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Liquid Chromatography-Tandem Mass Spectrometry Method for the Quantification of Enalapril and Enalaprilat in Human Plasma

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Abstract

A fast, perceptive, and highly discriminating Liquid chromatographic method with Mass spectroscopy was urbanized and validated for simultaneous determination of Enalapril and its major bioactive metabolite Enalaprilat in human plasma. Solid phase extraction process was used for the extraction of analytes from plasma. The chromatographic severance was achieved on Zorbax Eclipse; 150 × 4.6 mm, C18 5 µm column using a solvent system of acetonitrile and 0.1% v/v HCOOH in water with a ratio of 65:35 v/v at a flow rate of 0.8 mL/min. The analytes were detected in a positive ionization by multiple reactions monitoring mode. Mass transitions of m/z 377.10 → 234 for Enalapril, m/z 382.10 → 239.20 for Enalapril D5 and m/z 349 → 206 for Enalaprilat, m/z 354.20 → 211.20 for Enalaprilat D5 were used for quantification in plasma samples. The method exhibited a linear response with a Correlation co-efficient (r2) of >0.99 in the concentration range of 0.502-160.2 ng/mL for Enalapril and 0.506-161.5 ng/mL for Enalaprilat in human plasma. The mean recovery of Enalapril was 91.21% with a precision range of 1.72% to 5.06% and Enalaprilat was 90.85% with a precision range of 1.29% to 3.87%. The proposed method can be utilized for the quantification of Enalapril and Enalaprilat in human plasma in regular bioequivalence studies.

Keywords: LC-MS/MS; Enalapril; Enalaprilat d5; Solid phase extraction; LLOQ; LQ

Introduction

Enalapril is a prodrug acts as ACE inhibitor, efficiently used in the management of elevated blood pressure, congestive heart failure and diabetic kidney diseases. Enalapril, 1-[N-[(S)-1-Carboxy-3-phenylpropyl]-1-alanyl]-L-proline1'-Ethyl Ester (Figure 1), belongs to the class of substituted N-carboxy methyl peptides. Enalapril is metabolized after absorption by hydrolysis of the ethyl ester to active metabolite, Enalaprilat (Figure 2), which is a competitive angiotensin converting enzyme inhibitor (ACE), decreases formation of angiotensin-II their by decreases high blood pressure. Systolic heart failure death rate has been lowered by Enalapril and has been listed as one of the essential medicines by World Health Organization, needed in a basic health system [1]. Enalapril is recommended either single or in grouping with other medication for treatment of hypertension. Enalapril is generic and accessible globally under many brands. At present commercially marketed as Vasotec in US, Enaladex, Epaned and Renitec, Ednyt®, Innovace® in some other countries, and Enacard for veterinary use. The molecular formula is $C_{18}H_24N_2O_5$ with average mass of 348.399 Da. The physical appearance of material is an almost white to off white crystalline powder, Soluble in alcohol (0.08 g/mL), methanol (0.20 m/mL) with melting point 143-144.5°C and pKa value 2.97. Number of analytical techniques have been reported for the estimation of Enalapril or Enalapril maleate either uniquely or in conjuction with other antihypertensive agents in formulations, which include Capillary Electrophoresis, Flow injection analysis based on the genesis of ternary complex, RP-HPLC [2-4]. Enalapril and Enalaprilat are frequently determined in biological samples like plasma and urine. Therefore, the simultaneous detection of plasma concentrations is of most important for pharmacokinetic studies. Radioimmunoassay, GC–MS, and enzyme kinetics have been published for estimation in biological samples [5-7]. Recently liquid chromatographic technique coupled with mass spectrometric detection (LCMS/MS) has been widely used for quantitation of drug products in biological matrix. Different extraction techniques have been applied like solid phase extraction, Liquid-Liquid extraction and protein precipitation for the extraction of drug metabolites from biological fluids. A range of LC-MS/MS methods reported for quantification of Enalapril and Enalaprilat in biological matrixes either in single or in presence of other antihypertensive agents [8-20].

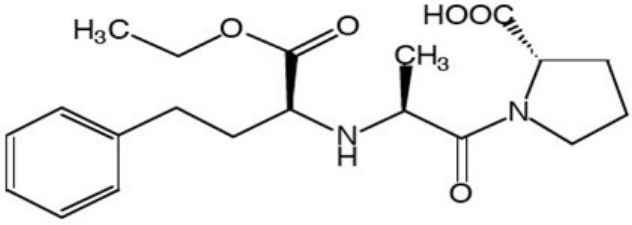

Figure 1: Structure of Enalapril.

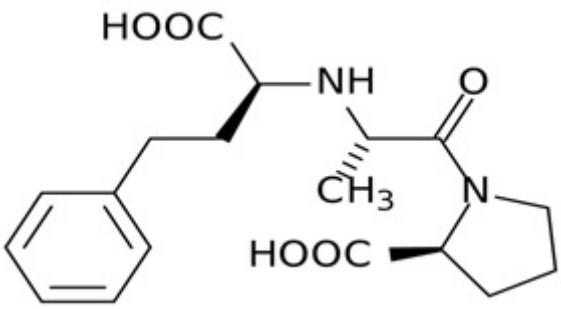

Figure 2: Structure of Enalapriat.

The objective of the follow a line of investigation was to develop and validate very simple, fast, quick and sensitive, Liquid Chromatography Mass Spectrometric method for the estimation of Enalapril and Enalaprilat in human plasma in presence of amLodipine in positive ion mode using Enalapril D5 and Enalaprilat D5 as internal standards.

Materials and Methods

Enalapril Maleate (99.95%) and Enalaprilat (99.95%) working standards were purchased from Ramdev Chemicals Pvt. Ltd. Enalapril D5 Maleate (98.43%) and Enalapriatl D5 (96.02%) internal standards were purchased from Clearsynth Labs (P) Ltd. Acetonitrile, Methanol HPLC grade solvents be procured from Fisher Scientific supplier. Formic acid (AR grade) reagent and Ammonia (25%) solution were obtained from Merck. Milli-Q water was prepared on Milliq Laboratory Plant (Millipore, Bedford. USA) and HPLC grade Water was from Rankem. Orpheus, C18 100 mg/1 mL SPE cartridges were from Orochem manufacturers.Serum was primed from Gandhi Hospital, Hyderabad.

Liquid chromatography

Shimadzu HPLC system (Model: BE-LC-11) equipped with autosampler was used for analysis. The Liquid chromatographic elution and separation was performed using Zorbax Eclipse 150 mm × 4.6 mm, C18 5 µm (Make: Agilent Technologies) column. The mobile phase composition was an isocratic mixture of 65% v/v Acetonitrile and 35% v/v Buffer (0.1% v/v formic acid in water). The Chromatographic system flow rate was maintained with 0.8 mL per min. The injection volume of analyte solution was 20 µL and the chromatographic run time was 3.5 minutes per each run.

Mass spectrometry

Ionisation and detection were carried out using API 4000 LC-MS/MS system (BE-MS-05) (Applied Biosystem, Sciex, Concord, Canada), equipped with a pneumatically assisted APCI(heated nebulizer) and ESI (electrospray) ionization source, which was operated in positive mode. The mass selective detector in the multiple reaction monitoring (MRM) mode was used to get highest probable selectivity and sensitivity.

The source dependent parameters were optimized as:

Ion spray voltage : 5500 V,

Ion transfer capillary temperature : 550°C,

GS1 and GS2 were 35 psi,

Dwell time per transition : 200 msec,

Gas flow : 8 L/min,

Curtain gas and Collision gas for Enalapril and Enalaprilat were 20 V and 6 V.

De clustering Potential (DP): 80 V and 85 v for Enalapril and Enalaprilat and 80 v and 70 v for Enalapril D5 and Enalaprilat D5.

Collision Energy (CE) : 27 V and 26 V for Enalapril and Enalaprilat and 28 V and 27 V for Enalapril D5 and Enalaprilat D5.

Collision Cell Exit Potential (CXP): 20 Vand 18 V for Enalapril and Enalaprilat and 17 V and 12 V for Enalapril D5 and Enalaprilat D5.

Entrance Potential (EP): 20 V.

Quantitative finding was performed in multiple reactions monitoring (MRM) mode using the precursor → product ion transitions of m/z 377.10 → 234 for Enalapril, m/z 382.10→ 239.20 for Enalapril D5 and m/z 349 →206 for Enalaprilat, m/z 354.20 → 211.20 for Enalaprilat D5 in plasma samples. The quantification was performed via peak-area ratio method. Data acquirement and processing were accomplished using Analyst software version 1.6.1.

Stock solutions of enalapril and enalaprilat

2.0 mg of Enalapril and Enalaprilat working standards were weighed separately and accurately, transferred into a 2 mL clean glass volumetric flask, dissolved in methanol and then made up the volume to produce a solution of 1 mg/mL.

Stock solutions of enalapril D5 and enalaprilat D5

2.0 mg of Enalapril D5 and Enalaprilat D5 working standards were weighed accurately and separately, transferred into a 2 mL clean glass volumetric flask, dissolved in methanol and then made up the volume to produce a solution of 1 mg/mL. The stock solutions were diluted to appropriate concentrations with a diluent for spiking into plasma to get calibration curve (CC) standards. Both the stock solutions were stored in refrigerator at 2–8°C.

Preparation of dilute solutions for calibration curve standards and quality control samples

The stock solutions of Enalapril and Enalaprilat were further diluted with diluent to give sequential concentrations of 0.5 to 160 ng/mL for Enalapril and Enalaprilat to form calibration curve standards. Quality control samples of Enalapril were prepared at concentrations of 0.5 ng/mL for LLOQ QC, 1.5 ng/mL for IQC, 20 ng/mL for MQC1, 80 ng/mL for MQC2, and 140 ng/mL for HQC. Quality control samples of Enalaprilat were prepared at concentrations of 0.5 ng/mL for LLOQ QC, 1.5 ng/mL for IQC, 20 ng/mL for MQC1, 80 ng/mL for MQC2, and 140 ng/mL for HQC.

Sample preparation

Sample preparation was performed by simple solid phase extraction method. The samples were thawed at room temperature and vortexed for complete mixing of the contents. 250 µL of the plasma sample was pipetted into RIA vial tubes, 25 µL of combined dilution of Internal

standards (500 ng/mL Enalapril and Enalaprilat) was added and vortexed, 100 µL of 20% formic acid solution was added and vortexed it. The sample mixture was loaded onto Orpheus C18 (100 mg/mL) cartridges, which were pre-conditioned with 1 mL of methanol followed by 1 mL Milli Q water (new cartridge for each sample). After applying the maximum pressure, the extraction cartridge was washed with 1 mL of 1% formic acid solution followed by 1 mL of Milli Q water. Then the sample was eluted with 1 mL of 1% ammonia solution in methanol and evaporated to dryness under gentle stream of nitrogen at 45°C for 20 minutes. The residue was reconstituted with 1 mL of mobile phase and injected into the LC-MS/MS system. Blank plasma samples were prepared with 25 µL of diluent and vortexed.

Buffer solution (0.1% v/v formic acid)

1 mL of formic acid was transferred to a 1000 mL volumetric flask, containing 999 mL of milli Q water and sonicated in an ultrasonicator for 5 minutes.

Mobile phase

A mixture of 1300 mL of methanol was transferred to a 2000 mL volumetric flask and 700 mL of 0.1% v/v formic acid buffer was added to it, mixed well and sonicated in an ultrasonicator for 5 minutes.

Diluent

A mixture of methanol and milli Q water was prepared in the ratio of 50:50 v/v, and then sonicated in an ultrasonicator for 5 minutes.

Rinsing solution (acetonitrile and water 50:50 v/v)

A mixture of acetonitrile and milli Q water was prepared in the ratio of 50:50 v/v and then sonicated in an ultrasonicator for 5 minutes.

1% v/v ammonia solution in methanol

1 mL of ammonia solution (25%) was transferred to a volumetric flask containing 99 mL of Milli Q water, mixed well and sonicated in an ultrasonicator for 5 minutes.

Washing solvent (5%v/v methanol)

50 mL of methanol was transferred to a 1000 mL volumetric flask containing 950 mL of Milli Q water, mixed well and sonicated in an ultrasonicator for 5 minutes.

20% v/v formic acid solution

10 mL of formic acid was transferred to a 50 mL reagent bottle containing 40 mL of Milli Q water, mixed well and sonicated for 5 minutes in an ultrasonicator.

System suitability solution

A mixture of analytes and internal standards were prepared for system suitability test. The test solution was prepared by mixing 25 µL of 1600 ng/mL of Enalapril and Enalaprilat and 50 µL of 500 ng/mL of Enalapril D5 and Enalaprilat D5 with 925 µL of mobile phase.

Results and Discussion

The chromatographic conditions as a function of analytes peak intensity/peak symmetry/analysis runtime. Separation was achieved on Zorbax Eclipse (150 × 4.6 mm, C18 5 µm) column using acetonitrile: 0.1% formic acid in a ratio of 65:35, v/v as a mobile phase at a flow rate of 0.800 mL/minute with a splitter in a ratio of 50:50 under isocratic condition. The auto Sampler Cooler Temperature was maintained at 10°C and Injection volume was used 20 µL. The total run Time was 3.50 minutes with a retention time of 1.60 ± 0.5 minutes for Enalapril, Enalapril D5, Enalaprilat and Enalaprilat D5 respectively. Sample preparation is a decisive step for sensitivity and recovery by LC-MS/MS methods. Protein precipitation (PPT), Liquid–liquid (LLE) and solid-phase extraction (SPE) techniques are frequently used for consistent recoveries of analytes from biological samples. SPE was adopted for the extraction of Enalapril and Enalaprilat from human plasma but the recoveries were not reported [8]. The recoveries of Enalapril and Enalaprilat in human plasma were only about 65% and 24% using LLE [10]. The consequential variation in extraction recoveries for Enalapril and Enalaprilat are due to the difference in hydrophobic nature between them. Even though recoveries of Enalapril and Enalaprilat with protein precipitation were improved compare to LLE but the sensitivity was not satisfactory (Figures 3 and 4) [11,18]. It is a helpful and rapid procedure to optimize lifetime of the equipment but does not enhance the analytical sensitivity which is important for low-volume LC-MS/MS assays [13].

In the present research work, a solid phase extraction was preferred for the extraction of the biological fluid owing to the greater purification performance if compared to PPT or LLE and also provided high recoveries for both the analytes compared to LLE [10]. Under the optimal LC–MS conditions, the obtained sensitivity was higher than that reported in the literature [11,18]. To accomplish desire sensitivity and selectivity, proper extraction procedure and suitably optimized LC-MS detection parameters were used. ESI source in positive ionization mode for MRM HPLC-MS-MS analyses was used to maximize sensitivity for both Enalapril and Enalaprilat consisting secondary amino and carboxyl functions. Parameters such as ESI source temperature, curtain gas and collision gas were optimized at 550°C, 20 V and 6 V to get highest intensity of protonated molecules of the two compounds. The precursor ions are readily fragmented to give stable and consistent product ions. The most abundant product ions for Enalapril at m/z value 234 and Enalaprilat at m/z value. The collision energy of Enalapril is 27 v and 26 v for Enalaprilat. Multiple reactions monitoring (MRM) was employed for quantification of Enalapril and Enalaprilat in human plasma using the precursor → product ion transition of m/z 377 → m/z 234, m/z349 → m/z 206, respectively. A dwell time of 200 msec per transition was adequate to have sufficient datapoints. The multiple-reaction monitoring mode (MRM) (+) chromatograms extracted from plasma are depicted in Figures 5 and 6 as shown, the retention times of Enalapril and Enalaprilat were 1.60 minute. The entire HPLC–MS analysis time was 3.5 minute per sample.

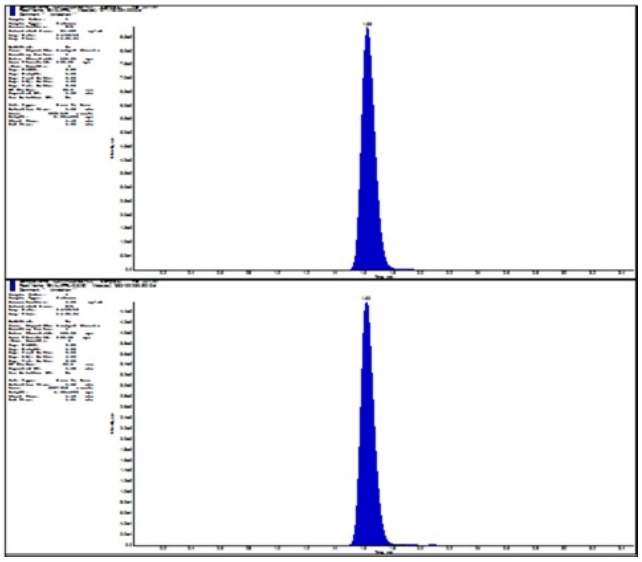

Figure 3: Representative chromatogram of an aqueous standard and internal standard mixture of Enalapril.

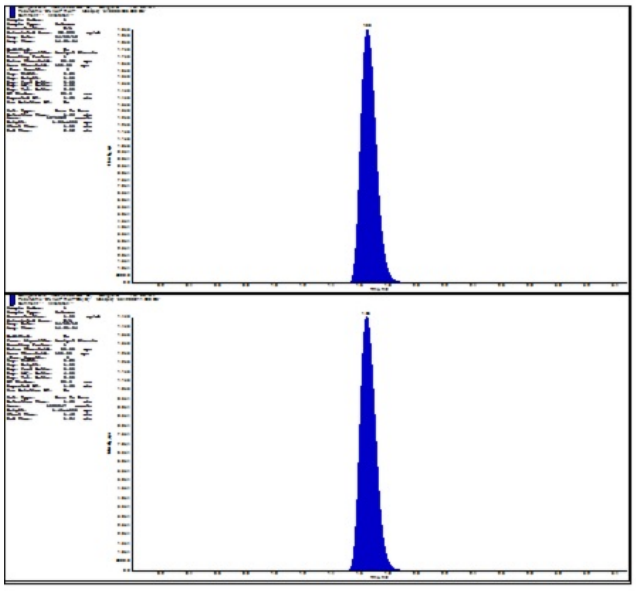

Figure 4: A representative chromatogram of an aqueous standard and internal standard mixture of Enalaprilat.

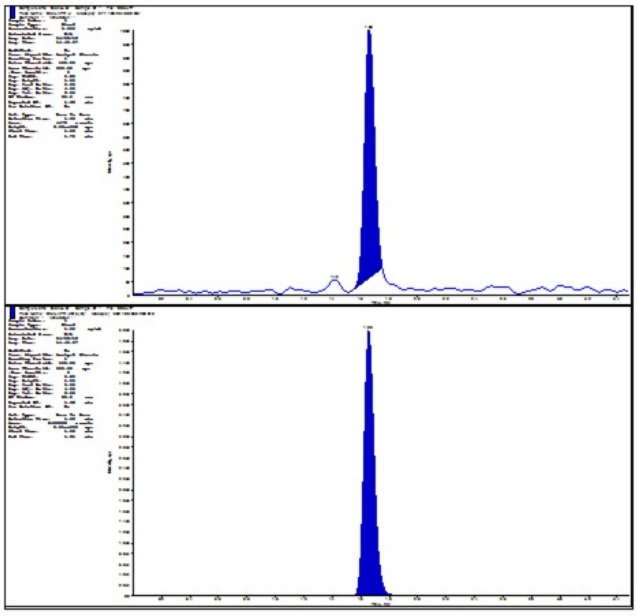

Figure 5: A representative chromatogram of blank plasma with internal standard sample of Enalapril.

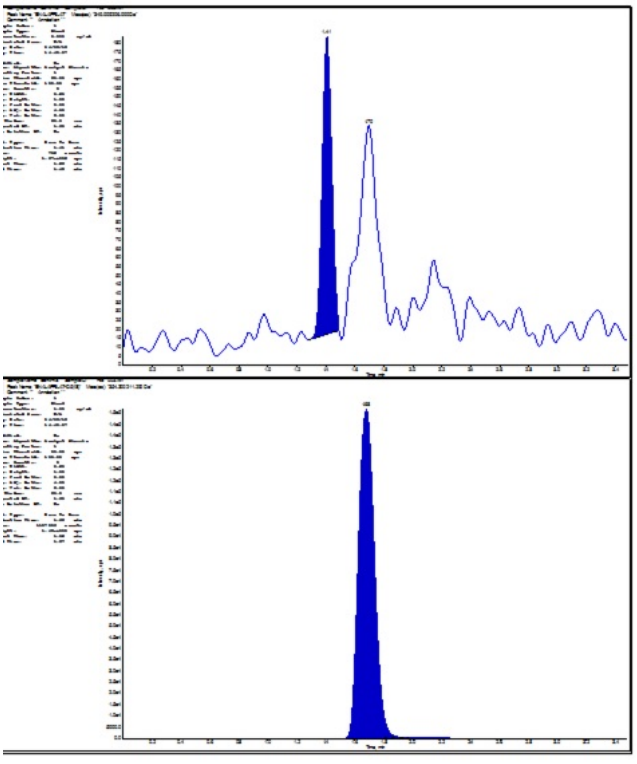

Figure 6: A representative chromatogram of blank plasma with internal standard sample of Enalaprilat.

Method validation

Selectivity: Free of any significant interference for Enalapril and Enalaprilat and internal standards from all eight human plasma samples was observed. There was no significant interference from endogenous components observed at their mass transitions. The chromatograms of blank plasma sample of Enalapril and Enalaprilat are given in Figures 7 and 8.

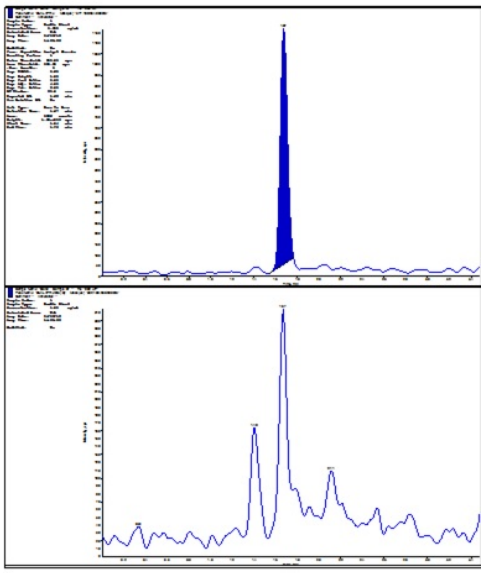

Figure 7: Representative chromatogram of blank plasma sample of Enalapril.

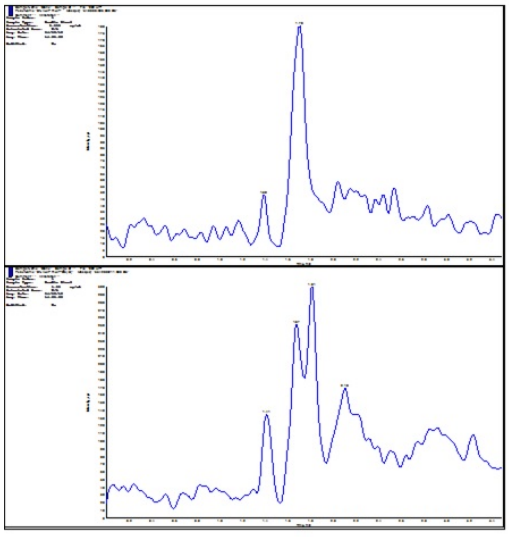

Figure 8: Representative chromatogram of blank plasma sample of Enalaprilat.

Carry over test: There was no significant carryover effect of analytes and internal standard was observed as response of interfering peaks in extracted blank plasma at the retention time of analytes and internal standards. A representative chromatogram of extracted blank plasma sample of Enalaprilat was shown in Figure 9.

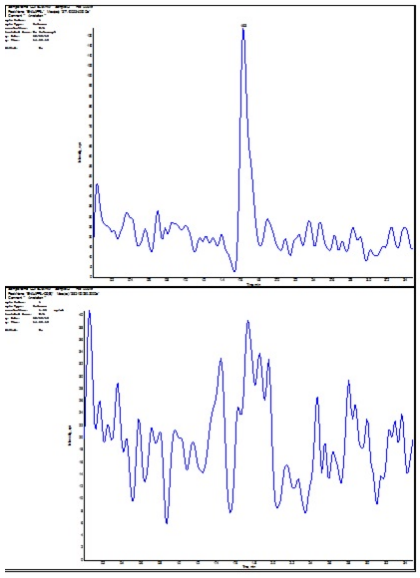

Figure 9: A representative chromatogram of extracted blank plasma sample analyzed for carryover test Enalapril.

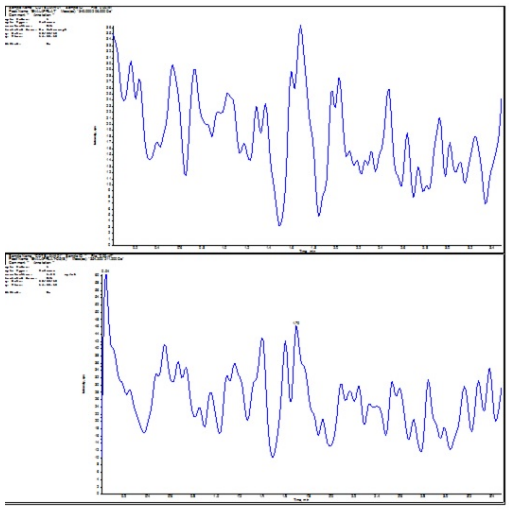

Figure 10: A representative chromatogram of extracted blank plasma sample analyzed for carryover test of Enalaprilat.

Linearity: The method shows linear response over the concentration range of 0.502 to 160.222 ng/mL for Enalapril and 0.506 to 161.518 ng/mL for Enalaprilat. A straight-line fit was made through data points by least square regression analysis. A representative linear regression equation of the process was: y=0.0231x+0.00481, for Enalapril and y=0.0142x+0.00211, for Enalaprilat, with x and y in place of concentration (in ng/mL) and response (in arbitrary units), respectively, and the regression coefficient (r) of 0.9965 for Enalapril

and 0.9996 for Enalaprilat. The representative calibration curves for Enalapril and Enalaprilat are illustrated in Figures 10-20.

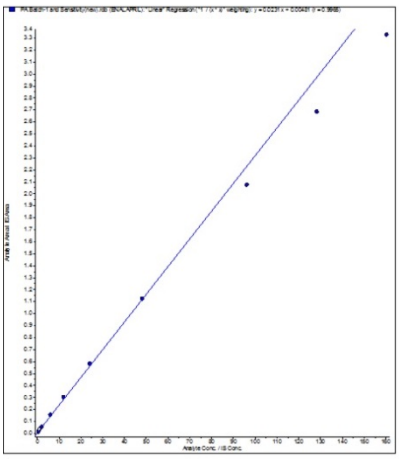

Figure 11: Representative calibration curve for regression analysis of Enalapril.

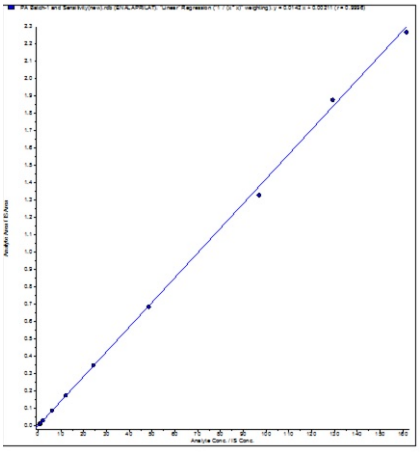

Figure 12: Representative calibration curve for regression analysis of Enalaprilat.

Precision and accuracy

Within-batch precision and accuracy: The %CV for Enalapril at LLOQ QC, IQC, MQC1, MQC2, and HQC were ranged from 2.95%-5.25%, 2.87%-10.82%, 1.21%-1.40%, 0.52%-1.46%, and 1.03%-2.08%, respectively. The % nominal for Enalapril at LLOQ QC, IQC, MQC1, MQC2, and HQC ranged from 91.42%-102.06%. Precision and accuracy results for Enalapril are shown in Table 1. The %CV for Enalaprilat at LLOQ QC, IQC, MQC1, MQC2, and HQC ranged from 4.49%-8.30%, 1.19%-4.39%, 1.32%-1.85%, 1.21%-2.29%, and 0.68%-0.78%, respectively. The % nominal for Enalaprilat at LLOQ QC, IQC, MQC1, MQC2, and HQC ranged from 84.73%-101.09%. Precision and accuracy results for Enalaprilat are shown in Table 2.

Intra-day (within run) precision and accuracy: Intra-day precision (%cv) for Enalapril at LLOQ QC, IQC, MQC1, MQC2, and HQC was 3.47%, 8.60%, 1.23%, 1.18% and 1.87%, respectively. The % of mean Accuracy for Enalapril for LLOQ QC, IQC, MQC1, MQC2, and HQC was 92.21%-101.87%, and 91.07%, respectively.

Intra-day precision for Enalaprilat for LLOQ QC, IQC, MQC1, MQC2, and HQC was 6.17%, 4.39%, 2.12%, 1.46% and 0.81%, respectively. % mean accuracy for Enalaprilat at LLOQ QC, IQC, MQC1, MQC2, and HQC was 88.00%, 92.79%, 99.84%, 98.11%, and 99.92%, respectively. Precision and accuracy results for Enalapril and Enalaprilat are shown in Table 3.

Inter day (between batch) precision and accuracy: Inter day precision for Enalapril at LLOQ QC, IQC, MQC1, MQC2, and HQC was 3.98%, 9.24%, 1.26%, 1.06%, and 1.61%, respectively. Accuracy for LLOQ QC, IQC, MQC1, MQC2, and HQC was 92.39%, 99.08%, 101.73%, 92.12%, and 91.19%, respectively. Inter day precision for Enalaprilat at LLOQ QC, IQC, MQC1, MQC2, and HQC was 6.73%, 4.01%, 1.86%, 1.71%, and 0.79%, respectively. Accuracy for LLOQ QC, IQC, MQC1, MQC2, and HQC was 88.31%, 91.72%, 99.73%, 98.05%, and 99.79%, respectively. Precision and accuracy results for Enalapril and Enalaprilat are shown in Table 4.

QC Level	LLOQ	IQC	MQC1	MQC2	HQC
Nominal conc.ng/mL	0.509	1.534	20.735	82.942	140.579
Mean measured Conc.ng/mL	0.4755	1.5058	21.1625	76.773	128.146
S.D.	0.01827	0.14557	0.26696	1.11924	2.33903
C.V.%	3.84	9.67	1.26	1.46	1.83
% Nominal	93.42	98.16	102.06	92.56	91.16
Number of injections	6	6	6	6	6
Mean measured Conc.ng/mL	0.4653	1.4453	20.9347	76.03	127.909

S.D.	0.01375	0.04155	0.2535	0.39273	2.66651
C.V.%	2.95	2.87	1.21	0.52	2.08
% Nominal	91.42	94.22	100.96	91.67	90.99
Number of injections	6	6	6	6	6
Mean measured Conc.ng/mL	0.47	1.5535	21.033	76.2537	128.507
S.D.	0.02467	0.16815	0.294	0.63052	1.31873
C.V.%	5.25	10.82	1.4	0.83	1.03
% Nominal	92.34	101.27	101.44	91.94	91.41
Number of injections	6	6	6	6	6

Table 1: Within batch precision and accuracy for Enalapril.

QC Level	LLOQ	IQC	MQC1	MQC2	HQC
Nominal conc.ng/mL	0.513	1.545	20.874	83.495	141.517
Mean measured Conc.ng/mL	0.4347	1.4005	20.5797	81.2615	140.847
S.D.	0.02432	0.04387	0.38072	1.04739	0.95503
C.V.%	5.6	3.13	1.85	1.29	0.68
% Nominal	84.73	90.65	98.59	97.32	99.53
Number of injections	6	6	6	6	6
Mean measured Conc.ng/mL	0.4682	1.4668	21.1018	82.5708	141.965
S.D.	0.021	0.06442	0.34952	1.0024	1.10559
C.V.%	4.49	4.39	1.66	1.21	0.78
% Nominal	91.26	94.94	101.09	98.89	100.32
Number of injections	6	6	6	6	6
Mean measured Conc.ng/mL	0.4563	1.384	20.7728	81.78	140.85
S.D.	0.03789	0.01647	0.27496	1.87464	1.03942
C.V.%	8.3	1.19	1.32	2.29	0.74
% Nominal	88.95	89.58	99.52	97.95	99.53
Number of injections	6	6	6	6	6

Table 2: Within batch precision and accuracy for Enalaprilat.

Name of the analytes	QC Level	Nominal conc.ng/mL	Mean (N=12) measured Conc.ng/mL	S.D	C.V.%	% Nominal
Enalapril	LLOQ	0.509	0.4704	0.01631	3.47	92.42
	IQC	1.534	1.5032	0.12934	8.6	97.99
	MQC1	20.735	21.1235	0.25916	1.23	101.87
	MQC2	82.942	76.4797	0.89989	1.18	92.21
	HQC	140.579	128.027	2.39462	1.87	91.07

Enalaprilat	LLOQ	0.513	0.4514	0.02785	6.17	88
	IQC	1.545	1.4337	0.06294	4.39	92.79
	MQC1	20.874	20.8408	0.44247	2.12	99.84
	MQC2	83.495	81.9162	1.19287	1.46	98.11
	HQC	141.517	141.406	1.14498	0.81	99.92

Table 3: Intraday (within run) Precision and Accuracy for Enalapril and Enalaprilat.

Name of the analytes	QC Level	Nominal conc.ng/mL	Mean (N=12) measured Conc.ng/mL	S.D	C.V.%	% Nominal
Enalapril	LLOQ	0.509	0.4703	0.0187	3.98	92.39
	IQC	1.534	1.5199	0.1405	9.24	99.08
	MQC1	20.735	21.0933	0.2661	1.26	101.73
	MQC2	82.942	76.4043	0.8081	1.06	92.12
	HQC	140.579	128.187	2.06783	1.61	91.19
Enalaprilat	LLOQ	0.513	0.4531	0.0305	6.73	88.31
	IQC	1.545	1.4171	0.0568	4.01	91.72
	MQC1	20.874	20.8181	0.3873	1.86	99.73
	MQC2	83.495	81.8708	1.3995	1.71	98.05
	HQC	141.517	141.221	1.113	0.79	99.79

Table 4: Inter Day (Between Batch) Precision and Accuracy for Enalapril and Enalaprilat.

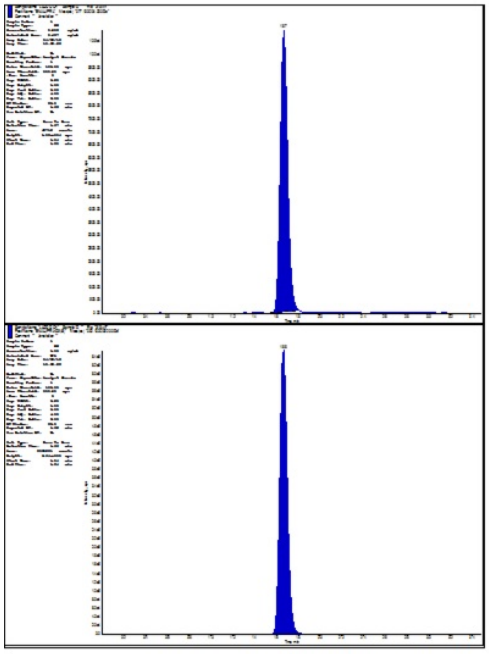

Figure 13: A representative chromatogram of lloq qc sample of Enalapril.

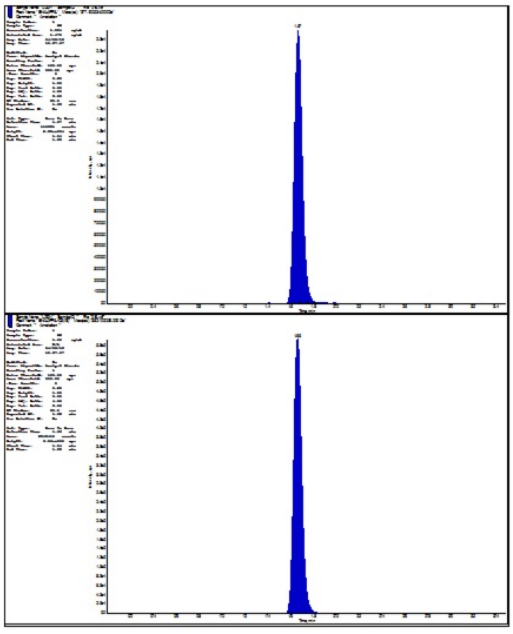

Figure 14: A representative chromatogram of IQC sample of Enalapril.

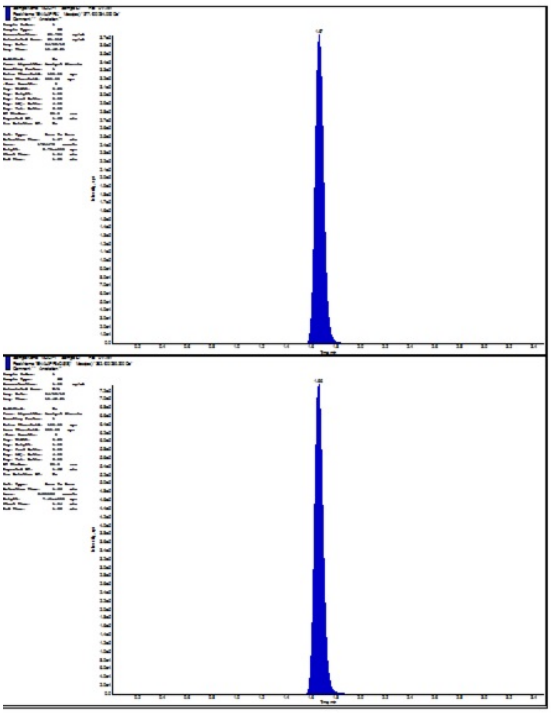

Figure 15: A representative chromatogram of MQC sample of Enalapril.

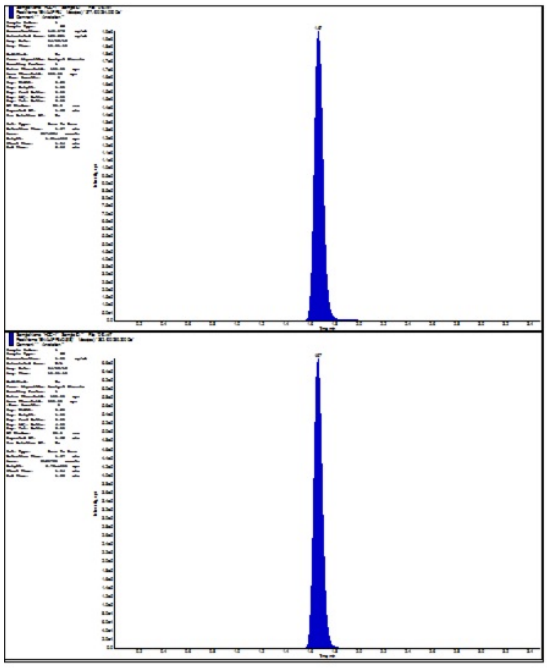

Figure 16: A representative chromatogram of HQC sample of Enalapril.

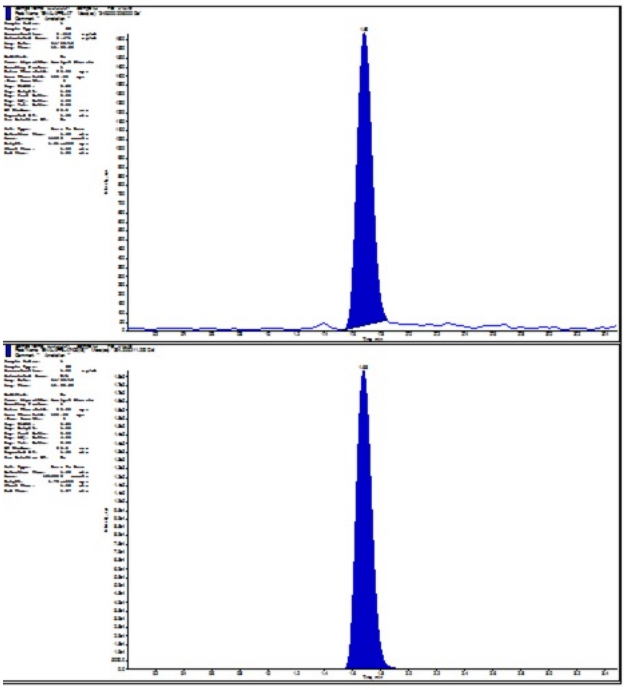

Figure 17: A representative chromatogram of lloq qc sample of Enalaprilat.

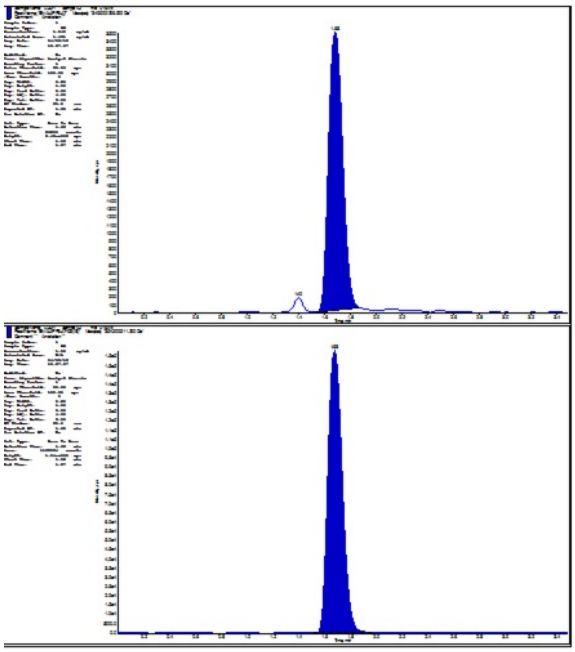

Figure 18: A representative chromatogram of IQC sample of Enalaprilat.

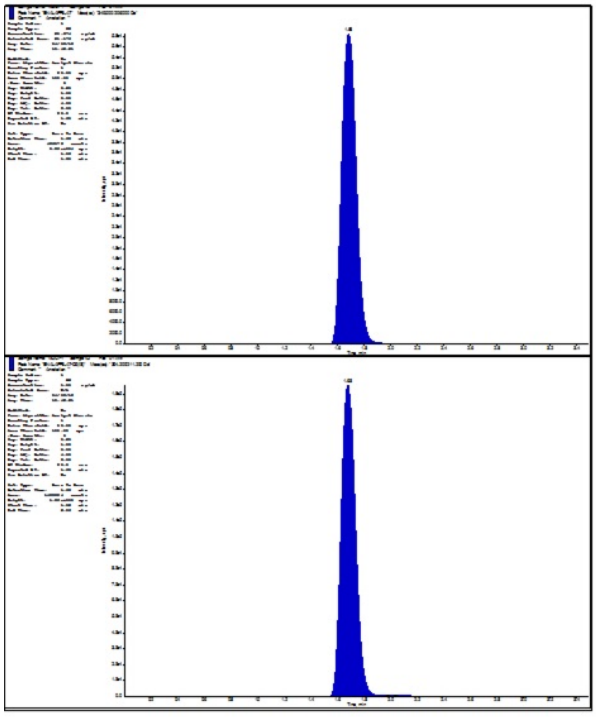

Figure 19: A representative chromatogram of MQC sample of Enalaprilat.

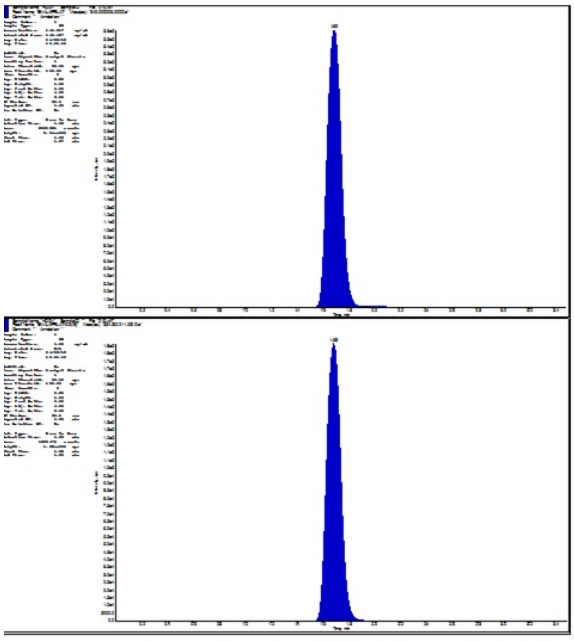

Figure 20: A representative chromatogram of HQC sample of Enalaprilat.

Recovery

The mean overall recovery of Enalapril was 91.21% with a precision range of 1.72% to 5.06%. Similarly, the mean overall recovery of Enalaprilat was 90.85% with a precision range of 1.29% to 3.87%. The mean recovery of internal standard Enalapril D5 was 88.51% with a precision ranging from 3.16% to 3.91%.The mean recovery of internal standard Enalaprilat D5 was 88.27% with a precision ranging from 2.56% to 2.79%. Recovery results are displayed in Tables 5 and 6.

Name of the analyte	QC Level	Extracted QC samples		Non extracted QC samples		% Recovery	Overall recovery	% Difference
		Mean area response (N=6)	% C.V.	Mean area response (N=6)	% C.V.			
Enalapril	IQC	82481.7	2.85	87211.5	5.06	94.58	91.21	6.01
	MQC2	4142139	5.06	4578406	3.81	90.47		
	HQC	6442250	1.83	7273829	1.72	88.57		
Enalaprilat	IQC	22146.7	1.84	24318	3.87	91.07	90.85	4.32
	MQC2	1185937	2.7	1276482	2.22	92.91		
	HQC	1871414	3.06	2112532	1.29	88.59		

Table 5: Recovery of Enalapril and Enalaprilat from human plasma.

Name of the analyte	QC Level	Mean area responses of extracted QC samples (N=18; 6 from each level)	C.V.%	Mean area response of non-extracted QC samples (N=18; 6 from each level)	C.V.%	% Recovery
Enalapril	IQC	2335263	3.91	2638269	3.16	88.51
	MQC2					
	HQC					
Enalaprilat	IQC	1033471	2.79	1170753	2.56	88.27

Table 6: Recovery of Enalapril d5 and Enalaprilat d5 from human plasma ruggedness (robustness).

Precision and accuracy result of both Enalapril and Enalaprilat for Ruggedness of a method was within the acceptance criteria. Within batch precision of Enalapril for LLOQ QC, IQC, MQC1, MQC2, and HQC was 5.25%, 10.82%, 1.40%, 0.83%, and 1.03% and accuracy was 92.34%, 101.27%, 101.44%, 91.94%, and 91.41%, respectively. Within batch precision of Enalaprilat for LLOQ QC, IQC, MQC1, MQC2, and HQC was 8.30%, 1.19%, 1.32%, 2.29%, and 0.74% and accuracy was 88.95%, 89.58%, 99.52%, 97.95%, and 99.53%, respectively. Ruggedness results are shown in Tables 7 and 8.

Calibration curve Standards	Enalapril			Enalaprilat		
	Nominal conc. ng/mL	Measured conc. ng/mL	% Accuracy	Nominal conc.ng/mL	Measured conc. ng/mL	% Accuracy
STD-1	0.502	0.463	92.23	0.506	0.521	103.01
STD-2	1.003	1.103	109.99	1.012	0.942	93.11
STD-3	2.007	2.171	108.2	2.023	2.038	100.72
STD-4	6.008	6.483	107.9	6.057	6.221	102.71
STD-5	12.017	12.602	104.8	12.114	12.213	100.82
STD-6	24.033	24.778	103.1	24.288	24.152	99.69
STD-7	48.408	47.534	98.89	48.455	48.237	99.55

STD-8	96.133	88.98	92.56	96.911	98.364	101.5
STD-9	128.178	118.036	92.09	129.214	127.855	98.95
STD-10	160.222	144.641	90.28	161.518	161.433	99.95
Calibration curve						
Slope	0.0234			0.0138		
Intercept	0.0047			0.0024		
Correlation Coefficient (r)	0.9963			0.9995		

Table 7: Concentration response linearity data of Enalapril and Enalaprilat for ruggedness.

Name of the analyte	QC Level	Nominal conc.ng/mL	Mean (N=12) measured Conc.ng/mL	S.D	C.V.%	% Nominal
Enalapril	LLOQ	0.509	0.47	0.02467	5.25	92.34
	IQC	1.534	1.5535	0.16815	10.82	101.27
	MQC1	20.735	21.033	0.294	1.4	101.44
	MQC2	82.942	76.2537	0.63052	0.83	91.94
	HQC	140.579	128.507	1.31873	1.03	91.41
Enalaprilat	LLOQ	0.513	0.4563	0.03789	8.3	88.95
	IQC	1.545	1.384	0.01647	1.19	89.58
	MQC1	20.874	20.7728	0.27496	1.32	99.52
	MQC2	83.495	81.78	1.87464	2.29	97.95
	HQC	141.517	140.85	1.03942	0.74	99.53

Table 8: Within batch precision and accuracy of Enalapril and Enalaprilat for ruggedness.

Room temperature standard stocks and spiking solution stability: The precision of stock solution stability of Enalapril and Enalaprilat at room temperature for 9 hours was 1.61% and 2.03% and the percentage of stability was found to be 95.22% and 96.27% respectively. The precision of stock solution stability of Enalapril D5 and Enalaprilat D5 at room temperature for 9 hours was 1.62% and 1.27% and the percentage of stability was found to be 98.84% and 100.32% respectively. The precision of spiking solution stability of Enalapril and Enalaprilat at room temperature for 9 hours was 1.89% and 2.33%. The percentage of stability was found to be 97.82% and 97.93% respectively. The precision of spiking solution stability of Enalapril D5 and Enalaprilat D5 at room temperature for 9 hours was 1.43% and 0.78%. The percentage of stability was found to be 97.10% and 98.91% respectively. Based on the results, both stock and spiking solutions of analytes and internal standards were stable at room temperature for 9 hours. The results are shown in Table 9.

Name of the Stock/ Spiking Solution	Stability samples			Comparison samples			% Stability
	Standard Stock dilution	Mean area of (N=6) Stability standard stock	CV %	Standard Stock dilution	Mean area of (N=6) Comparison standard stock	CV %	
Enalapril	996384.8	5181307	1.61	1001389	5468927	1.77	95.22
Enalaprilat	1000769	2653387	2.03	1000241	2754626	1.93	96.27

Enalapril D5	1007739	1488057	1.62	1009487	1508062	1.43	98.84
Enalaprilat D5	986605.5	1100007	1.27	1003409	1115227	1.44	100.32
Enalapril	1594.216	5323117	1.89	1602.222	5468927	1.77	97.82
Enalaprilat	500.384	2674699	2.33	500.384	2754626	1.93	97.1
Enalapril D5	1612.383	1474301	1.43	1615.179	1508062	1.43	97.93
Enalaprilat D5	493.303	1103083	0.78	493.303	1115227	1.44	98.91

Table 9: Room temperature standard stocks and spiking solution stability refrigerated stock solution stability.

The refrigerated stock solutions were found to be stable for 5 days. The precision of Enalapril and Enalaprilat was 1.28% and 1.24%. The percentage of stability was found to be 95.02% and 101.71%. The precision for refrigerated stock solution stability of Enalapril D5 and Enalaprilat D5 was 1.49% and 1.29%. The percentage of stability was found to be 95.83% and 101.23%.

Freeze-thaw stability

The percent nominal of Enalapril for IQC and HQC was 93.80% and 89.23% and the precision was 1.61% and 0.57%. Similarly, the percent nominal of Enalaprilat for IQC and HQC was 99.25% and 99.24% and the precision was 1.75% and 1.26% after five freeze-thaw cycles.

Bench top stability or short-term stability

Enalapril and Enalaprilat were found to be stable up to 11 hours. The percent nominal of Enalapril at IQC was 92.48% and at HQC 88.63% and the % CV was 1.22% and 1.17% respectively. Similarly, the percent nominal of Enalaprilat was 97.31 for HQC and 98.78% for HQC and the precision was 2.50% and 0.64% respectively.

Wet extract stability

Results demonstrate that the processed samples were stable for 45 hours at room temperature (20 ± 5°C). The percent nominal of Enalapril was 92.14% and 88.38% and the precision was 1.72% and 1.33%. Similarly, the percent nominal of Enalaprilat was 99.09% and100.38% and the precision was 2.10% and 1.98%.

Auto sampler stability

Results reveal that the processed samples were stable for 54 hours. The percent nominal of Enalapril was 91.73% and 88.68% and the precision was 1.84% and 0.79%. Similarly, the percent nominal of Enalaprilat ranged from 99.71% to 99.87% and the precision was 1.81% and 1.31.

Short-term plasma samples stability at -20°C

Percentage of Stability was calculated against freshly spiked quality control samples. The percent stability of Enalapril was 101.24% and 99.03% and the precision was 2.70% and 1.43%. Similarly, the percent stability of Enalaprilat was 99.04% and 98.39% and the precision was 2.10% and1.04%.

Re Injection stability

Results showed that the re injected samples were stable for 31 hours. The percent stability of Enalapril after 31 hours was 102.40% and 101.34% and precision was 7.95% and 2.57%. Similarly, the percent stability of Enalaprilat after 31 hours was 96.54%and 95.28% and precision was 3.20% and 1.09%.

Dilution integrity

The results of Enalapril and Enalaprilat demonstrate acceptable dilution integrity for two and four times. The precision and accuracy results of Enalapril, for a dilution factor of 2 were 1.69% and 92.81%, respectively. Similarly, for a dilution factor of 4 were 1.34% and 97.02%, respectively.

The precision and accuracy results of Enalaprilat, for a dilution factor of 2 were 1.26% and 98.80%, respectively. Similarly, for a dilution factor of 4 were 1.10% and 98.21%, respectively.

Matrix effect

No significant matrix effect was observed in all the eight batches for Enalapril and Enalaprilat at low (IQC) and high (HQC) concentrations. The precision (%CV) results of Enalapril for IS normalized matrix factor at IQC and HQC level was found to be 1.99% and 1.16%, respectively and mean IS normalized matrix factor was 0.998 for IQC and 0.999 for HQC. Similarly, the precision (%CV) results of Enalaprilat for IS normalized matrix factor at IQC and HQC level was found to be 3.90% and 1.02%, respectively and mean IS normalized matrix factor was 0.997 for IQC and 1.006 for HQC.

Concomitant drug effect

Acceptance results were observed for concomitant drugs spiked QC's of Enalapril and Enalaprilat. The precision and accuracy for LLOQ QC, IQC and HQC of Enalapril were 7.67%, 1.11% and 2.63%, and 92.34%, 94.90% and 88.93%, respectively. The precision and accuracy for LLOQ QC, IQC and HQC of Enalaprilat were 12.19%, 3.45% and 1.57%, and 92.95%, 88.13% and 98.96%, respectively.

Run size evaluation

160 QC's out of 160 QC's of run size evaluation for both Enalapril and Enalaprilat were within 15% of their respective nominal (theoretical) values and 24 QC's out of 24 QC's of freshly prepared QCs for both Enalapril and Enalaprilat were within 15% of their respective nominal (theoretical) values.

Stability in whole blood

Results demonstrate that the whole blood samples were stable for 3 hours at room temperature (20 ± 5°C). The percent stability of Enalapril at IQC level was found to be 101.23% with the precision range of 1.93%-2.42% and at HQC level was found to be 98.24% with the precision range of 2.52%-3.21% for 3 hours. Similarly, the percent stability of Enalaprilat at IQC level was found to be 101.02% with the precision range of 0.74%-1.53% and at HQC level was found to be 98.57% with the precision range of 1.32%-1.41% for 3 hours.

Conclusion

The main aim of this research is to develop simple, fast, selective, highly sensitive, robust, and reproducible bio-analytical method to quantify the Enalapril, Enalaprilat form human plasma. The benefit of using a solid phase extraction procedure in this research is owing to high extraction recovery and minimization of sample extraction time. For the quantitation, 250 µL of plasma sample has been used for this method and therefore decrease the quantity of blood withdrawn from volunteers during study. Projected method gives cleaner and consistent extraction without any significant matrix effect. Because of rapid sample preparation technology and short chromatographic run time is 3.5 minutes; more numbers of samples can be analyzed. The developed HPLC-MS/MS method to estimate Enalapril and Enalaprilat in K2EDTA human plasma is fully validated. Finally, this optimized bio-analytical method is trouble-free, perceptive, quick, and greatly selective, precise, accurate, stable and reproducible method. This method can be efficiently used to quantify Enalapril and Enalaprilat in human plasma of routine bioequivalence studies.

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