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ORIGINAL RESEARCH PAPER

Simultaneous determination of ezetimibe, atorvastatin and simvastatin using quadrupole LC-MS: Application to combined tablets and plasma after SPE

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ABSTRACT

A simple and sensitive liquid chromatography-mass spectrometric (LC-MS) method has been developed and validated for the simultaneous determination of ezetimibe (EZE), atorvastatin calcium (ATO), and simvastatin (SMV) in combined dosage forms and human plasma. Successful separation of the studied drugs was achieved on a Zorbax Eclipse Plus C18 column (3.0 × 150 mm, 5 µm) using a mobile phase consisting of acetonitrile and 0.1% formic acid in water (65:35, v/v) at a flow rate of 0.5 mL min^{-1}. Total run time was 9.3 min and diclofenac sodium was used as internal standard (IS). Positive selected ion monitoring (SIM) mode was applied where, the monitored ions were those at *m/z* values of 392.1, 559.3, 296.0, and 441.4 corresponding to EZE, ATO, IS, and SMV, respectively. The method was fully validated according to the ICH guidelines. The intraday and interday precision showed relative SD values not more than 1.77 and 1.99%; respectively. The limits of detection (LOD) were 0.25, 0.25, and 0.75 ng mL^{-1} while the limits of quantification (LOQ) were 1.25, 0.75, and 2.5 ng mL^{-1} for EZE, ATO, and SMV, respectively. The developed method was applied on two types of combined tablets concerning drug assay with mean percent recoveries within acceptable range. The method has been extended to the determination of the studied drugs in human plasma where, a solid phase extraction method was optimized for their extraction with percent recovery not less than 97%.

KEYWORDS

ezetimibe, atorvastatin, simvastatin, electrospray ionization mass spectrometry, plasma, combined dosage forms

INTRODUCTION

Atorvastatin calcium salt trihydrate (ATO) (Fig. 1C), Calcium (3R,5R)-7-[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoate trihydrate, and simvastatin (SMV) (Fig. 1B), (1S,3R,7S,8S,8aR)-8-[2-[(2R,4R)-4-Hydroxy-6-oxotetrahydro-2H-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8ahexahydronaphthalen-1-yl 2,2-dimethylbutanoate, are antihyperlipidemics from the group of 3-Hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly known as statins. They lower elevated low-density lipoproteins (LDL) cholesterol levels by inhibiting the first enzymatic step of cholesterol synthesis, resulting in a substantial reduction in coronary events and death from coronary heart diseases [1].

On the other hand, ezetimibe (EZE) (Fig. 1A), (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)azetidin-2-one, decreases the delivery

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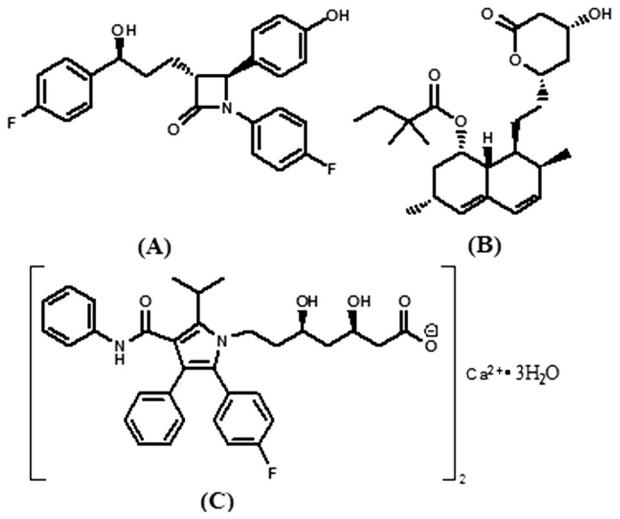

Fig. 1. The chemical structures of (A) EZE, (B) SMV and (C) ATO

of intestinal cholesterol to the liver by selectively inhibiting the intestinal absorption of dietary and biliary cholesterol in the small intestine, leading to increase in clearance of cholesterol from the blood. The use of combined anti-hyperlipidemic drugs was necessary to achieve the treatment goals in plasma lipid levels. A low dose of statins in combination with EZE achieves comparable or even greater LDL cholesterol reduction than a very-high-dose of statins [1].

Different LC-MS/MS methods were developed for the simultaneous determination of EZE and ATO in plasma after liquid-liquid extraction [2, 3]. Karanam et al. described an LC-MS/MS method to quantify EZE and SMV in rat plasma [4]. A pharmacokinetic study involving simultaneous determination of EZE, SMV, and SMV acid in human plasma was also reported [5]. Other HPLC-UV methods were established for the determination of EZE with ATO in human plasma [6] and EZE with SMV in rabbit serum [7]. Also, a reported spectrodensitometric method was developed for the simultaneous determination of a quaternary mixture containing niacin, ATO and bezafibrate with EZE or SMV in plasma [8]. Corresponding to the simultaneous determination of EZE with ATO or SMV in pharmaceutical formulations, several analytical methods were reported including, HPLC [9–11], UV spectrophotometry [10, 12], and HPTLC [13–14].

To the best of our knowledge, no methods have been reported regarding simultaneous single quad LC-MS determination of the three studied drugs with a versatile application on the two co-formulated dosage forms including EZE with ATO or SMV. Also, all reported plasma extraction methods were liquid–liquid extraction or SPE with low recovery and they were applicable for only one combination. The aim of the present study was to develop a sensitive LC-MS method for the simultaneous determination of the three drugs. The method was fully validated according to ICH guidelines [15]. Application of the proposed method on two different combined tablets was tested. The method was also applied for determination of the studied drugs in human plasma, where a solid phase extraction (SPE) method was optimized for the simultaneous extraction of the three studied drugs with a high percent of recovery.

EXPERIMENTAL

Chemicals and reagents

Simvastatin (≥97%) and atorvastatin calcium salt trihydrate (≥98%) and diclofenac sodium (≥98.5%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). EZE was obtained as a gift from Medical Union Pharmaceuticals (MUPs) (Ismailia, Egypt) and used as received without further purification. Atoreza®10/10 tablets (contain 10 mg EZE and 10.85 mg ATO equivalent to 10 mg atorvastatin per tablet) and Zocozet® 10/10 tablets (contain 10 mg EZE and 10 mg simvastatin per tablet), manufactured by Marcyrl Pharmaceutical Industries – Egypt, were purchased from the local market. All solvents used were of HPLC grade (Sigma–Aldrich, USA) and other chemicals were of analytical grade. Ultrapure water was used during all the analysis (Milli-Q Integral 3 Water Filtration System, Millipore Corp., Bedford, MA, USA). Blank drug-free human plasma was kindly provided by the internal plasma bank of King Abdulaziz University Hospital, Jeddah, Saudi Arabia.

Instrumentation

For the LC-MS analysis, Agilent 6120 Quadrupole MS coupled with a 1200 series HPLC (Agilent Technologies, Germany) was used. All data acquisition and processing were performed by ChemStation [Rev. B.04.02 (96)]. The HPLC system consisted of a solvent delivery module, a quaternary pump, an autosampler, and a column compartment. Zorbax Eclipse plus C18 column (3.0 × 150 mm, 5 µm) (Agilent Technologies, Palo Alto, CA, USA) was used for analytes separation. Chromabond C18 solid phase extraction columns (1 mL, 100 mg, Macherey Nagel, GmbH & Co., Duren, Germany) were used for extracting drugs from plasma samples. The Visiprep extraction manifold of 12 ports and the membrane vacuum pumps were from Waters. Screw-capped total recovery autosampler vials, 12 mm × 32 mm, were purchased from Waters (Milford, MA, USA) and used only with plasma samples. Calibrated micro-transfer pipette 25–250 µL, Brand, Wertheim, Germany was used.

Chromatographic and mass spectrometric conditions

A mobile phase consisting of acetonitrile and 0.1% formic acid in water (65:35, v/v) was eluted isocratically at a flow rate of 0.5 mL min^{-1}. The column was maintained at ambient temperature and the sample injection volume was 20 µL.

The MS parameters were optimized by performing flow injection analysis (FIAs) of EZE, ATO, SMV, and IS standard solutions (10 µg mL^{-1}, dissolved in the mobile phase). General MS adjustments were set as follows: capillary

voltage, 4,000 V; nebulizer pressure, 35 psig; drying gas flow, 12 L min^{-1}; desolvation temperature, 350 °C; Dwell, 71 msec; fragmentor, 135 v for EZE and ATO and 80 v for SMV and IS. Peak areas were calculated from extracted ion chromatogram of pre-defined ions to ensure the accuracy of the quantitative analysis.

Standard solutions preparation

ATO stock solution containing 1.0 mg mL^{-1} was prepared by dissolving 10.0 mg of ATO salt trihydrate in 10 mL of methanol. EZE, SMV and IS stock solutions were prepared by the same method. These solutions were stable for three weeks when kept protected from light in refrigerator (−20 °C). Working solutions were prepared by further dilution with the mobile phase.

Aliquots of the standard working solutions of EZE, ATO, and SMV were transferred into a series of 10-mL volumetric flasks followed by 1.5 mL of the IS working solution (1 μg mL^{-1}) and then solutions were made up to volume with the mobile phase. The final concentrations were within the range of 2.5–30 ng mL^{-1} for SMV, 1.25–30 ng mL^{-1} for EZE, and 0.75–30 ng mL^{-1} for ATO.

Application to dosage forms

Twenty Zocozet® tablets (10 mg EZE and 10 mg SMV per tablet) were weighed and powdered. In a screw capped test tube, a weight of the powdered tablets equivalent to one tablet was mixed with 10 mL of methanol, sonicated for 10 min and then filtered through nylon filtration disk (0.45 μm), the concentration of the filtrate was 1.0 mg mL^{-1} of EZE and SMV. This solution was further 100-fold diluted then aliquots of the diluted solution were transferred to a series of 10-mL volumetric flasks where, a 1.5 mL of the IS working solution was added. All solutions were made up to volume with the mobile phase to obtain final concentration of 10, 20, and 25 ng mL^{-1} for EZE and SMV. The same procedure was repeated with Atoreza® tablets (10 mg EZE and 10.85 mg ATO) to obtain final concentrations of 10, 20, and 25 ng mL^{-1} for EZE and 10.85, 21.70, and 27.13 ng mL^{-1} for ATO.

Application to spiked human plasma

Aliquots (1.0 mL) of human plasma were transferred into a series of screw capped test tubes. The plasma samples were spiked with working solutions of the studied drugs prepared by diluting the standard solution with ultrapure water. The spiked plasma samples were prepared to contain final concentrations in the range of 0.7–7.5 ng mL^{-1} for SMV and 0.5–7.5 ng mL^{-1} for EZE and ATO. The SPE C18 column was primarily conditioned by passing 3 mL of methanol then 2 mL of 3% formic acid in water (v/v). Thereafter they were loaded with 200 μL of spiked plasma followed by 50 μL of 3% formic acid in water (v/v) and 100 μL of IS solution (75 ng mL^{-1}). The vacuum was applied, and the tap was opened just to allow the sample to leave the solid support surface, and the tap was closed again and wait for 2 min. Then, the sample was washed with 1 mL of 3% formic acid in water (v/v). The retained compounds were eluted with 1 mL of acetonitrile followed by 1 mL of ethyl acetate. The extract was then evaporated to dryness at 40 °C under a stream of nitrogen. The residue was reconstituted in 200 μL of methanol, quantitatively transferred to the total recovery autosampler vial, evaporated with nitrogen gas at 40 °C using heating block and reconstituted in 50 μL of methanol. A 20-μL sample was injected for LC–MS analysis. A blank experiment was carried out simultaneously.

RESULTS AND DISCUSSION

The present study involved three major tasks including the development, validation and application of the proposed LC-MS method. The proposed method was applied for the determination of the three studied drugs in two combined dosage forms and plasma. The optimization of a SPE method for extracting the drugs from plasma was also involved.

Method optimization

Mobile phase composition. Several modifications in the mobile phase composition were performed to achieve the best chromatographic performance. These modifications involved changing the organic modifier type and ratio, the use of ammonium acetate buffer or formic acid with the organic modifier and changing the pH of ammonium acetate buffer. Longer retention times and peak broadening especially with SMV were observed upon using methanol or ethanol as organic modifier. So, acetonitrile was the organic modifier of choice giving symmetrical peaks within suitable time. Optimal chromatographic separation was achieved upon using acetonitrile with 0.1% formic acid in a ratio of 65:35.

Internal standard. Internal standard (IS) was used to improve the precision of the quantitative analysis where, phenazone, propylparapen, caffeine, rosuvastatin, and diclofenac sodium were tested as IS. In contrast to the other tested compounds, only diclofenac sodium was totally resoluted from the three drugs with a suitable retention time, so it was used throughout the study.

Choice of column. Several columns including Zorbax Eclipse plus C18, solvent saver (3.0 × 150 mm, 5 μm), Zorbax Eclipse plus C18, rapid resolution (4.6 × 100 mm, 3.5 μm) and Zorbax ODS, analytical (4.6 × 250 mm, 5 μm) were tested. The first column was the most suitable one since symmetrical peaks with good resolution were obtained. The three drugs were separated within longer run time using the second column and increasing the ratio of the acetonitrile in the mobile phase affected the resolution of EZE and ATO peaks. The third column showed marked peak broadening.

The MS parameters. The MS parameters were optimized to enhance the method sensitivity. Preliminary analysis of the three drugs and the IS applying the scan mode was

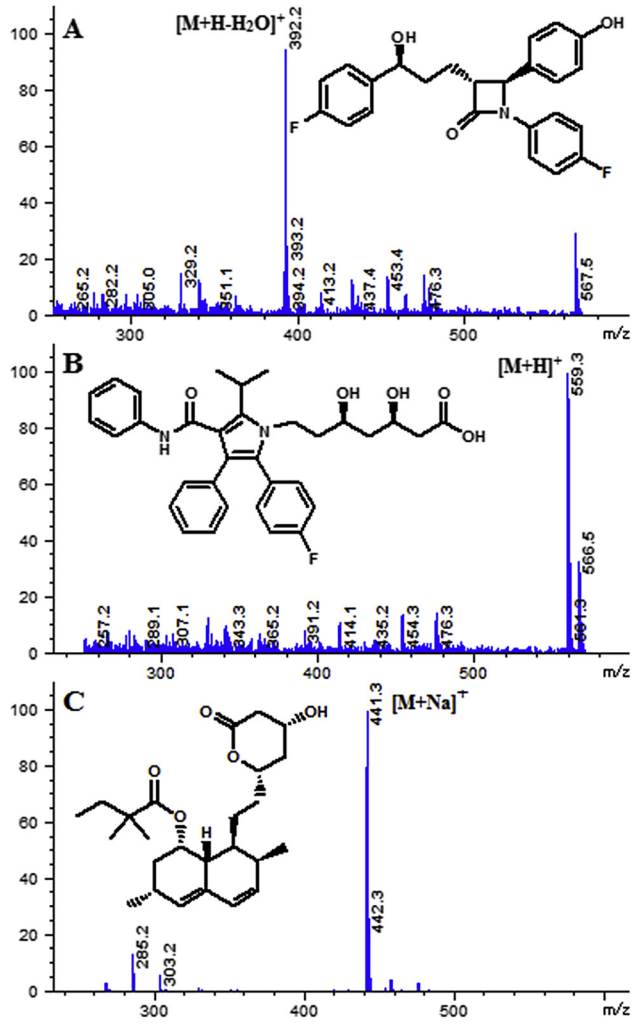

Fig. 2. Representative MS spectrums of EZE (A), ATO (B) and SMV (C) upon applying positive MS scan mode

performed to determine the *m/z* values of the fragments with the highest abundance (Fig. 2). This step was a starting point for the application of the SIM data acquisition mode to monitor only the selected *m/z* values. EZE, ATO, IS, and SMV were monitored at *m/z* values of 392.1, 559.3, 296.0, and 441.4 corresponding to $[M+H\text{-}H_2O]^+$, $[M+H]^+$, $[M]^+$, and $[M+Na]^+$, respectively. The next step was the flow injection analysis (FIAs) of the standard solutions (10 µg mL^{-1}, prepared in the mobile phase) where, multiple samples were injected within the same run, without a column to optimize the MS parameters. The studied drugs were sensitively detected upon adjusting the MS parameters as mentioned under the experimental section.

Validation

Linearity and range. A linear relationship was constructed by plotting the peak area ratio of the drug to the IS (y) against the respective drug concentration (x). Three replicate injections of seven calibration solution levels were performed. The relationship was found to be linear over a range of 2.5–30 ng mL^{-1} for SMV, 1.25–30 ng mL^{-1} for EZE and 0.75–30 ng mL^{-1} for ATO. Typically, the regression equations were:

$$y = 0.0395x - 0.0007 (r = 0.9998) \text{ for EZE,}$$
$$y = 0.0709x - 0.0147 (r = 0.9995) \text{ for ATO,}$$
$$y = 0.0812x + 0.0321 (r = 0.9999) \text{ for SMV.}$$

Statistical analysis of the data abridged in Table 1 proved the linearity of the method [16].

Limit of detection and limit of quantitation. Limit of detection (LOD) and limit of quantitation (LOQ) were experimentally determined as recommended by the ICH guidelines [15]. The LOD was taken as the lowest analyte amount which could be detected but not quantitated where, signal-to-noise ratio should be 3:1. On the other hand, LOQ was the lowest analyte amount that could be quantitatively determined in accurate and precise manner where, signal-to-noise ratio in this case is 10:1. The LOD values were 0.75 ng mL^{-1} for SMV and 0.25 ng mL^{-1} for EZE and ATO. The LOQ values were 1.25, 0.75 and 2.5 ng mL^{-1} for EZE, ATO, and SMV; respectively; with RSD values not more than 3.14% (n = 5).

LOD and LOQ were also calculated based on the slope and the SD of the response. They were calculated as 3.3 *Sa/b* and 10 *Sa/b* for LOD and LOQ respectively, where *Sa* is the SD of the response and *b* is the slope of the calibration curve. Calculated LOD values were 0.48, 0.54, and 0.27 ng mL^{-1} while, LOQ values were 1.46, 1.64, and 0.82 ng mL^{-1} for EZE, ATO, and SMV, respectively.

Accuracy. Solutions spiked with known amounts of EZE, ATO, and SMV were analyzed using the proposed method to evaluate its accuracy. Three different concentrations of each drug were determined in three replicates and the percent recovery of each drug was calculated. Recoveries of all drugs were found to be within the acceptable range, so the method was deemed to be accurate (Table 2). As recommended by ICH guidelines, the accuracy of the method in

Table 1. Analytical performance data for the determination of the studied drugs by the proposed LC-MS method

Parameter	EZE	ATO	SMV
Linearity range (ng mL^{-1})	1.25–30	0.75–30	2.5–30
Intercept (a)	−0.0007	−0.0147	0.0321
Slope (b)	0.0395	0.0709	0.0812
Correlation coefficient (r)	0.9998	0.9998	0.9999
S.D. of residuals ($S_{y/x}$)	8.140×10^{-3}	1.827×10^{-2}	9.785×10^{-3}
S.D. of intercept (S_a)	5.781×10^{-3}	1.168×10^{-2}	6.688×10^{-3}
S.D. of slope (S_b)	3.302×10^{-4}	6.890×10^{-4}	4.001×10^{-4}
LOD (ng mL^{-1})	0.25	0.25	0.75
LOQ (ng mL^{-1})	1.25	0.75	2.50

Table 2. Accuracy and precision data for the determination of EZE, ATO and SMV using the proposed LC-MS method (n = 9)

Parameter	Taken concentration (ng mL^{-1})	Found concentration (ng mL^{-1} ± SD)[a]	%RSD	% Error[b]	% Recovery
(1) EZE					
Intraday	10.00	10.03 ± 0.12	1.23	0.31	100.31
	17.50	17.30 ± 0.16	0.90	1.16	98.84
	25.00	24.99 ± 0.39	1.55	0.06	99.94
Interday	10.00	10.03 ± 0.15	1.54	0.33	100.33
	17.50	17.14 ± 0.14	0.82	2.04	97.96
	25.00	25.18 ± 0.18	0.71	0.72	100.72
(2) ATO					
Intraday	7.50	7.41 ± 0.11	1.46	1.26	98.74
	12.50	12.31 ± 0.07	0.59	1.55	98.45
	25.00	25.22 ± 0.05	0.19	0.88	100.88
Interday	7.50	7.32 ± 0.14	1.90	2.44	97.56
	12.50	12.31 ± 0.05	0.45	1.53	98.47
	25.00	24.87 ± 0.34	1.37	0.52	99.48
(3) SMV					
Intraday	5.00	4.98 ± 0.04	0.78	0.36	99.64
	20.00	20.15 ± 0.36	1.77	0.74	100.74
	12.50	12.49 ± 0.05	0.40	0.09	99.91
Interday	5.00	4.80 ± 0.06	1.21	3.95	96.05
	20.00	19.80 ± 0.32	1.63	0.99	99.01
	12.50	12.22 ± 0.24	1.99	2.26	97.74

[a] Mean of three determinations.
[b] % Error=[(taken concentration − mean found concentration) × 100]/taken concentration.

the determination of the studied drugs in their dosage forms was also evaluated [15]. The observed tablet assay results using the proposed method were compared with those obtained from a second, well characterized reference method [9, 17] (Table 3). The two reference methods were HPLC methods with UV detection. The first method was for the determination of ATO and EZE with detection at 254 nm while the other one was for the determination of SMV and EZE with detection at 240 nm.

Precision. The interday and intraday precision of the proposed method was determined by analyzing three replicates of three different concentrations of each drug for three successive days (n = 9). The relative SD values were not more than 1.77 and 1.99% for the intraday and interday precision; respectively (Table 2).

System suitability. Six replicate injections of a standard mixture solution containing 17.5 ng mL^{-1} of the studied

Table 3. Assay data for the analysis of EZE, ATO and SMV in combined dosage forms by the proposed and reference methods (n = 3)

	(Proposed method)						(Reference method) [9, 17]	
	Concentration taken (ng mL^{-1})		Concentration found (ng mL^{-1})		% Found ± SD		% Found ± SD	
Dosage form	EZE	SMV	EZE	SMV	EZE	SMV	EZE	SMV
Zocozet®	10.00	10.00	9.82	9.80	98.23 ± 0.81	98.00 ± 0.75	98.60 ± 0.24	97.89 ± 0.35
	20.00	20.00	19.78	19.58	98.90 ± 0.29	97.90 ± 0.17	99.40 ± 0.13	98.04 ± 0.71
	25.00	25.00	25.00	24.66	100.01 ± 0.56	98.64 ± 0.66	99.00 ± 0.25	98.16 ± 0.44
Mean ± SD					99.05 ± 0.90	98.18 ± 0.40	99.00 ± 0.40	98.16 ± 0.14
t					0.082 (2.78)	0.613 (2.78)		
F					5.051 (19.00)	8.808 (19.00)		
Atoreza®	*EZE*	*ATO*	*EZE*	*ATO*	*EZE*	*ATO*	*EZE*	*ATO*
	10.00	10.85	9.84	10.68	98.40 ± 0.48	98.47 ± 1.26	98.22 ± 0.65	99.87 ± 0.92
	20.00	21.70	19.98	21.52	99.90 ± 0.19	99.19 ± 1.01	100.02 ± 0.55	98.16 ± 0.85
	25.00	27.13	24.98	27.28	99.91 ± 0.66	100.57 ± 1.75	97.88 ± 0.24	99.03 ± 0.77
Mean ± SD					99.40 ± 0.87	99.41 ± 0.51	98.71 ± 1.15	99.02 ± 0.86
t					0.837 (2.78)	0.492 (2.78)		
F					1.752 (19.00)	1.555 (19.00)		

The figures between parentheses are the tabulated values of t and F at P = 0.05 [16].

Table 4. Chromatographic parameters for the LC-MS analysis for a mixture solution of EZE, ATO and SMV, Claimed concentration is 17.5 ng mL^{-1} (n = 3)[a]

Drug	tR (min)	$W_{h/2}$	K'	R	α	T	N
EZE	2.21	0.066	1.21			1.08	6256
ATO	2.46	0.076	1.46	2.06	1.21	1.10	5725
IS	3.25	0.084	2.25	5.82	1.54	1.07	8320
SMV	8.75	0.205	7.75	22.37	3.44	1.00	10,089

[a] tR: retention time, $W_{h/2}$:Peak width at half height, K': Capacity factor, R: resolution, α: selectivity coefficient, T: USP tailing factor and N: plate count.

drugs in methanol were performed to test the system suitability. The relative standard deviation values of the peak area ratio, the number of theoretical plates, the retention time and the USP tailing factor for the three drugs were not more than 1.64, 1.72, 0.12, and 1.78%; respectively. All the tested parameters for the proposed method showed RSD values lower than 2.00%, proving its suitability for the intended applications.

Specificity. Specificity of the method was established by verifying the purity of each drug peak. The results revealed that all the peaks were totally separated, and no co-eluted peaks were observed. The purity factors were within the calculated threshold limit. The use of SIM mode, where only the selected ions corresponding to the studied drugs were monitored ensured the selectivity of the method. Additionally, the peak areas were calculated from the extracted ion chromatogram (EIC) of each ion to avoid any interference. The LC-MS chromatographic performance parameters like tailing factor, resolution, selectivity coefficient, and plate count of the separated peaks were within the acceptable range as shown in Table 4 and Fig. 3.

The matrix effect on ions suppression or enhancement of the MS response has been studied. Six lots of blank human plasma from different sources were tested. The matrix factor (MF) of each analyte and the IS normalized MF were calculated for each lot. MF is the analyte peak area ratio in the presence of matrix (blank spiked with analyte after extraction) divided by the peak area ratio in absence of matrix (pure analyte solution). The ratio of the MF of the analyte to that of the IS is the IS normalized MF. Two analytes concentrations at 5.00 and 7.50 ng mL^{-1} were studied. The MF at 5.00 ng mL^{-1} was 0.99 ± 0.03, 0.98 ± 0.05, and 0.99 ± 0.06 for EZE, ATO, and SMV, respectively. The RSD of IS normalized MFs of the six plasma lots were not more than 4.93% for the three studied drugs. Thus, plasma matrix did not appear to interfere significantly with the method.

Robustness. The stability of the chromatographic parameters including peak area ratio, tailing factor, capacity factor and plate count after deliberate changes in the proposed method proved its robustness. The studied parameters were acetonitrile ratio in the mobile phase (65 ± 1% v/v), formic acid concentration (0.1 ± 0.02% v/v), the column temperature (25 ± 5 °C) and using other column of similar composition but from another lot. After changing each of these parameters, a mixture solution containing 17.5 ng mL^{-1} of the studied drugs was injected to observe any change in the performance parameters. The results proved the robustness of the proposed method against minor changes in its parameters.

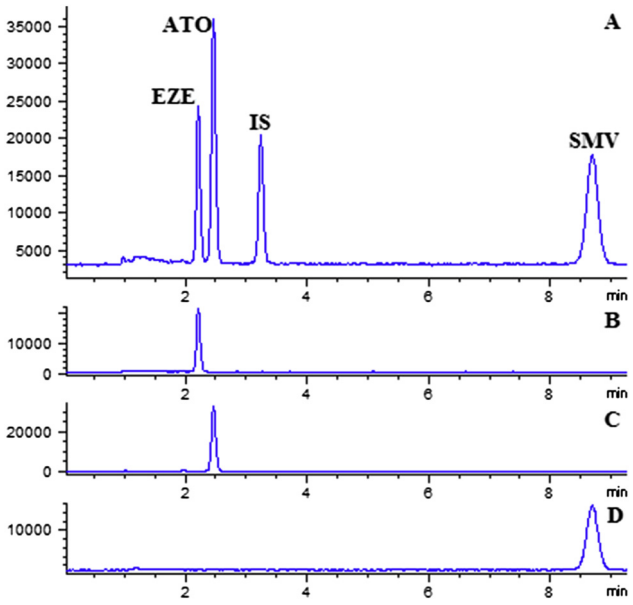

Fig. 3. Representative total ion chromatogram (TIC) for the LC-MS analysis of the studied drugs (A) and extracted ion chromatograms (EICs) of EZE (B), ATO (C) and SMV (D)

Stability of sample solution. The results revealed that the stock standard solutions were stable for at least three weeks when kept protected from light in refrigerator (−20 °C). Working standards and sample solutions were stable in the auto-sampler for at least 5 h at room temperature. The stability of these solutions was studied by performing the analysis and observing any changes in the chromatographic pattern, compared with freshly prepared solutions.

Applications

Analysis of commercial formulations. The proposed LC-MS method was applied for the simultaneous determination of EZE with SMV in Zocozet® tablets and EZE with ATO in Atoreza® tablets (Fig. 4). Three Working sample solutions

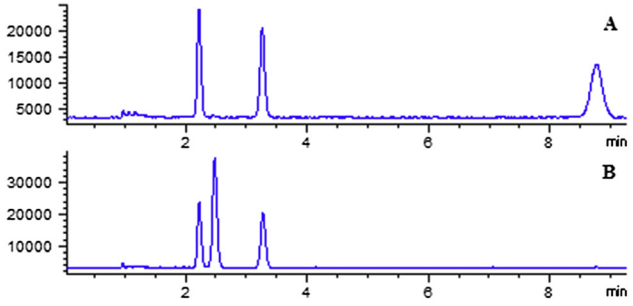

Fig. 4. Representative total ion chromatogram (TIC) for the LC-MS analysis of Zocozet® (A) and Atoreza® (B) tablets

Table 5. Application of the proposed method to the determination of the studied drugs in spiked human plasma

Concentration taken (ng mL^{-1})			Concentration Found (ng mL^{-1})			% Recovered		
EZE	ATO	SMV	EZE	ATO	SMV	EZE	ATO	SMV
1.25	1.25	1.25	1.27	1.23	1.21	101.89	98.21	97.04
5.00	5.00	5.00	4.90	4.99	4.86	98.01	99.85	97.11
7.50	7.50	7.50	7.43	7.35	7.57	99.11	98.00	100.91
Mean ± SD						99.67 ± 2.00	98.69 ± 1.01	98.35 ± 2.21
% RSD						2.01	1.03	2.25

were prepared as described under experimental section where, three replicate determinations of each solution were performed. The concentration of each drug was calculated from its corresponding regression equation depending on its peak area ratio. That was followed by comparing the results obtained by the proposed LC-MS method with other independent measurements series of a reported reference method [9, 17]. Statistical analysis [16] using Student's *t*-test and variance ratio *F*-test for these results showed no significant differences between the performance of the two methods regarding accuracy and precision respectively (Table 3).

Biological application. The sensitivity of the proposed method allowed the determination of the studied drugs in spiked human plasma. Following oral administration of a 20-mg dose of ATO to 8 h fasting volunteers, maximum plasma concentrations for the young volunteers reached 12.7 ng mL^{-1} within 1–2 h and 18.1 ng mL^{-1} for the elderly [18]. On the other hand, when EZE was given in a dose of 20 mg to healthy male volunteers, peak plasma concentrations of free, conjugated, and total EZE were 5.21, 61.2, and 64.2 ng mL^{-1} [19]. SMV is an inactive pro-drug which is metabolized to SMV β-hydroxy acid analog which is an inhibitor of HMC-CoA reductase [20]. When healthy male volunteers were administered a single 40 mg dose of SMV, the peak plasma concentration of the active inhibitors was 10.3 ng eq mL^{-1} and total inhibitors 34.5 ng eq mL^{-1} [21]. According to literature and the previously mentioned data, the concentration of the three drugs in plasma could be measured using the proposed method.

A linear relationship was established between the peak area ratio and the drug concentration in plasma after application of the described extraction method. The regression equations were:

$$y = 0.1528x + 0.0124 (r = 0.9994) \text{ for EZE}$$
$$y = 0.2749x - 0.003 (r = 0.9994) \text{ for ATO}$$
$$y = 0.3302x - 0.006 (r = 0.9998) \text{ for SMV}$$

where y is the peak area ratio and x is the concentration in ng mL^{-1}. The obtained results for the determination of EZE, ATO, and SMV in spiked plasma were abridged in Table 5. The intraday and interday accuracy and precision for the determination of the studied drugs in plasma was also studied. The percent recoveries were within the acceptable range with RSD values not more than 2.25 and 2.91% for intraday and interday precision, respectively.

Dilution integrity was studied to check if diluting plasma samples will affect the accuracy and precision of the proposed method or not. Six samples of human plasma containing 50 ng mL^{-1} of each drug were ten-fold diluted with blank plasma. The concentrations obtained after extracting and analyzing theses samples were compared with those of undiluted plasma sample containing 5 ng mL^{-1} of each drug. After ten-fold dilution of the six samples, the accuracy was not less than 96.87 ± 3.77% and the RSD value was less than 3.08% (n = 6). Therefore, the proposed method allowed dilution of concentrated samples that exceed the calibration curve range.

CONCLUSION

A sensitive validated single quad LC-MS method was developed for the simultaneous determination of EZE, ATO, and SMV. The three drugs were totally separated within a total run time equals 9.3 min with a resolution factor not less than 2.06. The method was successfully applied for the determination of the studied drugs in two combined tablet formulations. The high sensitivity of the developed method allowed the determination of the drugs in spiked human plasma where, a SPE method was optimized for extracting the three drugs from plasma. The good validation criteria of the proposed method rendered it applicable for the routine quality control purposes. Also, high percent recoveries from plasma made it suitable for application in bioequivalence and pharmacokinetic studies.

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