

## A HPLC-MS/MS METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS DETERMINATION OF BISOPROLOL AND ENALAPRIL IN THE PRESENT OF ENALAPRILAT IN HUMAN PLASMA

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### ABSTRACT

**Objective:** A highly specific, sensitive and rapid HPLC-MS/MS method has been developed and validated for the simultaneous quantification of bisoprolol 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 bisoprolol, enalapril, enalaprilat and IS (verapamil) occurred at ~1.01, 1.03, 0.96 and 1.09 min, respectively. A linear response function was established at 0.5-50 ng/ml for bisoprolol fumarate, 2-200 ng/ml for enalapril maleate, 1-100 ng/ml for enalaprilat dehydrate in human plasma. The intraday and interday accuracy and precisions were in the range of 0.311 %-0.647 % and 0.364 %-0.572 % for bisoprolol, 0.321 %-0.747 % and 0.390 %-0.673 % for enalapril, 0.221 %-0.547 % and 0.264 %-0.773 % for enalaprilat, respectively.

**Conclusion:** A new rapid method was developed for simultaneous determination of bisoprolol and enalapril in the present of enalaprilat in human plasma. The method was strictly validated according to the ICH guidelines. The information thus obtained from the study can be used for the full pharmacokinetic profiling in individuals.

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

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### INTRODUCTION

Bisoprolol is a synthetic, β<sub>1</sub>-selective (cardioselective) adrenoceptor blocking agent without significant membrane stabilizing activity or intrinsic sympathomimetic activity in its therapeutic dosage range. The chemical name of bisoprolol fumarate is 1-{4-[(2-isopropoxyethoxy) methyl] phenoxy}-3-(isopropyl amino) propan-2-ol (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].

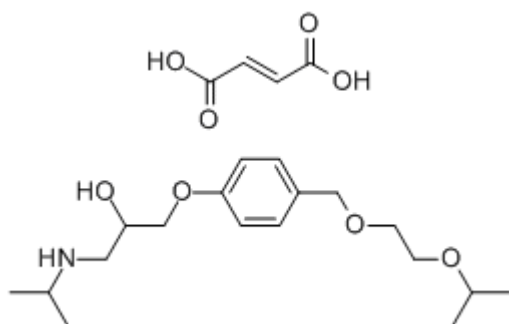


Fig. 1: Chemical structure of bisoprolol fumarate

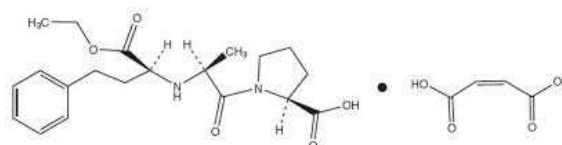


Fig. 2: Chemical structure of enalapril maleate

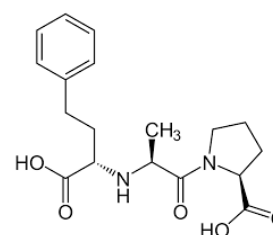


Fig. 2: Chemical structure of enalaprilat

In the contemporary literature, few bioanalytical methods were reported for quantification of bisoprolol. Dinga *et al.* reported an LC-electrospray ionization (ESI)-MS for determination of bisoprolol in human plasma. Plasma aliquots were processed using ethyl acetate liquid-liquid extraction method. Chromatographic separation of bisoprolol was achieved on ZORBAX SB-C18 column using gradient elution. The linearity range for bisoprolol in plasma was found to be 0.05-120 ng/ml. Total run time for this analysis was 5.0 min [3]. Similarly, Bhatt *et al.*

reported the determination of bisoprolol in human plasma by an LC-ESI-MS method. Plasma aliquots were processed using solid phase extraction methodology. Chromatographic separation of bisoprolol was achieved on BetaBasic 8 column using an isocratic elution. The linearity range was 0.5-70 ng/ml for bisoprolol in plasma [4]. Recently, Liu *et al.* reported a liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for the quantitation of bisoprolol in human plasma with a linearity range of 0.5-100 ng/ml. Plasma aliquots spiked with bisoprolol were processed using simple protein precipitation extraction method [5] followed by chromatographic separation of bisoprolol achieved on Capcell Pak C18 MG III column (100 mm × 2.0 mm, 5 μ), using gradient elution. Similarly, Hemavanthi G. *et al.* reported an LC-electrospray ionization (ESI)-MS for determination of bisoprolol and triamterene in human plasma. Chromatographic separation of bisoprolol was achieved on Welchrom XB C18, 50 × 4.6 mm0, 5 μm column using an isocratic mobile phase (2 mmol ammonium formate: acetonitrile, 70:30 v/v) at a flow rate of 0.60 ml/min. A linear response function was established at 2.04-210 ng/ml for both the analytes in human plasma. Total run time for this analysis was 3.5 min [6].

Bisoprolol fumarate can be determined by various methods such as ultraviolet, reverse phase high-performance LC (HPLC), LC-MS/MS, and high-performance thin-layer chromatography. Methods have been reported for analysis of bisoprolol fumarate in mono-or in combination with other drugs in pharmaceutical formulations [7-11]. From the literature survey, it is very clear that no method has been reported for simultaneous determination of bisoprolol and enalapril in the present of enalaprilat in human plasma by LC-MS/MS. As for quantification, LC-MS/MS seems to be more sensitive and precise for the simultaneous determination of bisoprolol and enalapril in the present of enalaprilat in human plasma. The proposed method is validated as per guidelines [12].

## MATERIALS AND METHODS

### Chemicals and reagents

Bisoprolol (purity 100.0 %), enalapril (purity 99.3 %), enalaprilat (purity 100.0 %), verapamil (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 was 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. Parameters of electrospray ionizer and MRM parameters are listed in table 1-2. The analytical data were processed by analyst software (version 1.5.2).

Table 1: Parameters of ionizer electrospray

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	326.435	116.3	50	46	260	11	27	20
Verapamil (IS)	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

Bisoprolol, enalapril, enalaprilat and IS have weighed accurately into volumetric flasks using an analytical microbalance. Approximately 1 mg/ml primary stock solutions of enalapril, 0.5 mg/ml primary stock solutions of enalaprilat, 0.25 mg/ml primary stock solutions of bisoprolol and 0.5 mg/ml primary stock solutions of verapamil (IS) solutions were prepared in methanol. The stock solutions were stored at -20 °C, which was found to be stable for 1 mo. The stock solutions of bisoprolol, 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 bisoprolol, enalapril, enalaprilat and IS, respectively. Calibration

standards' samples (0.5-50 ng/ml for bisoprolol fumarate, 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 bisoprolol, enalapril and enalaprilat.

Samples for the determination of precision and accuracy were prepared by spiking control human plasma in bulk with bisoprolol, enalapril and enalaprilat at appropriate concentrations (for bisoprolol fumarate 1.5 ng/ml low QC [LQC], 15.0 ng/ml medium QC [MQC], and 37.5 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 bisoprolol, enalapril, enalaprilat 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  $\mu$ l plasma, 20  $\mu$ l of IS was added. To this mixture, 300  $\mu$ l 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  $^{\circ}$ C. After centrifugation, approximately 50  $\mu$ l supernatant was aliquoted into, respectively, labeled autosampler vials, which were later placed in the autosampler at 15  $^{\circ}$ C $\pm$ 4  $^{\circ}$ C. 10  $\mu$ l of the sample was injected onto LC-MS/MS system for analysis.

### Method validation

A full validation according to the ICH guidelines [12] 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 (0.5-50 ng/ml for bisoprolol fumarate, 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/X<sup>2</sup> (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  $\pm$ 15% deviation from the nominal value except at LLOQ, which was set at  $\pm$ 20%.

### Recovery

The efficiency of bisoprolol, 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, bisoprolol was determined at LQC (1.51 ng/ml) and HQC (36.7 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 bisoprolol, 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 bisoprolol, 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  $\pm$ 15% deviation from the nominal value.

### Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing bisoprolol, enalapril, enalapril, at four different QC levels concentrations (for bisoprolol fumarate 1.5

ng/ml low QC [LQC], 15.0 ng/ml medium QC [MQC], and 37.5 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 bisoprolol, enalapril, enalaprilat in plasma samples under different conditions. 8 h bench top stability, processed samples stability (autosampler stability for 26 h at 10  $^{\circ}$ C), three cycles of freeze-thaw stability, 30 d of long-term stability at  $-80\pm 10$   $^{\circ}$ 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.,  $\pm$ 15% relative standard deviation [RSD]).

## RESULTS AND DISCUSSION

LC separation of bisoprolol and IS has been carried out using various mobile phases consisting of different aqueous solutions and methanol or acetonitrile as the organic phase. Hernandez *et al.* [13] used acetonitrile as an organic mobile phase to achieve shorter retention times and better resolution of bisoprolol and IS. The formic acid solution as an additive in water was used by Li *et al.* [14-16] to improve the sensitivity of MS detection.

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 bisoprolol, enalapril, enalaprilat, and IS. Various analytical columns were tested to obtain good and reproducible response within short run time. The resolution of peaks was best achieved with Discovery C18, 50  $\times$  2.1 mm, 5  $\mu$ m 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  $\mu$ l.

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. 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 bisoprolol, enalapril, enalaprilat in low-dose pharmacokinetic studies.

Bisoprolol, enalapril, enalaprilat eluted at  $\sim$ 1.01, 1.03 and 0.96 min, respectively. During a direct infusion experiment, the mass spectra for bisoprolol, enalapril, enalaprilat and IS revealed peaks at m/z 326.435, 377.397, 349.328 and 455.385, respectively as protonated molecular ions, [M+H]<sup>+</sup>. Typical multiple reaction monitoring chromatograms of bisoprolol, enalapril, enalaprilat and internal standard in dipotassium ethylenediaminetetraacetic acid human blank plasma are shown in fig. 4-5.

In the previous study, chromatographic separation of bisoprolol was achieved on Welchrom XB C18, 50  $\times$  4.6 mm0, 5  $\mu$ m column

using an isocratic mobile phase (2 mmol ammonium formate: acetonitrile, 70:30 v/v) at a flow rate of 0.60 ml/min. A linear response function was established at 2.04-210 ng/ml for analytes

in human plasma. Total run time for this analysis was 3.5 min [6]. In the present study, total run time for analysis was less than 2.0 min.

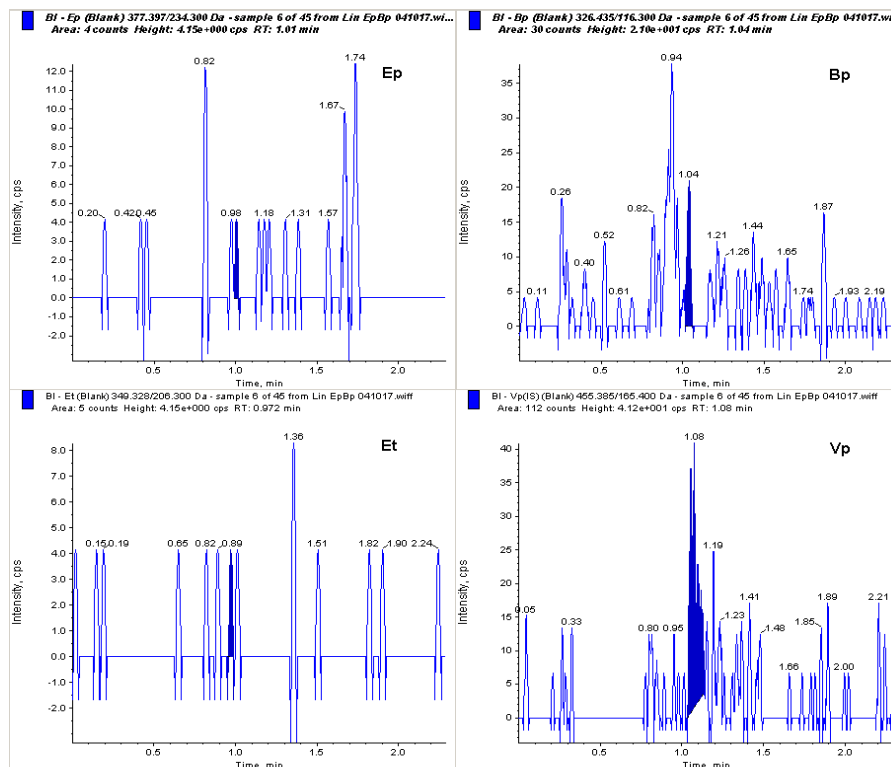


Fig. 4: Typical multiple reaction monitoring chromatograms of bisoprolol (right up panel), enalapril (left up panel), enalaprilat (left down panel) and internal standard (right down panel) in dipotassium ethylenediaminetetraacetic acid human blank plasma

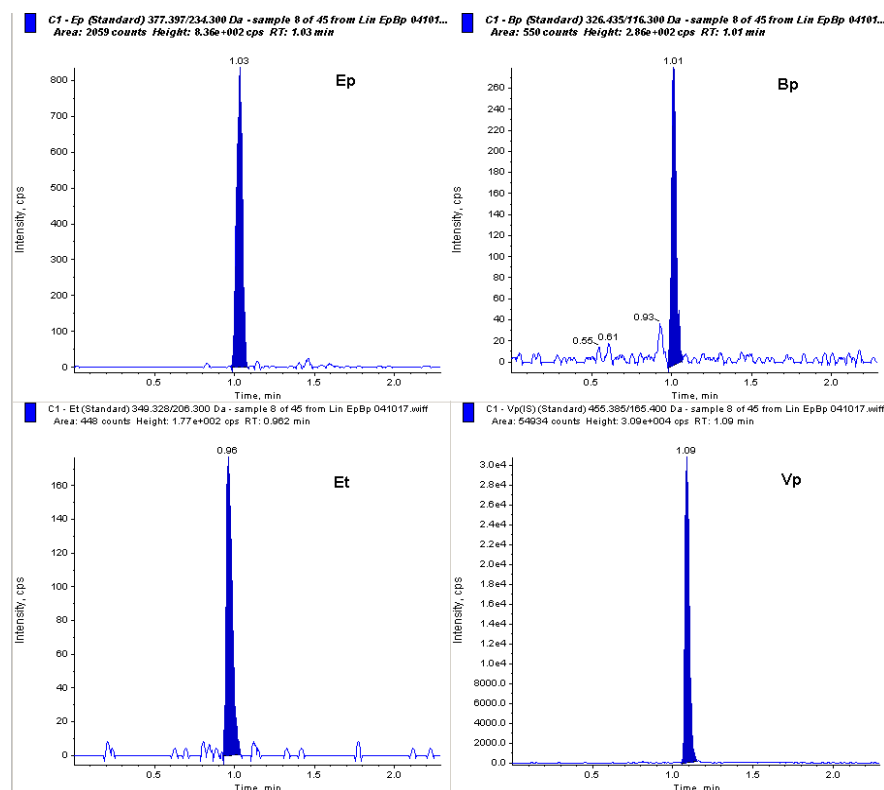


Fig. 5: Typical multiple reaction monitoring chromatograms of bisoprolol (right up panel), enalapril (left up panel), enalaprilat (left down panel) and internal standard (right down panel) in dipotassium ethylenediaminetetraacetic acid human blank plasma

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

### Specificity

Different lots of plasma were analysed to ensure that no endogenous interferences were present at the retention time of bisoprolol,

enalapril, enalaprilat. LLOQ level samples along with plasma blank from the respective plasma lots were prepared and analysed (table 3).

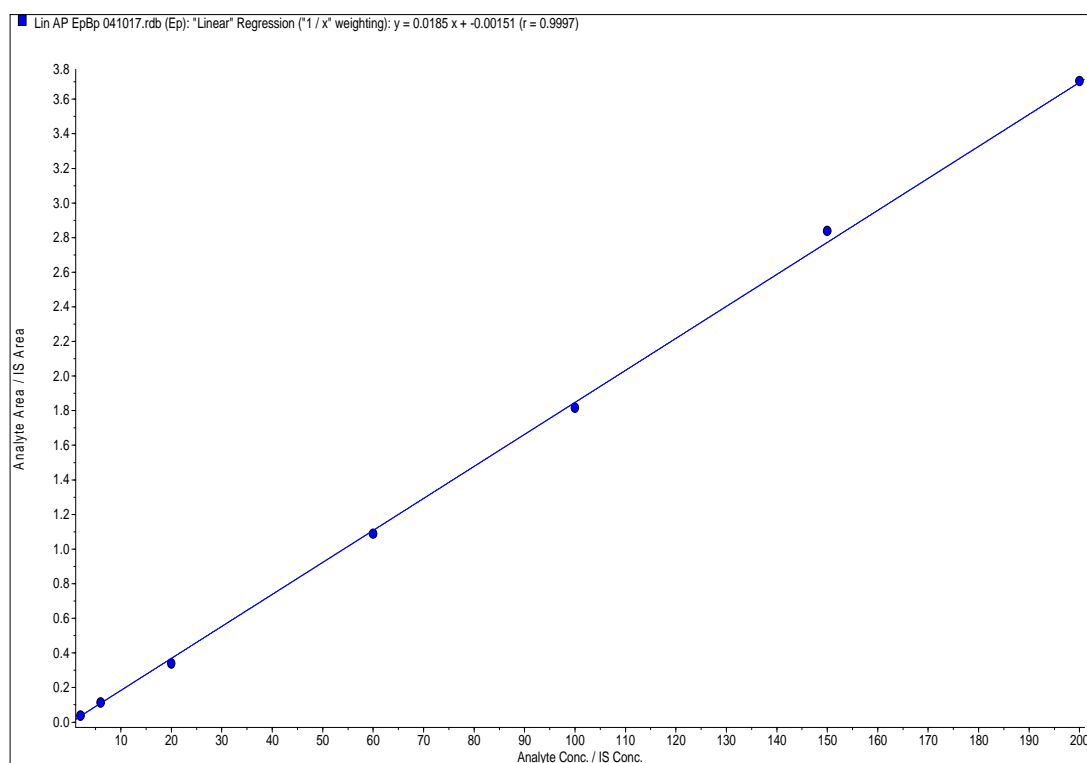
### Linearity

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.

**Table 3: Results of specificity for bisoprolol, enalapril, enalaprilat**

S. No.	Enalapril			% interference	Enalaprilat			% interference	Bisoprolol			% interference
	STD BL	LLOQ	Area		RT	STD BL	LLOQ		Area	RT	STD BL	
1	0	2190	1.03	NIL	0	394	0.97	NIL	0	557	1.01	NIL
2	0	2208	1.03	NIL	0	420	0.96	NIL	0	563	1.01	NIL
3	0	2098	1.03	NIL	0	394	0.97	NIL	0	570	1.01	NIL
4	0	2190	1.03	NIL	0	394	0.97	NIL	0	557	1.01	NIL
5	0	2190	1.03	NIL	0	503	0.96	NIL	0	554	1.01	NIL
6	0	2208	1.04	NIL	0	379	0.98	NIL	0	558	1.01	NIL
7	0	2098	1.03	NIL	0	382	0.96	NIL	0	551	1.01	NIL
8	0	2126	1.03	NIL	0	379	0.97	NIL	0	547	1.01	NIL
9	0	2190	1.03	NIL	0	501	0.97	NIL	0	543	1.01	NIL
10	0	2224	1.03	NIL	0	420	0.97	NIL	0	547	1.01	NIL

\*Average of triplicate injections, In all plasma blanks, the response at the retention time of bisoprolol, 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.



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

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

$Y=0.0185x+0.00151$  with coefficient of correction ( $r^2$ ) = 0.9997.

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

$Y=0.00732x+0.000198$  with coefficient of correction ( $r^2$ ) = 0.9998.

The calibration curve (fig. 8) (peak area ratio Vs Concentration) was linear over working range for bisoprolol fumarate of 0.5 to 50.00 ng/ml with 7 point calibration used for quantification by linear regression, shown in fig. 8. The regression equation for the analysis was

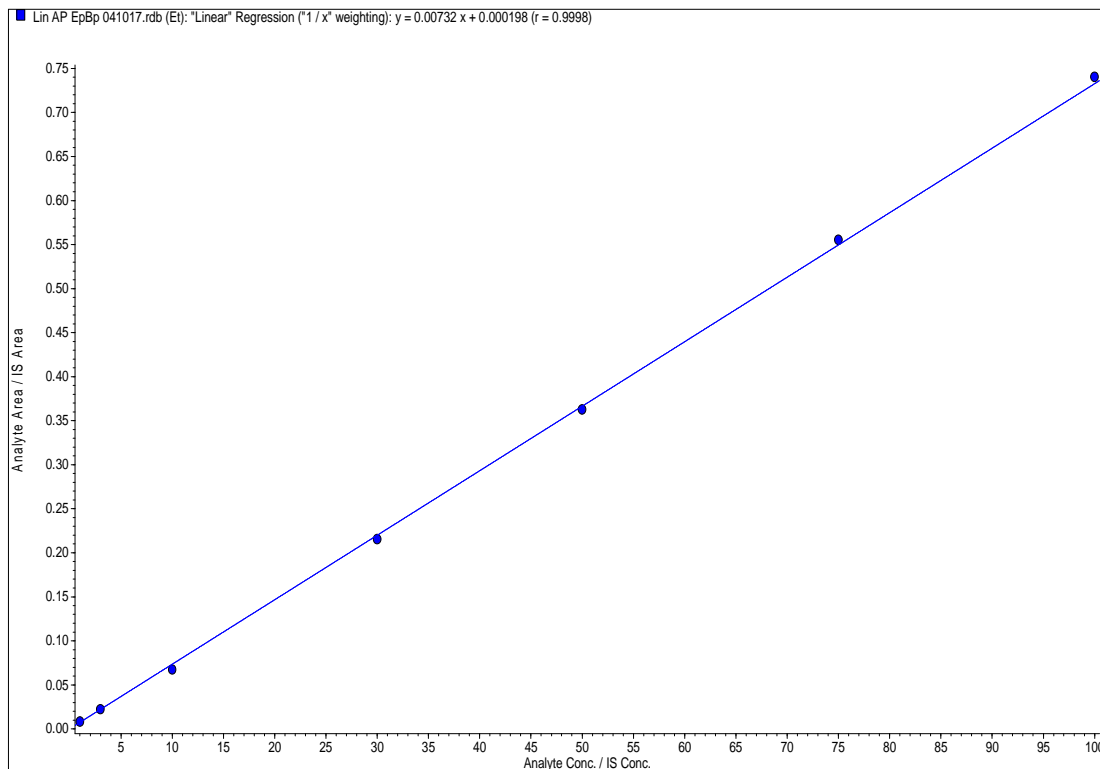
$Y=0.0195x+0.000763$  with coefficient of correction ( $r^2$ ) = 0.9997.

In the previous study, a linear response function was established at 2.04-210 ng/ml for bisoprolol in human plasma. [6]. In the present

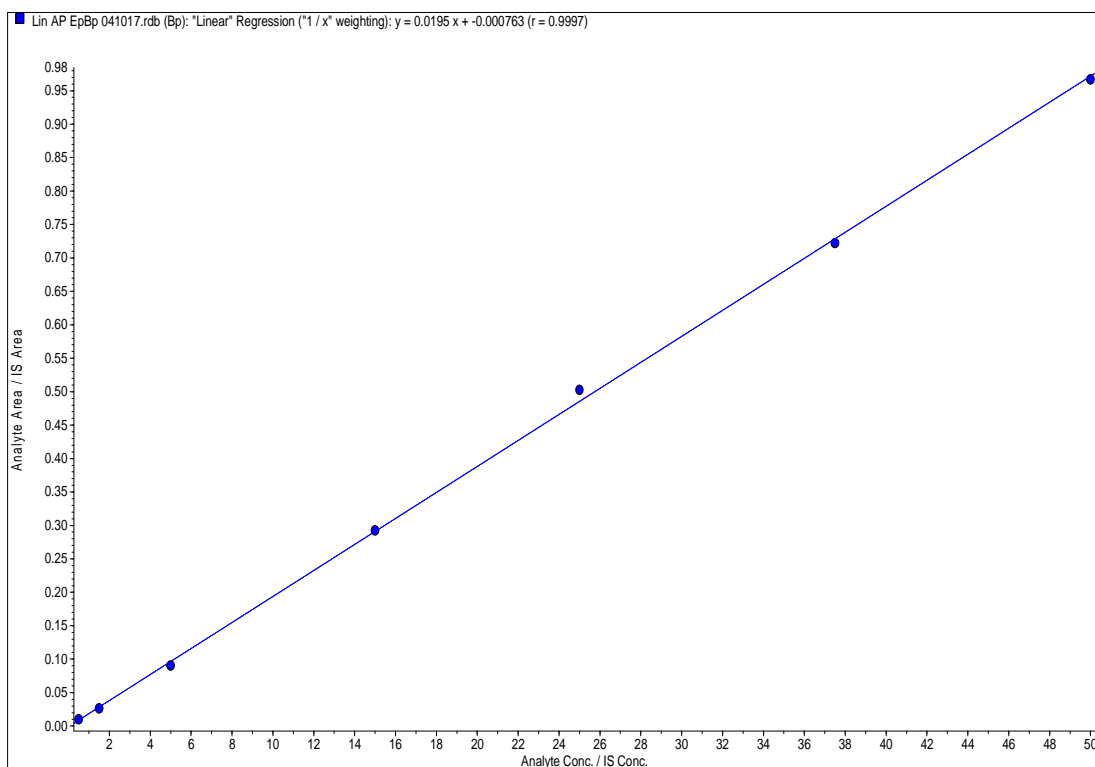
study, a linear response function was established at 0.5-50 ng/ml for bisoprolol fumarate, 2-200 ng/ml for enalapril maleate, 1-100 ng/ml for enalaprilat dehydrate in human plasma.

**Recovery**

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



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



**Fig. 8: The calibration curve of bisoprolol fumarate in human plasma**

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

S. No.	LQC	MQC	HQC
1	6.03	62.9	154
2	6.37	58.8	142
3	5.86	58.9	144
4	6.91	63.3	153
5	6.22	60.6	159
Mean	6.28	60.9	150
SD	0.402	2.14	7.16
% CV	6.4	3.5	4.8
% Mean Recovery	104.6	101.5	100.3

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

S. No.	LQC	MQC	HQC
1	3.00	31.2	78.6
2	2.89	27.6	73.9
3	3.04	28.4	73.4
4	3.09	30.7	76.2
5	2.93	31.3	77.1
Mean	2.99	29.8	75.8
SD	0.081	1.72	2.18
% CV	2.7	5.8	2.9
% Mean Recovery	99.7	99.4	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 bisoprolol for LQC, MQC and HQC

S. No.	LQC	MQC	HQC
1	1.51	15.1	37.3
2	1.57	15.9	34.6
3	1.46	14.8	35.6
4	1.42	15.6	36.9
5	1.57	15.3	39.1
Mean	1.51	15.3	36.7
SD	0.067	0.43	1.72
% CV	4.4	2.8	4.7
% Mean Recovery	100.4	102.3	97.9

\*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 104.6 %, 101.5 % and 100.3 %, for enalaprilat in LQC, MQC and HQC was 99.7 %, 99.4 % and 101.1 %, for bisoprolol in LQC, MQC and HQC was 100.4 %, 102.3 % and 97.9 % respectively.

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

The within-run coefficients of variation ranged between 0.311 % and 0.647 % for bisoprolol. The within-run percentages of nominal concentrations ranged between 99.82 % and 100.82 % for bisoprolol. The between-run coefficients of variation ranged between 0.364 % and 0.572 % for bisoprolol. The between-run

percentages of nominal concentrations ranged between 99.27 % and 100.76 % for bisoprolol. 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 0.321 % and 0.747 % for enalapril. The within-run percentages of nominal concentrations ranged between 99.93 % and 101.31 % for enalapril. The between-run coefficients of variation ranged between 0.390 % and 0.673 % for enalapril. The between-run percentages of nominal concentrations ranged between 99.47 % and 102.76 % for enalapril. Results are presented in table 8.

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

Day	Intra-day precision		Inter-day precision	
	Mean	R. S. D. %	Mean	R. S. D. %
1	99.82	0.311	100.76	0.364
2	100.41	0.647	99.27	0.390
3	100.82	0.336	100.53	0.572

\*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	99.93	0.321	102.76	0.564
2	101.31	0.747	99.47	0.390
3	100.82	0.376	100.13	0.673

\*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	0.221	100.16	0.264
2	100.31	0.547	99.87	0.490
3	100.62	0.336	101.33	0.773

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

The within-run coefficients of variation ranged between 0.221 % and 0.547 % for enalaprilat. The within-run percentages of nominal concentrations ranged between 99.13 % and 100.62 % for enalaprilat. The between-run coefficients of variation ranged between 0.264 % and 0.773 % for enalaprilat. The between-run percentages of nominal concentrations ranged between 99.87 % and 101.33 % for enalaprilat. Results are presented in table 9.

In the previous study, the intraday and interday accuracy and precisions were in the range of 1.12-7.87% and 1.26-6.36% and 1.46-6.13% and 1.65-7.34% for bisoprolol and triamterene, respectively [6]. In the present study, the intraday and interday accuracy and precisions were in the range of 0.311 %-0.647 % and 0.364 %-0.572 % for bisoprolol, 0.321 %-0.747 % and 0.390 %-0.673 % for enalapril, 0.221 %-0.547 % and 0.264 %-0.773 % for

enalaprilat, respectively. The assay values the occasions (intra-and inter-day) were found to be within the accepted limits.

#### Matrix effect

The lowest concentration with the RSD<20% was taken as LLOQ and was found to be 2.19 ng/ml for enalapril, 1.01 ng/ml for enalaprilat, 0.585 ng/ml for bisoprolol.

The % accuracy of LLOQ samples prepared with the different biological matrix lots was found 109.5 % for enalapril, 101.1 % for enalaprilat, 117.1 % for bisoprolol, which were found within the range of 80.00-120.00% for the seven different plasma lots. % CV for LLOQ samples was observed as 3.1%, 12.0 %, 7.0 % 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	2.18
2	2.17
3	2.14
4	2.31
5	2.16
Mean	2.19
SD	0.068
% CV	3.1
% Mean recovery	109.5

\* **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	0.924
2	0.973
3	1.22
4	0.93
5	1.01
Mean	1.01
SD	0.122
% CV	12.0
% Mean recovery	101.1

\* **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.

#### Stability

The predicted concentrations for bisoprolol (1.51 ng/ml and 36.7 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).



Table 12: Results of matrix effect of bisoprolol

S. No.	LLQC
1	0.547
2	0.581
3	0.655
4	0.571
5	0.572
Mean	0.585
SD	0.041
% CV	7.0
% Mean recovery	117.1

\* **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)
Bisoprolol-1.51	0 h	1.50±0.32	99.3	2.29
	7 h (bench-Top)	1.50±0.42	99.3	2.36
	22 h (in-injector)	1.50±0.52	99.3	2.60
	3 FT cycles	1.49±0.42	99.3	2.30
Enalapril-6.28	0 h	6.27±0.37	99.8	3.01
	7 h (bench-Top)	6.27±0.22	99.8	2.71
	22 h (in-injector)	6.26±0.42	99.6	3.36
	3 FT cycles	6.26±0.37	99.6	2.96
Enalaprilat-2.99	0 h	2.98±0.38	99.6	3.05
	7 h (bench-Top)	2.98±0.45	99.6	3.12
	22 h (in-injector)	2.97±0.67	99.3	3.43
	3 FT cycles	2.96±0.44	98.9	3.11
Bisoprolol-36.7	0 h	36.6±0.37	99.7	2.07
	7 h (bench-Top)	36.6±0.63	99.7	3.34
	22 h (in-injector)	36.5±0.32	99.4	2.32
	3 FT cycles	36.5±0.57	99.4	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.

## CONCLUSION

In summary, a highly sensitive, specific, reproducible, rapid and high-throughput LC-MS/MS assay was developed and validated to quantify bisoprolol and enalapril in the presence 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. The sample volume requirement supports the possibility to study the full pharmacokinetic profile in individuals. Furthermore, the rationale for selecting the combination of bisoprolol and enalapril is justifiable. 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.

## CONFLICTS OF INTERESTS

Declared none

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