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Original Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS DETERMINATION OF VERAPAMIL AND ENALAPRIL IN THE PRESENT OF ENALAPRILAT BY HPLC-MS/MS

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ABSTRACT

Objective: The main purpose of this study was to develop a simple, precise, rapid and accurate method for the simultaneous quantification of verapamil and enalapril in the present of enalaprilat in human plasma.

Methods: Analytes were extracted from plasma using a protein precipitation extraction method. Chromatography was achieved on Discovery C18, 50 × 2.1 mm, 5 µm column. Samples were chromatographed in a gradient mode (eluent A (acetonitrile–water–formic acid, 5: 95: 0.1 v/v), eluent B (acetonitrile–formic acid, 100: 0.1 v/v)). The initial content of the eluent B is 0%, which increases linearly by 1.0 min to 100% and to 1.01 min returns to the initial 0%. The mobile phase was delivered at a flow rate of 0.400 ml/min into the mass spectrometer ESI chamber. The sample volume was 5 µl.

Results: The total chromatographic run time was 2.0 min and the elution of verapamil, enalapril, enalaprilat and IS (bisoprolol) occurred at ~1.09, 1.03, 0.96 and 1.01 min, respectively. A linear response function was established at 1-100 ng/ml for verapamil hydrochloride, 2-200 ng/ml for enalapril maleate, 1-100 ng/ml for enalaprilat dehydrate in human plasma. The % accuracy of LLOQ samples prepared with the different biological matrix lots were found 99.2 % for enalapril, 103.4 % for enalaprilat, 90.6 % for verapamil, which were found within the range of 80.00-120.00% for the seven different plasma lots. % CV for LLOQ samples was observed as 4.9%, 5.9 %, 9.0 % respectively, which are within 20.00% of the acceptance criteria. The % mean recovery for enalapril in LQC, MQC and HQC was 103.6 %, 100.1 % and 102.4 %, for enalaprilat in LQC, MQC and HQC was 103.2 %, 98.0 % and 101.1 %, for verapamil in LQC, MQC and HQC was 97.9 %, 96.1 % and 98.6 % respectively.

Conclusion: A new rapid method was developed for simultaneous determination of verapamil and enalapril in the present of enalaprilat in human plasma. The method was strictly validated according to the ICH guidelines. Acquired results demonstrate that proposed strategy can be effortlessly and advantageously applied for routine examination of verapamil and enalapril in the present of enalaprilat in human plasma.

Keywords: Verapamil, Enalapril, Enalaprilat, HPLC-MS/MS, Validation, Human plasma

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INTRODUCTION

Bioanalytical methods for appraising drugs and their metabolites in biological samples play an important role in calculating and interpreting data from the studies on bioavailability, bioequivalence, and pharmacokinetics.

Verapamil hydrochloride is a first generation calcium channel blocker used for treatment of hypertension, angina pectoris and supraventricular tachyarrhythmias. Verapamil has been linked to a low rate of serum enzyme elevations during therapy and to rare instances of clinically apparent acute liver injury. The chemical name of verapamil is 2-(3,4-dimethoxyphenyl)-5-[2-(3,4-dimethoxyphenyl)ethyl-methyl-amino] -2-propan-2-ylpentanenitrile (fig. 1) [1].

Enalapril maleate is chemically described as (2S)-1-[(2S)-2-[[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl] amino] propanoyl] pyrrolidine-2-carboxylic acid (Z)-butenedioate (fig. 2). Enalapril maleate is a prodrug which metabolized rapidly in the liver to ethyl ester of a long-acting enalaprilat (fig. 3) which inhibits angiotensin-converting enzyme (ACE) in human subjects and animals [2, 3].

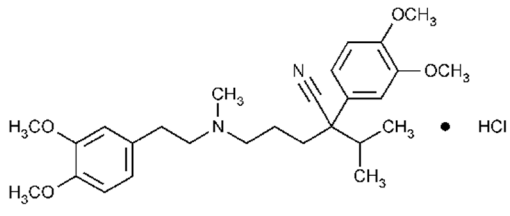

Fig. 1: Chemical structure of verapamil hydrochloride

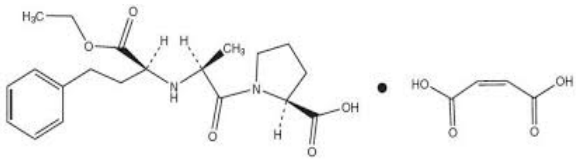

Fig. 2: Chemical structure of enalapril maleate

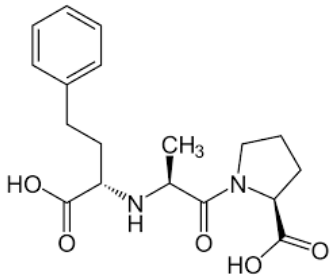

Fig. 3: Chemical structure of enalaprilat

In the contemporary literature, few bioanalytical methods were reported for quantification of verapamil and enalapril in the present of enalaprilat in human plasma [4-6]. Therefore, it was thought desirable to develop a simple, accurate, cheap and fast procedure that could be applied for the simultaneous determination of verapamil and enalapril in the present of enalaprilat in human

plasma, this study performed assay validations as per guidelines [7]. While this method with validation details were economical and applied for pharmacokinetic studies of verapamil and enalapril.

MATERIALS AND METHODS

Chemicals and reagents

Verapamil (purity 100.0 %), enalapril (purity 99.3 %), enalaprilat (purity 100.0 %), bisoprolol (Internal Standard) (purity 99.9 %) were purchased from Moehs Catalana, S. L., Spain, Zhejiang Huahai Pharmaceutical Co., Ltd, KHP, EDQM–Council of Europe. HPLC grade acetonitrile and methanol were purchased from CHROMASOLV, HPLC grade formic acid were purchased from Fluka. All other chemicals and reagents were of analytical grade. Microcaps® disposable micropipettes (50 µl, catalog number: 1-000-0500) were purchased from Drummond Scientific Company, USA. The control human dipotassium ethylenediaminetetraacetic acid (K2EDTA) plasma sample was procured from Red Cross Society, Ukraine.

Instrumentation and chromatographic conditions

A Shimadzu HT (Shimadzu, Japan) LC system equipped with degasser (DGU-14A), binary pump (LC-20ADXR) along with auto-sampler (SIL-20ACXR) was used to inject 5 µl aliquots of the processed samples on Discovery C18, 50 × 2.1 mm, 5 µm column maintained at 25±1 °C. Samples were chromatographed in a gradient mode (eluent A (acetonitrile–water–formic acid, 5: 95: 0.1 v/v), eluent B (acetonitrile–formic acid, 100: 0.1 v/v)). The initial content of the eluent B is 0%, which increases linearly by 1.0 min to 100% and to 1.01 min returns to the initial 0%. The mobile phase was delivered at a flow rate of 0.400 ml/min into the mass spectrometer ESI chamber. Paramatrs of electrospray ionizer and MRM parametrs are listed in table 1-2. The analytical data were processed by Analyst Software (version 1.5.2).

Table 1: Parameters of ionizer electrospray

S. No.	Parametr	Value
1	Polarity	Positive
2	Nebulizer Gas (NEB, Gas 1)	15
3	Curtain Gas (CUR)	8
4	Collision Gas (CAD)	4
5	IonSpray Voltage (IS)	5000
6	Temperature (TEM)	400
7	Turbo IonSpray Gas	8
8	Horizontal Position	5.3
9	Lateral Position	1.3

Table 2: Multiple reaction monitoring (MRM) parameters

ID	Parent, m/z	Daughter, m/z	Time, ms	DP, V	FP, V	EP, V	CE, V	CXP, V
Enalapril	377.397	234.3	50	41	210	11	29	20
Enalaprilat	349.328	206.3	50	46	220	11	27	34
Bisoprolol (IS)	326.435	116.3	50	46	260	11	27	20
Verapamil	455.385	165.4	50	61	320	11	39	28

*Abbreviations: DP, declustering potential; FP, focusing potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential

Standard solutions

Verapamil, enalapril, enalaprilat and IS were weighed accurately into volumetric flasks using an analytical microbalance. Approximately 1 mg/ml primary stock solutions of enalaprill, 0.5 mg/ml primary stock solutions of enalaprilat, 0.25 mg/ml primary stock solutions of bisoprolol (IS) and 0.5 mg/ml primary stock solutions of verapamil solutions were prepared in methanol. The stock solutions were stored at −20 °C, which were found to be stable for 1 mo. The stock solutions of verapamil, enalapril, enalaprilat were successively diluted with methanol and water to prepare secondary stocks and working solutions. Secondary stock solutions and working solutions were used to prepare calibration curve (CC) and quality control (QC) samples. Working stock solutions were stored at 4 °C for a week. Working stocks were used to prepare plasma calibration standards. A working IS solution (20 ng/ml) was prepared in acetonitrile: methanol (50:50 v/v). Blank human plasma was screened before spiking to ensure that it was free from endogenous interference at retention times of verapamil, enalapril, enalaprilat and IS, respectively. Calibration standards' samples (1-100 ng/ml for verapamil hydrochloride, 2-200 ng/ml for enalapril maleate, 1-100 ng/ml for enalaprilat dehydrate) were prepared by spiking the blank human K2EDTA plasma with appropriate concentration of verapamil, enalapril and enalaprilat.

Samples for the determination of precision and accuracy were prepared by spiking control human plasma in bulk with verapamil, enalapril and enalaprilat at appropriate concentrations (for verapamil hydrochloride 3.0 ng/ml low QC [LQC], 30.0 ng/ml medium QC [MQC], and 75.0 ng/ml high QC [HQC], for enalapril maleate 6.0 ng/ml low QC [LQC], 60.0 ng/ml medium QC [MQC], and 150.0 ng/ml high QC [HQC], for enalaprilat dihydrate 3.0 ng/ml low QC [LQC], 30.0 ng/ml medium QC [MQC], and 75.0 ng/ml high QC [HQC],) and 120 µL plasma aliquots were distributed into different tubes. All the samples were stored at −80 °C±10 °C.

Sample preparation

A simple protein precipitation extraction method was followed for extraction of verapamil, enalapril, enalaprilat at from human plasma. From the deep freezer, the required quantities of CC standards and QC samples were withdrawn. The samples were thawed at room temperature. To an aliquot of 100 µl plasma, 20 µl of IS was added. To this mixture, 300 µl of acetonitrile: methanol (50:50 v/v) was added and vortexed for 2 min, followed by centrifugation at 6000 rpm for 5 min at 4 °C. After centrifugation, approximately 50 µl supernatant was aliquoted into, respectively, labeled autosampler vials, which were later placed in the autosampler at 15 °C±4 °C. 10 µl of the sample was injected onto LC-MS/MS system for analysis.

Method validation

A full validation according to the ICH guidelines was performed for the assay in K2EDTA human plasma.

Specificity and selectivity

The specificity of the method was evaluated by analyzing human plasma samples from different lots to investigate the potential interferences at the chromatographic peak region for analytes and

IS. The acceptance criterion for the experiment was that should have<20% area response to that of the LLOQ level response in the same matrix. Two lots of hemolyzed plasma samples were also analyzed to ensure specificity against potential biological interferences.

Linearity

The points CC (1-100 ng/ml for verapamil hydrochloride, 2-200 ng/ml for enalapril maleate, 1-100 ng/ml for enalaprilat dehydrate) were constructed by plotting the peak area ratio of each analyte: IS against the nominal concentration of calibration standards in K2EDTA human plasma. Following the evaluation of different weighing factors, the results were fit into linear regression analysis using 1/X2 (X: Concentration) weighing factor. The CC should have a correlation coefficient (r) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were±15% deviation from the nominal value except at LLOQ, which was set at±20%.

Recovery

The efficiency of verapamil, enalapril, enalaprilat and IS extraction from human plasma was determined by comparing the responses of the analytes extracted from replicate QC samples (n=6) with those of neat standard solutions spiked in post-extracted plasma blank sample at equivalent concentrations by protein precipitation extraction method. Recovery of enalapril was determined at LQC (6.28 ng/ml) and HQC (150.00 ng/ml) concentrations, enalaprilat was determined at LQC (2.99 ng/ml) and HQC (75.8 ng/ml) concentrations, verapamil was determined at LQC (2.99 ng/ml) and HQC (75.8 ng/ml) concentrations whereas the recovery of IS was determined at a single concentration of 20 ng/ml.

Matrix effect

The effect of human plasma constituents over the ionization of verapamil, enalapril, enalaprilat, and IS was determined by post-column infusion method to evaluate matrix effect. Briefly, an infusion pump delivers a constant amount of analyte into LC system outlet entering to mass spectrometer inlet. To follow the analyte signal, the mass spectrometer was operated in MRM mode. The human plasma constituent sample extract was injected on LC column. A steady ion response was obtained as a function of time since the analyte was infused at a constant rate. Any endogenous compound that elutes from the column which causes a variation in ESI response of the infused analyte was seen as a suppression or enhancement in the response of the infused analyte. A separate experiment was performed with verapamil, enalapril, enalapril, and IS solutions, which were infused at a constant rate, and blank matrix sample injected through the LC. To evaluate matrix effect, different lots of human plasma were spiked with analyte concentration levels at LQC and HQC levels. According to guidelines, the acceptance criterion for each back-calculated concentration was±15% deviation from the nominal value.

Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing verapamil, enalapril, enalapril, at four different QC levels concentrations (for verapamil hydrochloride 3.0 ng/ml low QC [LQC], 30.0 ng/ml medium QC [MQC], and 75.0 ng/ml high QC [HQC], for enalapril maleate 6.0 ng/ml low QC [LQC], 60.0 ng/ml medium QC [MQC], and 150.0 ng/ml high QC [HQC], for enalaprilat dihydrate 3.0 ng/ml low QC [LQC], 30.0 ng/ml medium QC [MQC], and 75.0 ng/ml high QC [HQC]) in human plasma. The four-level QC samples on four different runs were performed to assess the interassay precision. The acceptance criteria for each back-calculated standard concentration were 85-115% accuracy from the nominal value except at LLOQ, which was set at 80-120%.

Stability experiments

Stability tests were conducted to evaluate the stability of verapamil, enalapril, enalaprilat in plasma samples under different conditions. 8 h bench top stability, processed samples stability (autosampler stability for 26 h at 10 °C), three cycles of freeze-thaw stability, 30 d of long-term stability at −80±10 °C were performed at LQC and HQC levels using six replicates at each level. Samples were considered stable if assay values' acceptance criterion was of accuracy (i.e., 85-115% from fresh samples) and precision (i.e.,±15% relative standard deviation [RSD]).

RESULTS AND DISCUSSION

In previous study, a rapid, sensitive, and highly selective liquid chromatography-tandem mass spectrometry method was developed and validated for simultaneous determination of enalapril and its major active metabolite enalaprilat in human plasma. The analytes were extracted from plasma samples by liquid-liquid extraction, separated on a Zorbax Extend-C^{18} column, and detected by tandem mass spectrometry with a Turbo IonSpray ionization interface. The method has a lower limit of quantification (LLOQ) of 0.1 ng/ml for both enalapril and enalaprilat. The chromatographic run time was 3.5 min [8].

In the present study, optimization and critical evaluation of mobile phase composition (gradient), flow rate, and analytical column were important to obtain good resolution of peaks of interest from the endogenous components, which in turn affect reproducibility and sensitivity of the method. Selection of chromatographic conditions for the proposed method was optimized to suit the preclinical pharmacokinetic studies. To ease the sample preparation in microtubes and to reduce the usage of solvent, the plasma volume was kept low. Initial feasibility experiments of a various mixture(s) of solvents such as acetonitrile, methanol and formic acid along with altered flow rates (in the range of 0.1-0.6 ml/min) were performed to optimize an effective chromatographic resolution of verapamil, enalapril, enalaprilat, and IS. Various analytical columns were tested to obtained good and reproducible response within short run time. The resolution of peaks was best achieved with Discovery C18, 50 × 2.1 mm, 5 µm column. Samples were chromatographed in a gradient mode (eluent A (acetonitrile–water–formic acid, 5: 95: 0.1 v/v), eluent B (acetonitrile–formic acid, 100: 0.1 v/v)). The initial content of the eluent B is 0%, which increases linearly by 1.0 min to 100% and to 1.01 min returns to the initial 0%. The mobile phase was delivered at a flow rate of 0.400 ml/min into the mass spectrometer ESI chamber. The injection volume was 5 µl.

The purpose of sample extraction optimization is mainly to achieve high extraction recovery with negligible or low matrix effects to improve sensitivity and reliability of LC-MS/MS analysis [9-16]. A poor extraction procedure decreases method robustness due to the presence of endogenous interference in the sample extracts, which are not efficiently cleaned up due to poor extraction procedure decreases the method robustness due to the endogenous interference in the sample extracts. With time-saving advantage and simplicity, the protein precipitation extraction method was chosen as an extraction method. The attained LLOQ was sufficient to quantify verapamil, enalapril, enalaprilat in low-dose pharmaco-kinetic studies.

Verapamil, enalapril, enalaprilat eluted at ~1.09, 1.03 and 0.96 min, respectively. During a direct infusion experiment, the mass spectra for verapamil, enalapril, enalaprilat and IS revealed peaks at m/z 455.385, 377.397, 349.328 and 326.435, respectively as protonated molecular ions, [M+H]+. Typical multiple reaction monitoring chromatograms of verapamil, enalapril, enalaprilat and internal standard in dipotassium ethylenediaminetetraacetic acid human blank plasmaare shown in fig. 4-5.

The total chromatographic run time was 2.0 min and the elution of verapamil, enalapril, enalaprilat and IS (bisoprolol) occurred at ~1.09, 1.03, 0.96 and 1.01 min, respectively.

Specificity

Different lots of plasma were analysed to ensure that no endogenous interferences were present at the retention time of verapamil, enalapril, enalaprilat. LLOQ level samples along with plasma blank from the respective plasma lots were prepared and analysed (table 3).

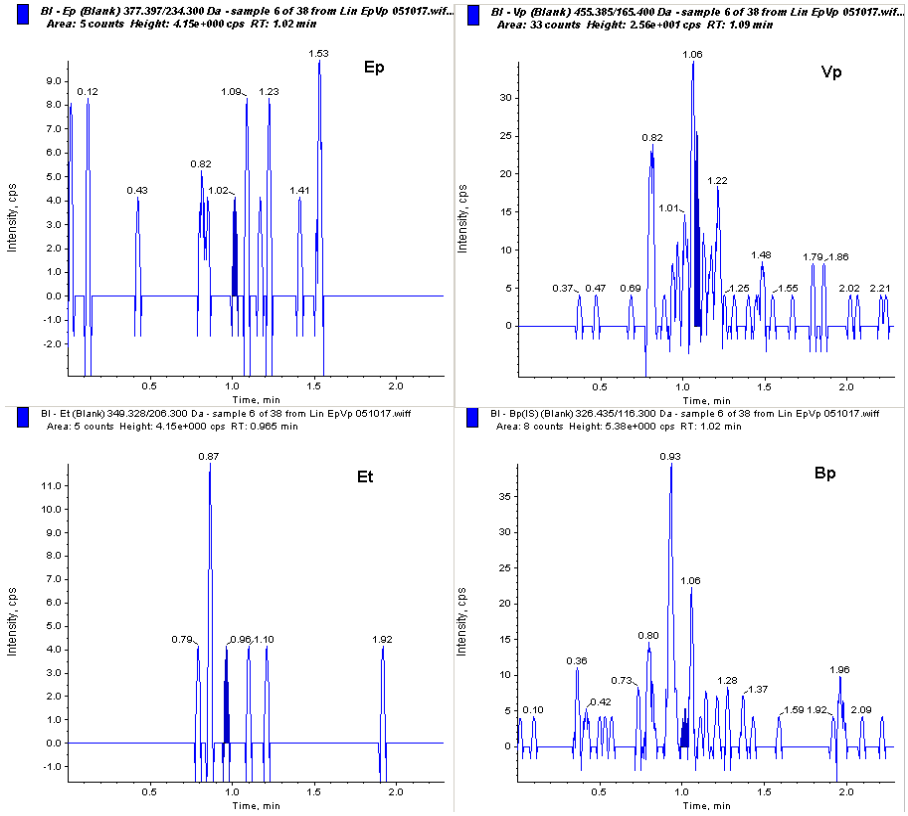

Fig. 4: Typical multiple reaction monitoring chromatograms of verapamil (right up panel), enalapril (left up panel), enalaprilat (left down panel) and internal standard (right down panel) in dipotassium ethylenediaminetetraacetic acid human blank plasma

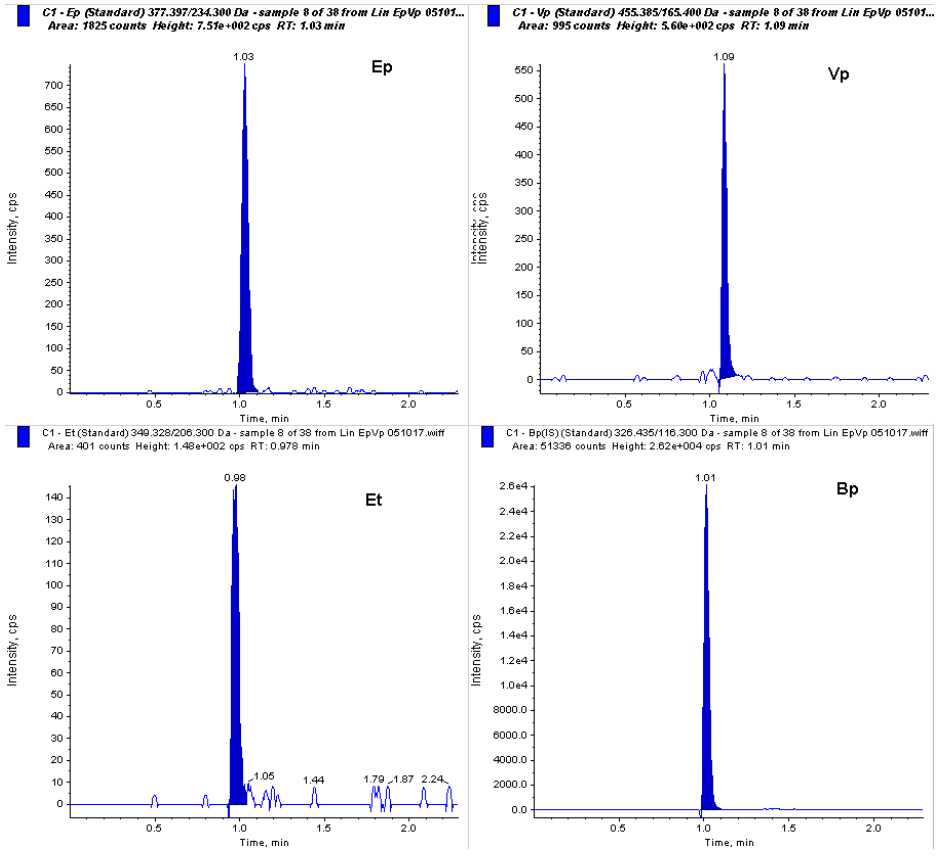

Fig. 5: Typical multiple reaction monitoring chromatograms of verapamil (right up panel), enalapril (left up panel), enalaprilat (left down panel) and internal standard (right down panel) in dipotassium ethylenediaminetetraacetic acid human blank plasma

Table 3: Results of specificity for verapamil, enalapril, enalaprilat

S. No.	Enalapril				Enalaprilat				Verapamil			
	STD BL	LLOQ		% Interference	STD BL	LLOQ		% Interference	STD BL	LLOQ		% Interference
		Area	RT			Area	RT			Area	RT	
1	0	1958	1.03	NIL	0	353	0.97	NIL	0	892	1.09	NIL
2	0	1831	1.03	NIL	0	322	0.96	NIL	0	817	1.09	NIL
3	0	1692	1.03	NIL	0	358	0.97	NIL	0	926	1.09	NIL
4	0	1888	1.03	NIL	0	372	0.97	NIL	0	978	1.08	NIL
5	0	1914	1.03	NIL	0	409	0.96	NIL	0	1024	1.09	NIL
6	0	1745	1.04	NIL	0	379	0.98	NIL	0	896	1.09	NIL
7	0	1902	1.03	NIL	0	382	0.96	NIL	0	902	1.08	NIL
8	0	1806	1.03	NIL	0	379	0.97	NIL	0	865	1.09	NIL
9	0	1896	1.03	NIL	0	348	0.97	NIL	0	910	1.09	NIL
10	0	1807	1.03	NIL	0	347	0.97	NIL	0	896	1.09	NIL

*Average of triplicate injections, In all plasma blanks, the response at the retention time of verapamil, enalapril, enalaprilat was less than 20% of LLOQ response and at the retention time of IS, the response was less than 5% of mean IS response in LLOQ.

Linearity

The calibration standard curves had a reliable reproducibility over the standard concentrations across the calibration range. The average regression (n=3) was found to be>0.997 for all analytes.

The calibration curve (fig. 6) (peak area ratio Vs Concentration) was linear over working range for enalapril maleate of 2 to 200.00 ng/ml with 7 point calibration used for quantification by linear regression, shown in fig. 6. The regression equation for the analysis was.

Y=0.0187x+0.000248 with coefficient of correction (r2) = 0.9993.

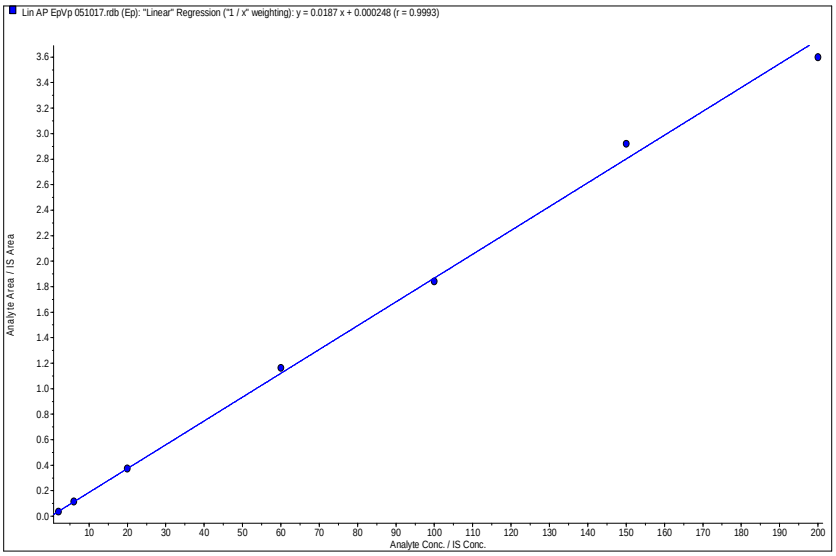

Fig. 6: The calibration curve of enalapril maleate in human plasma

The calibration curve (fig. 7) (peak area ratio Vs Concentration) was linear over working range for enalaprilat dihydrate of 1 to 100.00 ng/ml with 7 point calibration used for quantification by linear regression, shown in fig. 7. The regression equation for the analysis was.

Y=0.00833x+0.00133 with coefficient of correction (r2) = 0.9995.

The calibration curve (fig. 8) (peak area ratio Vs Concentration) was linear over working range for verapamil hydrochloride of 1 to 100.00 ng/ml with 7 point calibration used for quantification by linear regression, shown in fig. 8. The regression equation for the analysis was

Y=0.0162x+0.00391 with coefficient of correction (r2) = 0.9992.

Recovery

The % mean recovery for enalapril, enalaprilat and verapamil in LQC, MQC and HQC are listed in tables 4-6.

Table 4: The % mean recovery of enalapril for LQC, MQC and HQC

S. No.	LQC	MQC	HQC
1	6.06	61.5	152
2	6.02	62.6	158
3	6.74	55.7	155
4	6.27	59.2	155
5	6.00	61.2	147
Mean	6.22	60.0	154
SD	0.309	2.72	4.01
% CV	5.0	4.5	2.6
% Mean Recovery	103.6	100.1	102.4

***Abbreviations:** Lower quality control (LQC), middle quality control (MQC), higher quality control (HQC), Each value is represented as a mean±SD of 5 observations (n=5), SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0.

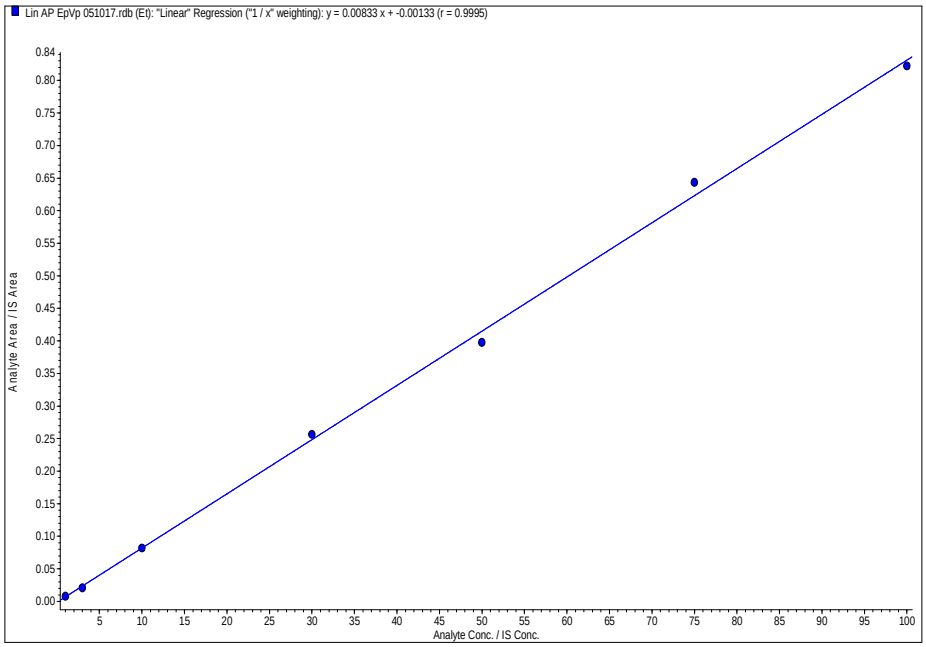

Fig. 7: The calibration curve of enalaprilat dihydrate in human plasma

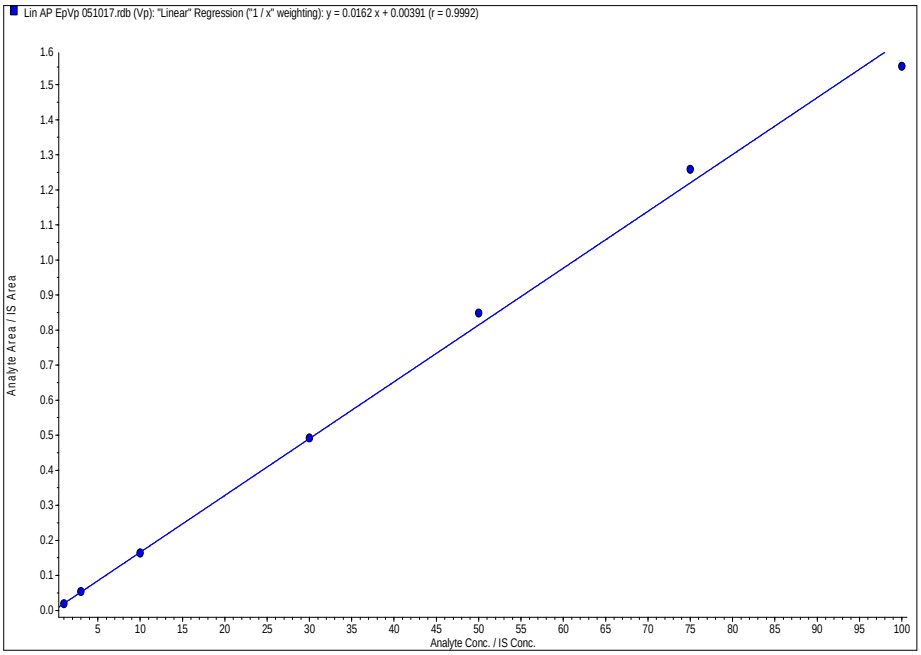

Fig. 8: The calibration curve of verapamil hydrochloride in human plasma

Table 5: The % mean recovery of enalaprilat for LQC, MQC and HQC

S. No.	LQC	MQC	HQC
1	3.49	30.6	76.8
2	2.96	29.8	77.2
3	3.30	26.7	72.8
4	2.94	29.2	78.3
5	2.80	30.7	73.9
Mean	3.10	29.4	75.8
SD	0.288	1.61	2.35
% CV	9.3	5.5	3.1
% Mean Recovery	103.2	98.0	101.1

***Abbreviations:** Lower quality control (LQC), middle quality control (MQC), higher quality control (HQC), Each value is represented as a mean±SD of 5 observations (n=5), SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0.

Table 6: The % mean recovery of verapamil for LQC, MQC and HQC

S. No.	LQC	MQC	HQC
1	3.10	28.5	73.3
2	2.92	30.8	76.2
3	2.90	27.4	74.9
4	3.01	28.3	72.0
5	2.76	29.2	73.3
Mean	2.94	28.8	73.9
SD	0.127	1.27	1.65
% CV	4.3	4.4	2.2
% Mean Recovery	97.9	96.1	98.6

***Abbreviations:** Lower quality control (LQC), middle quality control (MQC), higher quality control (HQC), Each value is represented as a mean±SD of 5 observations (n=5), SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0.

The % mean recovery for enalapril in LQC, MQC and HQC was 103.6 %, 100.1 % and 102.4 %, for enalaprilat in LQC, MQC and HQC was 103.2 %, 98.0 % and 101.1 %, for verapamil in LQC, MQC and HQC was 97.9 %, 96.1 % and 98.6 % respectively.

Intraday (within run) and Inter-day (between run) precision and accuracy

The within-run coefficients of variation ranged between 0.336 % and 0.617 % for verapamil. The within-run percentages of nominal concentrations ranged between 98.82 % and 100.62 % for verapamil. The between-run coefficients of variation ranged between 0.334 % and 0.612 % for verapamil. The between-run percentages of nominal concentrations ranged between 98.97 % and 101.76 % for verapamil. Results are presented in table 7. The assay values on both the occasions (intra-and inter-day) were found to be within the accepted limits.

The within-run coefficients of variation ranged between 0.351 % and 0.717 % for enalapril. The within-run percentages of nominal concentrations ranged between 99.13 % and 101.11 % for enalapril. The between-run coefficients of variation ranged between 0.343 % and 0.671 % for enalapril. The between-run percentages of nominal concentrations ranged between 99.17 % and 101.76 % for enalapril. Results are presented in table 8.

The within-run coefficients of variation ranged between 0.321 % and 0.541 % for enalaprilat. The within-run percentages of nominal concentrations ranged between 99.13 % and 101.12 % for enalaprilat. The between-run coefficients of variation ranged between 0.314 % and 0.663 % for enalaprilat. The between-run percentages of nominal concentrations ranged between 99.17 % and 101.03 % for enalaprilat. Results are presented in table 9.

Table 7: Intra-day and Inter-day precision data of verapamil

Day	Intra-day precision		Inter-day precision	
	Mean	R. S. D. %	Mean	R. S. D. %
1	98.82	0.378	101.76	0.334
2	100.41	0.617	98.97	0.390
3	100.62	0.336	100.53	0.612

*Each value is represented as a mean±SD of observations, SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0

Table 8: Intra-day and Inter-day precision data of enalapril

Day	Intra-day precision		Inter-day precision	
	Mean	R. S. D.%	Mean	R. S. D.%
1	99.13	0.351	101.76	0.514
2	101.11	0.717	99.17	0.343
3	100.82	0.376	100.13	0.671

*Each value is represented as a mean±SD of observations, SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0

Table 9: Intra-day and Inter-day precision data of enalaprilat

Day	Intra-day precision		Inter-day precision	
	Mean	R. S. D. %	Mean	R. S. D. %
1	99.13	0.321	100.77	0.314
2	100.31	0.541	99.17	0.490
3	101.12	0.396	101.03	0.663

*Each value is represented as a mean±SD of observations, SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0

Matrix effect

The lowest concentration with the RSD<20% was taken as LLOQ and was found to be 1.98 ng/ml for enalapril, 1.03 ng/ml for enalaprilat, 0.91 ng/ml for verapamil. The % accuracy of LLOQ samples prepared with the different biological matrix lots were found 99.2 % for enalapril, 103.4 % for enalaprilat, 90.6 % for verapamil, which were found within the range of 80.00-120.00% for the seven different plasma lots. % CV for LLOQ samples was observed as 4.9%, 5.9 %, 9.0 % respectively, which are within 20.00% of the acceptance criteria. Results are presented in tables 10-12.

Table 10: Results of matrix effect of enalapril

S. No.	LLQC
1	2.09
2	1.99
3	1.83
4	2.02
5	1.98
Mean	1.98
SD	0.097
% CV	4.9
% Mean Recovery	99.2

***Abbreviations:** Lower limit of quantification (LLOQ), Each value is represented as a mean±SD of 5 observations (n=5), SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0.

Table 11: Results of matrix effect of enalaprilat

S. No.	LLQC
1	1.01
2	0.951
3	1.03
4	1.06
5	1.12
Mean	1.03
SD	0.061
% CV	5.9
% Mean Recovery	103.4

***Abbreviations:** Lower limit of quantification (LLOQ), Each value is represented as a mean±SD of 5 observations (n=5), SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0.

Table 12: Results of matrix effect of verapamil

S. No.	LLQC
1	0.863
2	0.790
3	0.919
4	0.972
5	0.989
Mean	0.91
SD	0.082
% CV	9.0
% Mean Recovery	90.6

***Abbreviations:** Lower limit of quantification (LLOQ), Each value is represented as a mean±SD of 5 observations (n=5), SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0.

Table 13: Stability data of bisoprolol, enalapril, enalapril at QCs in human plasma

Nominal concentration (ng/ml)	Stability	Stability data		
		mean±SD °(n=6)	Accuracy (%)•	Precision (% CV)
Verapamil-2.99	0 h	2.98±0.41	99.6	2.29
	7 h (bench-Top)	2.98±0.33	99.6	2.36
	22 h (in-injector)	2.98±0.37	99.6	2.40
	3 FT cycles	2.97±0.45	99.33	2.33
Enalapril–6.28	0 h	6.27±0.37	99.8	3.08
	7 h (bench-Top)	6.27±0.22	99.8	2.51
	22 h (in-injector)	6.26±0.42	99.6	3.16
	3 FT cycles	6.26±0.37	99.6	2.16
Enalaprilat–2.99	0 h	2.98±0.38	99.6	3.12
	7 h (bench-Top)	2.98±0.45	99.6	3.07
	22 h (in-injector)	2.97±0.67	99.3	3.43
	3 FT cycles	2.96±0.44	98.9	3.11
Verapamil-75.8	0 h	75.5±0.43	99.6	2.07
	7 h (bench-Top)	75.5±0.53	99.6	3.34
	22 h (in-injector)	75.4±0.47	99.5	2.32
	3 FT cycles	75.5±0.51	99.5	3.01
Enalapril–150.0	0 h	149.9±0.51	99.9	3.15
	7 h (bench-Top)	149.7±0.31	99.8	2.32
	22 h (in-injector)	149.6±0.22	99.7	2.02
	3 FT cycles	149.2±0.64	99.4	3.24
Enalaprilat–75.8	0 h	75.5±0.53	99.6	3.08
	7 h (bench-Top)	75.3±0.27	99.3	2.04
	22 h (in-injector)	75.3±0.38	99.3	2.54
	3 FT cycles	75.1±0.57	99.0	2.84

°Back-calculated plasma concentrations; •Mean assayed concentration/mean assayed concentration at 0 h × 100. FT: Freeze-thaw, SD: Standard deviation, QC: Quality control, The results were found to be within the assay variability limits during the entire process.

Stability

The predicted concentrations for verapamil (2.99 ng/ml and 75.8 ng/ml), enalapril (6.28 ng/ml and 150.0 ng/ml), enalaprilat (2.99 ng/ml and 75.8 ng/ml) deviated within±15% of the fresh sample concentrations in a battery of stability tests namely, in-injector (22 h), bench-top (7 h), and repeated four freeze/thaw cycles stability (table 13).

CONCLUSION

In summary, a highly sensitive, specific, reproducible, rapid and high-throughput LC-MS/MS assay was developed and validated to quantify verapamil and enalapril in the present of enalaprilat in human plasma as per the regulatory guidelines. The present method involved a simple precipitation method of sample preparation, which gave consistent and reproducible recoveries. Acquired results demonstrate that proposed strategy can be effortlessly and advantageously applied for routine examination of verapamil and enalapril in the present of enalaprilat in human plasma. Hence, the combination was taken up for developing a bioanalytical method development and validation so that further it would be useful for performing pharmacokinetic studies.

AUTHOR CONTRIBUTION

All the work have been carried out by me

CONFLICT OF INTERESTS

Declared none

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