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Original Article

Application of a novel UPLC–MS/MS method for the pharmacokinetic/bioequivalence determination of atorvastatin and ezetimibe in human plasma

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ABSTRACT

Aims: The aim of this study is to determine the pharmacokinetic parameters of atorvastatin calcium (AT) and ezetimibe (EZ) in human volunteers following the administration of a single oral dose of two drug products containing both active ingredients.
Materials and methods: A novel, sensitive and selective ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) method was developed and validated for the quantification of AT and EZ in human plasma, following administration of a single dose comprising AT 40 mg and EZ 10 mg, using a non-blind, two-treatment, two-period, randomized, crossover design, in twenty four healthy human volunteers. Atorvastatin, ezetimibe and the internal standard etilefrine (IS) were extracted from plasma and analyzed on a reversed-phase C_{18} column under gradient conditions.
Results: The ion transitions monitored in multiple reaction-monitoring mode were 440.4, 271.25, 164.02 *m/z* derived from 559.57, 408.43, 182.12 *m/z* for AT, EZ and IS respectively. Calibration curves were generated over the range of 0.1–20 ng mL^{-1} for both drugs with values for coefficient of correlation greater than 0.999. The parametric point estimates and the 90% confidence intervals for ln-transformed AUC_{0-t}, $AUC_{0-\infty}$, and $c_{max,}$ were within commonly accepted bioequivalence range of 80–125% range, thus the results reveal that the bioequivalence between the two drug products could be concluded.
Conclusion: The method is very simple and allows obtaining a very good recovery of the analytes. The validated UPLC–MS/MS method has been applied to a pharmacokinetic/bioequivalence study.

Atorvastatin calcium (AT, Fig. 1a), a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is a lipid regulating drug. It is used to reduce LDL-cholesterol, apolipoprotein B and triglycerides. It is also used for primary prophylaxis of cardiovascular events in patients with multiple risk factors, including *diabetes mellitus*.[1] The typical dose of AT is 10–80 mg per day and it reduces 40–60% LDL.[2] AT is rapidly absorbed after oral administration. Extent of absorption increases in proportion to AT dose, indicating linear pharmacokinetics. The absolute bioavailability of AT (parent drug) is approximately 14% and the systemic availability of HMG-CoA reductase inhibitory activity is approximately 30%. The low

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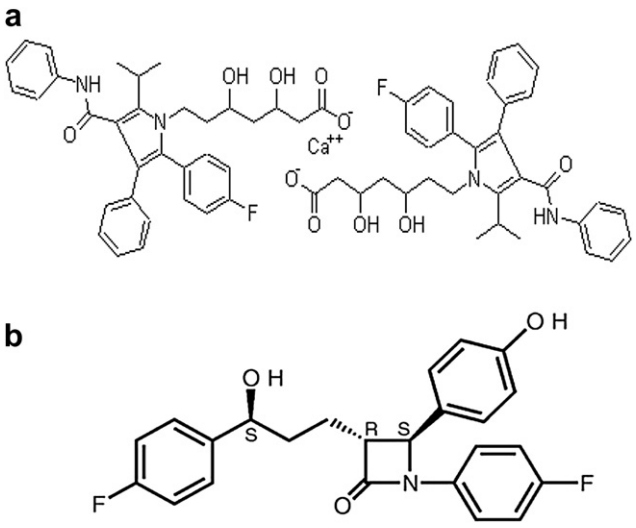

Fig. 1 – Chemical structures of (a)atorvastatin calcium AT, and (b)ezetimibe EZ.

systemic availability is attributed to presystemic clearance in gastrointestinal mucosa and/or hepatic first-pass metabolism. Plasma AT concentrations are lower (approximately 30% for c_{max} and AUC) following evening drug administration compared with morning.[2] AT is insoluble in aqueous solutions of pH 4 and below AT is very slightly soluble in distilled water, pH 7.4 phosphate buffer, and acetonitrile; slightly soluble in ethanol; and freely soluble in methanol.

Ezetimibe (EZ, Fig. 1b), a 1-(4-flurophenyl)-3(R)-[3(S)-(4-flurophenyl)-3-hydroxy propyl]-4(S) (4-hydroxyphenyl) azetidin-2-one, belongs to a group of selective and very effective cholesterol absorption inhibitors. It prevents transport of cholesterol through the intestinal wall by selectively blocking the absorption of cholesterol from dietary and biliary sources. This reduces the overall delivery of cholesterol to the liver.[3,4] EZ may be used alone or with other lipid regulating drugs. It is given in a usual dose of 10 mg once daily.[1] After oral administration, EZ is readily absorbed. There was no substantial deviation from dose proportionality between 5 and 20 mg. The absolute bioavailability of ezetimibe cannot be determined, as the compound is virtually insoluble in aqueous media suitable for injection.[1] EZ is a white, crystalline powder that is freely to very soluble in ethanol, methanol, and acetone and practically insoluble in water.

AT and EZ combinations are present in the market for some time now and several methods for their simultaneous evaluations in pharmaceutical products have been developed. These methods include TLC[5,6] spectrophotomety[7,8] and HPLC.[8–12] No methods have been reported for the simultaneous determination of AT and EZ in biological fluids using UPLC–MS/MS, followed by the application of this method in a bioequivalence study.

The aim of this work was to present a reliable UPLC–MS/MS method for the simultaneous determination of AT and EZ in human plasma with a low limit of quantification (0.1 ng mL^{-1}) to facilitate the pharmacokinetic and bioavailability studies of this combination in humans. The developed method was used to investigate the pharmacokinetic and bioequivalence study of commercially available combination product B *versus* the reference standard branded combination product A. The choice of this method, despite of its high cost, was due to its superior sensitivity, specificity and efficiency. The fast injection cycles, low injection volumes and negligible carryover together contributed to the speed and sensitivity of the UPLC analysis,[13] a quality that was highly appreciated in analysis of AT and EZ mixture in plasma.

1. Experimental

1.1. Reagents and chemicals

Standards of atorvastatin and ezetimibe were supplied and certified by ADWIA, Egypt (purity 99% and 99.5% respectively). The internal standard etilefrine was supplied and certified by DELTA Pharma, Egypt (purity 98.6%). Acetonitrile, formic acid, *tert*-butyl methyl ether and methanol, KH_2PO_4, Na_2HPO_4 were Merck products (Germany). Deionized bi-distilled water (Milli-Q® system, USA) was used. All other chemicals and solvents were of the highest analytical grade available. The human plasma used in the validation procedure was obtained from the holding company for biological products and vaccines (VACSERA, Egypt).

1.2. Liquid chromatography and mass spectrometry (MS)

Analytical separations were performed with an ACQUITY™ UPLC system equipped with a micro-vacuum degasser, binary gradient pumps, thermostatted autosampler, thermostatted column compartment, and an ACQUITY™ UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm), all obtained from Waters Corp. (USA). The column temperature was maintained at 40 °C. The mobile phase was 0.1% formic acid in water and acetonitrile mixture. The mobile phase was used in a gradient mode according to the profile shown in Table 1. The flow rate was adjusted to 0.7 mL min^{-1}. The mobile phase was filtered through a 0.22-μm membrane filter (Millipore, USA) before use. The autosampler temperature was kept at 10 °C and the samples were injected onto the column with an injection volume of 10 μL. The data acquisition run time was kept at 1.2 min for the mass spectrometer (MS). All data were collected and processed using Empower™ 2 Software (Waters Corp).

Mass spectra were acquired on a Quattro Premier XE™ Micromass® triple quadrupole mass spectrometer (Waters Corp.) with an electrospray ionization interface operated in positive and negative ion mode at source temperature 150 °C and desolvation temperature 480 °C. The operating conditions were optimized by flow injection of a mixture of all analytes as

Table 1 – Optimum gradient elution profile for the simultaneous separation of studied drugs.

	Time (min)	Flow rate	Formic acid (0.1%) in water	Acetonitrile
1	Initial	0.7	95	5
2	0.4	0.7	10	90
3	1.1	0.7	95	5

follows: nitrogen carrier gas flow 900 L h^{-1}, argon collision gas flow 0.1 mL min^{-1} cone voltage 50, 35 and 30 V for AT, EZ and IS with collision energy of 22, 16 and 12 V for AT, EZ and IS, respectively. The AT and EZ were quantified using multiple reaction monitoring (MRM) of the precursor ion and the related product ion using the internal standard method with peak area ratios. AT and IS were monitored using positive ionization mode while EZ was monitored using negative ionization mode. The mass transitions used for AT, EZ and the IS were *m/z* 559.57 → 440.4, 408.43 → 271.25 and 182.12 → 164.02, respectively (dwell time 0.08 s).

1.3. *Preparation of standards and quality controls*

Stock solutions of AT, EZ and the IS (100 μg mL^{-1}) were prepared daily in methanol. The AT and EZ standard solutions were serially diluted with methanol to reach a concentration of 10–200 ng mL^{-1}. 200 μL of the serially diluted solutions were added to 1.8 mL of drug-free plasma (originating from six different sources) to obtain concentrations of 0.1, 0.3, 0.5, 1, 3, 5, 10, and 20 ng mL^{-1}. The IS was diluted with methanol to 100 ng mL^{-1}. A calibration graph was derived from the peak area ratios of AT and EZ to the IS using a linear regression. Quality controls were prepared daily in human plasma (obtained from the holding company for biological products and vaccines, VACSERA), for low (0.2 ng mL^{-1} AT and EZ), medium (4 ng mL^{-1} AT and EZ), and high (15 ng mL^{-1} AT and EZ) concentrations to evaluate the precision and accuracy of the assay method.

1.4. *Volunteers samples pre-treatment*

Venous blood samples were collected in heparinized tubes and, within 30 min of collection, were centrifuged at 3500 rpm (Centurion Scientific LTD., West Sussex, UK) for 10 min at 4 °C. Plasma was transferred to clean cryovials and stored at −20 °C until analysis.

1.5. *Liquid–liquid extraction*

All samples and reagents were brought to room temperature on the day of analysis. Aliquots (500 μL) of volunteer samples, blank plasma, calibration samples and quality control (QC) solutions were transferred to 10-mL centrifuge tubes containing 200 μL of IS in methanol (100 ng mL^{-1}) and 100 μL of phosphate buffer (0.025 mol L^{-1}, pH 6.8). After vortex mixing for 1 min, 5 mL of *tert*-butyl methyl ether were added to each tube. All tubes were vortex-mixed for 2 min, and centrifuged at 3000 g for 5 min at room temperature. Then 4.5 mL of the upper organic layer were transferred to other labelled tubes and evaporated to dryness under vacuum in Eppendorf concentrator (Eppendorf 5301, Germany) at about 45 °C. The residue was reconstituted with 100 μL of mobile phase consisting of 0.1% formic acid in water and acetonitrile in a ratio of 95:5, vortex-mixed for 30 s and transferred to UPLC microvial where 10 μL of this solution were injected into the column.

1.6. *Method validation*

The method described above was validated with regard to linearity, sensitivity, accuracy, precision, specificity, percent recovery, dilution integrity, and stability according to accepted guidelines.[14,15]

1.6.1. *Linearity and sensitivity*

The calibration of AT and EZ was performed using a blank sample, a zero sample and eight calibration standards prepared in drug-free plasma originating from six different sources. Calibration curves were prepared daily prior to sample analysis by analyzing calibration standards ranging in concentration from 0.1 to 20 ng mL^{-1}. Calibration curves were plotted using the peak area ratio of AT and EZ to the IS *versus* the nominal concentration. Six calibration curves models were determined by calculating the linear regression (correlation coefficient, *R*), and by evaluating the back-calculated concentrations of the calibration standards. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was investigated.

Sensitivity was defined by the lower limit of quantitation (LLOQ), which was the concentration of AT and EZ at which the signal to noise (S/N) ratio was greater than 5 with acceptable accuracy and precision. This value was set as the lowest concentration in calibration curves.

The calibration models were accepted if the residuals were within ±20% at the lower limit of quantification (LLOQ) and within ±15% at all other calibration levels and if at least 2/3 of the standards met this criterion, including highest and lowest calibration levels.

1.6.2. *Precision and accuracy*

The within- and between-run precision (expressed as RSD %) and accuracy (expressed as %, *versus* nominal concentration) of the assay procedure were determined by analysis on the same day of a set of six different quality control samples at each of the lower (0.2 ng mL^{-1}), medium (4 ng mL^{-1}), and higher (15 ng mL^{-1}) levels and one set of six different quality control samples at the three concentration levels on three different occasions, respectively.

1.6.3. *Specificity*

Specificity tests were performed by a comparison of MRM chromatograms obtained from drug-free plasma samples from twenty four healthy volunteers with plasma spiked with AT and EZ 0.2, 4, and 15 ng mL^{-1}.

1.6.4. *Extraction recovery*

The recovery of AT and EZ from plasma using the liquid–liquid extraction procedure was evaluated by comparing mean analytes responses of triplicate analyses of three QC samples to mean analytes responses of the same concentrations with spiked samples in previously extracted blank plasma. The percent recovery of IS was calculated in a similar manner.

1.6.5. *Dilution integrity*

The ability to dilute samples with concentrations above the upper limit of quantification was also investigated. Three replicates of the high quality control were diluted five times in human plasma prior to sample processing and analysis. The

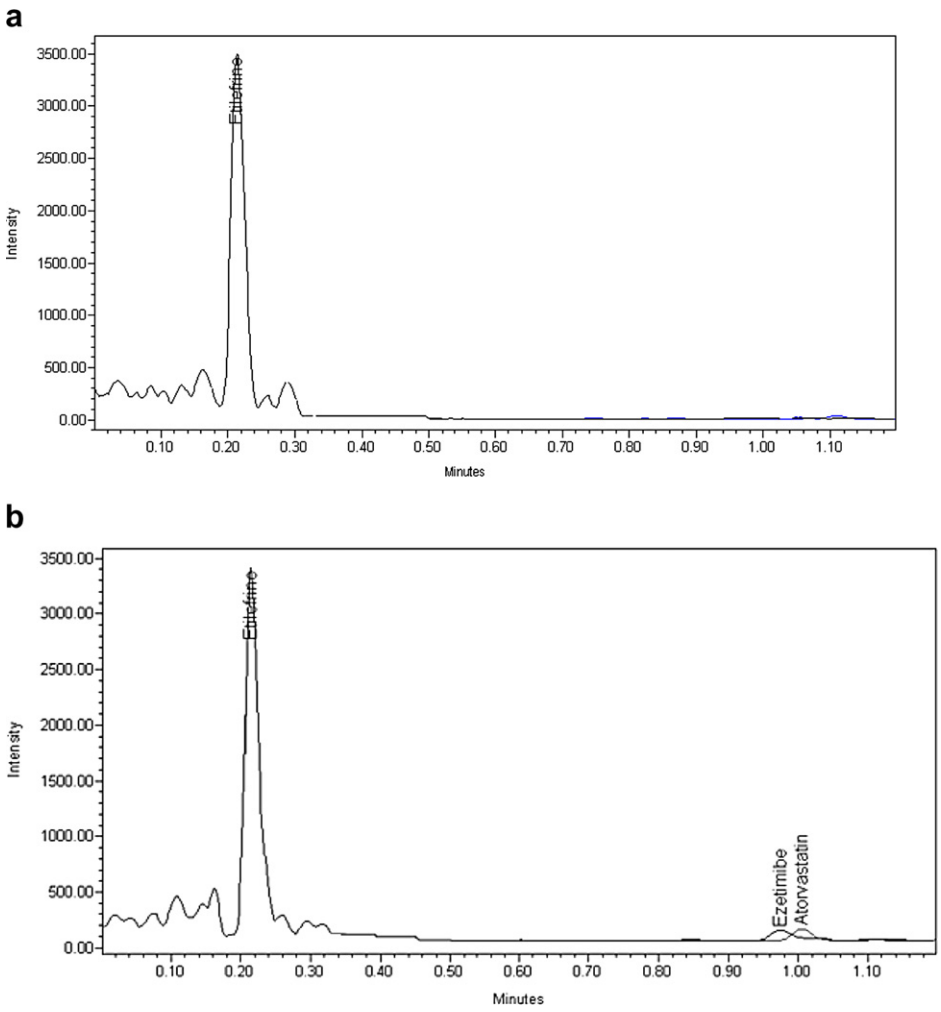

Fig. 2 – MRM chromatogram of extracted: (a) blank plasma with internal standard etilefrine, (b) 0.1 ng mL^{-1} AT and EZ (LOQ) from human plasma.

mean found concentration was compared with the nominal value.

1.6.6. *Stability*

The stability of the analytes in human plasma (expressed as % change) was investigated in four ways, in order to characterize each operation during the process of bioequivalence studies: short term stability (STS), post-preparative stability (PPS), freeze–thaw stability (FTS) and long-term stability (LTS). For all stability studies low, medium and high QC samples were used. Three replicates of QC samples at each level were prepared and left at room temperature for 24 h before processing (STS study). Other three replicates were prepared, immediately processed and kept at 10 °C then were injected after four days (PPS study). For the freeze–thaw stability, the QC samples were subjected to three cycles of freeze–thaw operations in three consecutive days then analyzed against a calibration curve of the day. For long-term stability three sets of QC samples were prepared, the first set was analyzed and calculated against calibration curve of the day. The other two sets were stored at −20 °C for 50 days then analyzed and calculated against calibration curve of the day.

1.7. *In vivo pharmacokinetic/bioequivalence study in healthy human volunteers*

1.7.1. *Study design and subjects*

The pharmacokinetics of AT and EZ from two commercially available combination products A and B was compared following the administration of single doses comprising AT 40 mg and EZ 10 mg, using a non-blind, two-treatment, two-period, randomized, crossover design.

Twenty-four healthy male volunteers participated in this comparative study after giving informed written consent and undergoing physical, complete haematological and biochemical examinations. They were randomly assigned to one of two groups of equal size. Their mean age was 34 ± 4 years, mean body mass was 71.4 ± 7.2 kg and mean height was 173.0 ± 4.5 cm. The study was approved by the Ethics Committee for protection of human subjects (Faculty of Pharmacy, Cairo University, Cairo, Egypt) and the protocol complies with the declarations of Helsinki and Tokyo for humans. Instructions were given to all subjects to abstain from taking medicines and smoking for 1 week before the beginning of the studies to the end of the test. All subjects fasted for at least

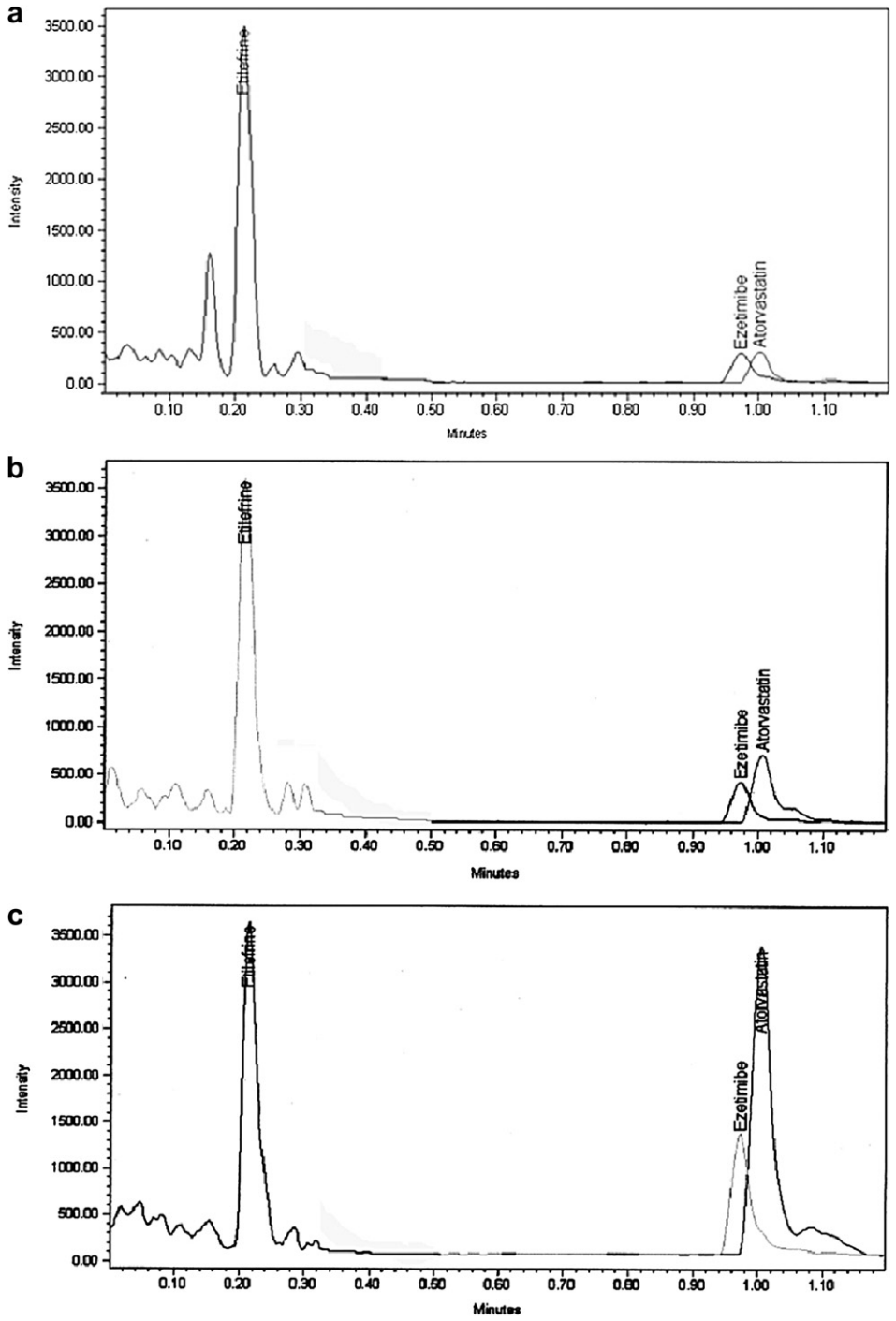

Fig. 3 – (a) MRM chromatogram of extracted AT, EZ and IS from human plasma: (a) 0.2 ng mL^{-1} (low QC), (b) 4 ng mL^{-1} (medium QC), (c) 15 ng mL^{-1} (high QC).

10 h before the study day[14] to facilitate the pharmacokinetic and bioavailability studies of this combination in humans. The study was performed in two phases: phase I, half the number of volunteers received product B (test formulation) and the remainder received product A (reference branded combination formulation). Both treatments were ingested with 200 mL of water. Food and drink (other than water, which was allowed after 2 h) were not allowed until 4 h after dosing and then a standard breakfast, lunch and dinner were given to all volunteers according to a time schedule. A washout period of one week separated the two phases. In the second phase, the reverse of randomization took place.

Each group was supervised by a physician who was also responsible for their safety and collection of samples during the trial. Adverse events were spontaneously reported or observed either by the volunteers or the physician and were recorded and evaluated. Venous blood samples (5 mL) were collected into heparinized tubes at the following set points:

Table 2 – Precision and accuracy of calibration samples for atorvastatin and ezetimibe in human plasma.

Nominal conc. (ng mL^{-1})	Atorvastatin		Ezetimibe	
	RSD (%)[a]	Accuracy (%)[a]	RSD (%)[a]	Accuracy (%)[a]
0.1	13.0	102.6	8.9	92.2
0.3	8.2	93.9	12.0	97.3
0.5	1.9	101.4	13.7	95.9
1.0	2.6	94.1	6.5	96.7
3.0	1.0	89.5	3.9	98.3
5.0	0.9	109.9	3.4	98.7
10.0	0.9	104.1	2.1	103.4
20.0	0.3	98.9	0.6	99.2

a $n = 6$.

Table 4 – Results of the stability studies.

Analyte	Concentration (ng mL^{-1})	STS[a,b]	PPS[a,c]	FTS[a,d]	LTS[a,e]
Atorvastatin	0.2	5.86	6.39	−6.74	4.69
	4	1.38	3.10	−10.92	−7.71
	15	8.03	−2.14	−14.49	−5.30
Ezetimibe	0.2	−3.57	5.10	6.04	−5.35
	4	−2.89	7.32	−7.91	−8.12
	15	4.32	2.79	−10.48	−8.82

a All values are represented as the % of change. All analyses performed in triplicate.
b STS: short term stability (24 h at room temperature).
c PPS: post-preparative stability (4 days at 10 °C).
d FTS: freeze–thaw stability (3 freeze–thaw cycles).
e LTS: long-term stability (after 50 days at −20 °C).

0 (pre-dose), 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 24 and 72 h after administration of each treatment. Samples were pretreated as previously mentioned.

1.7.2. Pharmacokinetic and statistical analysis

Pharmacokinetic analysis was performed by means of a model independent method using Kinetica™ 2000 computer program (USA). The maximum drug concentration (c_{max}, ng mL^{-1}) and the time to reach c_{max} (t_{max}, h) were obtained from the individual plasma concentration–time curves. The area under the curve from 0 to 72 h (AUC_{0-72}, ng h mL^{-1}) and to infinity ($AUC_{0-\infty}$, ng h mL^{-1}), were calculated as well.

Bioequivalence analysis was calculated based on the 90% confidence intervals for log-transformed AUC_{0-t}, $AUC_{0-\infty}$, and c_{max} according to the FDA guidance for in vivo bioequivalence studies.[14] In addition, analysis of variance (ANOVA) was used to test the difference between c_{max}, t_{max}, AUC_{0-t}, $AUC_{0-\infty}$, $t_{1/2}$ and k_{el} for the reference A and test B products.

2. Results and discussion

Measurements of AT, EZ and IS levels in samples of human plasma were made with a UPLC–MS/MS instrument in MRM scan mode. Solutions of AT, EZ and IS (1 μg mL^{-1}) were directly infused into mass spectrometer along with mobile phase (0.7 mL min^{-1}) and MS parameters were optimized to get maximum sensitivity for respective product ions. Both positive and negative electrospray ionization modes have been tried. Signal intensity obtained under ESI (+) was found to be higher than that under ESI (–) in the case of AT and IS, while the opposite was true in the case of EZ. Thus, positive ionization was used for AT and IS and negative ionization was used for EZ in our study. The precursor ions were set at *m/z* 559.57, 408.43 and 182.12 for AT, EZ and IS respectively to provide the best detection sensitivity. The fragmentation patterns of these ions under these conditions contained intense product peaks at *m/z* 440.4 for AT, 271.25 for EZ and 164.02 for IS. Therefore, the corresponding transitions associated with these product peaks were selected for MRM analysis.

A gradient mobile phase was used for the chromatographic separation of AT, EZ and IS. It consisted of 0.1% formic acid in water and acetonitrile at a flow rate of 0.7 mL min^{-1}. The retention time of AT was 1.01 min, EZ was 0.97 min while that of IS was approximately 0.22 min. The UPLC technique, with smaller column particle size (1.7 μm), separated AT, EZ and the IS within 1.2 min, significantly faster than previous LC methods.[8–12]

Upon utilizing the above conditions for the determination of AT and EZ in six different plasma sources, the absolute peak

Table 3 – Intra-day and inter-day precision and accuracy of quality control samples for atorvastatin and ezetimibe in human plasma.

Analyte	Concentration (ng mL^{-1})	Intra-day[a] ($n = 6$)		Inter-day[b] ($n = 18$)	
		RSD (%)[a]	Accuracy (%)[a]	RSD (%)[b]	Accuracy (%)[b]
Atorvastatin	0.2	5.6	97.8	7.2	96.1
	4	5.6	95.3	8.2	95.9
	15	4.8	94.8	6.5	93.7
Ezetimibe	0.2	4.2	92.3	7.0	92.0
	4	4.7	96.8	7.1	97.2
	15	3.6	93.2	6.8	93.8

a $n = 6$.
b $n = 18$.

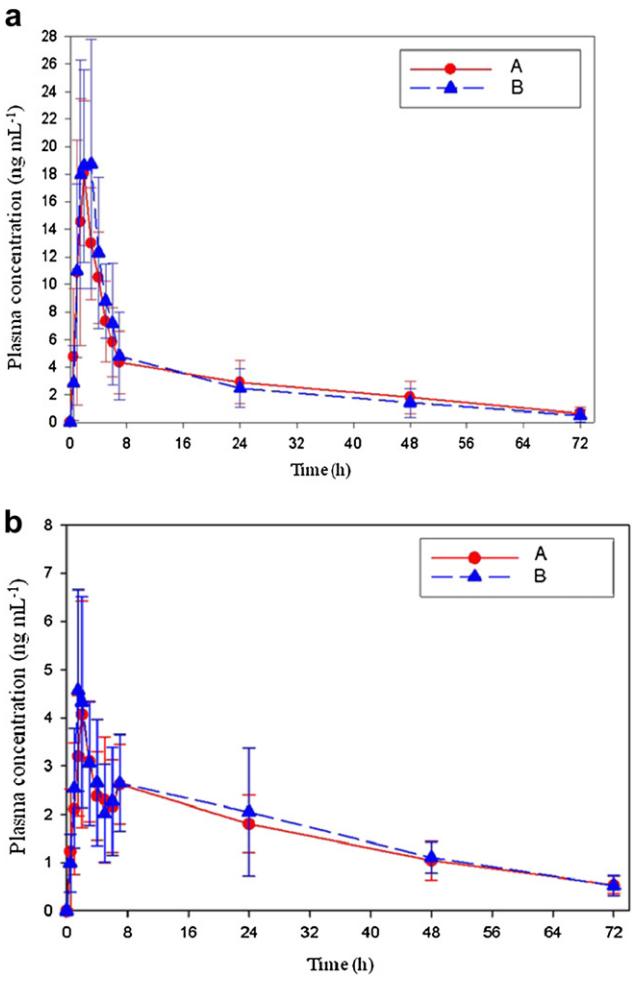

Fig. 4 – Mean plasma concentration *versus* time curve following the oral administration of products A and B (a) AT (b) EZ.

areas of analytes at the same concentration were different in different biofluid lots showing ionic suppression and suggesting the presence of matrix effect. Since the deuterated analogues of AT and EZ were not available therefore the quest arose for the presence of an internal standard that would overcome the matrix effect and give reproducible results with both drugs.

Several drugs from our laboratory that we knew from previous experience to show ionic suppression in similar systems have been tried. Etilefrine behaved in the same manner as the drugs in analysis and showed to be the most suitable IS in this method as the ratios of drug/IS for different plasma lots were not markedly different. Also the small RSD value of standard line slopes (1.72% for AT and 2.96% for EZ) indicated that the method is more reliable and free from relative matrix effect.[16] The reproducibility of results as shown in the validation data proved that etilefrine was suitable as an IS for both drugs despite of their different ionization modes.[17,18]

2.1. *Sample preparation and recovery*

Although the use of solid-phase extraction procedures reduces the matrix effect considerably, it increases overall time and cost of analysis. In the present method simple liquid–liquid extraction procedure, which was fast enough for high-throughput analysis, was optimized.

Knowing that AT is a member of the statins that are notoriously unstable and convert in solvents from open acid form to lactone form and *vice versa*, by non enzymatic reactions that are pH dependent, attempt was made to control this interconversion by adding phosphate buffer (pH 6.8). This is done before the sample extraction with the organic solvent to favour the acid form.[19–22]

2.2. *Validation*

2.2.1. *Recovery*

The good recovery of AT and EZ from plasma using the liquid–liquid extraction procedure proved that this extraction method reliably eliminated interfering material from plasma. The mean percent recovery values of AT were 94.4, 95.7 and 95.8% at low, medium and high quality control levels while that of EZ were 93.5, 95.0 and 92.6% at low, medium and high quality control levels respectively. The mean percent recovery of the IS at a concentration of 100 ng mL^{-1} was 90.9% with an acceptable precision (RSD < 8%).

Table 5 – Pharmacokinetic parameters of AT and EZ following the oral administration of "A" and "B" tablets.

Pharmacokinetic parameter	"A" tablets		"B" tablets	
	AT	EZ	AT	EZ
c_{max} (ng mL^{-1})[a]	21.28 ± 6.08	5.07 ± 1.81	23.34 ± 7.08	5.66 ± 2.10
Range (ng mL^{-1})	12.15–35.45	3.22–10.29	14.87–42.27	3.13–10.97
Median t_{max} (h)[a]	1.5	2.0	2.0	2.0
Mean t_{max} (h)	1.63 ± 0.37	2.44 ± 1.15	2.10 ± 0.66	2.73 ± 1.94
Range (h)	1.0–2.0	1.0–5.0	1.0–3.0	1.5–7.0
AUC_{0-t} (ng h mL^{-1})[a]	208.55 ± 91.40	106.07 ± 27.96	206.52 ± 88.59	112.15 ± 36.75
Range (ng h mL^{-1})	68.65–367.46	37.64–180.55	90.76–483.67	58.62–208.02
$AUC_{0-\infty}$ (ng h mL^{-1})[a]	230.68 ± 103.90	134.31 ± 44.34	227.17 ± 99.18	137.38 ± 42.98
Range (ng h mL^{-1})	71.69–421.14	49.42–274.60	93.65–534.10	71.75–222.31

a Mean ± SD, $n = 24$.

Table 6 – Bioequivalence confidence intervals of AT and EZ from product "B" *versus* the reference product "A".

Pharmacokinetic parameter	90% Confidence interval for means					
	Point estimate (%)		Upper limit (%)		Lower limit (%)	
	AT	EZ	AT	EZ	AT	EZ
c_{max} (ng mL^{-1})	109.59	110.71	123.54	124.90	95.64	96.52
AUC_{0-t} (ng h mL^{-1})	102.22	104.69	124.03	119.55	80.42	89.83
$AUC_{0-\infty}$ (ng h mL^{-1})	102.09	102.49	123.77	116.75	80.42	88.24

2.2.2. *Specificity*

Typical MRM chromatograms obtained from different plasma blank samples, plasma spiked with standard AT and EZ (0.2, 4, 15 ng mL^{-1}) and IS (100 ng mL^{-1}), are shown in Figs. 2 and 3. Retention times of AT, EZ and the IS were 1.01, 0.97 and 0.22 min, respectively. No significant interference from endogenous peaks was observed at these retention times.

2.2.3. *Linearity and lower limit of quantification (LLOQ)*

Calibration curves were linear in the concentration range of 0.1–20 ng mL^{-1} for both AT and EZ. The calibration curves were fitted by weighted least-squares linear regression. The precision and accuracy of calibration samples for AT and EZ in human plasma are given in Table 2. The mean ± SD of six standard curve slopes for AT and EZ were 1.069 ± 0.018 and 0.037 ± 0.001, respectively. The coefficient of determination (R^2) of the calibration curves was ≥0.999 for both analytes. The lowest limit of quantification was determined to be 0.1 ng mL^{-1} for both analytes with a signal to noise ratio of 5.8 and 7.1 for AT and EZ respectively (Fig. 2).

2.3. *Precision and accuracy*

The intra- and inter-day precision and accuracy of three quality control concentrations (0.2, 4, 15 ng mL^{-1}) are summarized in Table 3. For AT intra- and inter-day RSDs were less than 5.60 and 8.24%, respectively, whereas intra-day accuracy ranged from 94.80 to 97.78% with a mean of 95.9% and inter-day accuracy ranged from 93.6 to 96.10% with a mean of 95.2%. For EZ intra- and inter-day RSD was less than 4.73 and 7.13%, respectively. Intra-day accuracy ranged from 92.3 to 96.8% with a mean of 94.1% and inter-day accuracy ranged from 92.0 to 97.2% with a mean of 94.3%.

2.3.1. *Dilution integrity*

The ability to dilute samples with concentrations above the upper limit of quantification could be made with accuracy of 93.2% and precision less than 7.8% for AT and accuracy of 92.9% and precision less than 5.4% for EZ.

2.3.2. *Stability*

The stability of the two drugs under various conditions is shown in Table 4. Under all conditions tested, the two drugs proved to be stable. All results were within the acceptance criteria of ±15% deviation from the nominal concentration.

2.3.3. *Pharmacokinetic and bioequivalence analysis of atorvastatin and ezetimibe*

The mean plasma level of AT and EZ in both products A and B are shown in Fig. 4a and b. Table 5 shows the parameters for the non-compartmental pharmacokinetic analysis.

According to ANOVA results there is no significant sequence effect for both c_{max} and $AUC_{0-72\ h}$ indicating that the crossover design was properly performed. The parametric point estimates and the 90% confidence intervals for ln-transformed AUC_{0-t}, $AUC_{0-\infty}$, and c_{max}, (Table 6) were within commonly accepted bioequivalence range of 80–125% range, thus the results reveal that the bioequivalence between products A and B could be concluded.

3. Conclusions

A rapid, sensitive, and simple method for determining AT and EZ levels in human plasma was developed and validated. The UPLC–MS/MS method described herein reveals significant advantages over other techniques, including LC–MS/MS, due to the inherently increased column efficiency of UPLC, which resulted in complete analysis within 1.2 min with significantly lower limits of quantitation (0.1 ng mL^{-1}). To the best of our knowledge, this is the first UPLC–MS/MS method for the simultaneous determination of AT and EZ in human plasma. This fully validated method was an ideal tool for high-throughput analysis of plasma samples used in pharmacokinetic and bioequivalence study of AT and EZ between two market products.

Conflicts of interest

All authors have none to declare.

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REFERENCES

1. Martindale. *The Complete Drug Reference*. 36th ed. London: Pharmaceutical Press; 2009.
2. Page C, Hoffman B, Curtis M, Walker M, Sutter M. *Integrated Pharmacology*. 2nd ed. London: Mosby International Ltd; 2002.
3. Leitersdorf E. Selective cholesterol absorption inhibition: a novel strategy in lipid-lowering management. *Int J Clin Pract*. 2002;56:116–119.
4. Heek MV, Farley C, Compton DS. Ezetimibe selectively inhibits intestinal cholesterol absorption in rodents in the presence and absence of exocrine pancreatic function. *Br J Pharmacol*. 2001;134:409–417.
5. Dhaneshwar SS, Dhaneshwar SR, Deshpande P, Patil M. Development and validation of a method for simultaneous

densitometric estimation of atorvastatin calcium and ezetimibe as the bulk drug and in tablet dosage forms. *Acta Chromatogr*. 2007;19:141–148.
6. Chaudhari BG, Patel NM, Shah PB, Modi KP. Development and validation of a HPTLC method for the simultaneous estimation of atorvastatin calcium and ezetimibe. *Indian J Pharm Sci*. 2006;68:793–796.
7. Sonawane SS, Shirkhedkar AA, Fursule RA, Surana SJ. Simultaneous spectrophotometric determination of atorvastatin calcium and ezetimibe in tablets. *Indian J Pharm Sci*. 2007;69:683–684.
8. Sonawane SS, Shirkhedkar AA, Fursule RA, Surana SJ. Application of UV-spectrophotometry and RP-HPLC for simultaneous determination of atorvastatin calcium and ezetimibe in pharmaceutical dosage form. *Eurasian J Anal Chem*. 2006;1:32–41.
9. Chaudhari BG, Patel NM, Shah PB, Patel LJ, Patel VP. Stability-indicating reversed-phase liquid chromatographic method for simultaneous determination of atorvastatin and ezetimibe from their combination drug products. *J AOAC Int*. 2007;90:1539–1546.
10. Qutab SS, Razzaq SN, Ashfaq IK, Shuja ZA. Simultaneous determination of atorvastatin calcium and ezetimibe in pharmaceutical formulations by liquid chromatography. *J Food Drug Anal*. 2007;15:139–144.
11. Macwan JS, Ionita IA, Dostalek M, Akhlaghi F. Development and validation of a sensitive simple and rapid method for simultaneous quantitation of atorvastatin and its acid and lactone metabolites by liquid chromatography–tandem mass spectrometry LC–MS/MS. *Anal Bioanal Chem*. 2011;400:423–433.
12. Jackson A, D'Avolio A, Watson V, et al. Pharmacokinetics and safety of the co-administration of the antiretroviral raltegravir and the lipid-lowering drug ezetimibe in healthy volunteers. *J Antimicrob Chemother*. 2011;4:885–889.
13. Novakova L, Matysova L, Solich P. Advantages of application of UPLC in pharmaceutical analysis. *Talanta*. 2006;68:908–918.
14. Food and Drug Administration, Center for Drug Evaluation and Research. *Guidance for Industry, Bioavailability and Bioequivalence Studies for Orally Administered Drug Products – General Considerations*. Rockville: FDA, http://www.fda.gov/cder/guidance/5356fnl.pdf; 2003.
15. FDA. *Guidance for Industry – Bioanalytical Method Validation*, http://www.fda.gov/cder/guidance/5252fnl.pdf; 2001.
16. Matuszewski BK. Standard line slopes as a measure of a relative matrix effect in quantitative HPLC–MS bioanalysis. *J Chromatogr B*. 2006;830:293–300.
17. Zou J, Dai L, Ding L, et al. Determination of betamethasone and betamethasone 17-monopropionate in human plasma by liquid chromatography-positive/negative electrospray ionization tandem mass spectrometry. *J Chromatogr B*. 2008;873:159–164.
18. Durden DA. Positive and negative electrospray LC–MS/MS methods for quantitation of the antiparasitic endectocide drugs, abamectin, doramectin, emamectin, eprinomectin, ivermectin, moxidectin and selamectin in milk. *J Chromatogr B*. 2007;850:134–146.
19. Kearney AS, Crawford LF, Mehta SC, Radebaugh GW. The interconversion kinetics, equilibrium, and solubilities of the lactone and hydroxyacid forms of the HMG-CoA reductase inhibitor, CI-981. *Pharm Res*. 1993;10:1461–1465.
20. Jemal M, Xia YQ. Bioanalytical method validation design for the simultaneous quantitation of analytes that may undergo interconversion during analysis. *J Pharm Biomed Anal*. 2000;22:813–827.
21. Hoffmann M, Nowosielskia M. DFT study on hydroxy acid-lactone interconversion of statins: the case of atorvastatin. *Org Biomol Chem*. 2008;6:3527–3531.
22. Jemal M, Ouyang Z, Chen B, Teitz D. Quantitation of the acid and lactone forms of atorvastatin and its biotransformation products in human serum by high-performance liquid chromatography with electrospray tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 1999;13:1003–1015.