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A validated LC–MS/MS method for the quantification of amlodipine, bisoprolol, enalapril and enalaprilat—Application to pharmacokinetic study in healthy volunteers

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ABSTRACT

In this work, a highly sensitive and rapid liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the simultaneous determination of amlodipine (AML), bisoprolol (BIS) and enalapril maleate (ENA) in the presence of its metabolite enalaprilat (ENT) in real human plasma was developed and validated using felodipine, propranolol, ramipril, and captopril, as internal standards (IS), respectively. The prepared samples were chromatographed using Eclipse C_{18} column (4.6 × 100 mm, 5 μm) and the mobile phase was pumped in an isocratic mode consisting of acetonitrile: 0.01% formic acid (70:30, v/v) at a flow rate 0.7 mL min^{-1}. The detection was achieved on an API 4500 triple quadrupole mass spectrometer using multiple reaction monitoring (MRM) mode in the positive electrospray ionization interface. Linearity was obtained over a concentration range of 0.1–10, 0.5–50, 5–500 and 1–100 ng/mL for AML, BIS, ENA and ENT respectively, by applying weighted least-squares linear regression method ($1/x^2$). The accuracy and sensitivity of the proposed method allowed its application to determine the pharmacokinetics of these analytes in human male volunteers following a single oral administration.

1. Introduction

Hypertension affects at least one-quarter of the adult population in the United States and approximately 1.4 billion people worldwide. Uncontrolled hypertension is an important determinant of the incidence of coronary heart disease and stroke. The most commonly prescribed first-line antihypertensive agents include thiazide diuretics, Angiotensin Converting Enzyme (ACE) inhibitors, calcium channel blockers, β-blockers, and angiotensin II receptor antagonists [1]. Less than 15% of the hypertensive patients have their blood pressure adequately controlled [2]. Therefore, successful management of high blood pressure in the majority of hypertensive patients require the concurrent use of two or more medications. Fixed dose combination of antihypertensive agents was found to be an effective way to reduce cost and enhance patient adherence [3–5]. In this regard, a triple therapy combination of an ACE inhibitor, a calcium channel blocker, and a β_1-selective adrenoceptor blocking agent, recognized as an effective option due to their complimentary mechanism of action and thus give an additive blood pressure lowering effect [6].

Amlodipine besylate (AML), is a potent dihydropyridine calcium channel blocker and described chemically as 2-[(2-amino ethoxy)-methyl]-4-(2-cholophenyl)-1, 4-dihydro-6-methyl-3,5-pyridine dicarboxylic acid 3-ethyl-5-methyl ester benzosulfonate. Bisoprolol (BIS) is a potent cardioselective β1-adrenergic blocking agent, chemically described as the 1-{4-[(2-isopropoxyethoxy) methyl] phenoxy]-3-(isopropyl amino) propan-2-ol. Due to its cardioselectivity, BIS can be used safely in patients with severe asthma or chronic obstructive pulmonary disease (COPD). Enalapril maleate (ENA), (2S)-1-[(2S)-2-[[(1S)-1-(Ethoxycarbonyl)-3-phenylpropyl] amino] propanoyl] pyrrolidine-2-carboxylic acid (Z)-butenedioate, is a prodrug which undergoes rapid metabolism in the liver to enalaprilat (ENT) which inhibits ACE in human subjects and animals [7]. The molecular structures of these

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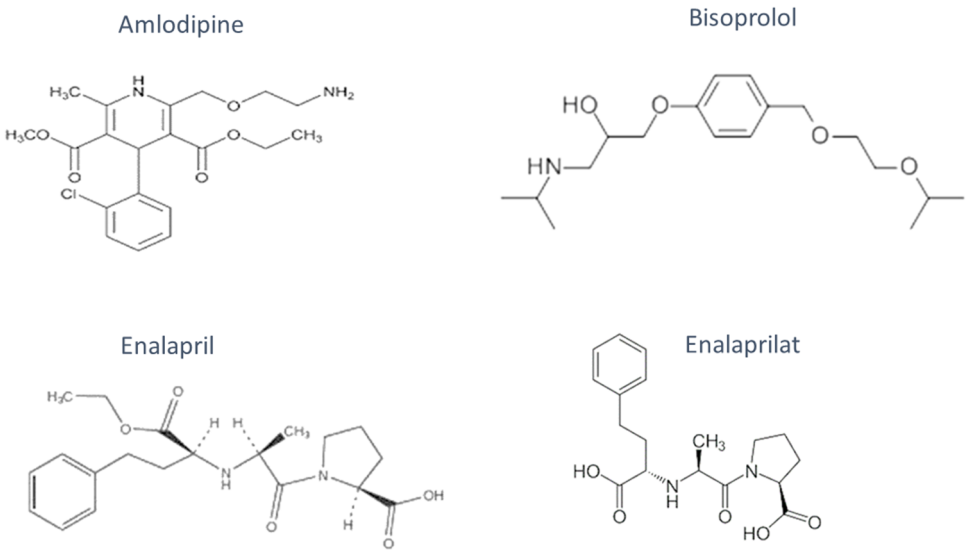

Fig. 1S. Chemical structures of the drugs under investigation in the study.

potent antihypertensive agents are demonstrated in Fig. 1S.

Although a number of methods have been reported for quantitative analysis of AML, BIS, ENA and ENT individually or in combination with other drugs, there is very few analytical methods reported for the simultaneous analysis of these drugs in a combined dosage formulation. The reported methods are UV spectrophotometric [8–12], spectrofluorimetric [13], HPLC [14–20], LC–MS/MS [21–28], HPTLC [29 30], and electrochemical methods [31]. However, and to the best of our knowledge, there are no reported methods that were applied in studying the pharmacokinetics of this potent drug combination in humans. Thus, the present work aimed to develop and validate a sensitive, selective and rapid LC–MS/MS method in the positive ionization mode for simultaneous analysis of AML, BIS, and ENA in presence of its metabolite ENT in real human plasma. The proposed LC–MS/MS method was applied successfully to a pharmacokinetic study of healthy Egyptian subjects with respect to FDA bioanalytical guidelines.

2. Experimental

2.1. Chemicals and reagents

AML (purity 99.7%) was purchased from Anek Prayog Pvt. Ltd. (Maharashtra – India), ENA (purity 99.4%) was purchased from Hunhm Zhejnng Hunhn Pharmaceuticals (Xunqiao – China), BIS (purity 100.1%) was purchased from Moehs Catalana (Barcelona – Spain), ENT (purity 99.9%) was purchased from Toronto Research Chemicals (North York, Canada). Ramipril, captopril, propranolol and felodipine were purchased from Pharco Pharmaceuticals (Cairo, Egypt). Acetonitrile, methanol, and formic acid (HPLC grade) were purchased from J.T. Baker (New Jersey, USA). Ammonium formate was obtained from Scharlau (Barcelona, Spain). A Milli-Q (Millipore Co., Germany) was used to obtain ultra-pure water with resistivity > 18 MΩ cm^{-1} at 25 °C and total organic carbon (TOC) < 5 ppb. Blank human plasma samples were obtained from Holding Company for Biological Products and Vaccines (Giza, Egypt) and stored at −80 °C until used.

2.2. Instruments

The quantification of AML, BIS, ENA, and ENT in human plasma was achieved using a chromatographic system Exion LC™ provided from (ABSciex, USA) coupled with an applied Biosystem API 4500 triple quadrupole mass spectrometry (ABSciex, Canada), equipped with an electrospray ionization source operated in positive ionization mode using multiple reaction monitoring (MRM). The source dependent parameters maintained for all analytes were: cone gas flow 50 L/h, desolvation gas flow 800 L/h, cone voltage 4 KV, and source temperature 600 °C. The collision of the gas maintained at 8 psi. Unit mass resolution was employed and the dwell time was set at 200 ms. All the MS/MS parameters were optimized at a flow rate of 10 μL min^{-1} by direct infusion of standards into the mass spectrometry. Analyst software Hotfixes version (ABSciex,1.6.3®) was used to control all parameters of HPLC and MS.

2.3. Liquid chromatographic and mass spectrometric conditions

Chromatographic separations were performed using Eclipse UPLC C_{18} column (4.6 × 100 mm, 5 μm) provided from Agilent Technologies (CA, USA) which was maintained at 30 °C. Samples (injection volume of 5 μL) were separated using an isocratic mobile phase consisted of acetonitrile and 0.01% formic acid (70:30, *v/v*) and was delivered at a flow rate of 0.7 mL min^{-1}. The detection of the ions at transition pairs was performed by MRM mode with positive electrospray ionization. The precursor-to-product ion pairs (Table 1) used for MRM were m/z 409.1 → 237.9 for AML, m/z 326.2 → 116 for BIS, m/z 377.1 → 234.1

Table 1
Linearity results for amlodipine (AML), bisoprolol (BIS) enalapril (ENA), and enalaprilat (ENT) in human plasma.

Analyte	Linear range (ng/mL)	Slope*	Intercept*	r^2*	LLOQ (ng/mL)
AML	0.1–10	0.0549 ± 0.017	0.0035 ± 0.0035	0.998 ± 0.001	0.1
BIS	0.5–50	0.771 ± 0.70	0.063 ± 0.036	0.998 ± 0.001	0.5
ENA	5–500	0.013 ± 0.005	0.01 ± 0.01	0.998 ± 0.0007	5.0
ENT	1–100	0.716 ± 0.023	0.107 ± 0.11	0.999 ± 0.002	1.0

LLOQ; Lower Limit of Quantification.
* data presented as mean ± standard deviation (n = 5).

for ENA, m/z 349.2 → 206 for ENT and m/z 386 → 340.3 for felodipine (IS), m/z 260.2 → 183 for propranolol (IS), m/z 417.2 → 234 for ramipril (IS), and m/z 218 → 116 for captopril (IS).

2.4. Preparation of calibration standards and quality control samples

Stock solution of the internal standards (ramipril, captopril, propranolol HCl and felodipine) were prepared in methanol at a concentration of 800 ng/mL and then stored in refrigerator at 4 °C. Stock solutions of the analytes of interest (enalapril, enalaprilat, bisoprolol and amlodipine) were prepared in methanol at a concentration of 1000 μg/mL. Working solutions of the analytes were obtained by serial dilution of stock solutions with 50% methanol to prepare working solutions with concentrations of 1.0–100 ng/mL for AML, 5.0–500 ng/mL for BIS, 50–5000 ng/mL for ENA, and 10–1000 ng/mL for ENT. All working solutions were stored at 4°C until used to prepare calibration standards and quality control (QC) samples.

Plasma calibration standards were prepared fresh on the day of analysis by adding 50 μL of working solutions to 450 μL of drug-free human plasma to yield a set of calibration standards in the range of 0.1–10 ng/mL for AML, 0.5–50 ng/mL for BIS, 5.0–500 ng/mL for ENA, and 1.0–100 ng/mL for ENT. Similarly, QC samples were prepared by spiking drug-free human plasma with working solutions to produce final concentrations covering the lower limit of quantification QC (LLOQ), lower concentrations (QCL), middle concentrations (QCM), and higher concentrations (QCH). The LLOQ, QCL, QCM, and QCH prepared were 0.1, 0.3, 3.0, and 8.0 for AML; 0.5, 1.5, 15.0, and 40.0 for BIS; 5.0, 15.0, 150.0, and 400.0 for ENA; 1.0, 3.0, 30.0, and 80.0 ng/mL for ENT, respectively. The calibration standards and QC samples were stored at −70 °C until further use.

2.5. Sample preparation

An aliquot (500 μL) of human plasma samples, calibration standards or QC samples was spiked with 50 μL of an IS (800 ng/mL in methanol) and vortex mixed for 4 min. Protein precipitation was carried out by adding 1.5 mL acetonitrile, and mixed for 4 min, then centrifuged at 4000 rpm at 5 °C temperature for 10 min. 1 mL of the clear supernatant was transferred to a Wassermann tube and diluted with 1 mL H_2O. A 5.0 μL was finally injected into the LC–MS/MS system for analysis.

2.7. Method validation

Validation of the bioanalytical proposed method was performed with respect to accuracy, precision, selectivity, carry-over, matrix effect, short term and long-term stability, and dilution integrity according to the acceptable criteria of the US Food and Drug Administration (FDA) guidelines for bioanalytical methods [32].

2.7.1. Selectivity

To evaluate the selectivity of the proposed method, blank human plasma from six different sources were analyzed to ensure the absence of chromatographic interference from endogenous plasma components at the respective retention times for the analytes and IS.

2.7.2. Linearity and range

Linearity was assessed by processing 6 calibration standards of each analyte on five different days. The used concentrations were 0.1–10, 0.5–50, 5–500 and 1–100 ng/mL for AML, BIS, ENA and ENT; respectively. Calibration curves were constructed by plotting peak area ratio of the transition pair of each drug with that of IS against the nominal concentration of calibration standards. The calibration plots were constructed using a weighted least-squares linear regression method using a weighted factor of $1/x^2$. The concentrations of all analytes and QC samples were calculated using full calibration curves created concurrently on each occasion. The blank samples and zero samples with IS were run with each calibration curve. The acceptance criteria for back-calculated standard concentrations were ±15% deviation from the nominal value except at the lower limit of quantification level (LLOQ), which was set at ±20%.

2.7.3. Carry-over

Blank human plasma samples were injected after the highest calibration standard of the analyte under investigation to make sure that it does not affect the precision and the accuracy of the suggested method. To investigate the carry-over for all four analytes, it should be not greater than20% of the peak response of the lower limit of quantification.

2.7.4. Accuracy and precision

Intra-day accuracy and precision were assessed through analysis of six replicates of QC samples at each of the LLOQ, QCL, QCM, and QCH within the same day. The inter-day accuracy and precision were evaluated by analyzing six replicates of QC samples on three consecutive days (n = 18). Accuracy was determined by calculating the ratio of the calculated mean concentration of the four QC samples to their respective nominal concentration. The accuracy was expressed as the percentage recovery with an acceptance criterion ±15% from the nominal values except at the lower level of quantification (LLOQ), which was set at ±20%. The precision was expressed as a percentage of relative standard deviation (%RSD) with an acceptance criterion ±15% from the nominal values except at the lower level of quantification (LLOQ), which was set at ±20%. Back calculation of the QC samples concentrations was done using different weighting factors to determine the best-fit for regression. Linearity range, accuracy, and precision were calculated using a weighting factor of $1/x^2$ which gave the best fit correlation between nominal concentration and detector response for the analytes under investigation.

2.7.5. Matrix effect and extraction recovery

Extraction recovery of AML, BIS, ENA, ENT and IS from human plasma were calculated by comparing the peak area of the extracted low, medium and high QC levels in six replicates with the response of un-extracted standards at equivalent concentrations. The matrix effects were demonstrated by comparing the peak areas obtained from samples where the extracted matrix was spiked with standard solutions to those obtained from the pure standard solution at equivalent concentrations. The efficiency was estimated by dividing matrix factor (ME) for each one of the analytes to the relative recovery (ME/RE × 100) [33]. All assays were performed in six replicates.

2.7.6. Stability

Analytes stability in human plasma was evaluated by analyzing QC samples at the low and high concentrations at different storing and handling conditions. Stability of the analytes in plasma during handling was assessed for 24 h at room temperature (bench top stability) and 12–24 h at 4 °C in the autosampler. Stability of the analytes during storing was assessed after 120 days of storage at −80 ± 10 °C, and after three cycles of freeze-thaw following freezing −80 ± 10 °C for 12 h and then allowed to thaw at room temperature before assay. All stability assays were assessed by three replicates.

2.7.7. Dilution integrity

This study was applied to ensure that analytes could be diluted with blank matrix without any effect to the final concentration. This experiment was performed using the high QC samples with concentrations of 8, 40, 400 and 80 ng/mL for AML, BIS, ENA, and ENT respectively. These samples were further diluted with blank human plasma two- and four- folds in six replicates for detecting the dilution integrity of the samples. The acceptance range should have accuracy of 100% ± 15%. and precision not greater than 15%.

2.8. Pharmacokinetic study

The purpose of the newly developed method was to investigate the pharmacokinetics after oral dose administration of AML, BIS, ENA, and ENT in adult healthy human volunteers. The experimental protocol and procedures of this study were reviewed and approved by the Institutional Review Board (IRB) of Zi Diligence Research Center (Cairo, Egypt) and in compliance with the Declaration of Helsinki. Three adult healthy male volunteers were enrolled in this study after giving their written informed consent and following clear explanation of the objectives and possible risks associated with study. Volunteers were asked to fast for at least 8 hours before the study. Blood samples were collected into a lithium heparin tubes prior to drug administration and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 24, 48, 72, and 96 h after a single oral administration of a tablet containing 5 mg AML, 10 mg BIS, and 10 mg ENA. Following immediate centrifugation of blood samples at 3500 rpm for 10 min at 4 °C min, then the plasma was transferred directly into 5 mL plastic tubes. The resultant plasma samples were stored at the study site in an ultra-deep freezer at -80 °C until reanalysis. A non-compartmental model was utilized to estimate the pharmacokinetic parameters for all studied drugs using Phoenix® WinNonlin® Version 6.3 (Certara, St. Louis, MO). The maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) following drug administration were obtained directly from the individual plasma–-concentration time data. The area under the concentration–time curve from time zero to infinity ($AUC_{0-\infty}$) was measured using linear trapezoidal summation with extrapolation. The terminal elimination rate constant (ke) was estimated by linear least square regression analysis of the terminal log-linear portion of plasma concentration

3. Incurred plasma samples reanalysis

According to the most recent regulatory guidelines, reanalysis of incurred samples following completion of initial analysis of all volunteers' plasma samples were performed. Measured concentration of all incurred plasma samples should be within ±20% of their initial concentration to ensure reproducibility of the method and absence of significant metabolic changes or variability in the plasma samples.

4. Results and discussion

4.1. LC–MS/MS method development

The initial phase of HPLC method development aimed to identify the optimum conditions for resolution and sensitivity. Electrospray ionization of the four analytes and their internal standards was carried out with positive ion scan mode because analytes have pyridine and/or amino groups (Fig. 1S) were readily protonated, and therefore, displayed favorable sensitivity in the positive ionization mode. Optimization of the chromatographic conditions was performed by careful investigation of the column type as well as mobile phase composition. To simultaneously separate four analytes and their internal standards, we used Eclipse® C18 column (4.6 × 100 mm, 5 μm) that was maintained at 30 °C with an injection volume of 5 μL. A simple aqueous acetonitrile mobile phase with 0.01% formic acid (70:30, v/v) was pumped under an isocratic condition with a flow rate of 0.7 mL/min. These optimum conditions resulted in peaks that were well defined and resolved free from tailing with retention times of 1.44, 1.18, 1.23, 1.24, 4.34, 1.16, 1.40 and 1.37 min for AML, BIS, ENA, ENT, felodipine (IS), propranolol (IS), ramipril (IS) and captopril (IS); respectively as shown in Fig. 1 and 2.

In order to improve the mass spectrometric and ionization mode conditions, further optimization of the mass spectrometric parameters including curtain gas, collision energy, ion-spray capillary voltage, and source temperature, were investigated to achieve the richest relative abundance of precursor-to-product ions. The optimized MS/MS ion source parameters are detailed in the method section, while the compound-dependent parameters are illustrated in Table 1S. The predominant ions $[M+H]^+$ for AML, BIS, ENA, ENT, felodipine, propranolol, ramipril, and captopril in the full scan mode Q1/ MS spectra were at m/z 409.1, 326.2, 377.1, 349.2, 386, 260.2, 417.2, and 218.1. The collision energy and other MRM parameters were optimized and the following product ions were selected 237.9, 116, 234.1, 206, 340.3, 183, 234, and 116 for AML, BIS, ENA, ENT, felodipine, propranolol, ramipril, and captopril, respectively.

Sample preparation is acritical step in bio-analysis. Therefore, the development of a method for extraction of plasma is considered as the cornerstone for any bioanalytical method. The literature reviews stated that only liquid-liquid extraction (LLE) and protein precipitation (PP) have been tried successfully to extract all these analytes simultaneously from their plasma samples. Thus, during initial trials these approaches were tested for selective extraction of the analytes using different solvent systems. Protein precipitation was studied using acetonitrile and methanol as precipitants in 3:1 and 2:1 (v/v) with respect to the sample, as the goal of plasma sample extraction optimization is to be able to reach the maximum extraction recovery and minimum matrix effects in order to enhance the sensitivity and accuracy of LC–MS/MS analysis. It was found that acetonitrile is the best precipitating agent.

4.2. Method Validation

4.2.1. Selectivity

Six drug-free plasma samples obtained from healthy volunteers were processed to validate the selectivity of the proposed bioanalytical method. Representative chromatograms clearly indicate the absence of significant interference peaks in blank human plasma samples (Figs. 1A and 2A) at the respective retention times of the analytes (Fig. 1B) and the internal standards (Fig. 2B). The retention times of AML, BIS, ENA, ENT, felodipine, propranolol, ramipril, and captopril were 1.44, 1.18, 1.23, 1.24, 4.34, 1.16, 1.40, and 1.37 min, respectively. Absence of interference peaks from endogenous substances indicates that our suggested method is selective for the analytes of interest in real human plasma.

4.2.2. Linearity

Calibration curves were constructed using 6 calibration standards in human plasma by plotting peak area ratios (peak area of the analyte of interest /peak area of the IS) vs concentration of the analyte of interest. The calibration curves were linear over concentration ranges of 0.1–10, 0.5–50, 5–500, and 1–100 ng/mL for AML, BIS, ENA, and ENT respectively, as shown in (Table 1). The coefficient of determination (r^2) was > 0.998 in all calibration curves (n = 5). The mean regression equations were $y = 0.0549x + 0.0035$ for AML, $y = 0.771x + 0.063$ for BIS, $y = 0.013x + 0.01$ for ENA, and $y = 0.716x + 0.107$ for ENT where y represents peak area ratio for analyte to internal standard and x represents the concentration of the analyte in ng/mL. Blank and zero samples were assayed in order to verify the absence of interference. The precision and accuracy of the five replicates of calibration curves were within the acceptable range as described below. The low limit of quantifation (LLOQ) for this method was found to be 0.1, 0.5, 5, and 1 ng/mL for AML, BIS, ENA, and ENT respectively based on a signal-to-noise ratio of 5:1 and accuracy of back-calculated concentration of ±20% deviation from the nominal value.

4.2.3. Carry-over

Drug-free human plasma samples were injected immediately after calibration standards at (ULOQ) for AML, BIS ENA, and ENT respectively. The carry-over in the blank samples was found to be <20% of LLOQ.

4.2.4. Accuracy and precision

To determine the intra- and inter-day accuracy and precision of the

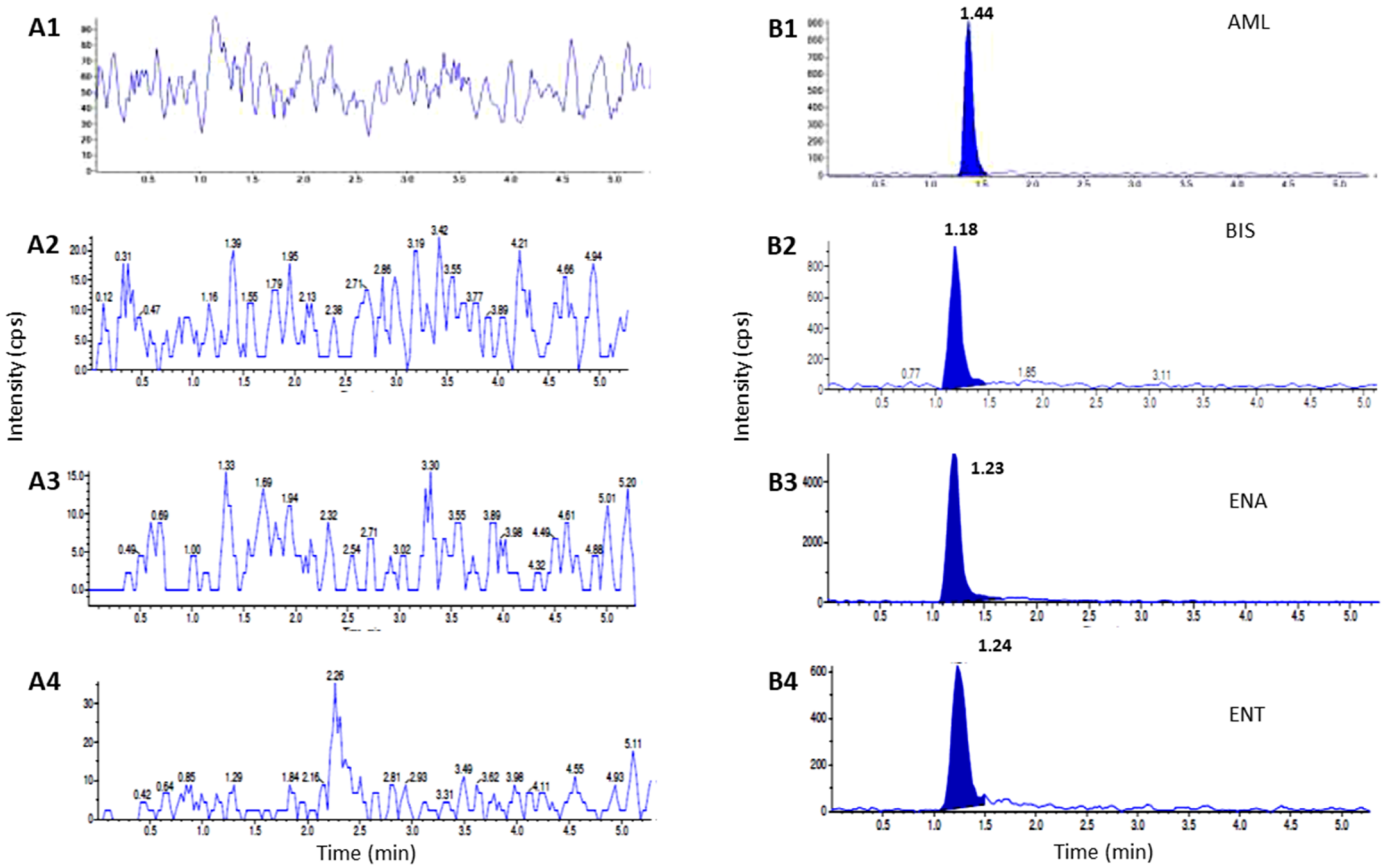

Fig. 1. Mass chromatograms of drug-free human plasma (A1, A2, A3, and A4) and human blank plasma spiked with AML (B1), BIS (B2), ENA (B3), and ENT (B4) at the LLOQ.

assay, a replicate set (n = 6 and 18, respectively) of LLOQ and three quality control concentrations at low (QCL), medium (QCM) and high (QCH)) concentration of the analytes in human plasma were analyzed. The accuracy and precision were calculated as described previously and the results are summarized in (Table 2). The intra-assay and inter-assay precision for the QC samples were below 15% in all cases. Inter- and intra-day accuracies, on the other hand, were in the range of 90–103, 99–106, 91–100%, and 91–108% for AML, BIS ENA, and ENT respectively (Table 2).

4.2.5. Matrix effect and Extraction recovery

The effect of plasma constituents over the ionization of all analytes and IS was evaluated by comparing the responses of the post-extracted plasma standard QC samples obtained from six different batches at three levels QCL, QCM and QCH with the analytes from neat samples at equivalent concentrations. The IS-normalized matrix factor CV% was found not exceeding 13.4% for all analytes indicating no significant matrix effect. The relative recovery was calculated by comparing the response ratio of peak area for the plasma samples spiked before extraction to the post-extraction samples. The recoveries across all QC levels were ranged from 51.60 to 120.40% for all analytes as presented in Table 2S.

4.2.6. Stability

These studies were undertaken to evaluate the stability of the analytes of interest in human plasma under expected conditions of storage, processing, and handling. Separate QC standards covering the low, medium and high ranges concentrations of the analytes were analyzed and left in the autosampler at 25 °C for one day, the obtained results were compared with those from the freshly prepared samples. The results depicted in Table 3S indicate that AML, BIS, ENA, and ENT to be stable in human plasma at room temperature for 24 h, in auto-sampler at 4 °C for 12–24 h, at −80 °C for 120 days, and after three freeze-thaw cycles.

4.2.7. Dilution integrity

To determine the dilution integrity, spiked human plasma samples were prepared at concentrations 8.00, 40.00, 400.0, and 80 ng/mL for AML, BIS ENA, and ENT respectively, then diluted two and four folds. Precision (% CV) and accuracy (mean % nominal) of six replicates were found to be 6.5–11% and 100.3–101%, respectively for samples with 2-fold dilution and 2.8–7.2% and 91.4–111.4%, respectively for samples with 4-fold dilution. The results are summarized in Table 4S.

5. Application to pharmacokinetic study

Due to its sensitivity and accuracy, the proposed method was applied successfully for determination of AML, BIS, ENA and its major metabolite enalaprilat in real human plasma for a pharmacokinetic study in three healthy male human volunteers, who were orally administrated a tablet containing AML (5 mg), BIS (10 mg) and ENA (10 mg) in a fed prohibited condition. Plasma concentrations of the analytes were detectable as early as 5.0 min after a single oral administration. The mean plasma concentration-time profiles are illustrated in Fig. 3 and the mean pharmacokinetic parameters for the investigated drugs are summarized in Table 3. The proposed sampling schedule covered the plasma concentration-time curve was long enough to provide a reliable estimate of the extent of exposure where $AUC_{0\text{-}t}$ covers more than 80% of $AUC_{0\text{-}\infty}$, therefore C_{max}, $AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$, k_e, $t_{1/2}$ were used for evaluation and accurate demonstration of pharmacokinetic parameters. The calculated C_{max} values were at least 12-fold higher than the reported LLOQ for the four analytes by our method. Therefore,

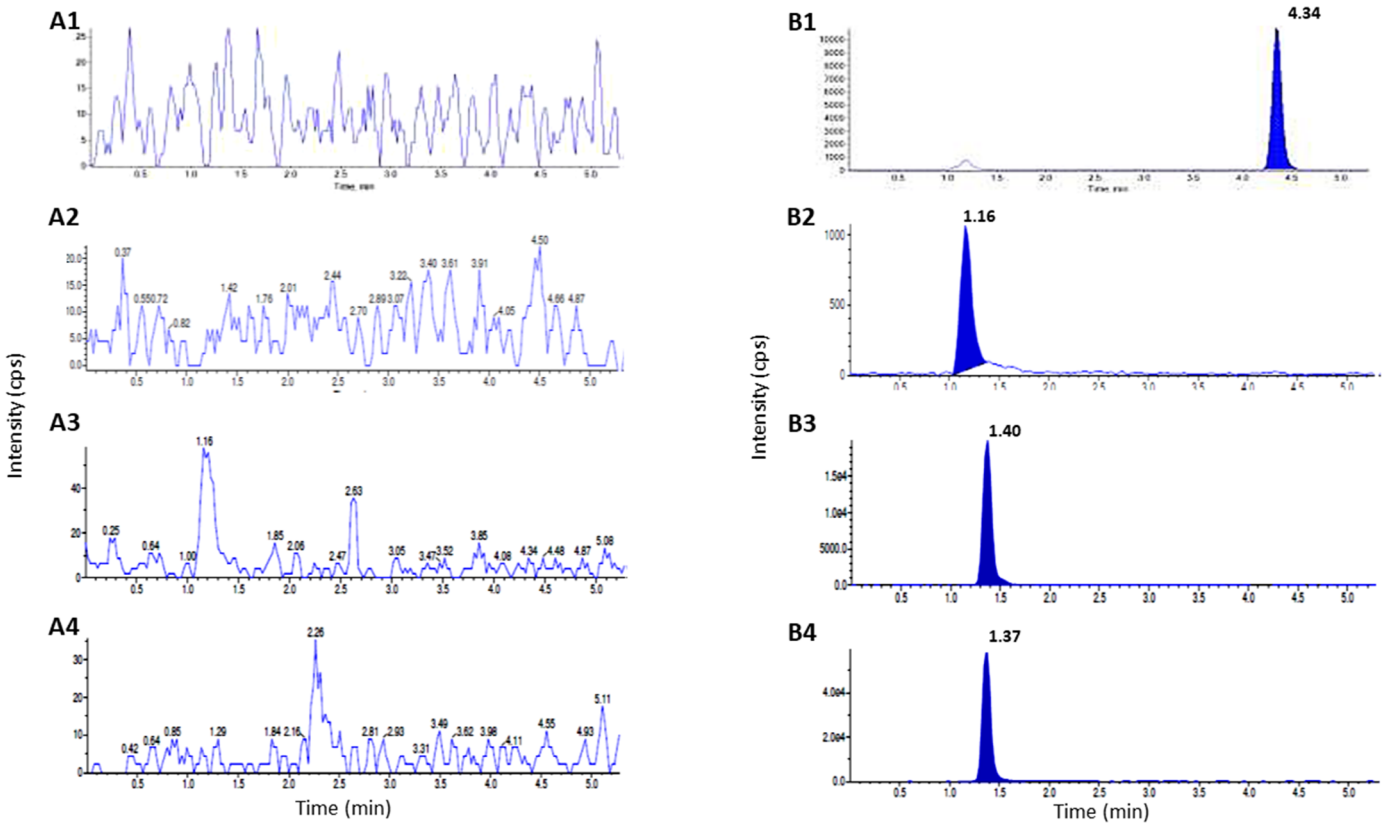

Fig. 2. Mass chromatograms of drug-free human plasma (A1, A2, A3, and A4) and human blank plasma spiked with the internal standards, felodipine (B1), propranolol (B2), ramipril (B3) and captopril (B4).

Table 2
Intra- and Inter-day accuracy and precision results for amlodipine, bisoprolol, enalapril and enalaprilat.

Analyte	Concentration (ng/ mL)		Intra-day[a]		Inter-day[b]	
			Mean (%)	CV (%)	Mean (%)	CV (%)
AML	LLOQ	0.10	90.20	5.30	93.80	10.6
	QCL	0.30	92.60	7.32	95.90	8.10
	QCM	3.00	102.7	8.24	100.1	8.71
	QCH	8.00	97.30	6.70	103.2	6.51
BIS	LLOQ	0.50	104.9	4.89	99.78	6.51
	QCL	1.50	105.8	7.44	104.2	7.56
	QCM	15.0	104.8	9.66	104.1	7.81
	QCH	40.0	102.4	7.70	103.0	6.04
ENA	LLOQ	5.00	93.77	12.9	91.10	6.65
	QCL	15.0	95.10	3.54	94.04	5.18
	QCM	150	99.69	3.00	95.82	5.79
	QCH	400	95.04	9.21	97.09	7.76
ENT	LLOQ	1.0	107.9	8.03	105.6	3.81
	QCL	3.0	91.81	7.31	97.51	8.10
	QCM	30.0	98.97	12.2	92.33	9.29
	QCH	80.0	100.9	8.41	104.6	5.57

LLOQ, Lower limit of quantification; QCL, quality control concentrations at low concentration; QCM, quality control concentrations at medium concentration; QCH, quality control concentrations at high concentration.

[a] $n = 6$.

[b] $n = 18$.

our method demonstrates sufficient reliability in determining the pharmacokinetics of these drugs following a single oral administration of therapeutic doses. Despite the low number of subjects recruited in this study, a large inter-individual variation was not observed (Fig. 3). In general, our estimated pharmacokinetic parameters were within 1-3 magnitude of the previously published data for the individual antihypertensive agents in this study. For example, and in agreement to our findings, several studies have shown that plasma concentrations of AML increase gradually to peak 6–8 h after a single oral administration [34]. Similarly, AML has shown a larger volume of distribution of 20–30 L/kg and slow metabolic elimination with a terminal half-life of 50–60 h [34,35].

6. Incurred plasma samples reanalysis

Reanalysis of study samples revealed plasma concentrations that were in the range of 3.7–14.1% of their initial concentration (Data not shown). Such finding indicates the absence of metabolic changes and instability of the measured analytes under the described validation conditions.

7. Conclusion

Here, we report a developed and validated LC–MS/MS method to simultaneously measure AML, BIS, ENA and its major metabolite enalaprilat in human plasma. Mass spectrometric and chromatographic conditions were optimized to ensure the simplicity, sensitivity, and reliability of the developed method. The excellent sensitivity and wide linearity range of the proposed method enable its successful application to measure plasma concentration of the four analytes in human subjects following a single oral administration of a tablet containing AML, BIS, and ENA. It is likely that the proposed method will be helpful in the analysis of plasma samples containing one or more of these analytes for routine pharmacokinetics or bioequivalence studies.

Table 3
Pharmacokinetic parameters for AML, BIS, ENA, and ENT after oral administration of a single dose in human subjects (n = 3).

	Analytes			
	Amlodipine	Bisoprolol	Enalapril	Enalaprilat
C_{max} (ng/mL)	3.6 ± 0.035	51.0 ± 4.6	69.3 ± 7.1	76.3 ± 2.5
T_{max} (h)	6.3 ± 2.9	1.5	1.5	2.5
T_{lag} (h)	0	0	0	0.5
$t_{1/2}$ (h)	105.5 ± 52	9.1 ± 0.3	7.3 ± 0.4	8.8 ± 1.1
AUC_{0-t} (ng h/mL)	132.5 ± 5.4	515 ± 10.3	147.5 ± 8.9	559 ± 16
$AUC_{0-\infty}$ (ng h/mL)	139.1 ± 7.2	523 ± 12.1	156.3 ± 6.5	572 ± 13
k (1/h)	0.008 ± 0.005	0.076 ± 0.002	0.095 ± 0.005	0.079 ± 0.01
Vz/F (L/kg)	118 ± 57	2.97 ± 0.02	7.4 ± 0.24	0.25 ± 0.04

AUC_{0-t}, area under the concentration-time curve from time 0 to last measurable concentration; $AUC_{0-\infty}$, area under the concentration-time curve from time 0 to infinity; C_{max}, maximum plasma concentration; T_{lag}, lag time, T_{max}, time required to achieve C_{max}, $t_{½}$ = elimination half-life; Vz/F, apparent volume of distribution.

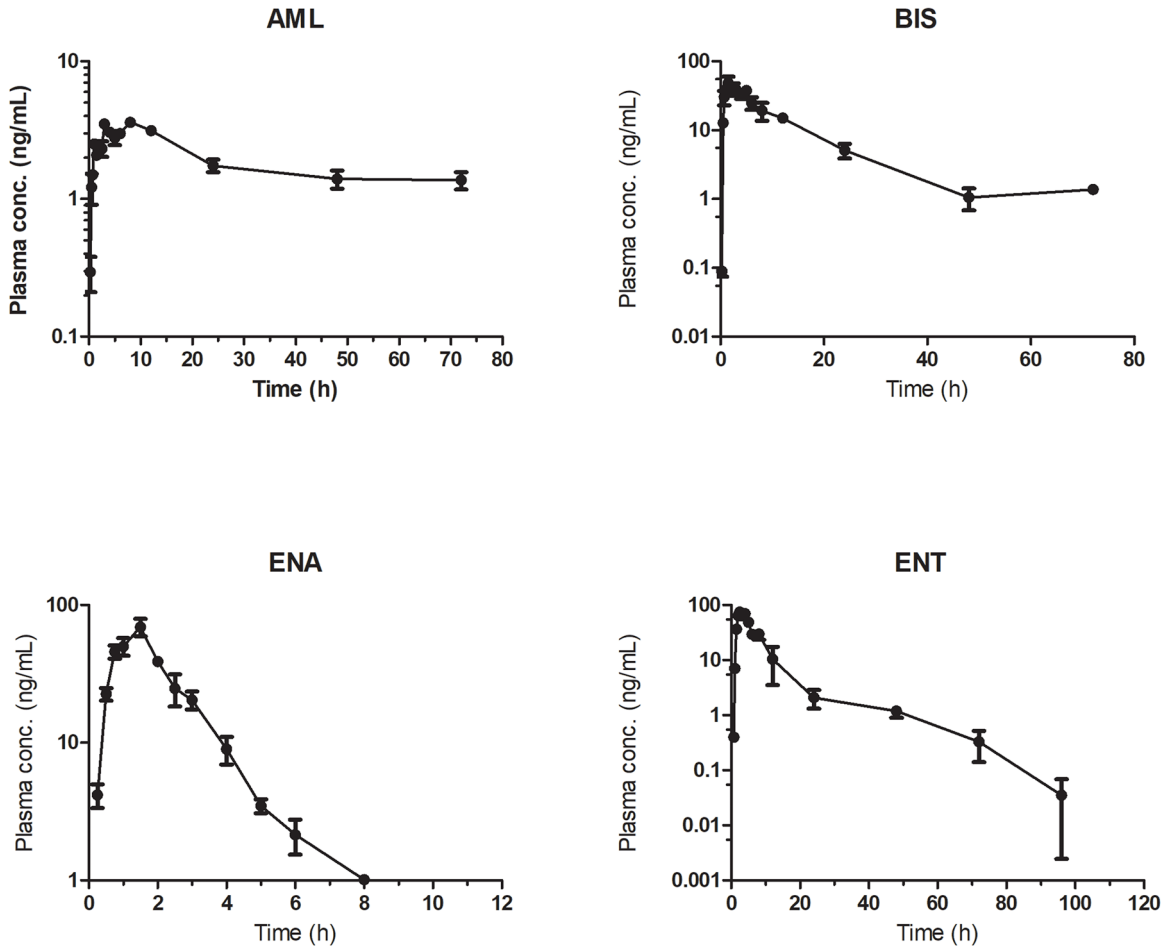

Fig. 3. Mean plasma concentration (±SD) vs. time curves following administration of single oral dose of 5 mg AML, 10 mg BIS, and 10 mg ENA.

CRediT authorship contribution statement

Liliya Logoyda: Conceptualization, Formal analysis, Writing - original draft. **Sergiy Kovalenko:** Methodology, Formal analysis. **Moustafa Gaafar:** Validation, Project administration, Writing - review & editing. **Ahmed M. Abdel-Megied:** Conceptualization, Methodology, Project administration, Formal analysis. **Fawzy A. Elbarbry:** Formal analysis, Writing - original draft, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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