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Simultaneous determination of amlodipine and atorvastatin with its metabolites; *ortho* and *para* hydroxy atorvastatin; in human plasma by LC–MS/MS

Mahmoud Yacoub[a], Ahmad Abu awwad[a], Mahmoud Alawi[b,*], Tawfiq Arafat[c]

[a] *Jordan Center for Pharmaceutical Research, P.O. Box 950435, Amman 11105, Jordan*
[b] *University of Jordan, Chemistry Department, P.O. Box 13003, Amman 11942, Jordan*
[c] *University of Petra, Faculty of Pharmacy, Amman, Jordan*

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ABSTRACT

A simple liquid chromatography/ion trap mass spectrometry method for the quantification of amlodipine and atorvastatin with its metabolites, *ortho* and *para* hydroxy atorvastatin, simultaneously in human plasma was developed. Analytes with internal standard were extracted by protein direct precipitation with acetonitrile. Adequate chromatographic separation was achieved using Phenomenex Synergi 4u polar-RP 80A (150 mm × 4.6 mm, 4 μm) column in the isocratic elution mode and the eluent was water/methanol (14:86%, v/v) adjusted by trichloroacetic acid to pH 3.2 which was delivered isocratically at constant flow rate of 0.50 mL/min. Standard solutions for the analytes were prepared using amlodipine besylate, atorvastatin calcium, *ortho*-hydroxy atorvastatin dihydrate monosodium salt, *para*-hydroxy atorvastatin disodium salt, and pravastatin sodium as an internal standard. The method validation intends to investigate specificity, sensitivity, linearity, precision, accuracy, recovery, matrix effect and stability according to USFDA guideline. Standard calibration levels were prepared by pooled human plasma to attain final dynamic range of 0.2–20.0 ng/mL for amlodipine, 1.5–150 ng/mL for atorvastatin, 1.0–100.0 ng/mL for *ortho*-hydroxy atorvastatin and 0.2–20.0 ng/mL for *para*-hydroxy atorvastatin. Clinical bioequivalence study was successfully investigated by the application of this validated bioanalytical method in order to evaluate bioequivalence of two commercial products 10 mg amlodipine/80 mg atorvastatin in a single dose. In this study, 29 healthy volunteers were participated in randomized, two periods, double blend, open label cross over design. Pharmacokinetic parameters of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ were calculated to compare a test product with CADUET® reference product.

1. Introduction

Amlodipine (Fig. 1A) and atorvastatin (Fig. 1B) are chemical compounds used in combination as antagonist or slow-channel blocker (antihypertensive/antianginal agent) [1] and cholesterol lowering agent, respectively [2].

CADUET® is a Pfizer's drug product, composed from 10 mg of long-acting calcium channel blocker amlodipine besylate and 80 mg of synthetic lipid-lowering agent atorvastatin calcium per tablet [3]. Amlodipine component of CADUET® inhibits the transmembrane influx of calcium ions into vascular smooth muscles and cardiac muscles. Atorvastatin component of CADUET is a selective, competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase.

In vivo atorvastatin is extensively metabolized to *ortho*-hydroxy atorvastatin (Fig. 1C) and *para*-hydroxy atorvastatin (Fig. 1D) derivatives and various beta-oxidation products [2].

For atotvaststin and its metabolites, the selected dynamic range in the standard calibration curve was considered data from the literatures with regard to atotvaststin bioavailability and its C_{max} in human body. Amlodipine dose of 10 mg, has a therapeutic value in the literatures [4] of about 8 ng/mL. For atorvastatin a dose of 80 mg, has a therapeutic value in the literatures [5] of about 65 ng/mL, 40 ng/mL for *ortho*-hydroxy atorvaststin and 5 ng/mL for *para*-hydroxy atorvastatin. These published therapeutic values are in agreement with our study findings that mentioned in Table 5.

Several analytical methods have been described for the determination of active ingredients (amlodipine and atorvastatin) each alone in human plasma and formulations. Amlodipine has been determined alone in plasma or in formulations using several techniques like capillary gas chromatography (GC) with electron capture detection [6], GC with electron-impact mass spectrometry (EI-MS) [7,8], high-performance liquid chromatography (HPLC) with amperometric detection [9], HPLC with UV detection [10],

* Corresponding author. Tel.: +962 777 483679; fax: +962 653 00253.
E-mail addresses: mahmood_yacoub@yahoo.com (M. Yacoub), ahmad_chem@hotmail.com (A.A. awwad), alawima@ju.edu.jo (M. Alawi), tarafat@uop.edu.jo (T. Arafat).

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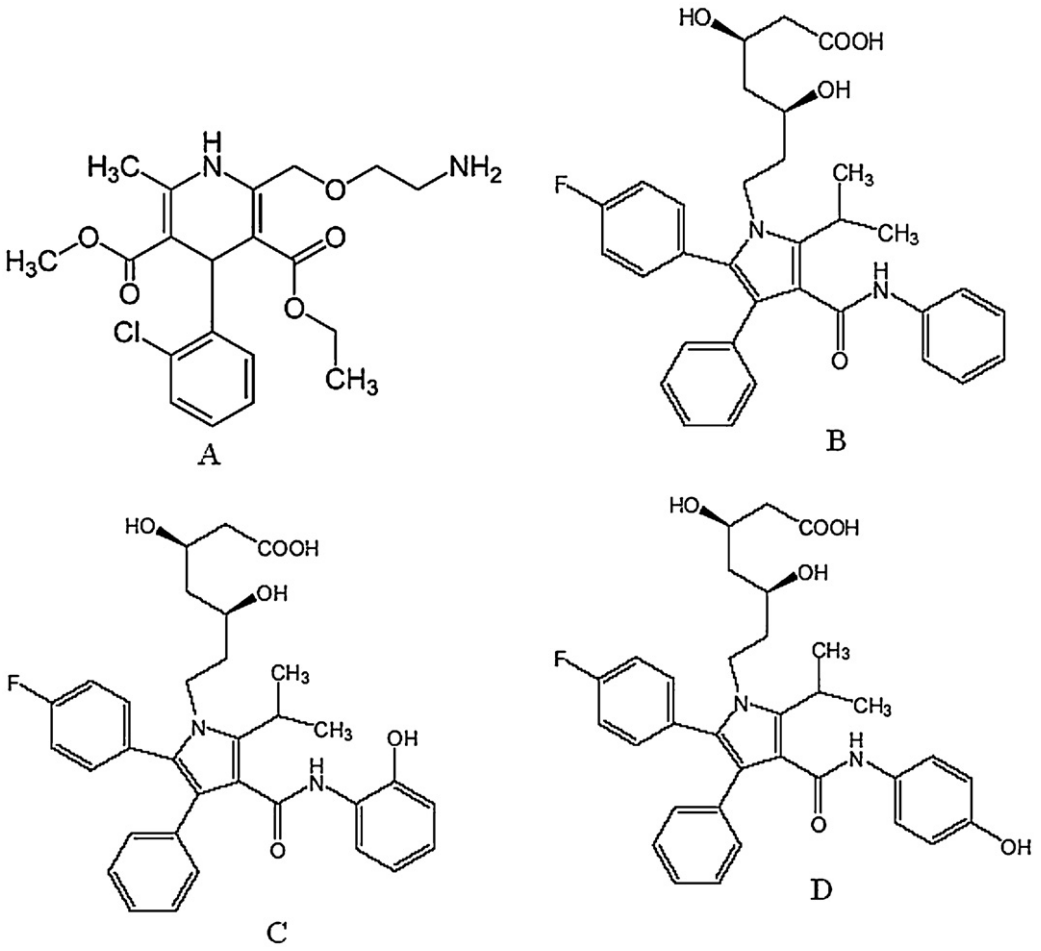

Fig. 1. Chemical structure of amlodipine (a); atorvastatin (b); *ortho*-hydroxy atorvastatin (c); *para*-hydroxy atorvastatin (D).

HPLC with fluorescence detection [11], liquid chromatography mass spectrometry (LC/MS) [12,13].

Atorvastatin has also been determined alone in plasma and formulation by different methods involving HPLC-UV detection [14], GC/MS and liquid chromatography electro spray ionization (LC–ESI-MS) [15] and high-performance thin layer chromatography (HPTLC)-UV detection [16].

Direct protein precipitation procedure has been published to quantify amlodipine alone in plasma samples [10]. Protein precipitation procedure is quick, simple and economical as compared to multiple steps sample processing procedures, like liquid–liquid or solid phase extraction. Atorvastatin has been also extracted by several techniques like liquid–liquid extraction [15], and solid phase extraction [17], but till date no published paper has been documented for the analysis of amlodipine and atorvastatin with its metabolites from direct precipitation of plasma samples.

Caduet® as a commercial product of amlodipine and atorvastatin with its metabolites have been determined in human plasma by separate bioanalytical methods, which is considered to be costly, time consuming and these multiple step procedures required like liquid–liquid and solid phase extraction, large plasma volume to be divided into two analytical volumes and two different analytical systems or more are needed to determine the active ingredients.

To the best of our knowledge, there is only one paper [18] deals with the simultaneous determination of atorvastatin, amlodipine and other compounds in human plasma, but it is necessary to establish a new analytical method for simultaneous determination of amlodipine and atorvastatin with metabolites. This study intends to develop and validate a new method for simultaneous determination of amlodipine and atorvastatin with its metabolites in human plasma by liquid chromatography ion trap mass spectrometry (LC ion trap MS/MS), and to apply the validated method in a bioequivalence study.

2. Experimental

2.1. Instrumentation

A Dionex ultimate 3000 RS HPLC system consisting of pump with on-line vacuum degasser, autosampler and column oven (Dionex Corporation, Germany) was used for constant solvent and sample delivery. An octapole ion trap mass spectrometer (LCQ FLEET, Thermo SCIENTIFIC Corporation, USA) equipped with an electrospray ionization (ESI) source (Finnigan™), protected by a built-in waste/detector switcher valve, was used for the analysis of targeted compounds. Data acquisition and processing were performed with Xcalibur® Data Management Software 2.0.7 (Thermo Scientific®). The best fit $1/x$ linear weighted function was applied for data back calculation.

2.1.1. HPLC conditions

Chromatographic separation was carried out on a Synergi polar column RP80A (150 mm × 4.6 mm, 4 μm, Phenomenex, USA) using an isocratic condition. The mobile phase consisted of water/methanol (14:86, v/v) adjusted to pH 3.20 with trichloroacetic acid (TCA) and delivered at a flow rate of 0.50 mL/min. The column temperature was maintained at 30 °C. The autosampler injection volume was fixed at 30 μL under 4 °C cooling system.

2.1.2. Mass spectrometric conditions

The mass spectrometer was operated in a positive ion mode and was optimized to detect the analytes at the highest intensity with ion source nitrogen gas flow rate of 70 and 40 arbitrary unit for sheath gas and auxiliary gas, respectively, ion spray voltage at 5.0 kV and ion transfer capillary temperature 350 °C. Five selected reaction monitoring (SRM) transitions (m/z 408.68 → m/z 238.00, amlodipine; m/z 559.09 → m/z 440.21, atorvastatin; m/z 575.07 → m/z 466.17, *ortho*-hydroxy atorvastatin; m/z 575.05 → m/z 466.18, *para*-hydroxy atorvastatin; m/z 447.11 → m/z 327.08, IS) were recorded and used for quantification. The optimized collision energy for the transitions was 50%.

2.2. Chemicals and reagents

Amlodipine besylate (purity as amlodipine = 72.20%) was obtained from JOSWE medical (Amman, Jordan), atorvastatin calcium (purity as atorvastatin = 91.20%) was obtained from Vitalife Laboratories (India), *ortho*-hydroxy atorvastatin dihydrate monosodium salt (purity as *ortho*-hydroxy atorvastatin = 84.20%) and *para*-hydroxy atorvastatin disodium salt (purity as *para*-hydroxy atorvastatin = 90.99%) were obtained from Toronto Research Chemical Inc (Canada). Pravastatin internal standard (I.S) (99.71%) was obtained from United Pharmaceuticals (Amman, Jordan).

The plasma blank sample was harvested from donors and collected through the Blood Bank. Plasma was obtained by centrifugation of blood treated with sodium heparin.

LC/MS-quality deionized water, methanol, acetonitrile and trichloroacetic acid were purchased from Merck (Darmstadt, Germany), Ammonia (Fisher, Germany), and all other chemicals were of analytical grade.

2.3. Standard solutions

Stock standard solutions of 1.0 mg/mL for amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin, *para*-hydroxy atorvastatin and pravastatin (IS) were prepared in methanol, and these solutions were stored in a refrigerator (4–8 °C). These solutions were further diluted in methanol/water (70:30% v/v) to give appropriate working solutions used to prepare the calibration curves and to do quality control tests.

2.3.1. Preparation of pravastatin working solution (IS)

0.125 mL from pravastatin stock solution (1 mg/mL) was taken into a 500-mL volumetric flask and diluted with acetonitrile. This solution was considered to be IS working solution which contains 0.25 μg/mL of pravastatin.

2.3.2. Preparation of working solution for amlodipine, atorvastatin and its metabolites (mix 4)

0.2 mL from amlodipine stock solution (1 mg/mL), 1.5 mL from atorvastatin stock solution (1.0 mg/mL), 1.0 mL from *ortho*-hydroxy atorvastatin stock solution (1.0 mg/mL), and 0.2 mL from *para*-hydroxy atorvastatin stock solution (1.0 mg/mL) were taken into a 10.0-mL volumetric flask, then filled to the mark using methanol:water (70:30, v/v). This solution was considered to be a working standard solution (W.S.) that contains 20.0 μg/mL of amlodipine, 150.0 μg/mL of atorvastatin, 100.0 μg/mL of *ortho*-hydroxy atorvastatin and 20.0 μg/mL *para*-hydroxy atorvastatin (Mix 4, Working standard solution).

Take 0.2 mL from amlodipine stock solution (1 mg/mL), 1.5 mL from atorvastatin stock solution (1 mg/mL), 1.0 mL from *ortho*-hydroxy atorvastatin stock solution (1.0 mg/mL), and 0.2 mL from *para*-hydroxy atorvastatin stock solution (1.0 mg/mL) was taken into volumetric flask, then diluted to 10.0 mL by 70% methanol which was considered to be a working solution (W.S) that contains 20.0 μg/mL of amlodipine, 150.0 μg/mL of atorvastatin, 100.0 μg/mL of *ortho*-hydroxy atorvastatin and 20.0 μg/mL *para*-hydroxy atorvastatin (Mix4 working solution).

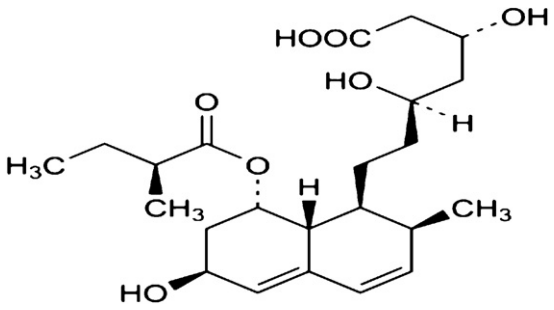

Fig. 2. Pravastatin (IS) Chemical Structure.

2.3.3. Standard calibration curves and quality control samples

Standard calibration concentrations for amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin were prepared in human pooled analytes-free plasma by adding small volumes of standard combined dilutions (a maximum of 5% of the total volume) in single dilution step for each level to yield final concentrations of 0.2, 0.4, 1.0, 2.4, 6.0, 12.0 and 20.0 ng/mL for amlodipine and *para*-hydroxy atorvastatin; 1.5, 3.0, 7.5, 18.0, 45.0, 90.0 and 150.0 ng/mL for atorvastatin and 1.0, 2.0, 5.0, 12.0, 30.0, 60.0 and 100.0 ng/mL for *ortho*-hydroxy atorvastatin. The various concentrations, which prepared to construct the calibration curves, were selected on basis of pharmacokinatic parameters (bioavailability and maximum concentration) of each analyte in human plasma, to attain the FDA guidance requirements [19].

Similarly, quality control samples were prepared in human pooled analytes-free plasma at concentration of 0.2 lower limit of quantification (LLOQ), 0.6 (low), 10.0 (mid) and 16.0 (high) ng/mL for amlodipine and *para*-hydroxy atorvastatin, 1.5 (LLOQ), 4.5 (low), 75.0 (mid) and 120.0 (high) ng/mL for atorvastatin and 1.0 (LLOQ), 3.0 (low), 50.0 (mid) and 80.0 (high) ng/mL for *ortho*-hydroxy atorvastatin.

The lowest concentration for calibration curves of amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin were considered to be LLOQ. All the calibration standard and QC plasma samples were divided into aliquots and stored in deep freezer at -40 ± 5 °C until analysis. Calibration curves were constructed from a blank sample (an analytes-free plasma sample processed without an I.S), a zero sample (an analytes-free plasma processed with I.S) and seven non-zero samples covering the total range including LLOQ.

2.4. Sample preparation

Protein plasma direct precipitation is the procedure of drugs extraction from their biological matrices, it took place by adding 300 μL acetonitrile (after spiking with 50 μL of 0.25 μg/mL pravastatin) to 200 μL plasma sample in an eppendorf tube, the mixture was vortexed for 30 s using a Vibrax Type VX-Z, VXR Basic Vortexer (IKA-Werke GmbH& Co. Staufen, Germany) and then centrifuged using Multitude Sigma1-15 (Sigma, Germany) for 15 min at 14,000 rpm (14,680 × g), The supernatant was transferred to an auto-sampler micro-vial and 30 μL was injected into the analytical column.

2.5. Bioanalytical method validation

Validation runs were conducted on three separate days, each validation run consisted of a set of spiked standard samples of seven

concentrations over the concentration range ($n = 5$, at each concentration), LLOQ, QC samples at three concentrations, low, medium and high ($n = 10$, each concentration), blank and zero samples. Calibration samples were analyzed from low to high concentration at the beginning of each validation run and the other samples were distributed randomly through the run, except the blank plasma samples which were placed after the high calibration sample. Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle is able to avoid any carry forward of injected sample in the subsequent runs. The stability and the freeze–thaw samples were analyzed on the day three along with other validation samples. Linearity was assessed by a weighted ($1/x$) least squares regression analysis. The calibration curve had to have a correlation coefficient (r) of 0.999 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%. At least 67% of non-zero standard should meet the above criteria including LLOQ and upper limit of quantitation [19].

To guarantee reliability and reproducibility of the assay for quantitation of amlodipine, atorvastatin, *ortho*-hydroxy

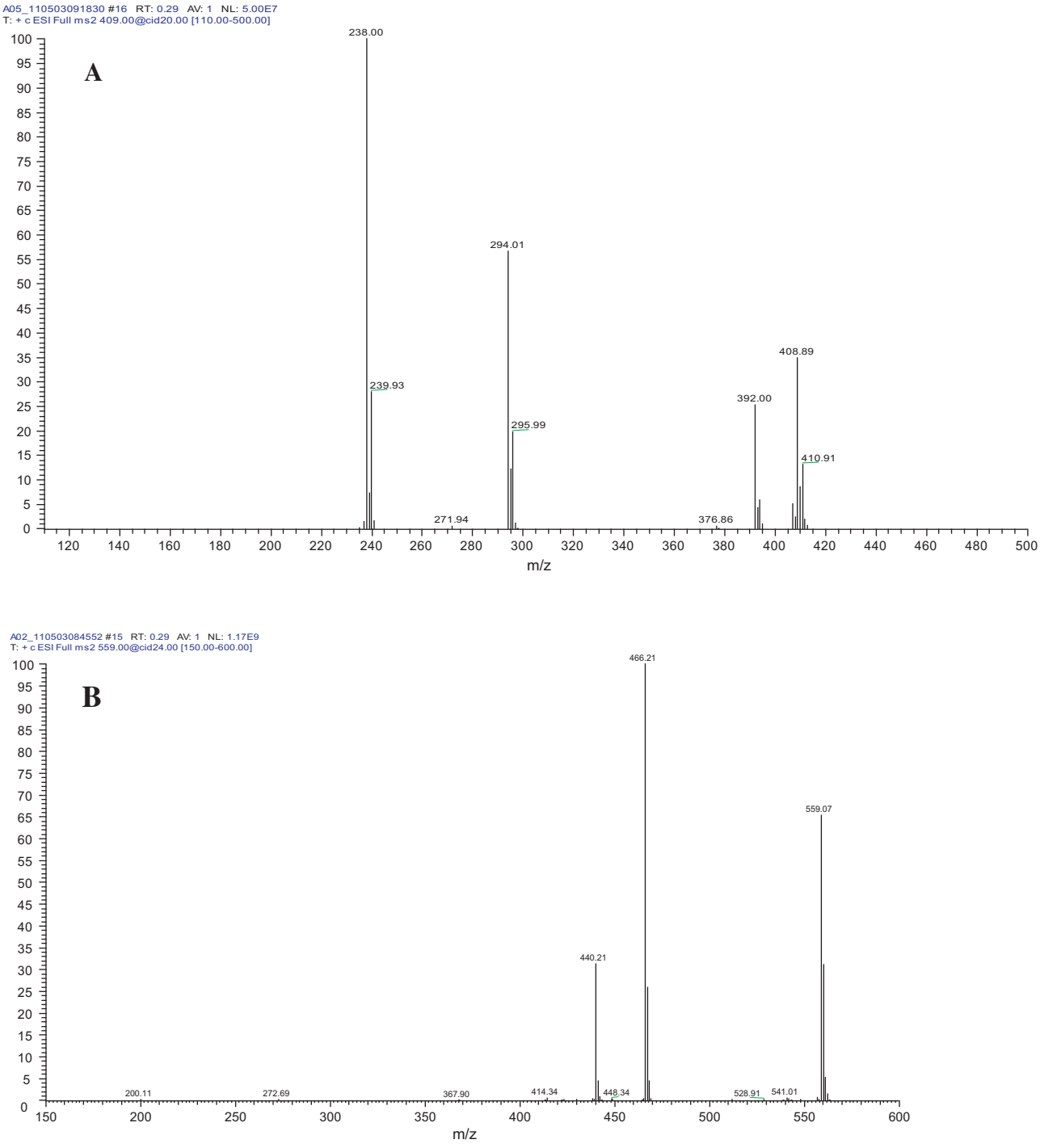

Fig. 3. (A) Precursor ion spectrum m/z for amlodipine with its fragmentation fashion. (B) Precursor ion spectrum m/z for atorvastatin with its fragmentation fashion. (C) Precursor ion spectrum m/z for *ortho*-hydroxy atorvastatin with its fragmentation fashion. (D) Precursor ion spectrum m/z for *para*-hydroxy atorvastatin with its fragmentation fashion. (E) Precursor ion spectrum m/z for I.S with its fragmentation fashion.

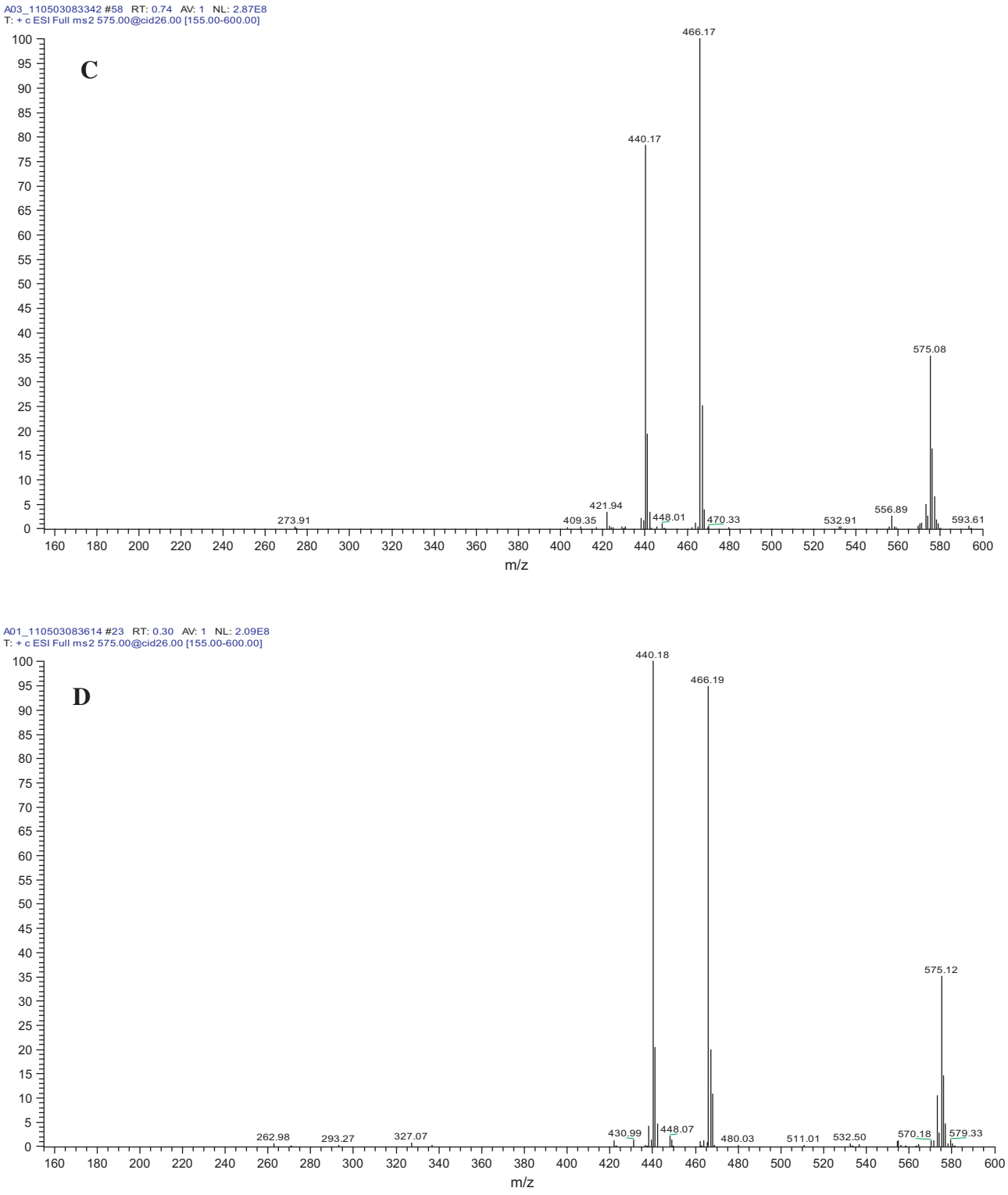

Fig. 3. (*Continued*).

atorvastatin and *para*-hydroxy atorvastatin, simultaneously in human plasma, the method was validated in concordance with the United State of Food and Drug Administration (FDA) guideline requirements. Validation of this procedure was performed in order to evaluate the method in terms of recovery, linearity of response, accuracy, precision, sensitivity, stability and specificity [19].

2.5.1. Accuracy and precision

Within-batch accuracy and precision evaluations were determined by analysis of 10 replicates quality control samples from each level. The between-batch precision and accuracy was determined by analyzing three sets of within-batch quality control sequence in three separate batches. The quality control samples

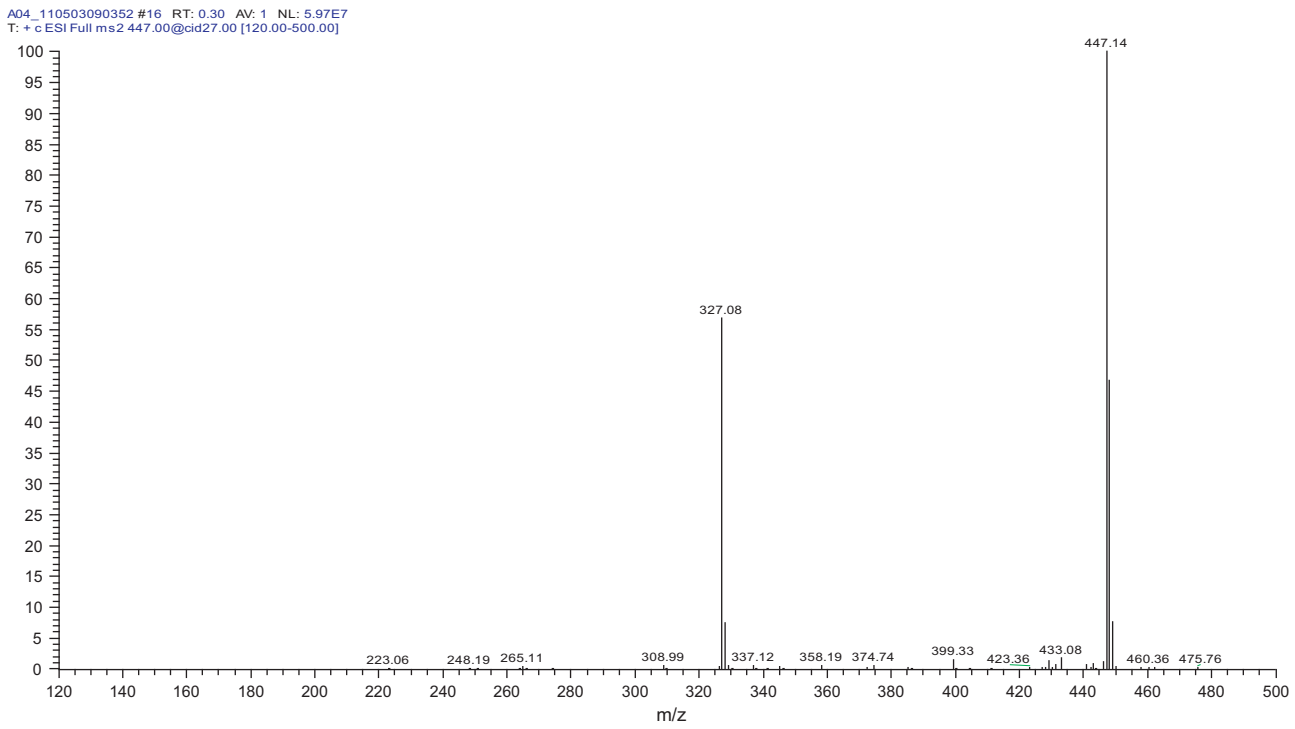

Fig. 3. (*Continued*).

were randomized daily, processes and analyzed in position either (a) immediately following the standard curve, (b) in the middle of batch or (c) at the end of the batch. The acceptance criteria for within- and between-batch precision and accuracy were 20% for LLOQ and 15% for the other concentrations.

2.5.2. Recovery and matrix effect

Recovery of amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin from the precipitation procedure was determined by a comparison of peak area of drugs in processed spiked plasma samples for (low, medium and high quality controls) with the peak area of drugs in unprocessed samples prepared by spiking supernatant drug free plasma samples with the same amount of analytes. Endogenous matrix components may change the efficiency of droplet formation or droplet evaporation, which in turns affects the amount of charged ion in the gas phase, which ultimately reaches the detector. Matrix effect was checked with six different lots of plasma.

Six samples each of low quality control (LQC), mid quality control (MQC) and high quality control (HQC) were prepared by directly spiking the analytes into mobile phase with or without the presence of supernatant from the different lots of plasma Ion suppression or enhancement was assessed by comparing the mean analyte peak area obtained from these sets of testing samples. It is considered there is no matrix effect if the deviation of the mean test responses were within 15% of freshly prepared or comparison samples (samples prepared in neat solution). Matrix effect was calculated [20,21] as per the following equation:

$$\text{Matrix effect} = \left[\frac{\text{analyte peak area of extracted plasma residue}}{\text{analyte peak area of neat solution}}\right] \times 100.$$

2.5.3. Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analytes in presence of other components in the sample. The specificity of the method was evaluated by screening six different lots of blank plasma. These lots were analyzed as blank and zero samples then compared with LLOQ to confirm lack of endogenous peaks.

2.5.4. Stability

The bench top stability was examined by keeping replicates of spiked plasma with low, mid and high quality control samples at room temperature for approximately 6 h. Freeze–thaw cycles stability of the samples were obtained over three freeze–thaw cycles, by thawing at room temperature for 1–2 h and refrozen for 12–24 h. Auto-sampler stability of the analytes were tested by analysis of the prepared low, mid and high quality control samples, which were stored in the auto-sampler tray for 24 h. Long-term stability was tested after storage of analytes for 4 months under approximately −40 °C deep freezer. For each concentration and storage condition, three replicates were analyzed in one analytical batch with freshly prepared calibration samples . The concentrations of analytes after each storage period were compared to the nominal concentrations of the samples.

2.5.5. Stock solution stability

The stability of stock solution was tested and established at room temperature for 6.00 hr and under refrigeration conditions (4–8 °C) for 4 months.

2.6. Clinical application

The developed and validated LC/MS method was applied to investigate a bioequivalence study of Caduet® as reference product versus the test product 10 mg amlodipine and 80 mg atorvastatin.

This study was conducted in compliance with the Declaration of Helsinki and in accordance with Good Clinical Practice [22]. The protocol was approved by the local institutional review board and written informed consent and consent form was obtained for all volunteers before study participation. The

subjects were selected upon inclusion/exclusion criteria. This study was designed as two-period, open-label, single-centre, randomized crossover to evaluate the bioequivalence of single doses for Caduet® (10 mg amlodipine and 80 mg atorvastatin) against test product. All subjects were between 18 and 50 years age and within body mass index range of 17.1 – 28.6 kg/m^2, and were subjected to a pre- and post-study safety examination. Blood samples for analysis were drawn (10 mL for each) pre-dose and 0.33, 0.66, 1.00, 1.33, 1.66, 2.00, 2.5, 3.00, 3.5, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00, 24, 48, and 72 h post dose. Venous blood samples were collected by direct venipuncture of an antecubital vein into tubes containing lithium heparin as anticoagulant, and then tubes with blood were immediately centrifuged at 4000 rpm, at room temperature, for 5.0 min. The separated plasma was transferred into polypropylene tubes, immediately stored frozen at −40 °C.

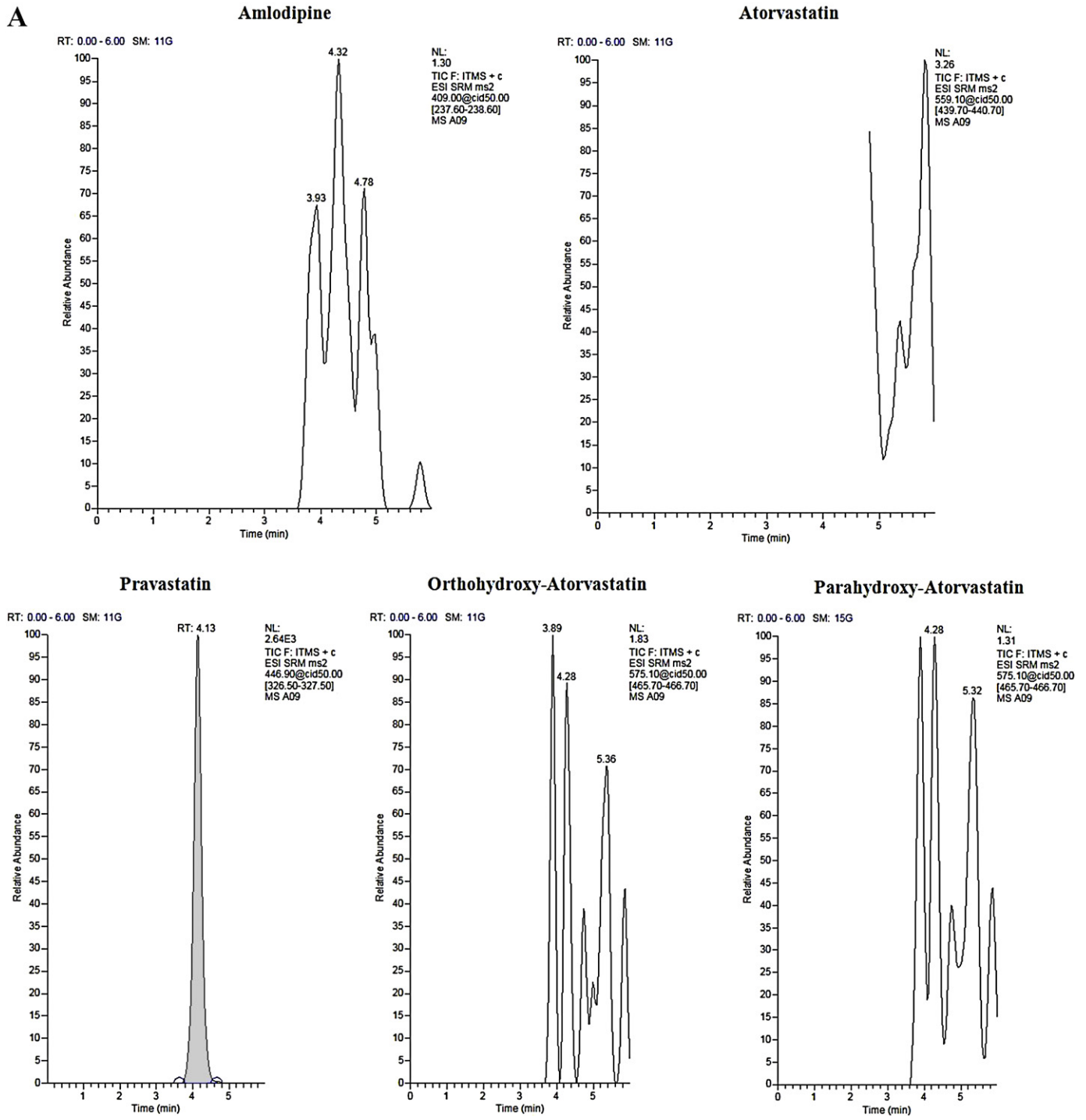

Fig. 4. (A) Blank plasma with IS (pravastatin) Chromatogram for amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin. (B) LLOQ plasma chromatogram for amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin, *para*-hydroxy atorvastatin and pravastatin (IS). (C) Unknown volunteer sample plasma chromatogram for amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin, *para*-hydroxy atorvastatin and pravastatin (IS).

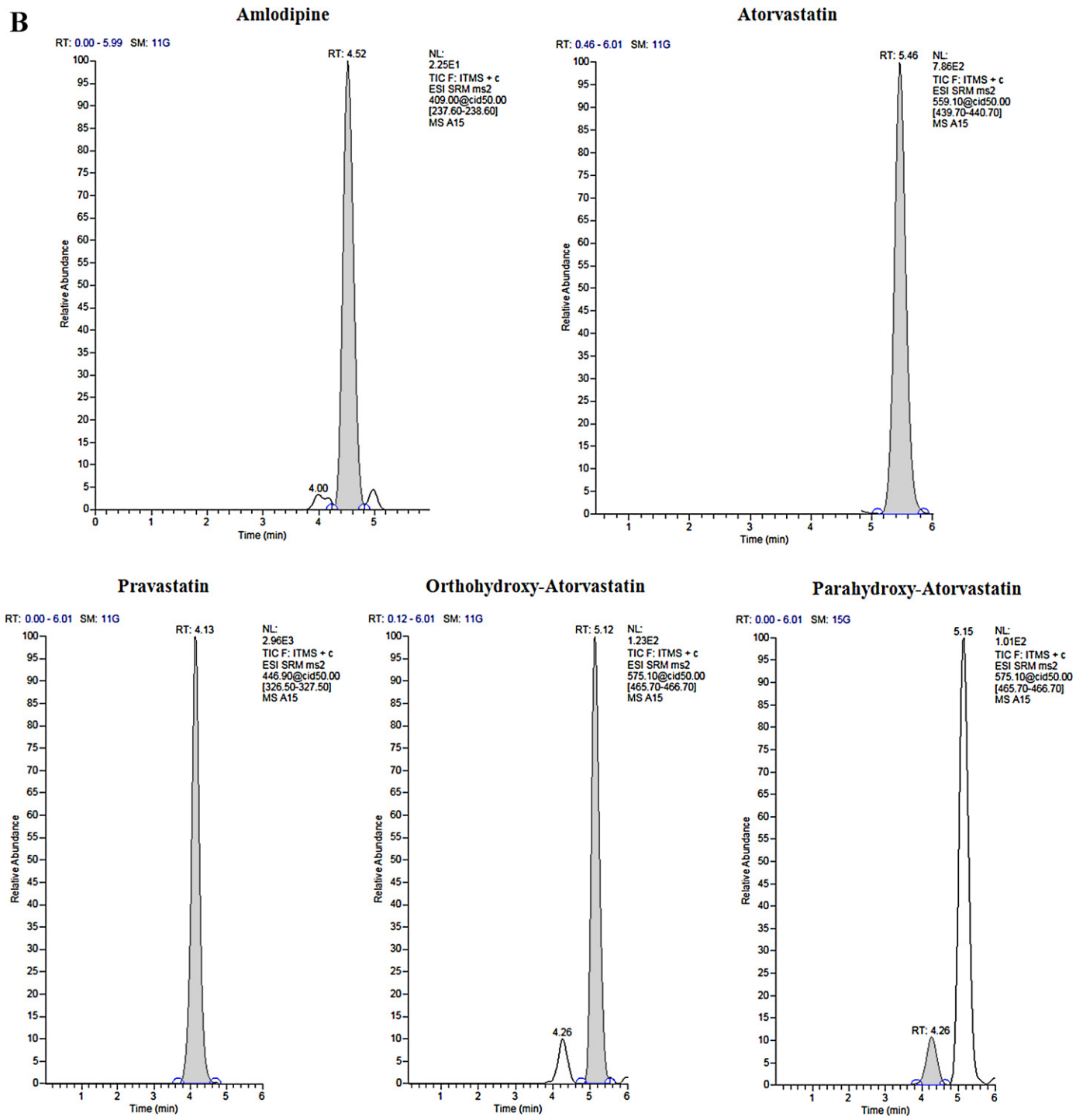

Fig. 4. (*Continued*).

3. Results and discussion

3.1. Internal standard

A stable analyte has to be used as an I.S to correct for the loss of analyte during sample preparation or sample inlet. Since such I.S is not available commercially, an alternative approach has been used. I.S chosen should match the chromatographic properties, recovery and ionization properties of the analytes [23]. Pravastatin (Fig. 2) was found to match these criteria and also serve our purpose of method development, therefore it was chosen as the I.S. Pravastatin has –COOH and –OH groups. It is ionizable either positively or negatively by protonation or deprotonation depending on the experimental conditions. Pravastatin was selected because it is from same group of statins [24]. The result indicates that the I.S did not alter or deteriorate the performance of the proposed method. Also the intensity of amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin molecular ion peaks in the mass spectrometric analysis remained unaffected as compared to others.

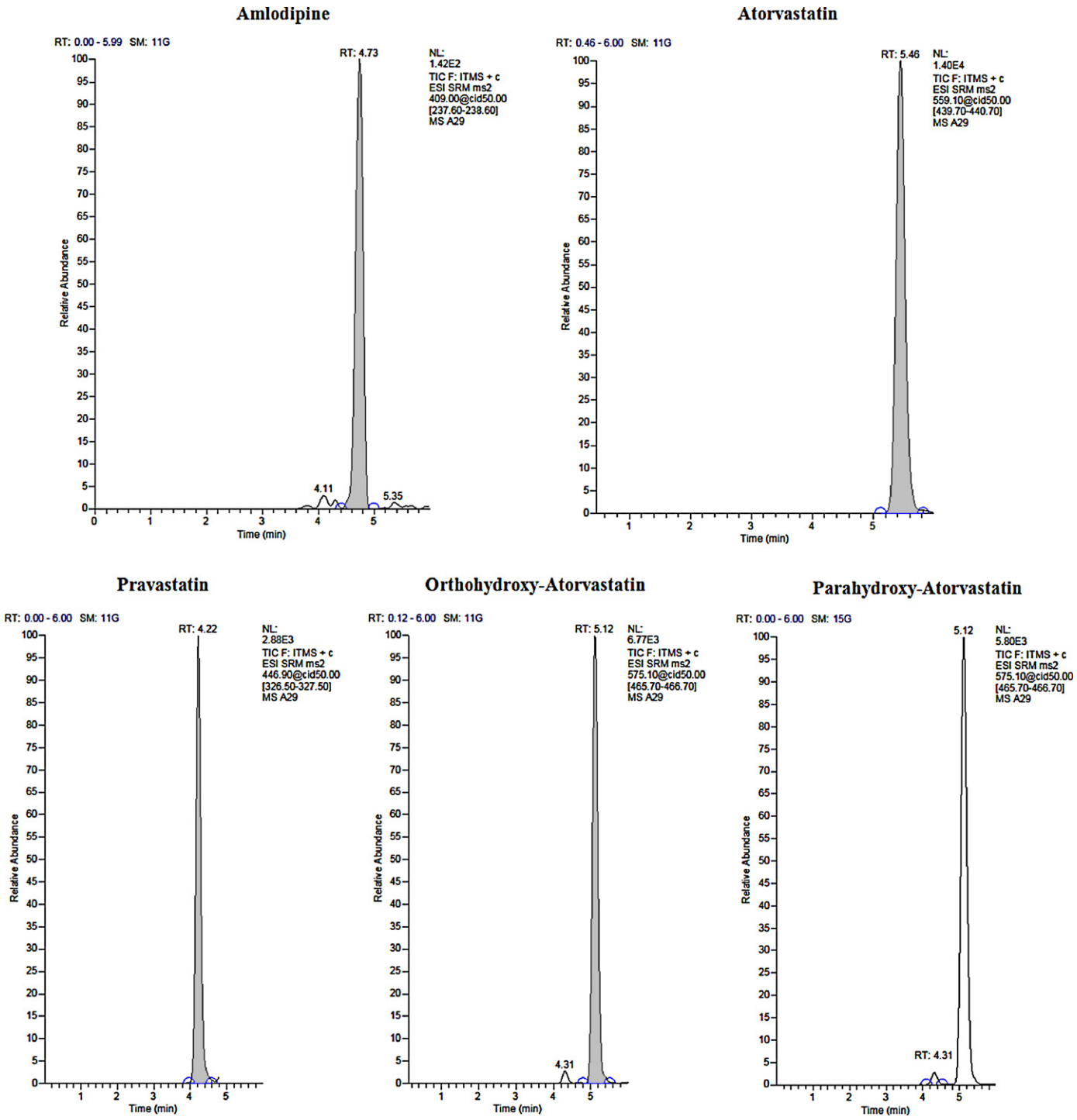

Fig. 4. (*Continued*).

3.2. LC–MS/MS analysis

LC–MS detection mode exhibits a high selectivity, and no interferences were observed. The positive MS [scan] mode mass spectrums shows protonated molecules $[M+H]^+$ in Fig. 3A–E for the most intense molecular ion precursor of the analytes amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin, *para*-hydroxy atorvastatin and I.S respectively with their product ions fragments under moderate collision energy. The major ions observed in the electrospray ionization (ESI) spectrum were at m/z 408.68 for amlodipine, m/z 559.09 for atorvastatin, m/z 575.07 for *ortho*-hydroxy atorvastatin, m/z 575.05 for *para*-hydroxy atorvastatin and m/z 447.11 for I.S A significant product ions fragments were observed in the SRM scan mode spectra that are m/z = 238.00, 440.21, 466.17, 466.18 and 327.08 for amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin, *para*-hydroxy atorvastatin and I.S, respectively.

3.3. Separation and chromatography

The chromatographic conditions were optimized through several trials to achieve good resolution, symmetrical peaks for the analytes and the I.S, as well as short run time. It was found

Table 1
Extrapolated linear regression equations and LLOQ of amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin, with dynamic range from replicate calibration curves ($n = 6$).

LLOQ (ng/mL)	Correlation coefficient	Linear regression equation	Dynamic range (ng/mL)	Compounds
0.2	0.99995	$y = 0.04770x - 0.00485$	0.2–20.0	Amlodipine
1.5	0.99997	$y = 0.14638x + 0.06329$	1.5–150.0	Atorvastatin
1.0	0.99984	$y = 0.06622x - 0.06690$	1.0–100.0	*ortho*-Hydroxy atorvastatin
0.2	0.99994	$y = 0.02733x - 0.00247$	0.2–20.0	*para*-Hydroxy atorvastatin

the optimum mobile phase of water/methanol with a ratio of 14/86% (v/v) adjusted using trichloroacetic acid to pH 3.20. A good chromatographic separation was needed for *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin, because they have the same chemical finger print mass spectrum for both precursor ions of parent molecules and daughter molecular fragments, so they could not be separated depending on the MS high selectivity property, herein a good enough separation was attained with shortest short run. The development of the current method was focused on the short run time to assure high throughput, with minimum matrix effects as well as good peak shapes. The retention times of amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin, *para*-hydroxy atorvastatin and I.S, respectively, were 4.5, 5.5, 5.1, 4.2 and 4.1 min, respectively.

Fig. 4A shows plasma zero concentration chromatogram; it is obviously clean and with no endogenous interfering peak compared to LLOQ chromatogram, which is presented in Fig. 4B with concentrations of 0.2, 1.5, 1.0 and 0.2 ng/mL for amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin, respectively. Fig. 4C chromatogram taken from participant volunteer in bioequivalence study, its measurement was 0.755, 34.855, 34.951 and 1.654 ng/mL for amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin, respectively.

3.4. Standard calibration curve and linearity

Standard calibration curve for each of amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin was defers form other by its regression linear function parameters as illustrated in Table 1. The linear curve data was best-fit to a straight line with a weighting factor of $1/x$ regression function, and it was used to calculate the concentrations of all samples throughout the batch.

3.5. Inter- and intra-day accuracy and precision

The measurements accuracy and variation between same QC level were studied in both inter-and intra-day precision by analyzing ten replicates for each QC level {LLOQ, low, mid and high} in the batch for amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin during 3 successive days. In Table 2 the analytical signal for all replicates of each concentration were measured and concentrations were back-calculated by employing the regression equation established on the corresponding day.

3.6. Recovery and matrix effect

From Table 3, the indication from extraction procedure for the analytes was a high recovery value from their biological matrix and it was acceptable at the studied concentration range. The biological matrix affected on the analytes by an acceptable factor. Measurement values for each QC level represented in the average of six replicates after subtraction of matrix factor from each QC level for amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin.

3.7. Specificity and sensitivity

The protein direct precipitation procedure was specified and sensitive for each of amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin, *para*-hydroxy atorvastatin and pravastatin, where both blank and zero samples that examined from six deferent lots of plasma were attained the required clean chromatogram for specific method.

3.8. Stability

The spiked plasma on bench top was stable for 6.00 h of waiting under room temperature before applying the precipitation

Table 2
Precision and accuracy of amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin in human plasma QC samples.

Analytes	Spiked concentration (ng/mL)	Intra-day ($n = 10$)			Inter-day ($n = 30$)		
		Concentration measured (ng/mL)	Precision (RSD, %)	Accuracy (%)	Concentration measured (ng/mL)	Precision (RSD, %)	Accuracy (%)
Amlodipine	0.2	0.200 ± 0.023	11.84	100.20	0.198 ± 0.023	11.62	98.95
	0.6	0.576 ± 0.026	4.83	96.06	0.593 ± 0.029	4.38	98.76
	10.0	10.422 ± 0.404	4.09	104.22	10.099 ± 0.553	5.05	100.99
	16.0	15.720 ± 0.830	5.57	98.25	15.799 ± 0.669	4.25	98.75
Atorvastatin	1.5	1.460 ± 0.069	4.98	97.36	1.521 ± 0.085	5.69	101.42
	4.5	4.565 ± 0.175	4.03	101.45	4.445 ± 0.185	3.77	98.78
	75.0	76.313 ± 3.087	4.26	101.75	75.850 ± 3.295	4.35	101.13
	120.0	115.464 ± 4.458	4.07	96.22	116.212 ± 4.149	3.68	96.84
ortho-Hydroxy atorvastatin	1.0	1.019 ± 0.058	6.04	101.89	1.053 ± 0.068	6.59	105.29
	3.0	2.923 ± 0.204	7.37	97.44	2.961 ± 0.145	4.77	98.71
	50.0	51.746 ± 2.282	4.65	103.49	51.009 ± 2.662	4.97	102.02
	80.0	81.655 ± 2.187	2.82	102.07	80.854 ± 3.042	3.12	101.07
para-Hydroxy atorvastatin	0.2	0.206 ± 0.021	10.89	102.89	0.201 ± 0.018	8.93	100.40
	0.6	0.610 ± 0.023	4.04	101.63	0.604 ± 0.023	3.81	100.75
	10.0	10.185 ± 0.381	3.94	101.85	10.032 ± 0.427	4.30	100.32
	16.0	15.924 ± 0.753	4.98	99.53	16.009 ± 0.725	4.72	100.05

Table 3
Matrix effects and recovery of amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin, *para*-hydroxy atorvastatin and pravastatin (IS) in human plasma.

Analytes	Spiked concentration (ng/mL)	Matrix effect (%, mean, $n = 6$)	Recovery (%, mean, $n = 6$)
Amlodipine	0.6	79.09	92.71
	10.0	79.75	106.76
	16.0	84.72	101.18
Atorvastatin	4.5	77.03	106.62
	75.0	79.97	100.26
	120.0	76.58	103.54
ortho-Hydroxy atorvastatin	3.0	75.84	103.18
	50.0	78.17	104.35
	80.0	80.05	104.29
para-Hydroxy atorvastatin	0.6	81.20	95.12
	10.0	84.36	105.96
	16.0	86.45	102.82
Pravastatin (IS)	250.0	80.24	99.19

Table 4
Stability of amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin in human plasma QC samples.

Analytes	Spiked concentration (ng/mL)	Storage at −40 °C for 4 month		Autosampler 4 °C for 24 h		Three-thaw cycles	
		Concentration measured (ng/mL)	Accuracy (%)	Concentration measured (ng/mL)	Accuracy (%)	Concentration measured (ng/mL)	Accuracy (%)
Amlodipine	0.6	0.626 ± 0.012	104.25	0.554 ± 0.029	92.40	0.640 ± 0.017	106.67
	10.0	9.715 ± 0.241	97.15	8.863 ± 0.100	88.63	9.676 ± 0.205	96.76
	16.0	15.815 ± 0.420	98.84	14.532 ± 0.496	90.82	15.572 ± 0.624	97.33
Atorvastatin	4.5	4.664 ± 0.283	103.65	4.492 ± 0.205	99.83	4.734 ± 0.362	105.21
	75.0	77.411 ± 1.775	103.22	77.769 ± 0.467	103.69	77.881 ± 2.905	103.84
	120.0	115.649 ± 7.827	96.37	119.263 ± 4.215	99.39	114.618 ± 9.324	95.52
ortho-Hydroxy atorvastatin	3.0	2.883 ± 0.099	96.12	3.022 ± 0.105	100.74	2.835 ± 0.104	94.48
	50.0	49.755 ± 1.410	99.51	50.110 ± 0.875	100.22	48.843 ± 1.385	97.69
	80.0	77.766 ± 2.286	97.21	81.920 ± 2.584	102.40	74.930 ± 3.847	93.66
para-Hydroxy atorvastatin	0.6	0.563 ± 0.028	93.88	0.606 ± 0.001	100.98	0.636 ± 0.023	106.00
	10.0	10.179 ± 0.303	101.79	10.006 ± 0.100	100.06	9.167 ± 0.465	91.67
	16.0	15.767 ± 0.356	98.54	16.313 ± 0.297	101.95	14.330 ± 0.342	89.56

procedure. Table 4 illustrates freeze–thaw cycles, auto-sampler and long-term stability test results with their storage condition.

4. Clinical study applications

This study was conducted in compliance with the Declaration of Helsinki and in accordance with Good Clinical Practice [22]. The protocol was approved by the local institutional review board and written informed consent and consent form was obtained for all volunteers before study participation.

After oral administration single dose of CADUET® tablet 10/80 mg reference product, the pharmacokinetic parameters C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ were calculated and presented in Table 5 to compare them versus test product. The mean plasma concentration

Table 5
Phamacokinetic parameters of amlodipine and atorvastatin after the administration of amlodipine/atorvastatin test and reference (10/80) mg tablet to 29 subjects.

Parameter	Amlodipine	Atorvastatin
C_{max} (ng/mL) for Caduet reference product	7.898	81.366
C_{max} (ng/mL) for test product	8.396	84.057
AUC_{0-t} (ng h/mL) for Caduet reference product	230.640	649.263
AUC_{0-t} (ng h/mL) for test	233.380	628.901
$AUC_{0-\infty}$ (ng h/mL) for Caduet reference product	–	682.832
$AUC_{0-\infty}$ (ng h/mL) for test	–	642.425
T_{max} (hr) for Caduet reference product	6.5	0.66
C_{max} (hr) for test product	6.5	0.66
$T_{1/2}$ (hr) for Caduet reference product	44.438	14.788
$T_{1/2}$ (hr) for test product	44.939	12.574

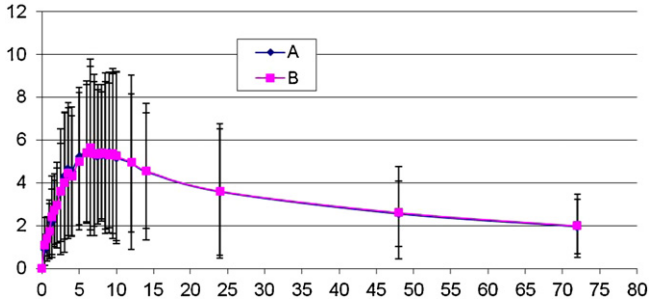

Fig. 5. Mean plasma concentration time profile after the administration of amlodipine/atorvastatin {test (B)} and {reference (A)} 10/80 mg tablet of 29 subjects for amlodipine.

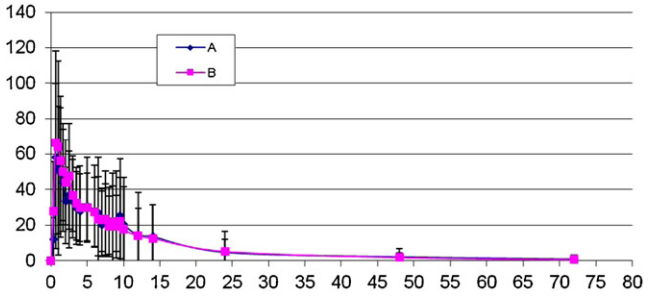

Fig. 6. Mean plasma concentration time profile after the administration of amlodipine/atorvastatin {test (B)} and {reference (A)} 10/80 mg tablet of 29 subjects for atorvastatin.

time profile of 29 subjects of amlodipine and atorvastatin for both products are presented in Figs. 5 and 6, respectively. These measured pharmacokinetic parameters are in agreement with those reported in the literatures [5,25].

5. Conclusions

The validated method has been successfully used to estimate amlodipine, atorvastatin, *ortho*-hydroxy atorvastatin and *para*-hydroxy atorvastatin in human plasma samples after oral administration of single dose of CADUET® reference product versus test drug.

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