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Original Research

Development, Validation, and Application for Simultaneous Assay of Amlodipine, Atorvastatin, and Ortho- and Para Hydroxy Atorvastatin as Metabolites in Human Plasma by Liquid Chromatography Tandem Mass Spectrometry

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ABSTRACT

Background: The objective of this study was to develop a liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantify amounts of amlodipine, atorvastatin, and two metabolites of atorvastatin (*ortho*- and *para*-hydroxy atorvastatin) in human plasma and to apply this method for a bioequivalence study.

Methods: An LC-MS/MS method was developed, validated, and applied to quantify amlodipine, atorvastatin and two metabolites of atorvastatin (ortho- and para-hydroxy atorvastatin) in human plasma. Rosuvastatin was used as an internal standard. LC-MS/MS with electrospray ionization (ESI) in positive ion mode, performed under the multiple reaction monitoring (MRM) mode, was used to analyze the analytes.

Results: The method was developed and fully validated with respect to selectivity, carryover, dilution integrity, and intra- and inter-day accuracy and precision according to US Food and Drug Administration (FDA) guidelines. Analytes were extracted from human plasma via a simple liquid-liquid extraction technique using a mixture of methyl *tert*-buthyl ether and ethyl acetate (1:1) after acidification with phosphoric acid. Mean extraction recovery for the analytes was between 70% and 99.95%, and matrix effects had only minor influence on precision.

Conclusion: The validated method was applied for a clinical bioequivalence study to evaluate the *in vivo* bioequivalence of two commercial products containing 5 mg amlodipine and 10 mg atorvastatin. 36 healthy subjects participated in this randomized, two-period, two-treatment, open label, crossover design study. Standard pharmacokinetic parameters were calculated to compare a test product to the CADUET® reference product. it is concluded that the two formulations are bioequivalent.

Keywords: Amlodipine; Atorvastatin; *Ortho*- and *para*-hydroxy atorvastatin; LC-MS/MS; Bioequivalence

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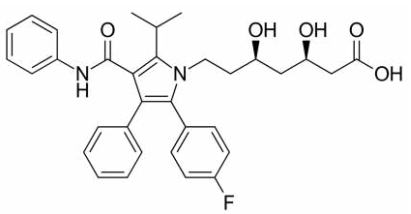
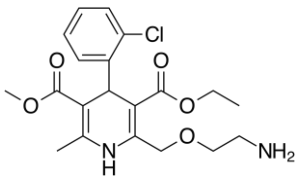
INTRODUCTION

Atherosclerosis is a leading cause of morbidity and mortality worldwide.[1] It is a multifactor disease, which must be treated with respect to each individual's overall cardiovascular risk. Hypertension and dyslipidemia are the two most important cardiovascular risk factors, which, together, cause an increase in coronary heart disease-related events.[2] Consequently, combined antihypertensive and lipid-lowering medications are used as a treatment regimen, which is expected to achieve treatment targets and reduce overall cardiovascular risk. However, the number of medications may be a barrier causing poor compliance among patients, despite clinical guidelines being implemented. Some recent studies have revealed that fixed-dose medicine combinations decreased the risk of non-compliance when compared with free-drug combination regimens.[3] The combination containing amlodipine (AML), an antihypertensive agent, and atorvastatin (ATO), a lipid-lowering medication, is designed to treat hypertension and dyslipidemia while enhancing patient adherence.

AML is a dihydropyridine calcium channel blocker approved for treating hypertension, used either alone or combined with other agents. It acts by inhibiting calcium entry through voltage-gated transmembrane L-type channels, thus decreasing intracellular calcium concentration and inducing vasodilation.[4] ATO is a lipid-lowering agent used to inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonate, a step in the cholesterol biosynthesis process. Inhibition of this enzyme decreases low-density lipoprotein cholesterol (LDL-C) and, thus, plays an important role in preventing atherosclerosis. ATO is extensively metabolized to *ortho*- and *para*-hydroxylated derivatives through cytochrome P450 3A4 metabolism, and around 70% of the HMG-CoA reductase inhibition is associated with its ortho- and para-hydroxylated metabolites.[5-6] Hence, the combination of amlodipine and atorvastatin reduces plasma cholesterol along with blood pressure, thereby providing a comprehensive control of atherosclerosis. This combination is available on the market as CADUET®, containing 5 mg of AML and 10 mg of ATO. The chemical structures of AML, ATO, *o*-ATO and *p*-ATO are shown in Figure 1.

Only a few reports have used LC-MS/MS to analyze AML and ATO,[7] as well as ATO and its two hydroxylated metabolites,[8] and only one study has used LC-MS/MS for the simultaneous quantification of AML, ATO, and its metabolites in human





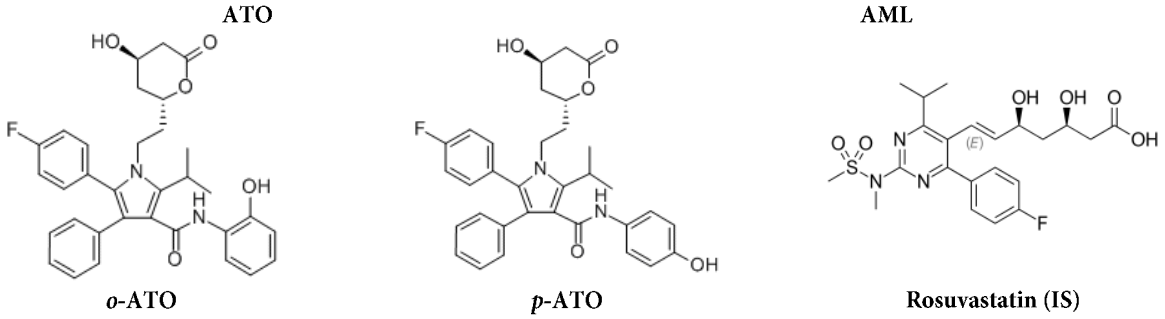


Figure 1. Chemical structures of atorvastatin (ATO), amlodipine (AML), *ortho*-hydroxy atorvastatin (*o*-ATO), *para*-hydroxy atorvastatin (*p*-ATO), and rosuvastatin as internal standard (IS)

plasma.[9] Therefore, the present authors' goal was to develop an easy and rapid LC-MS/MS method to quantify amlodipine, atorvastatin, and *ortho*- and *para*-hydroxy atorvastatin in human plasma, applying this method to evaluate the bioequivalence of a test product in comparison with the CADUET® reference product.

METHODS

Chemicals and reagents

The following reference standards were obtained from the Institute of Drug Quality Control, Ho Chi Minh City, Vietnam: atorvastatin calcium (92.20%), amlodipine besylate (100.43%), and rosuvastatin calcium (95.60%) as an internal standard (IS). The hydroxyl metabolites of atorvastatin, ortho-hydroxy atorvastatin dihydrate monosodium salt (95.00%) and para-hydroxy atorvastatin disodium salt (95.00%), were purchased from Toronto Research Chemicals (Canada). LCMS-grade acetonitrile, HPLC-grade methanol, and ethyl acetate were obtained from J.T. Baker (USA). LCMS-grade ammonium formate and methyl *tert*-buthyl ether (MTBE) were supplied by Fisher Scientific. Formic acid and phosphoric acid were of analytical grade and obtained from Prolabo and Merck, respectively. Blank human plasma sources were supplied by Blood Transfusion Hematology, Ho Chi Minh City, Vietnam, and stored at below -20°C prior to use.

Preparation of stock solutions, standards, and controls

Stock solutions of 0.3 µg/mL (for ATO and AML) and of 60 µg/mL (for *o*-ATO and *p*-ATO) were prepared at 20x the required concentrations in individual glass vials. Ten calibration standards were also prepared in blank human plasma at the following concentrations: 0.1, 0.3, 1.5, 2.4, 3, 6, 7.5, 15, 24, and 30 ng/mL for ATO and *o*-ATO; 0.1, 0.3, 0.75, 1.2, 1.5, 3, 3.75, 7.5, 12, and 15 ng/mL for ATO; and 0.025, 0.075, 0.15, 0.24, 0.3, 0.6, 0.75, 1.5, 2.4, and 3 ng/mL for p-ATO. The concentrations of the low, small, medium, and high quality controls (QCs) in blank human plasma were 0.1, 0.3, 15, and 24 ng/mL for ATO and *o*-ATO; 0.1, 0.3, 7.5, and 12 ng/mL for AML; and 0.025, 0.075, 0.15, and 0.24 ng/mL for *p*-ATO. The QCs were prepared separately from the calibration standards. Fresh calibration standards and QCs were prepared on each day of analysis during the validation and subject sample runs. A stock of IS solution at 100 µg/mL was prepared by dissolving an appropriate amount of 10 mg rosuvastatin in 100 mL methanol. On each day of analysis, an aliquot of the stock of IS solution was diluted in methanol to obtain the IS working solution (1000 ng/mL). Stock solutions of analytes and IS were stored at -20 ± 2°C, while calibration standards and quality control samples in plasma were kept at below -70°C.

Sample treatment

Prior to analysis, all frozen samples (subject samples, calibration standards, and QCs) were thawed and allowed to equilibrate at room temperature. 1 mL of the spiked plasma sample was pipetted into a glass tube, and 50 µL of the IS working solution was added. The sample was acidified with 50 µL of 1% phosphoric acid (v/v) and vortexed for 10 seconds before 3 mL of MTBE and ethyl acetate mixture (1:1) were added for liquid-liquid extraction and vortexed once more for 1 min. After centrifugation of the sample at 4,500 x g for 5 min, the organic layer was transferred into another tube and evaporated to dryness at 40°C under a gentle stream of nitrogen gas. The residue was reconstituted with 350 µL of methanol, vortexed for 1 minute, centrifuged for 5 minutes (4,500 x g; 0°C), and filtered through a 0.22 µm membrane filter. An aliquot of 5 µL was then injected into the LC-MS/MS system.

LC-MS/MS conditions

A Shimadzu UHPLC Nexera X2 and a Triple Quadrupole Mass Spectrometer LCMS-8040 were used for setting the reverse-phase liquid chromatographic conditions. The Shimadzu system consisted of an autosampler (SIL-30AC), two pumps (LC-30AD), a column oven (CTO-20A), and a controller (CBM 20A). The autosampler and oven temperatures were maintained at 5°C and 40°C, respectively. HPLC separation of analytes and the IS was performed using a Gemini® C18 column (150 × 2 mm; 3 µm). An isocratic mobile phase, composed of 10 mM ammonium formate pH 3.5 and acetonitrile (48:52, v/v), was delivered at a flow rate of 0.25 mL/min at an injection volume of 5 µL. The total chromatographic runtime was 6.5 min. The MS analysis was operated in positive ionization mode utilizing electrospray ionization (ESI). The interface voltage was set to 4,000 V; the heat block temperature was 400°C; the desolvation line temperature was 250°C; the nebulizer gas flow rate was 3 L/min; the drying gas flow rate was 15 mL/min; and the dwell time per transition was 100 ms. The multiple reaction monitoring (MRM) transitions for each analyte and IS, as well as their respective optimum MS parameters, including voltage potential (Q1, Q3) and collision energy (CE), are summarized in Table 1. Quantification was accomplished using peak area. Data acquisition and processing were performed using LabSolutions software for the LCMS-8040 system.

Table 1. Tandem mass-spectrometer main parameters

Analytes	MRM transitions m/z	Q1 (V)	CE (V)	Q3 (V)	Acquisition time (min)
ATO	559.25 → 440.20	-14	-11	-25	5.4
AML	409.10 → 238.05	-20	-22	-30	1.9
o-ATO	575.25 → 440.20	-20	-22	-30	4.6
p-ATO	575.25 → 440.20	-20	-23	-30	2.5
Rosuvastatin	482.20 → 258.05	-17	-35	-27	2.8

Method validation

This method was validated according to US Food and Drug Administration (FDA) bioanalytical method validation guidelines.[10]

Selectivity

Six individual, blank plasma samples were analyzed to investigate interference peaks at the retention time of each analyte. Selectivity was accepted if the blank response was less than 20% of the lower limit of quantitation (LLOQ) response for each analyte and less than 5% of the IS response for an IS.

Carryover

Carryover into injected blank plasma, immediately following injection of the upper limit of quantification (ULOQ), was assessed. Carryover was accepted if it was less than 20% of the LLOQ for an analyte and less than 5% of the IS response for an IS.

Calibration standard curve

The plasma samples, with a series of known concentrations, prepared as described above, were analyzed in three separate runs, and linear regression analysis was performed on known concentrations of each analyte against the area ratios of the corresponding peaks and the IS peaks. Then, the regression coefficient, slope, and y-intercept of the resulting linear calibration functions were determined.

Accuracy and precision

Four concentrations for each analyte (LLOQ and low, medium, and high QCs) were analyzed in six replicates per run. Three runs were carried out, and each run was conducted on a separate day. Intra-day (n = 6) and inter-day (n = 18) accuracy and precision was determined. Accuracy was accepted if the back calculated concentration was within 15% of the nominal concentration for each QC and 20% for the LLOQ. Precision was accepted if the coefficient of variation (CV) did not exceed 15% for each QC and 20% for the LLOQ.

Matrix effect

Plasma samples from six individuals were tested at high and low QC concentrations. The matrix effects for each analyte and IS were determined separately in each sample by calculating the ratio of the peak area in the post-extraction spiked plasma to the peak area in spiked methanol.

Recovery

Three series of six samples, with concentrations from high, middle, and low regions of the standard curve, were separately prepared and analyzed. Then, the ratios of the recorded peak area to the peak area resulting from directly injecting, in methanol, the solutions of the analytes with the same concentrations were determined as percentages in each case.

Dilution integrity

Six replicates of plasma samples were created at concentrations of 48 ng/mL (for ATO and *o*-ATO), 24 ng/mL (for *p*-ATO), and 3 ng/mL (for *p*-ATO). These plasma samples were diluted twofold in plasma and analyzed. The accuracy and precision of the diluted samples were considered acceptable within 15%.

Freeze and thaw stability

Low and high levels of QC plasma samples were kept at -70°C for 24 hours and thawed, unassisted, at room temperature. The cycles were replicated and, then, the samples were analyzed.

Short-term temperature stability

Low and high levels of QC plasma samples were reserved at room temperature for 6 hours before being analyzed.

Long-term stability

Low and high levels of QC plasma samples, retained at -70°C, were measured for a period of 88 days.

Post-preparative stability

The autosampler steadiness was steered, reanalyzing the extracted low and high QC samples retained under the autosampler situations (5°C) for 24 hours.

APPLICATION TO HUMAN SAMPLES

Bioequivalence study design

An open label, randomized, two-treatment, two-period, two-sequence, single-dose, crossover design was used to assess the pharmacokinetics and bioequivalence between 5 mg/10 mg AML/ATO test formulations (Vietnamese pharmaceutical company) and a reference formulation (CADUET® tablets manufactured by Pfizer Inc.). The study was conducted in accordance with the Declaration of Helsinki and the rules of good clinical trial practice following Circulars No. 29/2018/TT-BYT on "Regulations for Clinical Trials" and No. 03/2012/TT-BYT on "Guidelines for Clinical Trials" of the Vietnam Ministry of Health. The study protocol was approved by the Ethics Committee of the Institute of Drug Quality Control, Ho Chi Minh City.

36 healthy adult subjects, who had given informed consent, were selected for this study according to medical history, physical examination, vital signs (blood pressure, temperature, heart frequency), electrocardiogram, standard laboratory test results (blood cell count, biochemical profile, hepatitis B and C, HIV, urinalysis), and quick urine pregnancy test results for female subjects. Demographically, these subjects were: 20-28 years of age (mean 22.8±1.71 age), 148-179 cm in height (mean 161.9±8.50 cm), 44-76 kg in weight (mean 55.4±8.82 kg), and 18.1-26.6 kg/cm^2 in body mass index (mean 21.0±2.12 kg/cm^2). 20 subjects (55.6%) were male, and 16 (44.4%) were female. The subjects had no history of drug or alcohol abuse or hypersensitivity to the study drugs, nor had they taken any medication within two weeks of administration

of the study drugs. Alcohol, cigarettes, tobacco, and beverages containing caffeine were forbidden for 48 hours before and during the study. All subjects had given written, informed consent prior to study enrollment and were allowed to terminate their participation in the study at any time, without restrictions. Standard meals were served to the subjects at four and ten hours after drug administration.

Subjects were randomized to one of the two treatment sequences (Test-Reference or Reference-Test formulations) prior to study drug administration. Each subject received both formulations using a balanced crossover design according to the randomization schedule. Both the test and reference formulations were administered as a single oral dose of 5 mg AML and 10 mg ATO after an overnight fast of at least ten hours. There was a washout period of fourteen days between the two treatments, and each period lasted seven days. During the study, subjects were confined to the test center in the evening before the start of the study procedure on Day 1 and Day 15. They were also confined to staying within the test center for 24 hours after taking the study drugs. At the end of each period, subjects returned home and avoided vigorous exercise throughout the duration of treatment.

Blood samples were collected into dipotassium EDTA tubes through a catheter inserted in the antecubital vein prior to dosing (time 0) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 6, 7, 8, 10, 12, 24, 36, 48, 72, 96, and 120 hours after the dose in each period. The samples were immediately centrifuged at 1,470 × g for 10 min. Plasma was separated into two aliquots (approximately 1.2 mL each), transferred to labelled 1.5 mL polypropylene tubes, immediately frozen, and kept at -70°C until analysis.

Pharmacokinetic analysis

The pharmacokinetic parameters for AML, ATO, *o*-ATO, and *p*-ATO were determined using Equiv Test PK software. The C_{max} and T_{max} were read directly from the experimental data. The elimination rate constant (K_e) was obtained from the least-squared fitted terminal log-linear portion of the plasma concentration-time profile. The elimination half-life ($t_{1/2}$) was calculated as $0.693/K_e$. The total area under the plasma-concentration curve from time zero to the last measurable concentration (AUC_{0-t}) was calculated using the trapezoidal rule-extrapolation method. The extrapolated parameter AUC_{0-inf} (AUC from time zero to infinity) was calculated by adding the value of C_t/K_e to the calculated AUC_{0-t}, where C_t¬ was the last observed concentration higher than the LLOQ.

Incurred sample reanalysis (ISR)

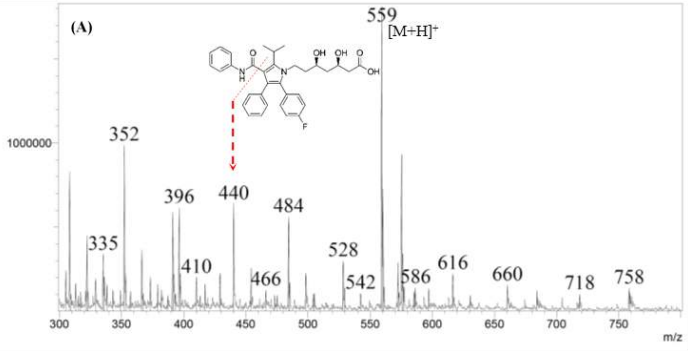
An incurred sample reanalysis (ISR) was performed on 125 sample points from the study population. The basic objective of the ISR was to reconfirm the initial concentrations and to prove that the assay was reproducible. The conformity of the original result with the ISR sample was calculated as % difference, which was required to be within 20% for at least two-thirds of the total ISR samples.

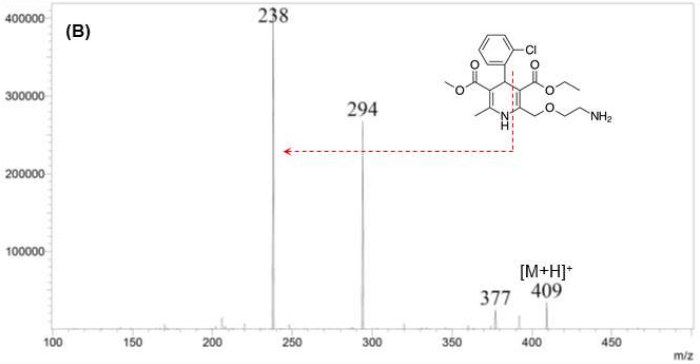
RESULTS

Optimization of LC-MS/MS conditions

During the method development, ESI was conducted in the positive ionization mode. The Q1 MS full scan spectra for AML, ATO, *o*-ATO, *p*-ATO, and rosuvastatin (IS) predominantly contained protonated precursor $[M+H]^+$ ions at *m/z* 409.10, 559.25, 575.25, 575.25, and 482.20, respectively. The most abundant production in Q3 MS spectra for ATO, AML, *o*-ATO, *p*-ATO, and IS was observed at 440.20, 238.05, 440.20, 440.20, and 258.05, respectively. Figure 2 shows the Q3 MS spectra *m/z* of the analytes and IS. The MS/MS parameters were systematically optimized for each analyte and IS to obtain a consistent and adequate response. A dwell time of 100 ms was sufficient.

The chromatographic conditions were investigated by considering the column type, mobile phase component, pH of the buffer and strength, and flow rate. These parameters were, thus, changed to obtain a symmetric peak shape, a short run time, minimum matrix interference, and solvent consumption. Based on the outcome of various trials, the Gemini® C18 (150 × 2 mm; 3 µm) column was selected. The mobile phase consisting of acetonitrile – 10 mM ammonium formate buffer pH 3.5, adjusted with formic acid (52:48, v/v), at a flow rate of 0.25 mL/min ensured separation of ATO, AML, *o*-ATO, *p*-ATO, and IS at the retention times of 5.4 min, 1.9 min, 4.6 min, 2.5 min, and 2.8 min, respectively, in a total runtime of 6.5 min. Figure 3 shows the chromatograms of the blank plasma and analytes at the MQC level with IS. The blank plasma chromatogram was obviously clean, with no endogenous interfering peak at the retention time of analytes and IS.





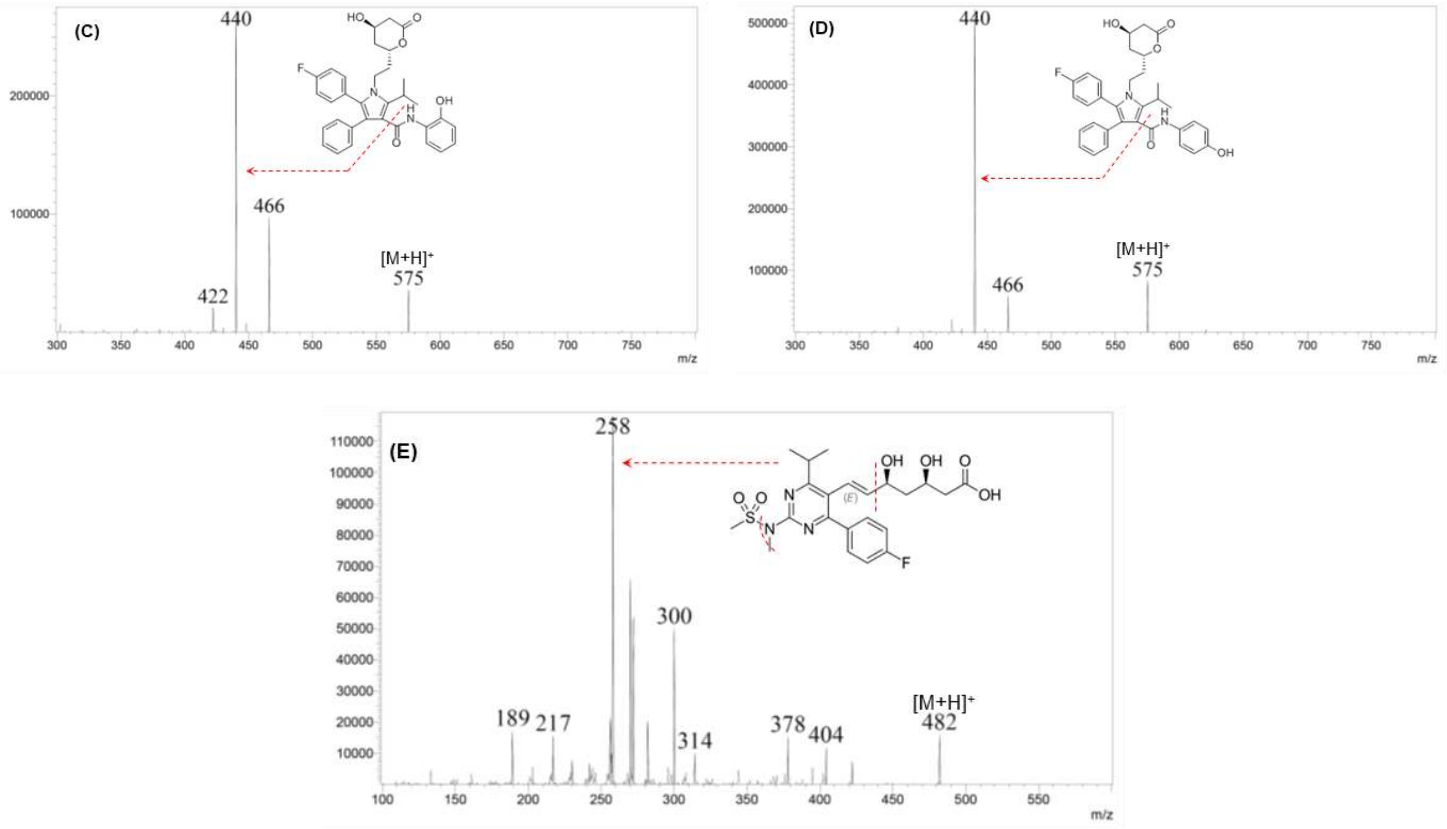
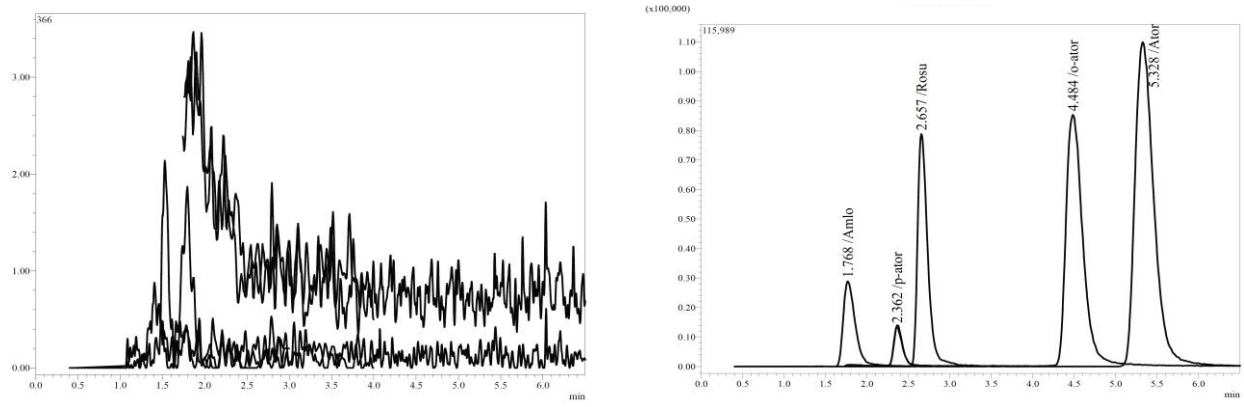


Figure 2. Precursor ion spectrum (*m/z*) for atorvastatin (A), amlodipine (B), *ortho*-hydroxy atorvastatin (C), *para*-hydroxy atorvastatin (D), and rosuvastatin (E) in positive ionization mode



(A) (B)

Figure 3. Chromatograms of blank plasma (A) and analytes at MQC level with IS (B)

Extraction procedure

Protein precipitation (PP) and liquid-liquid extraction (LLE) are routine sample pre-treatment strategies. During the initial stages of method development, PP was carried out using methanol and acetonitrile as agents, but it failed to achieve a very clean extract and produced high background noise with poor sensitivity. Therefore, LLE was desired because this technique can, not only purify, but also concentrate the sample. LLE was initiated with MTBE, ethyl acetate, and a mixture of MTBE and ethyl acetate (1:1, v/v). MTBE was not suitable as it showed low recoveries for all analytes. Ethyl acetate was superior for AML and ATO, but not for *o*-ATO, *p*-ATO, and IS. The mixture of MTBE and ethyl acetate (1:1, v/v) was adopted for good extraction recoveries in comparison with each extractor solvent alone.

The extraction was also investigated under neutral, acidic, and basic conditions. For the alkalified solution, sodium hydroxide, at various strengths (0.1 - 0.5 M) and volumes, was tested. The results, however, showed low extraction recoveries for all analytes. Acidified solution (1% phosphoric acid and 1% formic acid) was also investigated. Phosphoric acid generated higher recoveries compared to formic acid and was chosen for further investigation at different added volumes. The results showed a decrease in extraction recovery of AML from 88.03% to 47.62%, while the recoveries of other analytes and IS increased when the volumes of phosphoric acid ranged from 25 to 100 µL. Therefore, 50 µL of 1% phosphoric acid was

chosen in the sample procedure to achieve satisfactory recoveries for all analytes. Although the used extraction procedure was not the best for AML, it was excellent for ATO and its metabolites, which should be determined with high sensitivity due to their low plasma concentration levels.

Therefore, based on the investigation, MTBE - ethyl acetate (1:1, v/v) in the presence of 50 µL of 1% phosphoric acid was finally assumed in the present work.

Method validation

The bioanalytical method described here met full validation criteria for calibration curve, selectivity, carryover, accuracy, precision, matrix effect, stability, and dilution integrity in accordance with US-FDA guidelines. The results of the method validation are provided in Table 2.

Carryover evaluation was carried out to ensure that it did not affect the accuracy and precision of the method. No carryover (0.0%) was observed during the carryover experiment. There was also no response at the retention time of analytes and IS after subsequent injection of ULOQ.

The calibration curve was validated for all analytes over the following ranges: 0.1 - 15 ng/mL (for AML), 0.1 - 30 ng/mL (for ATO and *o*-ATO), and 0.025 - 3 ng/mL (for *p*-ATO). The calibration lines were drawn to give the following linear regression equations: y = (0.1005±0.0132)x - (0.0015±0.0066); y = (0.1130±0.0231)x - (0.0197±0.0029); y = (0.0484±0.0041)x - (0.0130±0.0001); and y = (0.1953±0,0203)x - (0.0022±0,0017) for AML, ATO, *o*-ATO, and *p*-ATO, respectively, where y was the peak area ratio of the analyte and IS, with x as the concentration of the analyte. The correlation coefficient square (R^2) was more than 0.99, while the accuracy for the calibration curve standards ranged from 89.83% - 114.88%; 87.15% - 100.14%; 81.68% - 103.31%; and 88.37% - 117.90% for AML, ATO, *o*-ATO, and *p*-ATO, respectively, which met the requirements. The LLOQ in the standard curve, which can be measured with acceptable accuracy and precision, was 0.1 ng/mL (for AML, ATO and *o*-ATO) and 0.025 ng/mL (for *p*-ATO) at a signal-to-noise ratio (S/N) of more than five.

The intra- and inter-day accuracy and precision of the LLOQ and QCs for all analytes were within an acceptable range. The intra-day precision (% CV) varied from 2.15% to 9.16%, and the accuracy was within 98.43% - 112.99% for all analytes. Similarly, the inter-day (% CV) varied from 1.87% - 8.00%, and the accuracy was within 94.90% - 111.47%.

Following a twofold dilution, the accuracy for all analytes was within 97.40% - 109.90%, and the precision (% CV) ranged from 1.97% - 7.30%, which was within the acceptance limit; therefore, dilution up to two times for volunteer samples higher than the ULOQ was acceptable. However, none of the subject samples measured showed concentrations above the ULOQ.

The stability of the analytes and IS in human plasma and stock solutions was investigated under various conditions. Analytes were stable for up to six hours at room temperature and for three freeze and thaw cycles at below -70°C. Spiked plasma samples were found stable for a period of up to 88 days after long-term stability experiments.

Autosampler stability was carried out and found that the spiked samples were stable up to 24 hours without significant loss of the analytes at 5°C.

Analyte responses were stable in plasma on the benchtop (room temperature for six hours), after three freeze-thaw cycles, after 88 days of storage (at -70°C), and in the autosampler (24 hours, set at 5°C). Moreover, analyte responses in stored stock were within 15% of the fresh solution responses, which was considered acceptable.

Application

The developed and validated method was applied to determine the concentrations of AML, ATO, *o*-ATO, and *p*-ATO for a bioequivalence study with a single fixed dose of a test and reference tablet formulation containing 5 mg/10 mg AML/ATO in 36 healthy Vietnamese subjects. This study was conducted in compliance with the Declaration of Helsinki and in accordance with Good Clinical Practice. The protocol was approved by the local institutional review board, and informed consents were signed by all subjects before study participation. The mean-concentration time curve of reference and test for AML, ATO, *o*-ATO, and *p*-ATO in human plasma after oral administration of the reference and test formulations under fasting conditions are shown in Figure 4. In Table 3, the mean pharmacokinetic parameters of AML, ATO, *o*-ATO, and *p*-ATO are summarized

Table 2: Summary of validation results

Property		QC	AML	ATO	*o*-ATO	*p*-ATO	IS
Selectivity	Analyte (% LLOQ)	-	0.0	0.0	0.0	0.0	
	Internal standard (% IS)	-	0.0	0.0	0.0	0.0	
Carryover	Analyte (% LLOQ)	-	0.0	0.0	0.0	0.0	
	Internal standard (% IS)	-	0.0	0.0	0.0	0.0	0.0
Calibration standard curve	R squared (R^2)	-	0.9963	0.9994	0.9985	0.9988	
Intra-day accuracy and precision	Accuracy (%)	LLOQ	103.97	106.60	101.29	106.22	
		LQC	105.55	105.82	109.01	106.80	
		MQC	112.99	104.66	102.60	98.93	
		HQC	112.75	105.12	105.39	98.43	
	Precision, CV (%)	LLOQ	4.44	3.00	6.55	9.16	
		LQC	6.21	8.20	4.37	6.78	
		MQC	2.15	4.18	3.57	3.55	
		HQC	2.41	4.89	4.21	3.58	
Inter-day accuracy and precision	Accuracy (%)	LLOQ	107.71	104.26	105.45	101.79	
		LQC	105.50	106.52	103.34	101.51	
		MQC	111.47	100.97	99.31	95.89	
		HQC	108.53	99.47	98.47	94.90	
	Precision, CV (%)	LLOQ	3.85	3.15	3.42	4.17	
		LQC	2.03	1.52	4.77	4.88	
		MQC	1.87	4.00	6.35	5.37	
		HQC	3.40	6.28	8.00	3.61	
Matrix effect	Precision, CV (%)	LQC	4.71	5.05	8.55	10.75	
		HQC	3.75	5.34	5.20	5.66	
Recovery	Mean	LQC	70.51	98.87	99.95	78.25	91.36
		MQC	89.24	99.12	95.98	82.52	79.38
		HQC	84.42	99.70	80.05	77.22	77.44
	Precision, CV (%)	LQC	8.16	7.88	6.14	6.92	2.77
		MQC	2.55	4.31	2.58	2.46	7.05
		HQC	4.88	2.16	2.94	5.62	6.99
Dilution integrity	Accuracy (%)	-	109.90	97.40	105.07	105.82	
	Precision, CV (%)	-	1.97	6.67	7.30	4.45	
Free-thaw stability	% of nominal concentration	LQC	94.97	90.22	97.63	104.71	
		HQC	103.95	90.85	88.69	90.64	
At room temperature for 6 hours	% of nominal concentration	LQC	105.14	94.54	99.30	103.77	
		HQC	99.32	90.50	100.21	94.76	
In autosampler for 24 hours	% of nominal concentration	LQC	104.87	101.17	101.18	96.40	
		HQC	105.38	102.35	110.81	99.36	
Long-term stability	% of nominal concentration	LQC	91.18	94.05	100.48	107.38	
		HQC	94.32	88.70	103.37	101.60	

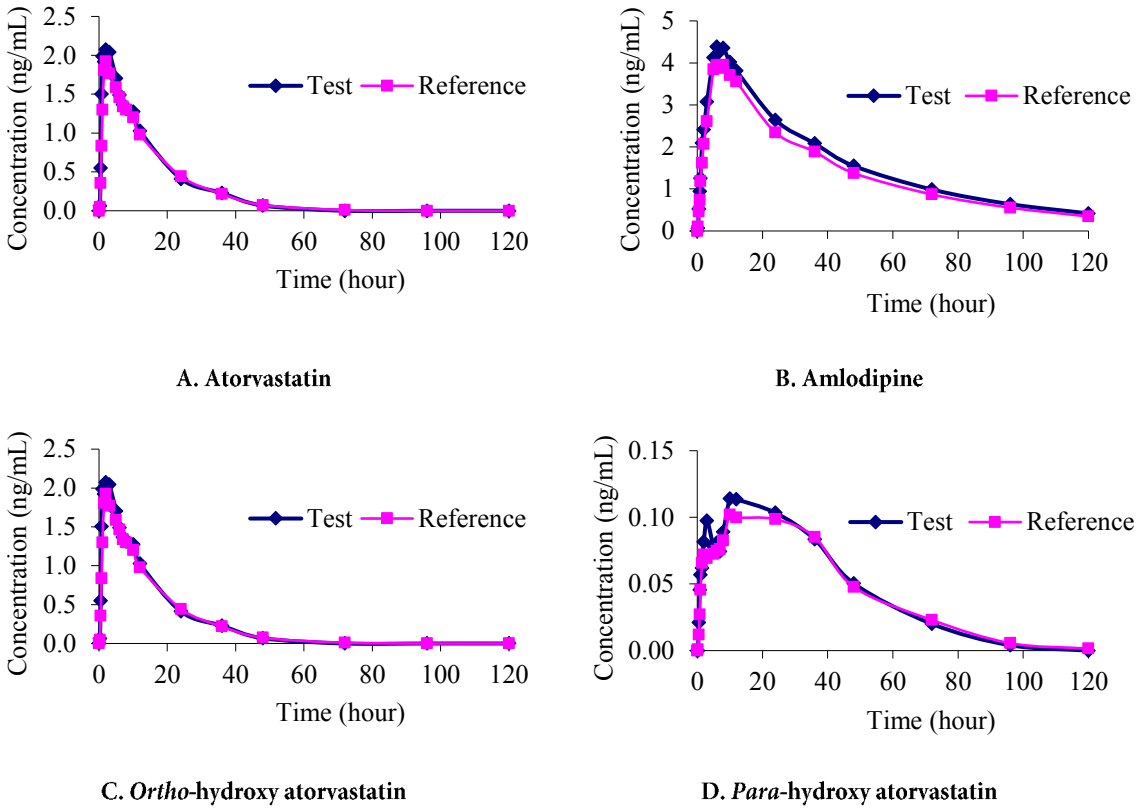


Figure 4. Mean plasma concentration-time curves of atorvastatin (A), amlodipine (B), ortho-hydroxy atorvastatin (C), and para-hydroxy atorvastatin (D) after oral administration of 5 mg amlodipine and 10 mg atorvastatin fixed dose tablet formulation to 36 healthy Vietnamese subjects

Table 3. Mean pharmacokinetic parameters (± SD) of 36 healthy Vietnamese subjects after oral administration of 5 mg amlodipine and 10 mg atorvastatin fixed dose tablet formulation

Parameter	Product	C_{max} (ng/mL)	T_{max} (hours)	$t_{1/2}$ (hours)	AUC_{0-t} (h.ng/mL)	AUC_{0-inf} (h.ng/mL)
Atorvastatin	T	6.028±4.10	1.01	12.30	29.387±13.11	32.041±13.23
	R	5.328±3.00	1.11	10.34	28.064±11.83	30.252±12.02
Amlodipine	T	4.789±1.19	7.03	40.60	192.982±53.41	172.731±46.42
	R	4.431±1.19	7.69	39.14	218.609±64.93	193.965±53.89
O-atorvastatin	T	2.765±1.93	3.16	12.57	31.527±13.62	34.335±14.15
	R	2.487±1.21	2.67	12.98	30.289±11.32	33.206±11.96
P-atorvastatin	T	0.162±0.10	14.51	25.27	5.000±2.32	6.287±2.63
	R	0.147±0.07	14.27	32.98	4.901±2.79	6.515±3.15

Abbreviations: T = test; R = reference; C_{max} = maximum plasma concentration; T_{max} = time point of maximum plasma concentration; $t_{1/2}$ = half-life of drug; AUC_{0-t} = area under the plasma concentration-time curve from zero hours to 120 hours; AUC_{0-inf} = area under the concentration-time curve from zero hours to infinity; SD = standard deviation

DISCUSSION

In a previous publication, Yacoub et al. have proposed a liquid chromatography/ion trap mass spectrometry method for quantifying AML and ATO with its metabolites in human plasma, using pravastatin as an internal standard. PP with acetonitrile was used as the extraction method. The mean recoveries were 100.22% (for AML), 103.47% (for ATO), 103.94% (for *o*-ATO), 101.3% (for *p*-ATO), and 99.19% (for IS). The analytes and IS were separated on a Phenomenex Synergi 4u polar-RP 80A (150 mm × 4.6 mm; 4 µm) column. The mobile phase was water/methanol, adjusted to pH 3.2 and isocratically delivered at a flow rate of 0.5 mL/min; the total runtime was approximately 6 min. The method linearity range was 0.2-20 ng/mL for AML, 1.5-150 ng/mL for ATO, 1-100 ng/mL for *o*-ATO, and 0.2-20 ng/mL for *p*-ATO. This method was also applied for a bioequivalence study of two commercial products containing 10 mg amlodipine and 80 mg atorvastatin in healthy volunteers.[9]

The present study reports another novel, simple, economical, and rapid method for simultaneous quantification of

amlodipine, atorvastatin, and its two metabolites by liquid chromatography tandem mass spectrometry. The process involved LLE with methyl tert-butyl ether - ethyl acetate (1:1) as the agent. The recovery was 81.39%, 99.23%, 91.99%, 79.33%, and 82.73% for AML, ATO, *o*-ATO, *p*-ATO, and IS, respectively. One IS (rosuvastatin) was capable of monitoring all analytes in their chromatographic separation, which was performed on Gemini® C18 column (150 × 2 mm; 3 µm). The mobile phase was composed of 10 mM ammonium formate pH 3.5 and acetonitrile (48:52, v/v) and was delivered at a flow rate of 0.25 mL/min. The total chromatographic runtime was 6.5 min, which was short enough to be used for routine analysis, in which large numbers of samples must be quantified. This method was established to be linear in the range of 0.1 - 15 ng/mL (for AML), 0.1 - 30 ng/mL (for ATO and *o*-ATO), and 0.025 - 3 ng/mL (for *p*-ATO). The accuracy and precision at LLOQ were within the acceptable range, revealing that the method had prodigious sensitivity.

This lower LLOQ was considered to be adequate enough for studying the bioequivalence of formulations containing AML and ATO at lower strengths (5 mg AML and 10 mg ATO).

CONCLUSION

The authors have developed and validated a novel, sensitive assay for quantifying amlodipine, atorvastatin, and two metabolites of atorvastatin (*ortho*- and *para*-hydroxy atorvastatin) in human plasma. The described method offers several advantages, such as a simple extraction procedure and a short chromatographic runtime, which makes the method suitable for analyzing large sample batches resulting from the study of products containing amlodipine and atorvastatin.

CONFLICT OF INTEREST

The authors have declared that they have no conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, et al. Heart disease and stroke statistics-2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation. 2008; 117: 25-146.
2. Jukema JW, van der Hoorn JW. Amlodipine and atorvastatin in atherosclerosis: a review of the potential of combination therapy. Expert Opin Pharmacother. 2004; 5: 459-468.
3. Bangalore S, Kamalakkannan G, Parkar S, Messerli FH. Fixed-dose combinations improve medication compliance: a meta-analysis. Am J Med. 2007; 120: 713-719.
4. Krum H. Critical assessment of calcium antagonists. Aust Fam Physician 1997; 26: 847-848.
5. Black AE, Hayes RN, Roth BD, Woo P, Woolf TF. Metabolism and excretion of atorvastatin in rats and dogs. Drug Metab Dispos. 1999; 27: 916-923.
6. Malhotra HS, Goa KL. Atorvastatin: an updated review of its pharmacological properties and use in dyslipidaemia. Drugs. 2001; 61: 1835-1881.
7. Pilli NR, Inamadugu JK, Mullangi R, Karra VK, Vaidya JR, Rao JV. Simultaneous determination of atorvastatin, amlodipine, ramipril and benazepril in human plasma by LC-MS/MS and its application to a human pharmacokinetic study. Biomed Chromatogr. 2011; 25: 439-449.
8. Yang Y, Xu Q, Zhou L, Zhong D, Chen X. High-throughput salting-out-assisted liquid–liquid extraction for the simultaneous determination of atorvastatin, ortho-hydroxyatorvastatin, and para-hydroxyatorvastatin in human plasma using ultrafast liquid chromatography with tandem mass spectrometry. J Sep Sci. 2015; 38: 1026-1034.
9. Yacoub M, Awwad AA, Alawi M, Arafat T. 2013. Simultaneous determination of amlodipine and atorvastatin with its metabolites; ortho and para hydroxy atorvastatin; in human plasma by LC–MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2013; 36-47: 917-918.
10. FDA. Bioanalytical method validation guidance for industry. Rockville, MD: FDA; 2013.