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ORIGINAL ARTICLE

Simultaneous determination of ezetimibe and simvastatin in rat plasma by stable-isotope dilution LC-ESI–MS/MS and its application to a pharmacokinetic study

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Abstract A simple, sensitive and specific liquid chromatography–tandem mass spectrometry method was developed for simultaneous quantification of ezetimibe and simvastatin in rat plasma. The deuterium isotopes: ezetimibe d_4 and simvastatin d_6 were used as internal standards for ezetimibe and simvastatin, respectively. MS/MS detection involved a switch of electron spray ionization mode from negative to positive at retention time 3.01 min. Samples were extracted from plasma by liquid–liquid extraction using tertiary butyl methyl ether. Chromatographic separation was achieved with Agilent Eclipse XBD-C_{18} column using mobile phase that consisted of a mixture of ammonium acetate (pH4.5; 10 mM)–acetonitrile (25:75 v/v). The method was linear and validated over the concentration range of 0.2–40.0 ng/mL for simvastatin and 0.05–15.0 ng/mL for ezetimibe. The transitions selected were *m/z* 408.3→271.1 and *m/z* 412.0→275.10 for ezetimibe and ezetimibe d_4, and *m/z* 419.30→285.20 and *m/z* 425.40→199.20 for simvastatin and simvastatin d_6. Intra- and inter-batch precisions for ezetimibe were 1.6–14.8% and 2.1–13.4%; and for simvastatin 0.94–9.56% and 0.79–12%, respectively. The proposed method was sensitive, selective, precise and accurate for the quantification of ezetimibe and simvastatin simultaneously in rat plasma. The method was successfully applied to a pharmacokinetic study by oral co-administration of ezetimibe and simvastatin in SD rats.

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1. Introduction

Hypercholesterolemia is one of the important risk factors for coronary artery disease and also a major cause of death in the industrialized and highly developed countries. Reduction in the total cholesterol and

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low density lipoprotein cholesterol (LDL) decreases the chances of coronary artery disease. Serum cholesterol is obtained both endogenously from biosynthesis and exogenously from the diet. Combination therapy with two or more hypolipidemics that act by different mechanisms is often more useful than treatment with single agent. Ezetimibe (EZE) localizes in the small intestine and acts at the brush border, thus inhibits the passage of dietary and biliary cholesterol across the intestinal wall [1]. Simvastatin (SV) hydrolyzes to form a β, Δ-dihydroxy acid, which is an active metabolite, structurally similar to β-hydroxy-β-methylglutaryl coenzyme A (HMG-Co A), a key intermediate in cholesterol biosynthesis. Hydrolyzed simvastatin competes for HMG-CoA reductase, this leads to decrease in cholesterol biosynthesis [2]. Co-administration of EZE and SV resulted in a dose dependent reduction of low density lipoprotein (LDL) and total cholesterol with no effect on high density lipoprotein (HDL) cholesterol and triglycerides. Thus combination therapy exerts synergistic effect by different mechanisms in reducing serum cholesterol derived from different routes. Hence co-administration of these two agents has beneficial effects in the treatment of hypercholesterolemia and thus in reducing the risk of coronary artery disease [3].

During preclinical and clinical investigations large numbers of biological samples are generated for quantitative analysis. This demands an efficient, reliable and rapid analytical method for the analysis of such a large pool of samples. Liquid chromatography–mass spectroscopy (LC–MS/MS) is one such analytical techniques which meets the above criteria. Several methods were reported for the quantification of EZE separately in biological fluids by LC–MS/MS [4,5], gas chromatography–mass spectroscopy [6], and with its phases I and II metabolites by high performance liquid chromatography (HPLC) with UV detection [7]. Similar methods were reported for the determination of SV individually in plasma samples by HPLC [8], with simvastatin acid [9–11] and with metoprolol [12] by LC–MS/MS. The available methods are used either for the determination of SV or EZE individually or for the determination of these analytes along with its metabolites or with other drugs. Simultaneous determination of EZE and SV from biological fluids has not been reported in the literature. Hence the aim of our work was to develop a simple, sensitive, rapid and validated bioanalytical method for the simultaneous quantification of EZE and SV in rat plasma.

Present work focuses on the development of rapid and reliable bioanalytical method for simultaneous quantification of EZE and SV in rat plasma using EZE d_4 and SV d_6 as deuterated internal standards respectively. Further this method was applied to a pharmacokinetic (PK) study in rats. In terms of combination EZE and SV were ionized efficiently in the negative and positive ionization modes, respectively. This demands the switch of electron spray ionization mode from negative to positive during analysis and separate internal standard for each analyte. The developed method is expected to provide a benchmark for pharmacokinetic, bioavailability and bioequivalence studies of EZE and SV.

2. Materials and methods

2.1. Chemicals and reagents

The reference standards EZE and SV were obtained from Dr. Reddy Labs, Hyderabad, India and internal standards of deuterium isotopes; EZE d_4, and SV d_6 were purchased from Sigma Aldrich, India. HPLC grade ammonium acetate and acetonitrile were purchased from Merck, India. All other chemicals are of AR grade from Merck, India.

2.2. Instrumentation

Liquid chromatographic system consisting of an Agilent 1200 series, an auto sampler and API 4000 Q Trap mass spectrometer, electrospray ionization (ESI) source in the negative as well as the positive ion mode for both analytes and internal standards (IS) with multiple reaction monitoring (MRM) was employed during the study.

2.3. Chromatographic conditions

Agilent Eclipse XDB-C_{18} column (4.6 mm × 150 mm, 5 μm) with a column oven temperature of 45.0 °C was employed. The mobile phase used was ammonium acetate (pH 4.5; 10 mM)–acetonitrile (25:75, v/v). The flow rate was 0.75 mL/min and injection volume was 20 μL with a total run time of 7 min.

2.4. Mass spectrometric conditions

ESI in both positive and negative ionization modes with MRM was employed to acquire the mass spectra. In mass spectrometry, the parameters including temperature, flow rate of curtain gas and collision gas and collision energy were optimized.

2.5. Preparation of solutions

Primary stock solutions of 1 mg/mL each of EZE, EZE d_4, SV and SV d_6 were prepared in methanol and stored at 2–8 °C. From primary stock solutions, stock dilutions of EZE ranging from 2.5 to 750 ng/mL and SV from 10 to 2000 ng/mL were made with 60% acetonitrile in water (diluent). From this, a series of eight mixed calibration curve (CC) standards were prepared. Concentrations ranging from 0.05 to 15.0 ng/mL for EZE (0.05, 0.1, 0.25, 0.75, 2.0, 5.0, 10.0 and 15.0 ng/mL) and 0.2 to 40.0 ng/mL for SV (0.2, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0 and 40.0 ng/mL) were prepared in K_2EDTA (potassium salt of ethylene diamine tetra acetic acid) rat plasma using above stock dilutions and labeled them as CC1 to CC8. QC samples were prepared at the concentrations of 0.05, 0.15, 1.00, 6.00 and 12.00 ng/mL for EZE and 0.2, 0.6, 2.0, 15.0 and 32.0 ng/mL for SV in K_2EDTA rat plasma and labeled them as lower limit of quantitation (LLOQ), low concentration (LQC), medium concentration 1 (MQC1), medium concentration 2 (MQC2), and high concentration (HQC) quality control samples, respectively. This IS stock solution was diluted with 60% acetonitrile in water solution to get a concentration of 0.5 μg/mL of EZE d_4 and 0.25 μg/mL of SV d_6.

2.6. Extraction procedure

The blank, calibration curve standards and quality control samples were withdrawn from the freezer and allowed to thaw. After post-thawing, the samples were vortexed to ensure complete mixing of the contents. 20 μL of 60% acetonitrile in water was added to a vial labeled as plasma blank. 20 μL each of SV d_6 (0.250 μg/mL) and EZE d_4 (0.50 μg/mL) were added to all pre-labeled vials and labeled as plasma blank with IS, calibration curve standards (CC1–CC8) and quality control samples (LLOQ, LQC, MQC1, MQC2 and HQC). To these vials, either 100 μL of blank plasma or spiked plasma was added as per the requirement. The samples were vortexed to ensure complete mixing of the contents. To all the vials, 2.5 mL of tertiary butyl methyl ether (TBME) was added and were kept on shaker for 15 min and centrifuged at 4000 rpm at

20 °C for 10 min. The supernatant organic layer was transferred to pre-labeled vials. This layer was evaporated under a stream of nitrogen gas at 45 °C. The residue was reconstituted with 0.20 mL of mobile phase for plasma samples. These samples were vortexed and loaded in auto-injector vials. 20 µL of samples was injected onto LC–MS/MS system.

2.7. Method validation

To meet its requirements, the developed bio-analytical method was validated according to FDA guidance [13]. The selectivity was determined by checking the blank plasma obtained from six lots.

Samples were processed according to the above procedure and injected into the LC–MS/MS system in order to determine the extent of interference from endogenous sample components at the retention times of EZE, EZE d_4, SV and SV d_6. Any interfering compounds those eluted along with the analyte (free ezetimibe/simvastatin) were compared with the respective mean peak area responses of six extracted LLOQ samples (prepared from any one of the six plasma lots).

2.7.1. Linearity

To determine linearity, eight mixed calibration curve standards were prepared over the concentration range of 0.05–15.0 ng/mL for EZE and 0.20–40.0 ng/mL for SV ($n=3$). Calibration curves were plotted between ratios of analyte to IS concentration on the *x*-axis *versus* peak area ratio on the *y*-axis. The lowest concentration on the calibration curve with the detector response five times larger than blank plasma response (precision of $\leq 20\%$ and accuracy of $\pm 20\%$) was considered as LLOQ.

2.7.2. Recovery

Recovery of EZE and SV was determined at low, medium and high QC concentrations ($n=6$) by comparing responses from extracted plasma samples with that from unextracted plasma samples. Recovery of internal standards EZE d_4 and SV d_6 was also determined in the same way.

2.7.3. Accuracy and precision

Accuracy and precision was determined for both intra- and inter-batch QC samples. Intra-batch accuracy and precision was determined by analyzing six replicates within the same batch for all the five QC samples. Inter-batch accuracy and precision was determined by analyzing six replicates (from each batch) of QC samples of two different batches. Precision was determined and expressed as percent coefficient of variation (%CV) and it should be $\leq 15\%$ in all cases except at LLOQ, where it should not exceed 20%. Accuracy was determined by comparing the mean value with the actual value and it should be $\pm 15\%$ of the actual value except at LLOQ, where it should not exceed $\pm 20\%$ of the actual value [13].

2.7.4. Matrix effect

Matrix effect was determined for both the analytes at low and high QC concentrations by spiking the samples to the extracted plasma. Matrix factor (MF) was determined by comparing peak response in the presence of matrix ions (spiked samples) with that in the absence of matrix ions (aqueous standards at low and high QC concentrations).

$$\mathrm{MF} = \frac{\text{Peak response in the presence of matrix ions}}{\text{Mean peak response in the absence of matrix ions}}$$

2.7.5. Stability studies

Stability studies were carried out for low and high QC samples as long term and short term stability, freeze–thaw stability and stability of processed samples [13]. Long term and short term stability were determined by keeping the samples at −20 °C for 1 month and at room temperature for 6 h. Freeze–thaw stability was assessed by storing the samples at −20 °C for 24 h and then thawing at room temperature. After such three freeze–thaw cycles the stability was determined. Stability of the processed samples was determined by keeping the samples in an auto sampler at 20 °C for 24 h. In all cases the samples are said to be stable if the nominal values obtained are within $\pm 15\%$ of the back calculated calibration curve concentrations.

2.8. Application of LC–MS/MS method to a pharmacokinetic study

The developed LC–MS/MS method was successfully applied to a pharmacokinetic study by co-administration of EZE and SV to six male Sprague-Dawley (SD) rats by oral route using BD syringe attached with oral gavage needle (size 18) at the dose of 1 and 8 mg/kg body weight, respectively. Required quantity of test item (3.03 mg EZE and 30.17 mg SV) was accurately weighed and transferred to a mortar and triturated with 2% (w/w) Tween 80 using a pestle to make a smooth paste. Then, 0.5% (w/w) carboxymethyl cellulose (CMC) was gradually added and suspended to make a required concentration (1 mg/mL of EZE and 8 mg/mL of SV). The final suspension was kept under continuous stirring till the dose administration.

Approximately 0.25 mL of blood samples from each anesthetized (isoflurane) rat at pre-determined time intervals was collected from the retro-orbital plexus using a capillary tube into pre-labeled eppendorf tubes containing 10% of K_2EDTA anticoagulant (20 µL). The time intervals for the sample collection were 0 (Predose), 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h (post-dose). The total blood volume collected from each rat was ~2.2–2.4 mL which does not exceed the maximal recommended blood volume of 20% (3.2 mL for a 250 g body weight rat) [14]. Plasma was obtained by centrifuging blood samples at 3000 rpm for 10 min. The obtained plasma samples were transferred into pre-labeled microcentrifuge tubes and stored at −20 °C. All the samples were analyzed by the developed method. Pharmacokinetic parameters were calculated by non-compartmental analysis by using Win Nonlin® 6.2 software. Concentrations obtained from the above bio-analytical method were compiled.

3. Results and discussion

3.1. Method optimization

Chromatographic conditions were optimized by using different ratios of acetonitrile-buffer and methanol-buffer as mobile phase and by employing various columns such as Hypersil, Chromosil and Agilent Eclipse. Better chromatographic separation was achieved with Agilent Eclipse XBD-C18 column using mobile phase consisting of a mixture of ammonium acetate (pH 4.5;

10 mM)–acetonitrile (25:75, v/v). These conditions provided a good peak shape to both analytes and IS and signal to noise ratio was also good at LLOQ.

Quadrupole mass spectrometer was employed; here parent ion was selected by first quadrupole (Q1) followed by fragmentation of the parent ion with N_2 gas in the collision chamber (Q2) and

Table 1 Optimized MRM conditions of mass spectroscopy applied in the method development.

Parameter	SV	SV d_6	EZE	EZE d_4
Declustering potential	60	60	−67	−67
Entrance potential	10	10	−10	−10
Collision energy	18	18	−20	−20
Collision cell exit potential	8	8	−6	−6
Ion spray voltage	5500	5500	−4500	−4500

All values are in volts.

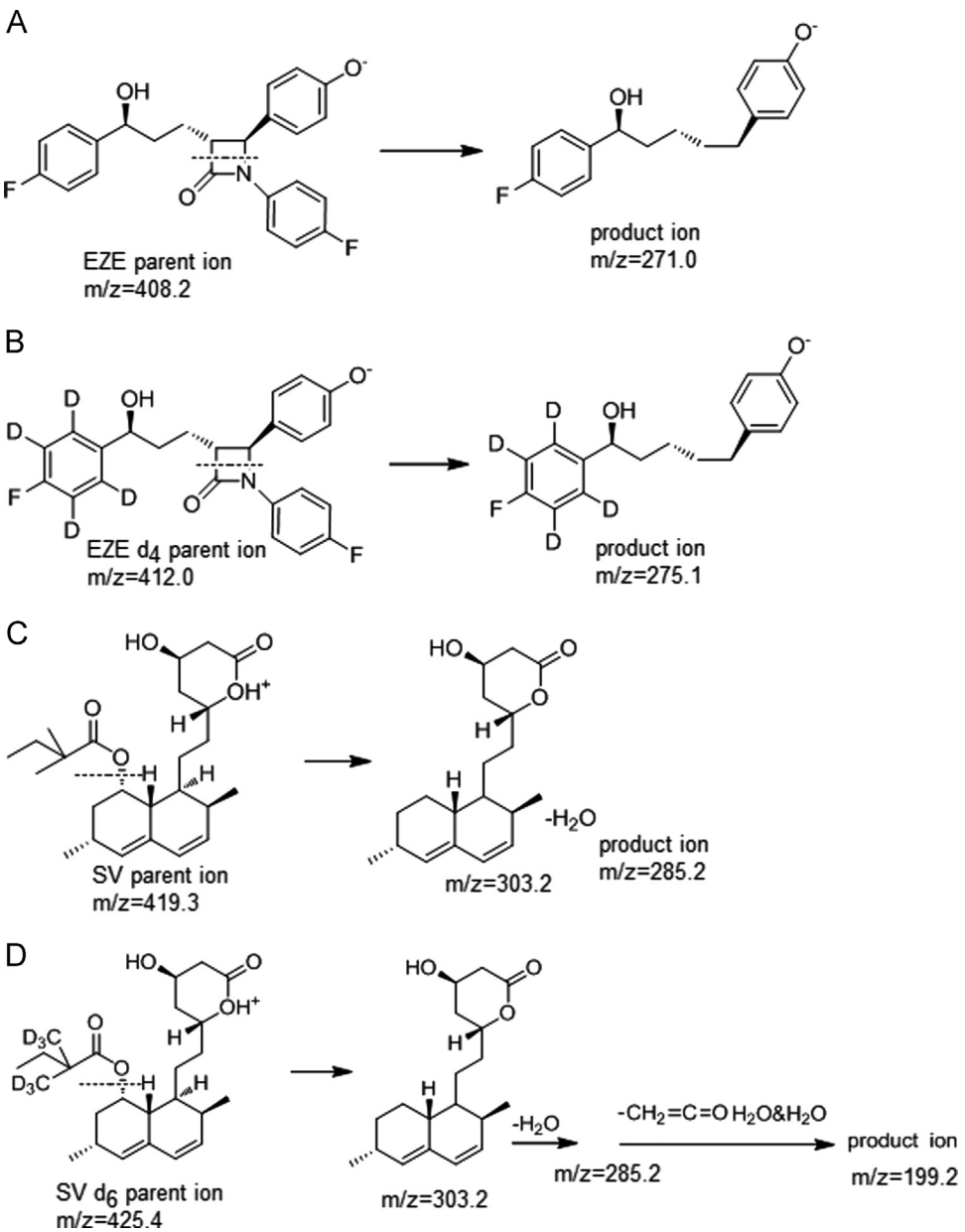

Fig. 1 Product ions of (A) ezetimibe (EZE) $(M-H)^-$, (B) ezetimibe d_4 (EZE d_4) $(M-H)^-$, (C) simvastatin (SV) $(M+H)^+$ and (D) simvastatin d_6 (SV d_6) $(M+H)^+$.

then full product ion scan in the third quadrupole (Q3). In mass spectrometry, the parameters including temperature, flow rate of curtain gas (20 psi), collision gas (medium), gas-1 (35.0 psi), gas-2 (45.0 psi) and temperature (450.0 °C) were optimized to obtain maximum response of the fragment ion. The optimized MRM conditions of mass spectroscopy applied in the method development are shown in Table 1.

Internal standard selected should always have similar chromatographic and ionization properties, and recovery of the analyte. Based on these properties deuterated analogs of EZE and SV (EZE d_4 and SV d_6) were chosen as internal standards. EZE and EZE d_4 were ionized efficiently in the negative ionization mode and precursor ions formed were of $(M-H)^-$. The SV and SV d_6 were ionized efficiently in the positive ionization mode and $(M+H)^+$ ions were formed. Hence there was a switch from negative to positive ionization mode at retention time (RT) 3.01 min. In the case of EZE and EZE d_4 fragmentation pattern is the same and it takes place at azetidine ring; and the formation of product ion was at *m/z* 271.10 and 275.10 amu respectively. Fragmentation of SV takes places at the ester side chain [15], followed by loss of water molecule leads to formation of product ion at *m/z* 285.20. For SV d_6 further loss of the neutral molecule (CH_3COOH or $CH_2{=}C{=}O$ and H_2O) leads to the formation of a product ion at *m/z* 199.20. Parent to product ion transitions selected were *m/z* 408.3 → 271.10 and *m/z* 412.0 → 275.10 for EZE and EZE d_4 respectively; *m/z* 419.30 → 285.20 and *m/z* 425.40 → 199.20 for SV and SV d_6 respectively, as shown in Fig. 1. Total chromatographic runtime was 7.0 min with RT of 2.32 and 2.31 min for EZE and EZE d_4; 5.98 and 5.91 min for SV and SV d_6 respectively, as shown in Figs. 2 and 3.

ESI in both positive and negative modes was employed to detect both analytes. But the presence of endogenous sample components may affect the ionization of analytes either by ion suppression or enhancement during ESI. This in turn affects the sensitivity, precision and recovery of the analyte. The extent of this effect mainly depends upon the extraction procedure and is also compound dependent. Compared to protein precipitation LLE is helpful in producing a clean sample spectroscopically and avoids introduction of plasma components and non volatile materials. Hence LLE was employed to extract the analyte and IS from plasma. Different solvents like hexane, dichloromethane, diethyl ether and TBME were tried as solvents. Efficient extraction of both analytes and IS was obtained by addition of 2.5 mL of TBME to 100 µL of plasma. Clean chromatograms were obtained with no significant matrix

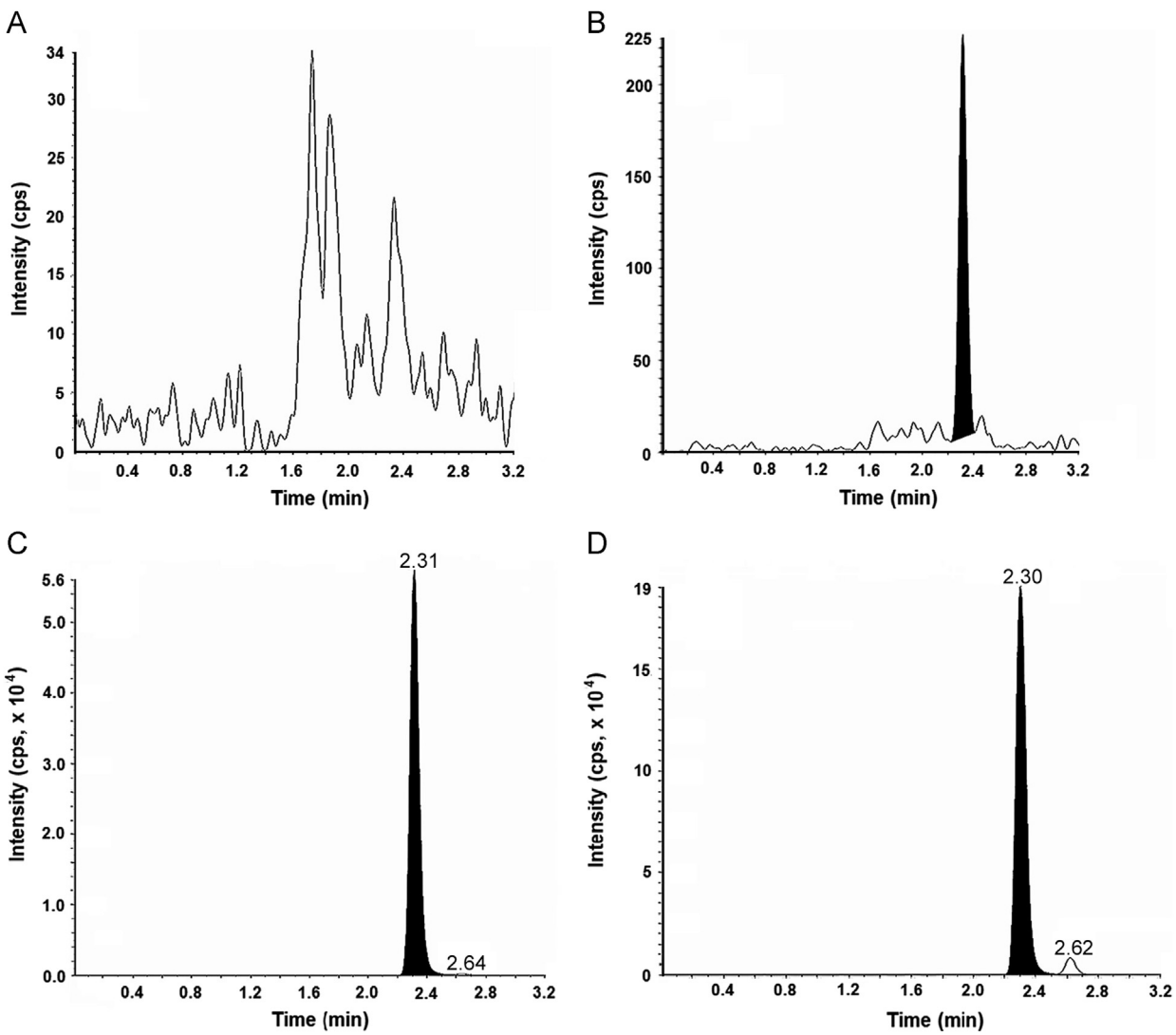

Fig. 2 Chromatograms of spiked ezetimibe with rat plasma. (A) blank plasma, (B) LLOQ of ezetimibe, (C) ULOQ of ezetimibe and (D) ezetimibe d_4.

effects as shown in Figs. 2 and 3. Recoveries of analyte and IS were consistent and reproducible. LLE procedure adopted using TBME was found to be suitable for simultaneous extraction of all the four components from rat plasma.

3.2. *Method validation*

Selectivity of the method was determined by comparing chromatograms of blank plasma from six different lots. No significant

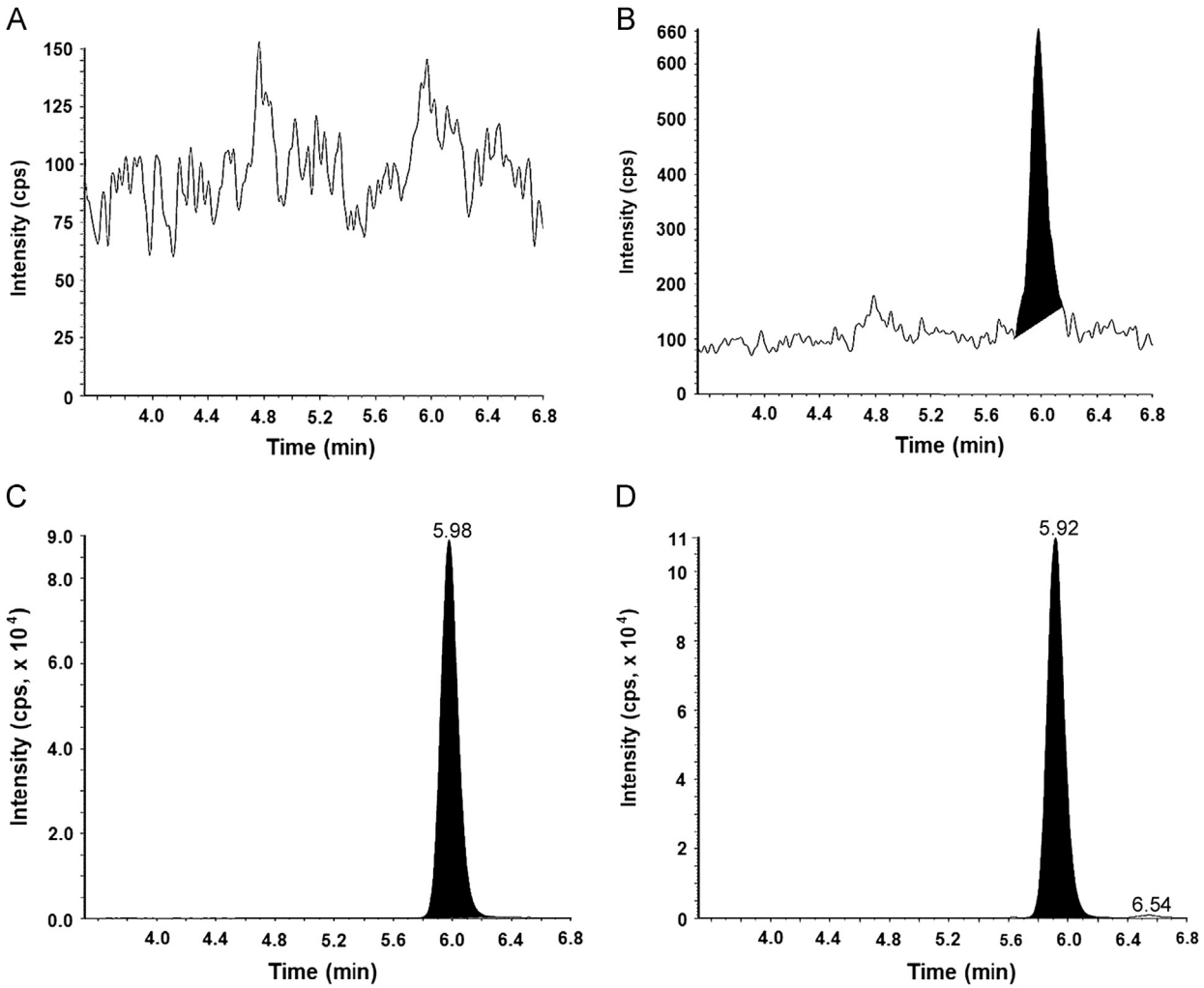

Fig. 3 Chromatograms of spiked simvastatin with rat plasma (A) blank plasma, (B) LLOQ of simvastatin, (C) ULOQ of simvastatin and (D) simvastatin d_6.

Table 2 Recovery of EZE and SV from matrix samples ($n=6$).

Analyte	Conc. (ng/mL)	Mean recovery (ng/mL)	CV (%)[a]	RE (%)[b]
EZE	0.15	0.14	11.49	−6.67
	6.0	5.79	8.12	−3.50
	12.0	11.27	7.34	−6.10
SV	0.6	0.51	5.05	−15.00
	15.0	14.02	1.73	−6.50
	32.0	31.2	2.37	−2.50
EZE d_4	500.0	451.4	6.32	−9.70
SV d_6	250.0	229.9	7.10	−8.10

[a]Coefficient of variation.
[b]Relative error.

interfering peaks were observed at the retention times of analytes and IS. The peak areas of blank samples at the RT of analytes were $\leq 20\%$ and those at RT of IS were $\leq 5\%$ compared to peak area of extracted LLOQ samples. Chromatograms of blank plasma, blank plasma spiked with EZE, SV, EZE d_4 and SV d_6 are shown in Figs. 2 and 3.

The method was linear over the range of 0.20–40.0 ng/mL for SV and 0.05–15.0 ng/mL for EZE. A straight line fit was made through the data points by $1/x^2$ weighting method and was found to be best fit for EZE and SV. The regression equations were calculated as $y=0.0203x+0.0000647$ for EZE and $y=0.0214x+0.000901$ for SV. The observed correlation coefficient (R^2) was greater than 0.999 in both the cases. Hence the method is linear in the stated range. The LLOQ of EZE and SV were 0.05 and 0.2 ng/mL respectively. Such low concentrations are suitable for pre-clinical and clinical PK studies.

The recovery of EZE and SV from rat plasma was determined by comparing peak areas from extracted standard samples with those from unextracted standard samples. The percentage recovery of EZE was 93.9–96.5% and for SV it was 93.5–97.5%. The percentage recovery of EZE d_4 and SV d_6 was 90.27% and 91.94% respectively. The results are shown in Table 2.

Both inter- and intra-batch precision and accuracy were determined and expressed as %CV (coefficient of variation) and % accuracy respectively. The %CV observed for EZE for the inter-batch QC samples was 2.10–13.46% and for intra-batch QC samples it was from 2.0% to 14.82%. The %CV observed for SV in the inter-batch QC samples was ranged from 0.79% to 12.00% whereas in the intra-batch QC samples it was from 0.57% to 12.39%. The % accuracy observed for EZE for inter- and intra-batch QC samples was 92.9–108% and 92.9–112% respectively. The % accuracy observed for SV for inter- and intra-batch QC samples ranged from 94.69% to 102.50% and 92% to 103% respectively. The results are summarized in Table 3.

To determine the extent of interference between analytes and biological matrix, the overall precision of matrix factor (MF) was determined. The MF as calculated by the %CV was found to be less than 15% for both analytes, which was within the acceptance criteria. The results are shown in Table 4.

Stability studies were performed under a variety of conditions and the results are given in Table 5. The analytes were stable at room temperature (25–30 °C) for a minimum of 6 h; unaltered to a significant extent at −20 °c for 1 month and good % recoveries were obtained. In an auto sampler kept at 20 °C samples of analytes were stable for 24 h. The freeze–thaw stability results indicated that analytes were stable for three cycles. Results showed that plasma samples under study can be freezed and thawed without compromising the reliability of the samples.

Table 3 Intra- and inter-batch precision and accuracy of EZE and SV ($n=6$).

Analyte	Conc. (ng/mL)	Intra-batch			Inter-batch		
		Amt. conc. ±SD[a] (ng/mL)	CV (%)[b]	RE (%)[c]	Amt. conc. ±SD[a] (ng/mL)	CV (%)[b]	RE (%)[c]
EZE	0.05	0.049±0.004	8.16	−2	0.049±0.003	6.12	−2
	0.15	0.168±0.025	14.82	12	0.162±0.220	13.46	8
	1.0	0.93±0.02	2.21	−7.2	0.93±0.02	2.16	−7.1
	6.0	5.63±0.09	1.64	−6.08	5.72±0.12	2.10	−4.67
	12.0	11.43±0.28	2.42	−4.77	11.46±0.24	2.13	−4.5
SV	0.2	0.206±0.002	9.56	3.17	0.195±0.023	12.00	−2.5
	0.6	0.619±0.024	3.83	3.2	0.615±0.023	3.85	2.5
	2.0	2.002±0.019	0.96	0.1	1.988±0.035	1.77	−0.6
	15.0	14.53±0.17	1.15	−3.16	14.56±0.13	0.89	−2.93
	32.0	30.22±0.28	0.94	−5.57	30.30±0.24	0.79	−2.19

[a]Standard deviation.
[b]Coefficient of variation.
[c]Relative error.

Table 4 Matrix effect of EZE and SV in rat plasma ($n=6$).

Analyte	Conc. (ng/mL)	Peak response ratio in the absence of matrix ions	Peak response ratio in the presence of matrix ions	Matrix factor±SD[a]	CV (%)[b]
SV	0.6	0.0154	0.0307	1.009±0.008	0.76
	32	0.0304	1.5941	0.982±0.013	1.32
EZE	0.15	0.0065	0.0063	0.964±0.039	3.99
	12	0.4943	0.4949	1.001±0.013	1.27

[a]Standard deviation.
[b]Coefficient of variation.

3.3. *Pharmacokinetic study results*

Blood was collected for 48 h at different time points and the plasma samples were analyzed using the developed method. The following pharmacokinetic parameters were primarily calculated by non-compartmental analysis and the results are shown in Table 6. Peak plasma concentration attained by the drug (C_{max}), time required to attain peak plasma concentration (T_{max}), area under the curve $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$, plasma clearance (Cl_f) and time it takes for a test item undergoing decay to decrease by half ($t_{1/2}$) were determined. Chromatograms in rat plasma after oral administration of EZE and SV and plasma concentration *versus* time profiles are given in Fig. 4. Analytes were detectable in the blood for up to 8–12 h only, after that they were not detectable and attained zero concentrations. Most of the PK parameters were variable and also standard deviation values were also higher due to intra and inter-animal variability. Ezetimibe is rapidly absorbed and extensively metabolized and oral bioavailability is low, due to extensive conjugation and biliary excretion. Thereby, inter and intra-animal variability often seems with ezetimibe in rats [16]. Simvastatin is a lactone which is readily hydrolyzed to the corresponding hydroxyacid and following oral administration simvastatin undergoes extensive first-pass effect in the liver thereby availability of the drug in the systemic circulation is very low. The developed LC–MS/MS method was successfully applied to a pharmacokinetic study following co-administration of EZE and SV (1 mg/kg and 8 mg/kg respectively) in SD rats.

4. Conclusions

The combination of EZE and SV is widely available and also used in the treatment of hypercholesterolemia. There is no published literature found for the simultaneous estimation of these drugs from biological samples. Therefore development of such methods is in most need for the quantification of these drugs simultaneously. Proposed LC–MS/MS method in electron spray ionization with MRM mode was found to be rapid, sensitive, and reliable for the quantification of EZE and SV from rat plasma simultaneously. The developed method was successfully applied to a pharmacokinetic study in rats. This method can be applied in clinical studies, bioavailability and bioequivalence studies.

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Table 5 Stability data of EZE and SV of samples maintained at various conditions ($n=6$).

Stability studies	Analyte	Conc. spiked (ng/mL)	Mean recovery ±SD[a] (ng/mL)	RE (%)[b]	CV (%)[c]
Short term (6 h)	SV	0.6	0.54±0.04	−9.50	8.10
		32.0	29.90±1.68	−6.56	5.60
	EZE	0.15	0.14±0.01	−6.66	4.92
		12.0	10.84±0.76	−9.66	6.99
Long term (−20 °C, 1 month)	SV	0.6	0.54±0.03	−9.33	6.10
		32.0	29.02±1.83	−9.30	6.31
	EZE	0.15	0.14±0.01	−6.66	5.09
		12.0	11.03±0.57	−8.08	5.17
Freeze–thaw (−20 °C and RT, 24 h and 3 cycles)	SV	0.6	0.54±0.04	−10.16	7.58
		32.0	30.61±1.16	−4.34	3.80
	EZE	0.15	0.143±0.004	−4.66	3.07
		12.0	10.89±0.62	−9.25	5.65
Processed samples (24 h)	SV	0.6	0.547±0.035	−8.83	6.51
		32.0	29.63±1.04	−7.25	3.52
	EZE	0.15	0.140±0.005	−6.66	3.94
		12.0	10.89±0.62	−9.27	5.71

[a]Standard deviation.
[b]Relative error.
[c]Coefficient of variation.

Table 6 Pharmacokinetic parameters following oral co-administration of EZE (1 mg/kg) and SV (8 mg/kg) to male SD rats ($n=6$).

Parameter	EZE (Mean±SD)	SV (Mean±SD)
C_{max} (ng/mL)	0.26±0.14	7.58±5.50
T_{max} (h	4.0±9.5	0.92±0.59
$AUC_{(0-t)}$ (ng h/mL)	1.75±1.68	11.12±18.99
$AUC_{(0-\infty)}$ (ng h/mL)	2.33±3.97	11.25±7.47
Cl_f (mL/h/kg)	0.35±0.25	0.92±0.45
$t_{1/2}$ (h)	0.15±7.68	0.43±0.60

SD: Standard deviation.

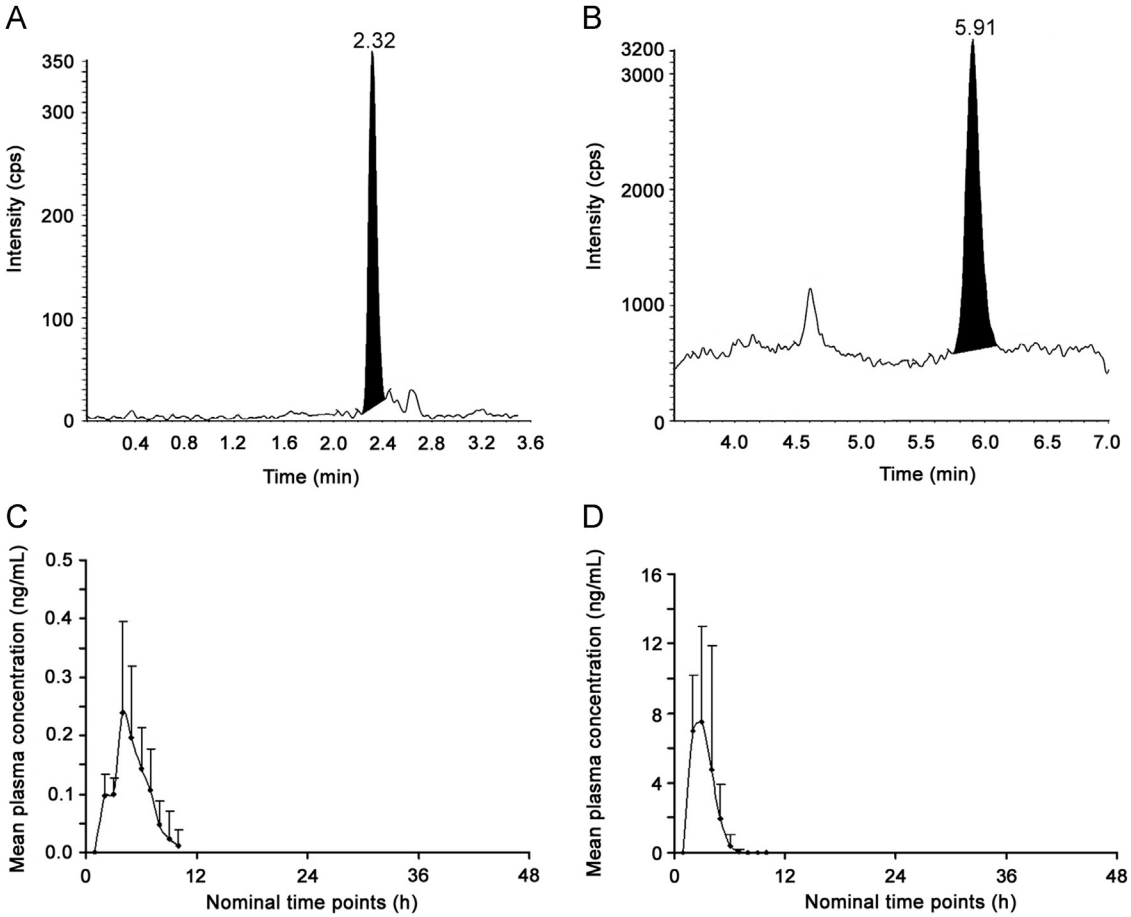

Fig. 4 Chromatograms in rat plasma (A) ezetimibe and (B) simvastatin after oral administration of 1 mg/kg of ezetimibe and 8 mg/kg of simvastatin and mean plasma concentrations of (C) ezetimibe and (D) simvastatin during pharmacokinetic study (means ± SD, $n = 6$).

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