENP, ENPT, and IS were investigated in the stock and working solutions, in plasma during storage, during processing, after four freeze-thaw cycles, and in the final extract. Stability samples were compared with freshly processed calibration standards and QC samples. Analyte and IS were considered stable when the change of concentration is ±10% with respect to their initial concentration.

Table 3 A. Summary of stability data of ENP

Experiment name	QC level	Mean accuracy	Mean Precision (%CV)	percent change	Stability Duration
Bench top	LQC	93.44	2.58	0.98	07 h
	HQC	98.54	0.62	1.50	
Freeze thaw	LQC	93.58	2.18	3.82	4 cycles
	HQC	97.07	0.22	−2.20	
Auto sampler	LQC	93.34	3.55	−1.48	48.3 h
	HQC	98.56	1.15	−0.52	

Table 3 B. Summary of stability data of ENPT

Experiment name	QC level	Mean accuracy	Mean Precision (%CV)	percent change	Stability Duration
Bench top	LQC	94.68	3.53	−1.01	07 h
	HQC	97.41	2.08	2.52	
Freeze thaw	LQC	91.80	4.70	−0.48	4 cycles
	HQC	94.93	0.41	−3.35	
Auto sampler	LQC	103.15	7.24	5.77	48.3 h
	HQC	99.29	1.62	0.12	

The % CV of ENP at LQC and HQC levels for freeze-thaw stability, bench-top stability and auto sampler stability samples were 2.18, 2.58, and 3.55 and 0.22, 0.62, and 1.15, respectively, whereas the % CV of ENPT at LQC and HQC levels were 4.74, 3.53, and 7.24 and 0.41, 2.08, and 1.62, respectively

Summary of stability data were presented in Tables 3A and 3B.

Calibration curve parameters

The summary of calibration curve parameters was as follows. For ENP the mean slope and y-intercepts were 0.034933 (Range: 0.033100 to 0.036900) and 0.00177 (Range: 0.001650 to 0.001830) respectively. The mean correlation coefficient, r was 0.9972 (Range: 0.9963 to 0.9981).

For ENPT the mean slope and y-intercepts were 0.011433 (Range: 0.011200 to 0.011600) and 0.000362 (Range: 0.000231 to 0.000487) respectively. The mean correlation coefficient, r was 0.9973 (Range: 0.9967 to 0.9980).

Application

The above-described fully validated method was applied to determine the concentration time profile following single dose administration of ENP in healthy human volunteers. The chromatograms obtained from analysis of real samples are presented in Figures 5A and 5B for ENP and ENPT. After LC-MS/MS analysis, the plasma concentration of ENP and ENPT for all volunteers at times (0.0) and at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 9.0, 12.0, 16.0, 24.0, 36.0, 48.0, and 72.0 h

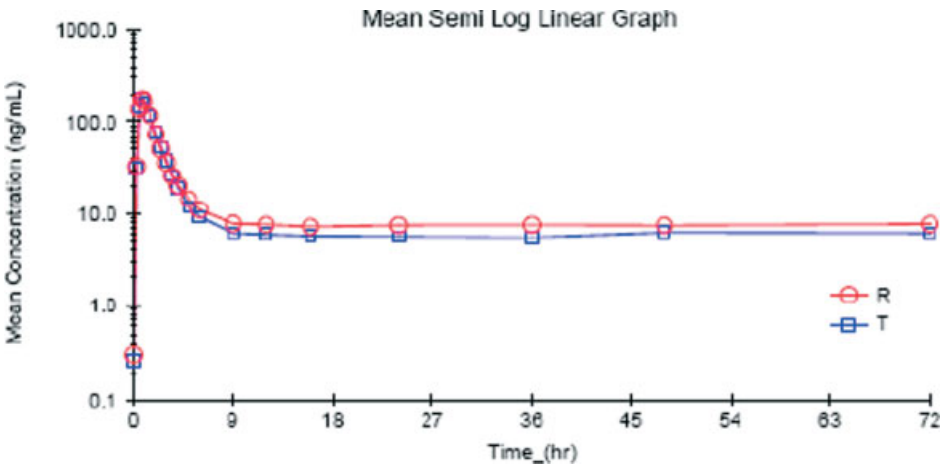

Figure 5A. Mean semi log linear graph of concentration versus time of enalpril in human plasma from 36 volunteers receiving a single oral dose of 20 mg enalapril maleate tablet as test and reference.

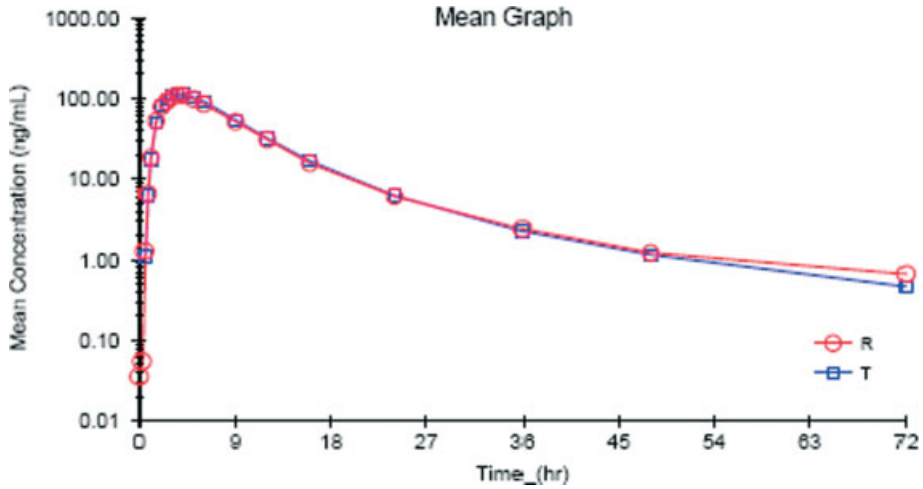

Figure 5B. Mean semi-log linear graph of concentration versus time of enalrilat in human plasma from 36 volunteers receiving a single oral dose of 20 mg enalapril maleate tablet as test and reference.

thereafter for the test and reference products were measured. The C_{max} for both test and reference products for ENP were 195.218 ± 60.467 ng/mL and 188.600 ± 58.738 ng/mL, AUC $_{0-t}$ for both test and reference products were 729.947 ± 1192.846 ng × h/mL and 725.629 ± 1200.800 ng × h/mL, AUC $_{0-\infty}$ for both test and reference products were 2846.011 ± 9313.886 ng × h/mL and 1697.745 ± 3593.657 ng × h/mL, T_{max} for both test and reference products were 0.883 ± 0.311 h and 0.812 ± 0.211 h, $t_{1/2}$ for both test and reference products were 44.586 ± 117.683 h and 32.056 ± 93.154 h and K_{el} for both test and reference products were 0.547 ± 0.346 h^{-1} and 0.518 ± 0.310h^{-1}.

On the other hand, the C_{max} for both test and reference products for ENPT were 118.870 ± 35.756 ng/mL and 113.731 ± 38.385 ng/mL, AUC $_{0-t}$ for both test and reference products were 1082.416 ± 320.112 ng × h/mL and 1043.522 ± 320.112 ng × h/mL, AUC $_{0-\infty}$ for both test and reference products were 1107.910±309.037 ng × h/mL and 1096.150±326.812 ng × h/mL, T_{max} for both test and reference products were 3.833 ± 0.863 h and 3.848±0.667 h, $t_{1/2}$ for both test and reference products were 11.936 ± 9.806 h and 12.073 ± 10.074 h and K_{el} for both test and reference products were 0.094±0.059 h^{-1} and 0.088±0.046 h^{-1}.

Conclusion

A rapid, simple, sensitive, selective, precise, and accurate LC-MS/MS method for simultaneous determination of ENP and its metabolite, ENPT, in human plasma, over a range of 0.064–431.806 ng/mL for ENP and 0.064–431.720 ng/mL for ENPT, was developed and validated. This method requires only 0.400 mL of biological samples, owing to simple sample preparation and short run time (2.0 min), it allows high sample throughput. The method was successfully applied to a single dose 20 mg tablet bioequivalence study of ENP and its major metabolite, ENPT.

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