SIMULTANEOUS DETERMINATION OF ENALAPRIL, ENALAPRILAT AND HYDROCHLOROTHIAZID IN HUMAN PLASMA BY LC-MS/MS METHOD

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Received April 21st, 2025

Accepted May 26th, 2025

Abstract: *A high and specific method using ultra performance liquid chromatography tandem mass spectrometry (LC/MS-MS) for simultaneous determination of enalapril (EPR), enalaprilat (EPL) and hydrochlorothiazide (HCT) in human plasma was developed. Enalapril-d5 (EPR-d5), enalaprilat-d5 (EPL-d5) and hydrochlorothiazide-13C,d2 (HCT-13C,d2) were utilized as internal standards. Analytes were extracted from plasma via protein precipitation using acetonitrile combined with sample acidification with phosphoric acid and sample freezing. Liquid chromatography was performed on Luna C18 (2) - HST; (50 x 3 mm; 2.5 µm) column with gradient program of 0.1% formic acid and methanol as mobile phase at a flow rate of 0.3 mL/min. The Thermo vantage UPLC-MS/MS was operated under the multiple-reaction monitoring (MRM) mode using the ESI+ mode with transitions at (m/z) 377.2 → 234.2 for EPR, 382.2 → 239.2 for EPR-d5, 349.1 → 206.1 for EPL, 354.1 → 211.1 for EPL-d5 and the ESI- mode with transitions at (m/z) 295.9 → 205.1 for HCT and 298.9 → 206.1 for HCT-13C,d2. The method has a rapid analysis time, low LLOQ value and standard curves were found to be linear in the wide range 1.25 to 250 ng/mL for EPR; 0.75 to 150 ng/mL for EPL and 1.0 to 200 ng/mL for HCT. The method's precision and accurary are within acceptable limit. The validated method has been successfully used to determine enalapril, enalaprilat and hydrochlorothiazide concentrations in healthy adult volunteers and demonstrate its applicability to bioavailability/bioequivalence studies of enalapril maleate and hydrochlorothiazide combination preparations.*

Keywords: enalapril, enalaprilat, hydrochlorothiazide, plasma, LC-MS/MS.

1. INTRODUCTION

Enalapril (EPR) is a prodrug that, after absorption, is metabolized into enalaprilat (EPL) - an angiotensin-converting enzyme (ACE) inhibitor with vasodilatory effects. It reduces peripheral resistance, sodium and water retention, and thus lowers blood pressure [1]. EPR is commonly formulated as enalapril maleate salt. Hydrochlorothiazide (HCT) increases the excretion of sodium and chloride ions along with water, producing an antihypertensive effect. However, its blood pressure-lowering effect manifests slowly, typically after 1 - 2 weeks, whereas the diuretic effect occurs more rapidly, often within a few hours. HCT can enhance the effects of other antihypertensive drugs [1].

Currently, numerous combination formulations containing enalapril maleate and HCT are available on the market for the treatment of hypertension. After oral administration of a single dose of the enalapril maleate/HCT 20 mg/12.5 mg tablet, the maximum plasma concentrations of EPR, EPL, and HCT are quite low, approximately 120 - 140 ng/mL for EPR, 70 - 80 ng/mL for EPL, and 60 - 80 ng/mL for HCT [2 - 5]. Therefore, quantifying EPR, EPL, and HCT in human plasma samples requires a suitable extraction method and a highly sensitive analytical technique such as LC-MS/MS. Furthermore, there is currently no published method in Vietnam for the simultaneous quantification of these three compounds in human plasma.

Thus, to meet the requirements for bioequivalence evaluation of combination formulations of enalapril maleate/hydrochlorothiazide and based on existing equipment, we conducted a study to develop a method for the simultaneous quantification of EPR, EPL, and HCT in human plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2. EXPERIMENTAL

2.1. Equipments, Instruments, Chemicals, and Reference Standards

2.1.1. Equipments and Instruments

All equipments and instruments were managed and calibrated according to ISO/IEC 17025 and GLP standards, including: TSQ Vantage mass spectrometry system (Thermo, USA); Mettler Toledo analytical balance (Switzerland, precision d = 0.01 mg); - 35°C ultra-low freezer (Panasonic, Japan); refrigerated centrifuge (Sigma 4-16KS, Germany); nitrogen evaporator, vortex mixer, water purification system, etc.

Instruments: volumetric flasks, glass extraction tubes, glass pipettes, and 2 mL centrifuge tubes, etc all conforming to Class A standards.

2.1.2. Chemicals and Reference Standards

Reference standards included enalapril maleate (National Institute of Drug Quality Control, Lot No. C0221237, 99.9% original purity), enalaprilat dihydrate (Toronto Research Chemicals, Lot No. 10-ABY-19-1, 98% original purity), and hydrochlorothiazide (National Institute of Drug Quality Control, Lot No. C0219308.02, 99.8% original purity).

Internal standards included: enalapril-d5 maleate (EPR-d5, Toronto Research Chemicals, Lot No. 16-SCC-93-1, 94% original purity), enalaprilat-d5 dihydrate (EPL-d5, Toronto Research Chemicals, Lot No. AC-109-A, 98% original purity), and hydrochlorothiazide-13C,d2 (HCT-13C,d2, Toronto Research Chemicals, Lot No. 1-MJA-136-2, 98% original purity).

Solvents and chemicals were of analytical/HPLC and mass spectrometry grade.

2.2. Study Subjects and Methods

2.2.1. Study Subjects

Blank plasma samples: Containing anticoagulant Na_2EDTA and free from EPR, EPL, and HCT, provided by the 108 Military Central Hospital. Spiked plasma samples: Blank plasma spiked with known concentrations of EPR, EPL, and HCT standards.

Volunteer plasma samples: Plasma from volunteers who took enalapril maleate/hydrochlorothiazide tablets (20 mg/12.5 mg) as part of a bioequivalence study.

2.2.2. Research Methods

2.2.2.1. Method Development

**** Selection of Internal Standards***

In LC-MS/MS analysis, stable isotope-labeled internal standards are preferred due to their physicochemical similarity to the analytes, which minimizes errors during sample preparation and analysis. Thus, EPR-d5, EPL-d5, and HCT-13C,d2 were selected as internal standards for EPR, EPL, and HCT, respectively.

**** Mass Spectrometry Optimization***

Standard solutions of EPR, EPL, and HCT (500 ng/mL) were individually introduced into the mass spectrometer to determine appropriate MS parameters. Ionization modes (ESI±), precursor ion detection, and optimization of source parameters such as ionization voltage, spray temperature, nitrogen gas flow rate, and ion funnel voltage were investigated to maximize ion signal intensity and stability.

Fragmentation of precursor ions in the second quadrupole was also studied to identify product ions for each analyte and optimize collision energy settings.

**** Chromatographic Condition Optimization***

Based on the physicochemical properties of the analytes, references [3 - 4] and current conditions, various chromatographic conditions were evaluated using the following columns: Hypersil Gold C18 (50 x 2.1 mm, 1.9 µm); Luna C18 (2) - HST (50 x 3 mm, 2.5 µm); Acquity C18 (50 x 2.1 mm, 1.7 µm); Hypersil Gold C18 (100 x 2.1 mm, 1.9 µm); Acquity C18 (100 x 2.1 mm, 1.7 µm) using different mobile phase systems: organic solvents (methanol or acetonitrile) in combination with buffer solutions (0.1% formic acid, 10 mM ammonium formate, 5 mM ammonium acetate, 7.5 mM ammonium bicarbonate) at various ratios to achieve sharp, symmetrical peaks and short run times of EPR, EPL and HCT.

**** Plasma Sample Preparation Optimization***

Based on the physicochemical properties (logP values: EPR = 0.19 [8], HCT = - 0.07 [9], EPL = - 0.7 [10]), liquid-liquid extraction was deemed unsuitable. Thus, different sample preparation methods such as: protein precipitation using methanol (MeOH) or acetonitrile (MeCN), protein precipitation combined with liquid-liquid extraction using dichloromethane, and protein precipitation combined with plasma freezing were investigated using blank and spiked plasma samples at LLOQ levels. The goal was to achieve symmetric, high-response peaks for EPR, EPL, and HCT.

2.2.2.2 Method Validation

The simultaneous quantification method for EPR, EPL, and HCT in human plasma was validated following US - FDA [6], EMA [7], and ICH [8] guidelines for LC-MS/

MS bioanalytical methods, including these parameters: specificity and selectivity, calibration curve and linearity range, lower limit of quantification (LLOQ), intra- and inter-day accuracy and precision, matrix effect, carry-over, effects of hemolyzed and lipemic plasma recovery of analytes and internal standards, and stability of analytes in plasma.

3. RESULT AND DISCUSSION

3.1. Development of the Analytical Method

3.1.1 *Optimization of Mass Spectrometry Conditions*

The procedure was carried out as described in section 2.2.2.1, with the results as follows: EPR and EPL exhibited higher fragment signal intensity in the positive ion mode as [M+H]+ with m/z values of 377.2 and 349.1, respectively. HCT showed stronger signal intensity in the negative ion mode as [M−H]− with an m/z of 295.9. Further fragmentation of the selected precursor ions yielded high and stable product ions used for quantification with m/z values of 234.2, 206.1, and 205.1, corresponding to EPR, EPL, and HCT, respectively. The same procedure was applied to determine the mass spectrometry parameters for the respective internal standards. The results are summarized in Table 1.

3.1.2. Optimization of Chromatographic Conditions

- *Mobile phase investigation*: Different mobile phase systems were evaluated as described in section 2.2.2.1. The results showed that using a methanol – 0.1% formic acid system provided high and stable responses for EPR, EPL, and HCT, with more symmetrical peak shapes compared to other systems.

- *Column investigation*:

Using the selected mobile phase, various chromatographic conditions were tested. It was observed that when operating in both ionization modes simultaneously (ESI+) and (ESI−), the signal intensities of EPR, EPL, and HCT were less stable than when each mode was operated separately. Therefore, the analysis process was divided into two segments: operating in the negative ion mode (ESI−) for HCT, and in the positive ion mode (ESI+) for EPR and EPL. Thus, it was necessary to develop conditions to separate the HCT and EPR, EPL peaks.

When investigating different chromatographic columns, the results showed that increasing the formic acid concentration improved the separation of HCT and EPR, EPL peaks. Among the tested columns, the Luna C18 (2) - HST (50 × 3 mm, 2.5 µm) column provided better separation between HCT and EPR, EPL peaks compared to the others. However, increasing the buffer concentration caused EPR, EPL peak broadening and reduced response. Therefore, a gradient program was investigated to improve the peak shape and response of EPR, EPL. The gradient program (Table 2) showed symmetrical peaks with high response with clear separation between HCT and EPR, EPL peaks.

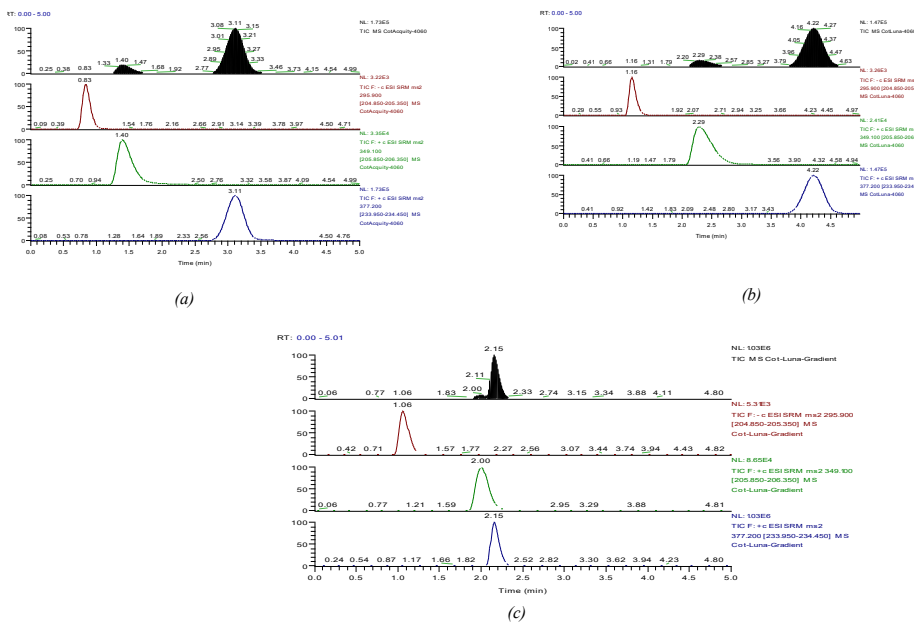


Figure 1. *Chromatograms of EPR, EPL, and HCT standards on columns: Acquity C18 (50 x 2.1 mm, 1.7 µm) (a), Luna C18 (2) - HST (50 x 3.0 mm, 2.5 µm) (b) and Luna C18 (2) – HST, gradient program (c)*

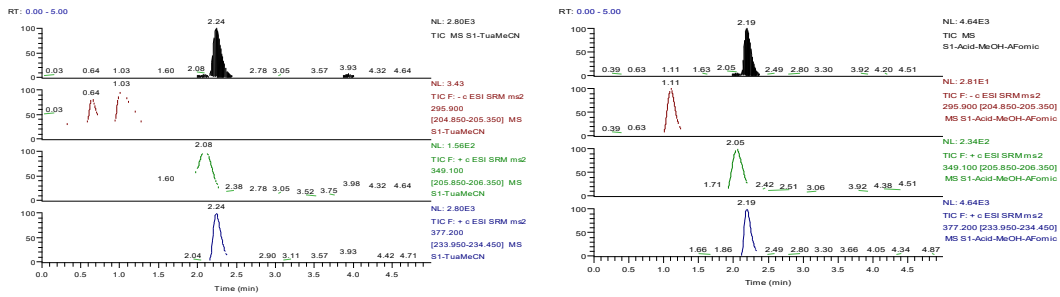
3.1.4. Selection of Sample Preparation Method

An evaluation of protein precipitation methods as described in section 2.2.2.1 showed that:

Simple protein precipitation using MeOH or MeCN provided a quick sample preparation process. However, no HCT peak response was observed, and the EPL peak had poor shape.

Protein precipitation combined with liquid-liquid extraction using dichloromethane: no HCT peak response was observed.

Protein precipitation using MeCN combined with sample freezing, followed by collecting the supernatant, evaporating under a nitrogen stream to dryness, and dissolving the residue in the sample solvent yielded detectable peaks for all three analytes. However, the recovery of EPL was low, and its peak exhibited tailing compared to the sample matrix. Further investigation revealed that acidifying the sample with phosphoric acid increased EPL recovery and improved its peak shape, thus, samples were acidified with phosphoric acid prior to processing.



(a) (b)

Figure 2. *Chromatogram of plasma sample precipitated with MeCN (a), acidified plasma sample precipitated with MeCN combined with freezing (b)*

**** Summary:*** Based on the above evaluations, a procedure has been developed for the simultaneous quantification of EPR, EPL, and HCT in human plasma using LC-MS/MS, as follows:

- Mass Spectrometry Conditions:

Table 1*. MS detector parameters for quantification of EPR, EPL, HCT, and internal standards*

Analytes / ***Parameters***	**HCT**	**HCT-13C,d2**	**EPR**	**EPR-d5**	**EPL**	**EPL-d5**
Ionization mode	ESI(-)	ESI(-)	ESI(+)	ESI(+)	ESI(+)	ESI(+)
Ionization voltage (Volt)	3000	3000	3000	3000	3000	3000
Ion source temperature (°C)	300	300	300	300	300	300
Capillary temperature (°C)	300	300	300	300	300	300
Collision energy (Volt)	23	23	18	18	16	16
Precursor ion (m/z)	295.9	298.9	377.2	382.2	349.1	354.1
Product ion (m/z)	205.1	206.1	234.2	239.2	206.1	211.1

- Chromatographic Conditions:

+ Column: Luna C18 (2) - HST; (50 x 3 mm; 2.5 µm).

+ Mobile phase: Methanol and 0.1% formic acid, gradient program as described in Table 2.

Table 2. Gradient program

Time (min)	MeOH (%)	0.1% formic acid (%)
0	40	60
0.3	40	60
0.75	80	20
2.0	80	20
2.1	40	60
5.0	40	60

+ Flow rate: 0.3 mL/min

+ Injection volume: 5 µL

+ Autosampler temperature: room temperature

- Sample Preparation Method:

+ Stock standard solutions: Prepared separately in methanol at concentrations of 1000 µg/mL for EPR, EPR-D5, EPL, IS-EPL, HCT, and IS-HCT.

+ Working internal standard solution: use EPR-D5, IS-EPL và IS-HCT stock standard solution to prepare working internal standard solutions in methanol : water (1 : 1, v/v) at concentrations of 1800 ng/mL (EPR-d5), 1200 ng/mL (EPL-d5), and 1500 ng/mL (HCT-13C,d2).

+ Calibration standards and QC samples in plasma: Calibration standards containing EPR, EPL, and HCT at concentrations of 1.25; 2.5; 12.5; 25; 50; 125; 212.5; 250 ng/ml (EPR), 0.75; 1.5; 7.5; 15; 30; 75; 127.5; 150 ng/mL (EPL) và 1; 2; 10; 20; 40; 100; 170; 200 ng/mL (HCT). QC samples prepared independently at three levels: LQC: 3.75 ng/mL (EPR), 2.25 ng/mL (EPL) and 3 ng/mL (HCT); MQC: 100 ng/mL (EPR), 60 ng/mL (EPL) and 80 ng/mL (HCT); HQC: 187.5 ng/mL (EPR), 112.5 (EPL) and 150.0 ng/mL (HCT).

- Sample Processing Procedure:

Take 0.3 mL of plasma sample, add 20 µL of working internal standard solution, add 30 µL of 1 M phosphoric acid and 1 mL of acetonitrile, vortex at 1700 rpm for 1 minute. Centrifuge at 11,000 rpm (RCF: 13,528g) for 5 minutes. Place the sample in a deep freezer at - 70°C. After 15 minutes, remove and thaw at room temperature. Transfer 0.5 mL of the clear supernatant to a labeled extraction tube. Evaporate to dryness under a nitrogen stream. Dissolve the residue in 0.5 mL of methanol - 0.1% formic acid (1 : 1, v/v) and transfer to the injection vial.

- Result Calculation Method:

The concentrations of EPR, EPL, and HCT in the test samples are determined based on the peak area ratios of EPR/EPR-d5, EPL/EPL-d5, and HCT/HCT-13C,d2 obtained from the chromatograms of the test samples and the corresponding calibration curves analyzed under the same conditions.

3.2. Analytical Method Validation

3.2.1. Specificity - Selectivity of the Method

Blank plasma samples (PS) including: six different lots of blank PS, hemolyzed blank PS, lipemic blank PS, and spiked PS containing internal standards and EPR, EPL, HCT at concentrations of 1.25 ng/mL, 0.75 ng/mL, and 1.0 ng/mL, respectively (LLOQ samples), were analyzed according to the developed method.

In the chromatograms (Figure 3) of blank PS, no peaks were observed at 1.07 minutes (retention times of HCT and HCT-13C,d2); 2.02 and 2.05 minutes (retention times of EPL-d5 and EPL); 2.21 and 2.28 minutes (retention times of EPR and EPR-d5). These peaks were observed only in the chromatograms of spiked PS (Figure 4). Therefore, the method demonstrates adequate specificity and selectivity for EPR, EPL, HCT, and the internal standards, in accordance with regulatory guidelines for bioanalytical method validation.

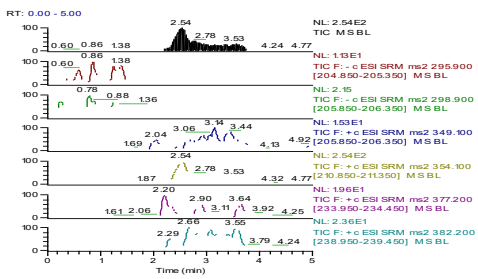


Figure 3. Chromatograms of blank PS

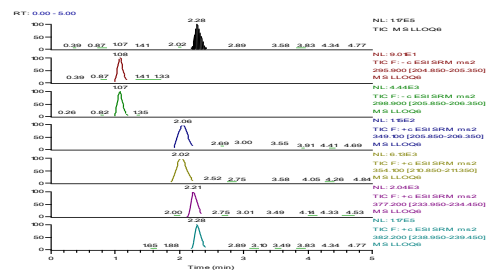


Figure 4. Chromatograms of spiked PS containing EPR (1.25 ng/mL), EPL (0.75 ng/mL), HCT (1.0 ng/mL) and internal standards

3.2.2. Calibration Curve and Linear Range

PS containing EPR standards at concentrations ranging from 1.25 ng/mL to 250 ng/mL, EPL standards from 0.75 ng/mL to 150 ng/mL, and HCT standards from 1.0 ng/mL to 200 ng/mL, along with their respective internal standards, were analyzed according to the developed procedure. The correlation between the concentrations of EPR, EPL, HCT in the samples and their corresponding peak area ratios (EPR/EPR-d5; EPL/EPL-d5; HCT/HCT-13C,d2) was determined using linear regression, applying a weighting factor (1/concentration²). The results of the linearity assessment are presented in Table 3, and the residual plots of the calibration curves are shown in Figure 5.

Table 3. *Results of linearity assessment of the method*

APIs	Concentration range (ng/ml)	Calibration curve	Accuracy (%) Min - max	Regression Equation (y = ax+b) Correlation Coefficient (r)
EPR	*1.25 – 250*	*1*	*96.3 – 104.8*	y = 0.0158678x – 0.000576896; r = 0.9993
		2	91.6 – 107.0	y = 0.0160636x + 0.000260999; r = 0.9981
		3	96.4 – 107.0	y = 0.0157106x + 0.001782620; r = 0.9991
		4	96.3 – 104.6	y = 0.0158516x + 0.000558724; r = 0.9994
		5	94.9 – 102.5	y = 0.0162364x + 0.000100713; r = 0.9995
EPL	*0.75 – 150*	*1*	*97.0 – 104.9*	y = 0.0252072x – 0.002817030; r = 0.9994
		2	89.5 – 105.3*	y = 0.0259006x + 0.000497942; r = 0.9974
		3	93.8 – 108.0	y = 0.0250199x – 0.000414463; r = 0.9987
		4	94.8 – 108.0	y = 0.0247846x – 0.003454460; r = 0.9981
		5	95.7 – 103.1	y = 0.0246877x – 0.001770020; r = 0.9995
HCT	*1.0 – 200*	*1*	*95.6 – 106.3*	y = 0.0197598x – 0.002795790; r = 0.9988
		2	89.7 – 110.4	y = 0.0199007x – 0.000703135; r = 0.9969
		3	94.9 – 105.6	y = 0.0197018x – 0.001821070; r = 0.9990
		4	97.0 – 107.8	y = 0.0200923x – 0.000238352; r = 0.9993
		5	93.4 – 104.2	y = 0.0205800x – 0.001085910; r = 0.9994

(*): Exclude S2 point due to accuracy falling outside the acceptable range of 85% – 115%

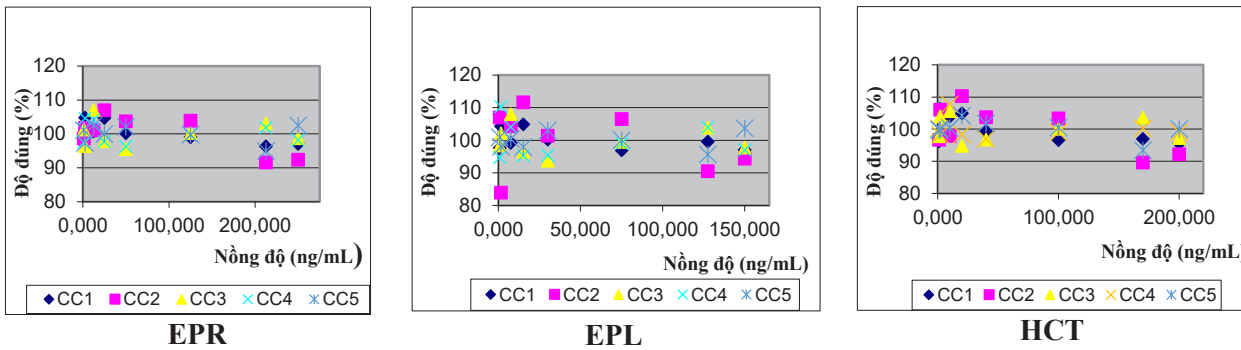


Figure 5. *Residual plots of the experimental calibration curves using the weighting model $1/x^2$*

The validation results show that within the concentration ranges of 1.25 - 250 ng/mL for EPR, 0.75 - 150 ng/mL for EPL, and 1.0 - 200 ng/mL for HCT, there is a linear correlation between the concentrations of EPR, EPL, HCT and their respective peak area ratios (EPR/EPR-d5; EPL/EPL-d5; HCT/HCT-13C,d2), with a correlation coefficient (r) approximately equal to 1. The concentrations of EPR, EPL, HCT determined from the calibration curve, when compared to the theoretical values, fall within the acceptable range (80% - 120% for the lowest concentration and 85% - 115% for the remaining concentrations), in accordance with bioanalytical method validation guidelines.

3.2.3. Lower Limit of Quantification (LLOQ) of the Method

Zero samples (blank PS containing only internal standards) and PS containing EPR, EPL, and HCT at concentrations of 1.25 ng/mL, 0.75 ng/mL, and 1.0 ng/mL, respectively (LLOQ samples), were analyzed across 3 batches, with 6 samples per batch. The results for LLOQ are presented in Table 4.

Table 4. *Results for LLOQ determination*

Analytes	EPR			EPL			HCT		
Batch	1	2	3	1	2	3	1	2	3
Zero sample peak area (n = 1)	12	21	45	NF	NF	NF	6	5	6
Mean LLOQ sample peak area (n = 6)	4565	4236	4255	343	339	260	77	86	106
LLOQ/Zero ratio	380.4	201.7	94.5	-	-	-	12.8	17.1	17.7
Accuracy (%) (n = 6)	111.7	106.2	93.9	118.4	101.7	101.9	113.8	108.9	97.1
CV (%) (n = 6)	2.0	3.6	3.6	6.7	9.2	11.0	10.7	13.4	5.5
Accuracy (%) (n = 18)	103.9			107.3			106.6		
CV (%) (n = 18)	7.9			11.3			12.1		

NF: No peak detected; (-): Not applicable

The validation results show that at the retention times corresponding to EPR, EPL, and HCT, the mean LLOQ response was more than 5 times the response of the zero sample. The average accuracy of each batch and all three batches was within the 80% - 120% range, the precision (CV%) was less than 20%, meeting the acceptance criteria for lower limit of quantification in bioanalytical methods.

3.2.4. Accuracy and Precision of the Method

Accuracy and precision were assessed at four concentration levels: LLOQ (EPR: 1.25 ng/mL, EPL: 0.75 ng/mL, HCT: 1.0 ng/mL), LQC (EPR: 3.75 ng/mL, EPL:

2.25 ng/mL, HCT: 3.0 ng/mL), MQC (EPR: 100.0 ng/mL, EPL: 60.0 ng/mL, HCT: 80.0 ng/mL) and HQC (EPR: 187.5 ng/mL, EPL: 112.5 ng/mL, HCT: 150.0 ng/mL). At each concentration level, 6 samples were prepared and analyzed across 3 different days. The concentrations of EPR, EPL, and HCT in the samples were determined using calibration curves under the same analytical conditions. The validation results for the method's accuracy and precision are presented in Table 5.

Table 5. *Results of intra-day and inter-day accuracy and precision validation*

Analytes		Accuracy (%)				CV (%)			
		LLOQ	*LQC*	*MQC*	*HQC*	*LLOQ*	*LQC*	*MQC*	*HQC*
EPR	Day 1 (n = 6)	111.7	109.5	108.4	106.4	2.0	9.1	4.8	4.8
	Day 2 (n = 6)	106.2	103.2	102.1	101.9	3.6	5.3	3.4	3.2
	Day 3 (n = 6)	93.9	105.2	104.3	107.9	3.6	4.0	3.1	2.2
	Inter-day precision	103.9	106.0	105.0	105.4	7.9	6.7	4.5	4.2
EPL	Day 1 (n = 6)	118.4	108.3	103.0	102.0	6.7	9.8	4.3	4.9
	Day 2 (n = 6)	101.7	100.3	99.3	98.8	9.2	6.4	2.0	5.0
	Day 3 (n = 6)	101.9	107.8	100.5	102.8	11.0	5.6	3.7	3.3
	Inter-day precision	107.3	105.5	100.9	101.2	11.3	7.9	3.6	4.5
HCT	Day 1 (n = 6)	113.8	111.2	108.6	105.7	10.7	7.6	5.2	5.8
	Day 2 (n = 6)	108.9	105.8	105.1	104.5	13.4	5.8	3.8	3.4
	Day 3 (n = 6)	97.1	109.1	106.0	107.1	5.5	8.2	3.8	2.1
	Inter-day precision	106.6	108.7	106.6	105.8	12.1	7.2	4.3	4.0

The validation results show that, at low, medium, and high concentration levels, the method's mean accuracy falls within the 85% - 115% range (except for LLOQ samples, which are within 80% - 120%). The intra- and inter-day precision (CV%) is less than 15% (except for LLOQ with CV% < 20%), meeting the acceptance criteria for accuracy and repeatability of bioanalytical methods as recommended by the US - FDA guidelines.

3.2.5. Matrix Effect

The matrix effect was evaluated at two concentration levels: LQC and HQC, using six different lots of plasma. The matrix effect factor for each analyte and internal standard was determined by calculating the ratio of analyte/IS response in different plasma matrices to that in solvent. The ratio of analyte matrix effect to the corresponding internal standard matrix effect was calculated for each matrix. The coefficient of variation (CV%) of these ratios was assessed to determine the variability in matrix effects for each analyte. The matrix effect evaluation results are shown in Table 6.

Table 6. *Results of matrix effect evaluation*

Concentration level	EPR		EPL		HCT	
	MF_{EPR}/MF_{IS}	*CV (%)*	MF_{EPL}/MF_{IS}	*CV (%)*	MF_{HCT}/MF_{IS}	*CV (%)*
LQC (n = 6)	1.042	5.9	1.055	5.2	1.011	9.7
HQC (n = 6)	1.066	0.7	1.072	1.1	1.049	2.0

The validation results show that matrix effects across different plasma lots were within acceptable limits (CV% < 15%), indicating that the analytical method meets the bioanalytical method requirements for drug analysis in biological matrices.

3.2.6. Recovery

The recovery of EPR, EPL, and HCT was evaluated at three concentration levels: LQC, MQC, and HQC, by comparing the analyte/IS response ratio in extracted samples with the corresponding ratio in unextracted samples. The recovery results are presented in Table 7.

Table 7. *Results of EPR, EPL and HCT recovery in plasma*

Concentration level	EPR			EPL			HCT		
	% Recovery	*CV (%)*		*% Recovery*	*CV (%)*		*% Recovery*	*CV (%)*	
		(*)	*(**)*		*(*)*	*(**)*		*(*)*	*(**)*
LQC (n = 6)	83.8	5.4	9.9	41.7	9.8	9.7	91.7	5.1	10.4
MQC (n = 6)	83.9	6.2	8.6	42.5	7.5	9.3	92.6	5.8	8.8
HQC (n = 6)	86.5	6.3	11.6	40.9	8.2	11.7	93.1	6.6	11.5

(*): Results in extracted samples; (**): Results in unextracted samples

The validation results showed that although the recovery of EPL was lower than that of EPR and HCT, the recovery of all three analytes were below 115%, and the differences between concentration levels did not exceed ± 15%. The %CV values between the analyte and IS responses in both extracted and unextracted QC samples at each concentration were all below 15%. Thus, the analytical method meets the requirements for drug analysis in biological fluids.

3.2.7. Effect of Lipemia and Hemolysis

A batch of 4% hemolyzed plasma and a batch of lipemic plasma (with triglyceride concentration of approximately 300 mg/dL) were prepared. LQC and HQC samples were prepared in the above hemolyzed and lipemic plasma, with 6 samples per concentration. The concentrations and accuracy of the hemolyzed and lipemic plasma samples were determined and compared with the nominal concentrations. The results are shown in Table 8.

Table 8. *Results of the study on the effects of lipemia and hemolysis*

Stability	Sample	% Accuracy			CV (%)		
		EPR	*EPL*	*HCT*	*EPR*	*EPL*	*HCT*
Effect of lipemia	LQC	104.9	100.4	104.9	2.5	5.0	2.7
	HQC	105.6	99.0	107.8	4.4	3.4	3.0
Effect of hemolysis	LQC	103.1	98.7	99.7	4.4	7.1	3.9
	HQC	106.5	102.7	107.2	1.7	3.1	3.5

3.2.8. Stability of Analytes in Plasma

The stability of EPR, EPL, and HCT in plasma was studied using LQC and HQC sample batches. Stability was evaluated by comparing the concentrations of EPR, EPL, HCT in samples stored under specific conditions with the theoretical concentrations. Stability was also assessed during and after the sample processing procedure, including autosampler stability and post-evaporation residue stability. The results are shown in Table 9.

Table 9. *Stability study results of EPR, EPL, and HCT*

Stability	Sample	% Stability			CV (%)		
		EPR	*EPL*	*HCT*	*EPR*	*EPL*	*HCT*
After 6 freeze–thaw cycles	LQC	111.4	89.6	105.7	3.1	2.4	1.4
	HQC	98.2	105.7	100.1	2.7	1.4	2.1
Short-term stability (21 h 25 min; room temperature)	LQC	111.5	100.0	106.1	2.4	2.3	5.2
	HQC	95.0	88.7	96.7	2.2	1.9	2.2
Long-term stability (125 days; -35°C)	LQC	109.0	106.9	110.6	7.9	7.5	6.9
	HQC	95.6	87.2	95.3	1.7	0.3	2.3
Stability during sample processing	LQC	105.6	106.0	110.2	3.5	8.8	4.4
	HQC	101.6	101.8	106.2	2.0	1.6	2.1
Post-evaporation residue stability (1.5 h; room temperature)	LQC	109.6	102.2	112.0	2.8	4.9	4.2
	HQC	105.9	102.0	109.7	1.7	1.7	2.9

The validation results indicate that the % stability of EPR, EPL, HCT in PS ranged between 85 - 115%, and all CV values were below 15% under various storage conditions, meeting the requirements for bioanalytical methods.

3.3. Application of the developed method for quantification of Enalapril, Enalaprilat, and Hydrochlorothiazide in plasma samples from human volunteers

Application of the established method for the simultaneous quantification of EPR, EPL, and HCT concentrations in the plasma of two volunteers after oral administration of one combined tablet containing 20 mg of enalapril maleate and 12.5 mg of hydrochlorothiazide under fasting conditions. Blood samples were collected at: 0 hour (before drug administration), 0.25 (15 minutes); 0.5 (30 minutes); 0.75 (45 minutes); 1; 1.5; 2; 2.5; 3; 3.5; 4; 5; 6; 8; 10; 12; 24; 36; 48; and 72 hours after administration. The study protocol was approved by the Ethics Committee of the National Institute of Drug Quality Control.

The concentrations of EPR, EPL, and HCT in the plasma samples of the two volunteers at the specified time points before and after drug administration are presented in Table 10. Representative chromatograms of plasma samples from the volunteers are shown in Figures 6 and 7.

Table 10. *Concentration results of EPR, EPL, and HCT in the plasma of volunteers and pharmacokinetic parameters*

Time points (hours)	EPR		EPL		HCT	
	Volunteer 01	*Volunteer 02*	*Volunteer 01*	*Volunteer 02*	*Volunteer 01*	*Volunteer 02*
0.0	BQL	BQL	BQL	BQL	BQL	BQL
0.25	35.020	9.316	BQL	BQL	6.272	1.235
0.5	134.938	177.815	1.921	1.179	46.826	29.281
0.75	126.382	166.290	12.360	9.846	72.593	53.363
1	107.767	135.104	36.505	26.851	102.196	72.510
1.5	55.687	91.640	68.150	64.904	84.367	77.656
2	34.678	66.990	91.902	82.527	79.843	76.038

Time points (hours)	EPR		EPL		HCT	
	Volunteer 01	*Volunteer 02*	*Volunteer 01*	*Volunteer 02*	*Volunteer 01*	*Volunteer 02*
2.5	23.054	43.877	102.674	97.712	71.285	68.394
3	13.861	29.007	101.569	97.942	66.774	54.717
3.5	7.331	16.016	89.340	95.433	47.614	46.922
4	4.503	11.766	84.367	94.812	44.230	46.622
5	2.526	5.329	62.878	80.120	23.735	34.314
6	1.425	3.134	43.810	71.834	16.087	28.243
8	BQL	BQL	27.776	49.581	12.370	20.369
10	BQL	BQL	16.225	27.489	9.237	14.740
12	BQL	BQL	8.995	15.948	5.588	10.138
24	BQL	BQL	2.498	2.600	2.994	4.384
36	BQL	BQL	2.045	1.205	1.271	1.720
48	BQL	BQL	1.450	BQL	BQL	BQL
72	BQL	BQL	BQL	BQL	BQL	BQL
t_{max} (h)	0.50	0.50	2.50	3.00	1.00	1.50
C_{max} (ng/mL)	134.938	177.815	102.674	97.942	102.196	77.656
AUC_{last} (h.ng/mL)	188.419	278.505	650.128	795.270	451.610	525.782
AUC_{inf} (h.ng/mL)	190.896	282.494	664.839	803.579	472.209	549.051
$t_{1/2}$ (h)	1.20	0.88	7.03	4.78	11.23	9.38

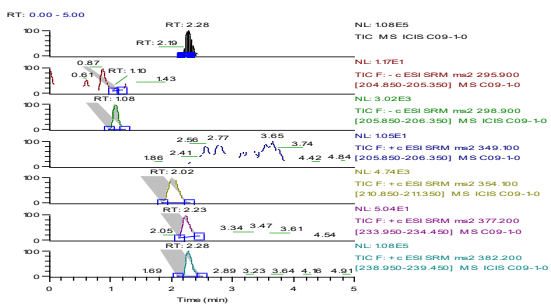


***Figure 6**. Chromatograms of PS from the volunteers 01 before drug administration*

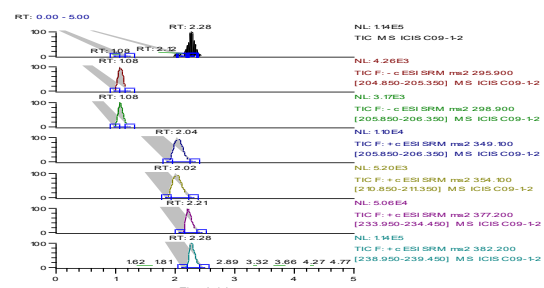


***Figure 7**. Chromatograms of PS from the volunteers 01 after 2 hours of drug administration*

The analysis results in the volunteers showed:

In the chromatograms of the volunteers' PS before drug administration (Figure 6), the responses at the retention times corresponding to EPR, EPL, and HCT were all less than 20% of the LLOQ sample response. Therefore, the plasma matrix did not affect the selectivity of the method and was consistent with the results of method validation.

The peak plasma concentrations (C_{max}) of the two volunteers fell within the calibration range for all three analytes, so sample dilution was not required during analysis. This confirms that the established calibration range is appropriate for EPR, EPL, and HCT. Two LQC and MQC

samples were within the concentration range of the volunteer plasma samples, ensuring compliance with the requirements for bioanalytical sample analysis. The pharmacokinetic parameters such as C_{max}; t_{max} of the two volunteers were similar to those reported in published reference [3 - 5].

4. CONCLUSION

A method for the simultaneous quantification of enalapril, enalaprilat, and hydrochlorothiazide in human plasma was successfully developed using UPLC/MS-MS with a short analysis time (5.0 minutes), lower limits of quantification of 1.25 ng/mL, 0.75 ng/mL, and 1.0 ng/mL, linear range of 1.25 - 250 ng/mL, 0.75 - 150 ng/mL, and 1.0 - 200 ng/mL for EPR, EPL and HCT respectively. The accuracy and precision met the requirements for bioanalytical methods as specified by the US-FDA, EMA, and ICH. With its wide linear range and low lower limits of quantification, the method is applicable for use in bioavailability and bioequivalence studies of combination drugs containing enalapril maleate and hydrochlorothiazide at various dosage strengths.

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