Article

Development and Validation of an LC–MS-MS Method for the Simultaneous Determination of Simvastatin, Simvastatin Acid and Ezetimibe in Human Plasma and Its Application to Pharmacokinetic Study in the Indian Population

Sathish Babu Munaga1,2,*, Rajani Kumar Valluru2, Phani Bhushana Reddy Bonga2, V. Sumathi Rao3, and Hemanth Kumar Sharma3

1Department of Pharmaceutical Sciences, J.N.T. University, Kukatpally, Hyderabad 500072, India, 2Axis Clinicals Limited, 1-121/1, Miyapur, Hyderabad 500 049, India, and 3Aurobindo Pharma Research Center, Bachupalli, Hyderabad, India

*Author to whom correspondence should be addressed. Email: munaga.sathish@gmail.com

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Abstract

A simple, selective, sensitive and high-throughput liquid chromatography–tandem mass spectrometry (LC–MS-MS) method has been developed and validated for the simultaneous quantification of simvastatin (SS), simvastatin acid (SSA, active metabolite of SS) and ezetimibe (EZM) in K2 EDTA containing human plasma, using simvastatin D6, simvastatin acid D3 and ezetimibe D4 as internal standards (ISTDs), respectively. A volume of plasma sample of only 400 µL was processed by the solid phase extraction technique; then 20 µL of processed sample was run on a Phenomenex, Kinetix XB C18, 150 × 4.6 mm, 5 µm column using an isocratic mobile phase consisting of 10 mM ammonium formate buffer (pH 4.0 ± 0.3): acetonitrile (27 : 73, v/v) with a run time of 6.3 min. The precursor and product ions of SSA, EZM and their ISTDs were monitored on a triple quadrupole instrument operated in the negative ionization mode, and SS was monitored in the positive mode. The method was validated over a concentration range of 0.2–80 ng/mL for SS, 0.1–60 ng/mL for SSA and 0.05–15 ng/mL for EZM. The method has been successfully applied in clinical pharmacokinetic study in the Indian population. The Cmax, AUC0–inf and Tmax values obtained in our study were 10.61 ± 5.287, 77.58 ± 29.367 and 1.62 ± 0.436 for EZM; 69.74 ± 45.274, 190.71 ± 107.271 and 1.74 ± 0.480 for SS; and 25.36 ± 23.576, 139.24 ± 131.653 and 3.95 ± 0.671 for SSA, respectively.

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 low-density lipoprotein (LDL) cholesterol 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 a single agent (1).

EZM, 1-(4-Fluorophenyl)-3(3)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2 azetidinone (Figure 1), and SS, (++)-1S,3R,7S,8S,8aR]-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]-1-naphthyl-2,2-dimethyl-butan-2-one [Figure 1], combination therapy is used with a proper diet to treat high cholesterol and triglyceride (fats) levels
EJM is a cholesterol absorption inhibitor, localizes in the small intestine and acts at the brush border, thus inhibiting the passage of dietary and biliary cholesterol across the intestinal wall; it is therapeutically beneficial drug that works by inhibiting the protein transporters on small intestinal brush border, which are involved in the active transport of cholesterol. In addition, it also inhibits phytosterol absorption. Specifically, it appears to bind to a critical mediator of cholesterol absorption, the Niemann-Pick C1-Like 1 (NPC1L1) protein in the gastrointestinal tract epithelial cells as well as in hepatocytes. In addition to this direct effect, decreased cholesterol absorption leads to an upregulation of LDL receptors on the surface of cells and an increased LDL-cholesterol uptake into cells, thus decreasing levels of LDL in the blood plasma, which contribute to atherosclerosis and cardiovascular events (4).

Clinical studies have shown that co-administration of ezetimibe (EZM) with statins could provide an additional reduction in LDL-cholesterol as well as total cholesterol (5). In addition, it also inhibits phytosterol absorption. A combination dosage form containing simvastatin (SS) and EZM was approved by the FDA in July 2004 for the treatment of hyperlipidemia (6). This distinct mechanism of action results in a synergistic cholesterol-lowering effect, when used together with statins that inhibit cholesterol synthesis by the liver. SS is an inactive hydrophobic lactone prodrg, which is metabolized in vivo to several more polar, pharmacologically active compounds, most notably the corresponding hydroxy acid form, simvastatin acid (SSA) (7).

After oral administration, EZM is rapidly absorbed and extensively conjugated to EZMG. EZM (free EZM—active form) and EZMG (conjugate) are the major drug-derived compounds detected in plasma. SS is a prodrug that is hydrolyzed to its active β-hydroxycacid form after administration, which is a specific inhibitor of 3-hydroxy-3-methylglutarly-coenzyme A (HMG-CoA) reductase, the enzyme that catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in the biosynthetic pathway for cholesterol (8, 9). Since EZM does not influence the activities of CYP450 enzymes, significant pharmacokinetic interactions with other medications including stains, fibrates, digoxin and warfarin have not been found (10).

Several published analytical methods are available for the estimation of SS and SSA in human plasma and in pharmaceutical formulations (4, 11–21). Yang et al. (14), Suellen Cristina et al. (7) and Khaled Alakhal (22) have developed an LC–MS method for the determination of only SS in human plasma. Abdulrahman et al. (23) developed a method called the Novel and simple LC–ESI-MS-MS method for the bioanalysis of SS and SSA in rat plasma. Sireesha et al. (1) developed a simultaneous LC–MS–MS method for the determination of only EZM and SS in rat plasma. Zhang et al. (13) developed an LC–MS–MS method for simultaneous determination of the SS and SSA in human plasma using an automated liquid–liquid extraction technique, which is less selective and more susceptible to matrix effects in mass spectrometric analysis. Jemal et al. (16) developed an LC–MS–MS method for the simultaneous determination of SS and SSA in human plasma. Various other methods were developed and validated on these drugs either individually or in two-drug combinations like Vierordt’s method (24), HPLC-UV (25), micellar electrokinetic capillary chromatography (26), SS and EZM by only HPLC (5), and only SS by LC–ESI-MS–MS (27).

But none of these consist of triple analyte estimation, namely of EZM, SS and SSA. Nan-Nan et al. (28) developed a simultaneous LC–MS–MS method for the determination of EZM, SS and SSA in human plasma and focused on pharmacokinetics and safety information on the Chinese population, with precise information on analytical methodology without details on method development and validation.

A literature survey revealed that several analytical methods that have been reported for the simultaneous estimation of the above-mentioned drugs individually or in combination with some other drugs in biological samples as well as in pharmaceutical dosage forms: UV spectrophotometry, HPLC and LC–MS–MS (5). All the above reported methods were based on the estimation of EZM, SS and SSA alone or in combination with other drugs, but none of these consist of triple analyte estimation, namely of EZM, SS and SSA. However, most of the available methods have limitations such as long run time, poor resolution, uneconomical and low sensitivity. Owing to the above-mentioned reasons, indeed an attempt has been made to develop a simple, precise, accurate, reproducible and robust liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the simultaneous determination of EZM, SS and SSA in human plasma which can be readily applied for the bioequivalence/pharmacokinetic studies in the Indian population.

As a part of it, we developed and validated an isocratic LC–MS–MS method with polarity switching, using a simple and reproducible solid phase extraction (SPE) procedure for the simultaneous quantification of EZM, SS and SSA in human plasma with a simple single-step extraction method, minimum run time and minimum processed volume of plasma, which has not been reported in previous publications.

EZM rapidly metabolizes to its phenoxyl glucuronide (EZMG) and the current method was developed to estimate free EZM, SS and SSA. During validation, the linearity range for each of the three analytes was considered to support the expected maximum concentration of the respective analytes as per the applicable regulatory requirements (29, 30). Deuterated internal standards (ISTDs) were used for all the three analytes to control the matrix effects during the mass spectrometric analysis. The developed method was successfully used to study the pharmacokinetics of SS and EZM combination tablets (80 + 10 mg), in healthy Indian human volunteers under fed conditions after administering a single dose of 10/80 mg of EZM/SS tablet.

**Experimental**

**Chemicals and reagents**

The reference standard of SS was procured from USP, and the working standards of SSA and EZM were purchased from Vivan Life Sciences.
Pvt Ltd (Mumbai, India). The working standards of simvastatin D6 (SSD), simvastatin acid D3 (SSAD), ezetimibe D4 (EZMD) and EZMG were procured from Clearsynth Labs Pvt Ltd (Mumbai, India). All the used standards have purity ≥99%. LC-MS grade methanol, acetonitrile and n-hexane were purchased from Merck Specialities Pvt Ltd (Mumbai, India). HPLC type I water was obtained from a Milli-Q A10 gradient water purification system (Millipore, Bedford, MA, USA). Drug-free human plasma containing K₂ EDTA anticoagulant was obtained from Laxmi Sai Clinicals (Hyderabad, India). Strata-X polymeric (30 mg, cm⁻²) SPE cartridges were purchased from Phenomenex Inida Pvt Ltd.

Instrumentation
A modular HPLC system (Shimadzu, Kyoto, Japan) consisting of a binary LC-20AD prominence pump, DGU-20A3 solvent degasser, CTO-ASvp column oven and high-throughput SIL. HTC autosampler was used for the analysis. Mass spectrometric detection was performed using a API-4000 triple quadrupole mass spectrometer (MDS SCIEX, Toronto, Canada) equipped with a turbo ionspray interface. Quantification was performed in the multiple reaction monitoring (MRM) mode with polarity switching. Analyst software version 1.5 was used for controlling the hardware and data handling.

Chromatographic conditions
Chromatographic separation was performed on a Phenomenex, Kinetix XB C18, 150 × 4.6 mm, 5 µm analytical column. An isocratic mobile phase consisting of 10 mM ammonium formate buffer (pH 4.0 ± 0.3): acetonitrile (27 : 73, v/v) was delivered at a flow rate of 1 mL/min. The autosampler was set at 10°C and the injection volume was 20 µL. The column oven temperature was maintained at 35°C. The total chromatographic run time was 6.3 min.

Mass spectrometric conditions
The entire run time of 6.3 min was divided into two periods; in the first 4.2 min (Period I) the turbo ion spray interface (TIS) was operated in the negative ionization mode to study the parent → product ion (m/z) transitions of SSA, EZM and their ISTDs. From 4.2 to 6.3 min (Period II), the TIS was operated in the positive ionization mode to study the ion transitions of SS and its ISTD. The details of the specific MRM detection applied for each analyte and ISTD are summarized in Table I. Declustering potential, entrance potential, collision energy and collision exit potential (CXP) were all optimized to allow the result with highest possible signal transduction with low background noise. The pressure of drying gas was set at 45 psi and the temperature was kept at 450°C. The ion spray voltage was set at −4,500 V in the negative mode and at 5,500 V in the positive mode. The pressure of collision gas (nitrogen) was maintained at 6 psi and the quadrupoles 1 and 3 were set at low mass resolution with a dwell time of 200 ms.

Preparation of calibration standards and quality control samples
Individual standard stock solutions of SS, SSA and EZM and ISTDs were prepared by dissolving their accurately weighed amounts in methanol to give a final concentration of 0.2 mg/mL. All the solutions were stored in the refrigerator at below 10°C and were brought to room temperature before use. Working solutions of analytes and ISTDs were prepared by appropriate dilution of their stock solutions in 50% methanol. The working solutions were stored at room temperature and were prepared on a day-to-day basis.

K₂ EDTA anticoagulant blank plasma collected from nonsmoking healthy volunteers was screened individually and pooled before use. Calibration standards and quality control (QC) samples were prepared by spiking (1.5%) working solutions in screened blank plasma. Calibration standards were prepared at concentrations of 0.2, 0.4, 1, 4, 20, 40, 64 and 80 ng/mL for SS, at 0.1, 0.2, 0.5, 2.5, 10, 29, 48 and 60 nmg/mL for SSA and at 0.05, 0.1, 0.25, 1, 4, 8, 12 and 15 ng/mL for EZM.

QC samples were prepared at 0.201 lower limit of quantification quality control (LLOQC), 0.599 low quality control (LQC), 33 medium quality control (MQC) and 61 ng/mL high quality control (HQC) for SS, at 0.101 (LLOQC), 0.299 (LQC), 25 (MQC) and 46 mg/mL (HQC) for SSA and at 0.05, 0.1, 0.25, 1, 4, 8, 12 and 15 mg/mL for EZM.

Sample preparation
A 400 µL aliquot of each plasma sample was transferred to a 5-mL polypropylene tube and 50 µL of ISTD solution (containing 25 ng/mL of EZMD and 100 ng/mL of SSD and SSAD) was added, followed by 0.4 mL of 100 mM sodium acetate buffer (pH 4.0 ± 0.3), and the resulting samples were briefly mixed on a vortex and were subjected to SPE. On the positive pressure SPE unit, Strata-X cartridges (30 mg/cm²) were conditioned with 1 mL of methanol and equilibrated with 1 mL of Milli-Q water. After dispensing of plasma samples, washing of the cartridges was performed with 1 mL of Milli-Q water, 1 mL of n-hexane followed by 1 mL of 30% methanol. Finally, the cartridges were eluted with 0.4 mL of elution solution (10 mM ammonium formate buffer, acetonitrile 20 : 80, v/v; pH 4.0 ± 0.3), the elutes were centrifuged at 4,000 rpm for 5 min and the solution was transferred into pre-labelled autosampler vials. During thawing and processing steps, the samples were maintained at below 10°C with the use of wet ice.

Table I. Detection Masses for Analytes and ISTDs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ionization mode</th>
<th>Retention time (min)</th>
<th>Q1 mass</th>
<th>Q3 mass</th>
<th>Dwell time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>Positive</td>
<td>4.7</td>
<td>419.2</td>
<td>199.0</td>
<td>200</td>
</tr>
<tr>
<td>SSA</td>
<td>Negative</td>
<td>3.2</td>
<td>435.2</td>
<td>319.2 and 115.0</td>
<td>200</td>
</tr>
<tr>
<td>EZM</td>
<td>Negative</td>
<td>1.8</td>
<td>408.1</td>
<td>271.0</td>
<td>200</td>
</tr>
<tr>
<td>SSD</td>
<td>Positive</td>
<td>4.7</td>
<td>425.3</td>
<td>199.3</td>
<td>200</td>
</tr>
<tr>
<td>SSAD</td>
<td>Negative</td>
<td>3.2</td>
<td>438.4</td>
<td>319.2</td>
<td>200</td>
</tr>
<tr>
<td>EZMD</td>
<td>Negative</td>
<td>1.8</td>
<td>412.3</td>
<td>275.0</td>
<td>200</td>
</tr>
</tbody>
</table>

Method Validation
A complete method validation of SS, SSA and EZM in human plasma was done by following the USFDA and EMEA guidelines (29, 30). Validation runs were performed to evaluate selectivity, sensitivity, linearity, precision, accuracy, recovery, matrix effect, dilution integrity and sample stability. Each validation run was organized with a set of spiked standards, blank (with ISTD and without ISTD) and QC samples as per the validation parameters. Standard samples were...
analyzed at the beginning of the run and QC samples were distributed consistently throughout the validation runs.

Selectivity
Selectivity of the method toward endogenous and exogenous components of plasma was evaluated in 14 different human plasma lots including two hemolyzed and two lipemic lots. The blank plasma lots were extracted (without the addition of ISTD) and injected for LC–MS-MS detection. Later, selectivity in each lot was evaluated by comparing the blank peak responses against the mean peak response observed in plasma spiked with the lower limit of quantification (LLOQ) samples (n = 6). The potential for interference from concomitant drugs was also investigated by spiking low QC samples with aceclofenac, ibuprofen, paracetamol, ranitidine and ondansetron.

Linearity
The linearity of the method was assessed using three calibration curves analyzed on 3 different days. Each plot was associated with an eight-point non-zero concentrations spread over the dynamic range. A linear least-squares regression analysis with a weighting factor of 1/X² for EZM and SSA, and 1/X for SS was performed on peak area ratios versus analyte concentrations. Peak area ratios for plasma spiked calibration standards were proportional to the concentration of analytes over the established range.

Precision and accuracy
Intrabatch (within day) and interbatch (between day) precision and accuracy were evaluated at four distinct concentrations (LLOQ, LQC, MQC and HQC) for each analyte. Precision and accuracy at each concentration level were evaluated in terms of %CV and relative error. The extraction recovery of SS, SSA and EZM was determined at LQC, MQC and HQC levels. The relative recoveries were evaluated by comparing the peak areas of extracted samples (spiked before extraction) with those of unextracted samples (blank extracts spiked after extraction).

Matrix effect
The matrix effect was checked at low and high QC levels using 8 different blank plasma lots (including two hemolytic and two lipemic lots). The matrix factor for analyte and ISTD was calculated in each lot by comparing the peak responses of post-extraction samples (blank extracts spiked after extraction) against those of equivalent aqueous samples, prepared in the mobile phase. The ISTD normalized matrix factor in each lot was later evaluated by comparing the matrix factor of analyte and ISTD.

Stability
The stability of analytes in both aqueous solutions and biological matrix was evaluated after subjecting them to different conditions and temperatures that could be encountered during regular analysis. Stability in plasma was evaluated in terms of freeze–thaw stability, benchtop stability, long-term stability and extracted sample stability. Freeze–thaw stability was evaluated after six freeze (at −70°C)–thaw (at room temperature) cycles. Benchtop stability was assessed at 10°C and the long-term stability was evaluated at both −70° and −20°C. Stability in whole blood was evaluated at 10°C. All the matrix-related stability assessments were made at LQC and HQC levels by comparing the stability sample concentrations against nominal concentrations. The stability of analytes in both stock solutions and working solutions was assessed at 1–10°C. All comparisons were made against freshly prepared stock solutions or working solutions (7).

During routine analysis, each analytical run was organized with a set of standard samples, a set of QC samples in duplicate and plasma samples to be determined. Every day before the start of the analytical run, system suitability was evaluated by injecting six replicates of LLOQ sample and two replicates of LLOQ sample, to check the precision of the system and chromatography. System suitability was considered acceptable when the coefficient of variation for response ratios was <4.0%.

Results
Method development
For consistent and reliable estimation of analytes, it was necessary to give equal importance to optimize the extraction procedure as well as chromatographic and mass spectrometric conditions. All analytes (Figure 1) were tuned in both positive and negative ionization modes using the electrospray ionization technique; however, based on sensitivity, the negative ionization mode was selected for SSA and EZM and the positive ionization mode was selected for SS. The Q1 and MSMS scans were performed using the infusion technique, and further compound and gas parameters were optimized in flow injection analysis. More emphasis was given to optimize chromatographic conditions, as it is necessary to have sufficient chromatographic resolution between SSA, EZM and SS to switch the mass spectrometer polarity during analysis. The [M−H]⁻ peaks were observed at m/z 408.1 for EZM and at m/z 435.2 for SSA, and the [M +H]⁺ peaks for SS were observed at m/z 419.2. Abundant and consistent product ions (Figure 2) were found at m/z 271.0 for EZM and at m/z 199.0 for SS. For SSA, multiple product ions (at m/z 319.2 and 115.0) were selected to achieve the required sensitivity. The pressure of collision gas at a set point of 6 psi resulted in maximum response for all three analytes. A 10% change in source temperature, nebulizer gas, and curtain gas and ion spray voltage did not affect the signal intensities of the mass spectrum. A dwell time of 200 ms was used for each MRM channel, and both Q1 and Q3 quadrupoles were operated in the low resolution mode.

Different types of analytical columns were tested in C18 and C8 phases, and finally a fused core silica column (Phenomenex, Kinetix XB C18, 150 × 4.6 mm, 5 μm) was selected as it produced more symmetrical peaks with good chromatographic resolution and in a reasonable run time. The use of 10 mM ammonium formate buffer in the mobile phase resulted in consistently high peak responses for the analytes and lesser concentration buffer has shown low chromatographic resolution.

As the method was intended to quantify three analytes and as all the three analytes are not able to produce reproducible intensity in the single ionization mode (positive or negative), it was considered to quantify EZM and SSA in the negative ionization mode and SS in the positive ionization mode. To achieve the quantification of all the three analytes in a single run with both negative and positive ionization modes, a novel polarity-switching process was utilized. A run time of 6.3 min was considered essential in order to switch effectively between negative and positive ionization modes in each run.

In the extraction procedure, the SPE technique was selected to avoid unintended matrix effects. HLB cartridges with a basic protocol resulted in low recovery for SSA and EZM, which is later redressed by incorporating an n-hexane wash before washing with 30% methanol solution. A direct elution technique was used to minimize the processing time and this does not affect the column life. To avoid interconversion of SS and SSA, pH of the mobile phase, sample
pretreatment buffer and elution solution were maintained on the acidic side and, in addition, the samples were maintained at below 10°C during sample processing and analysis. EZMG was found relatively stable and no interconversion was identified to free EZM under the employed chromatographic and processing conditions. Deuterated ISTDs were used for all the three analytes to control the matrix effects generally observed during the mass spectrometric analysis of endogenous matrix samples.

Selectivity

Selectivity of the method in human K2 EDTA plasma was evaluated in 14 individual matrix lots along with two hemolysed and two lipemic lots. Peak responses in blank lots were compared against those of spiked LLOQ, and negligible interference was observed at the retention time of analytes and ISTDs. Figures 3–5 demonstrate the selectivity of the method with the chromatograms of blank plasma without ISTD, blank plasma with ISTD and LLOQ samples, respectively.

Figure 2. Production ion spectra of EZM (A), EZMD (B, ISTD), SSA (C), SSAD (D, ISTD), SS (E) and SSD (F, ISTD).
Figure 3. Representative chromatograms of EZM (A), SSA (B), SS (C), EZMD (D, ISTD), SSAD (E, ISTD) and SSD (F, ISTD) in blank plasma.

Figure 4. Representative chromatograms of EZM (A), SSA (B), SS (C), EZMD (D, ISTD), SSAD (E, ISTD) and SSD (F, ISTD) in blank plasma with ISTDs.
Linearity and sensitivity
The linearity of each calibration curve was determined by plotting the peak area ratio (y) of analyte to ISTD versus the nominal concentration (X) of analytes. Calibration curves were linear from 0.2 to 80 ng/mL for SS, 0.1 to 60 ng/mL for SSA and 0.05 to 15 ng/mL for EZM with \( r^2 \) values >0.9981. The \( r^2 \) values, slopes and intercepts were calculated from three intra- and interday calibration curves using weighted (1/\( X^2 \)) linear regression for SSA and EZM and using 1/\( X \) weighting for SS. The observed mean back-calculated concentrations with accuracy (%RE) and precision (%CV) are presented in Table II.

The LLOQ for determination of analyte was found to be 0.2 ng/mL for SS, 0.1 ng/mL for SSA and 0.05 ng/mL for EZM. At LLOQ (\( n = 6 \)), accuracy (%RE) for all the three analytes were in the range of −5.2 to 7.5 with a %CV of ≤8.7. At LLOQ, the mean signal-to-noise ratios were found to be 111:1, 169:1 and 84:1 for SS, SSA and EZM, respectively.

Precision and accuracy
Precision and accuracy was evaluated by performing three intra- and interday precision and accuracy runs, each consisting of six replicates of QC samples at four concentrations (LLOQQC, LQC, MQC and HQC). The intra- and interbatch precision was ≤16.2 for SS, SSA and EZM with accuracy (%RE) between −18.8 and 8.0. The precision and accuracy results are presented in Table III.

Matrix effect
Co-elution matrix components can suppress or enhance ionization, but might not result in a detectable response in matrix blanks due to.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal concentration (ng/mL)</th>
<th>Mean(^a) (ng/mL)</th>
<th>%CV</th>
<th>%RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>0.200</td>
<td>0.215</td>
<td>8.7</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>0.401</td>
<td>0.395</td>
<td>1.8</td>
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<td></td>
<td>1.002</td>
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<td>4.007</td>
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<td>80.14</td>
<td>80.825</td>
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<td>SSA</td>
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<td>60.154</td>
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<td>15.070</td>
<td>1.8</td>
<td>0.4</td>
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</table>

\(^a\)Mean of three replicates at each concentration.

%CV, percentage coefficient of variation; %RE, percent relative error.
selectivity of MS detection; however, they can affect the precision and accuracy of the assay. Therefore, the potential for variable matrix-related ion suppression was evaluated in eight independent sources (containing two hemolytic and two lipemic lots) of human plasma, by calculating the IS normalized matrix factor. The mean IS normalized matrix factor for all analytes ranged between 0.99 and 1.01 with a %CV of ≤4.8, as given in Table IV.

Extraction recovery and dilution integrity
The extraction recovery of analyte from K2 EDTA plasma was determined by comparing the peak response of plasma samples (n = 6) spiked before extraction with that of plasma samples spiked after extraction. The mean recovery of SS, SSA and EZM was found to be 82.4, 81.7 and 87.3%, respectively, with %CV across the three levels ranging between 5.6 and 10.4%, as illustrated in Table V.
The dilution integrity experiment was carried out at two times the upper limit of quantitation (ULOQ) concentration for all the three analytes. After the 1:4 dilution the mean back-calculated concentration for dilution QC samples was within 85–115% of the nominal value with a %CV of ≤6.2. Similarly, low QC samples spiked with concomitant drug were quantified within 15% of the nominal value with a %CV of ≤4.7.
Stability
Stability evaluations were performed in both aqueous and matrix-based samples. All analytes and ISTDs in the stock solution were stable for 66 days at 1–10°C and stock dilutions in 50% methanol were stable up to 48 h at 1–10°C. Stability in matrix was established up to 63 days at both −70 and −20°C. The results of stability evaluations are given in Table VI.

Stability evaluation in matrix was performed against freshly spiked calibration standards. SS, SSA and EZM were stable up to 17 h at the benchtop at a temperature of below 10°C and over 6 freeze-thaw cycles. The processed samples were stable up to 47 h in the autosampler at 10°C. No significant degradation or interconversion of analytes was observed over the stability duration and conditions.

Stability in whole human blood was evaluated at both low and high QC levels by comparing the mean response ratio of stability samples against the comparison samples. The analytes were stable up to 2.5 h in whole human blood at below 10°C.

Application
The validated method was successfully applied to ‘An open label, randomized, two treatment, two sequence, two period, crossover, single dose, oral comparative bioavailability study of EZM and SS 10 mg/80 mg tablets’ conducted on 18 healthy male human volunteers under fed conditions. The study protocol was approved (Protection Plus IEC/ACL/425/14; date: 5 December 2014) by the Independent Ethics Committee named Protection Plus Ethics Committee situated at flat no. 503, OSRN Floor, above ICICI bank, Yad kumar chambers, Gangaram, Huda Bus Stop, Chanda Nagar, Hyderabad-500050, Andhra Pradesh, India with registration numbers ECR/106/Indt/AP/2013 from DCGI and IRB00007186 from FDA. This registration shall be in

Figure 6. Representative chromatograms of EZM (A), SSA (B), SS (C), EZMD (D, ISTD), SSAD (E, ISTD) and SSD (F, ISTD) in real subject sample.

Figure 7. Mean plasma concentration versus time profiles of EZM (A), SSA (B) and SS (C) after oral administration of SS and EZM (80 and 10 mg) tablets to 18 healthy Indian male volunteers under fed condition.
force for a period of 3 years from the date of issue. All subjects provided written informed consent. The study was conducted in accordance with ICH-GCP and the provisions of the Declaration of Helsinki.

The venous blood samples were withdrawn pre-dose (0.00) and at 0.08, 0.17, 0.25, 0.33, 0.66, 1.00, 1.33, 1.66, 2.00, 2.33, 2.66, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 7.00, 8.00, 10.00, 12.00, 16.00, 20.00, 24.00, 36.00, 48.00 and 72.00 h post-dose using K3 EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 2,500 rpm, 4°C for 10 min and the plasma was collected. The collected plasma samples were stored at −70°C until use. Plasma samples were spiked with IS and processed using the extraction procedure described previously. Samples of all the 18 volunteers along with calibration standards and QC samples were analyzed in 6 days, and the precision and accuracy of calibration and QC samples were within acceptable limits. The chromatograms of SS, SSA and EZM in real subject samples are presented in Figure 6. Results of incurred sample reanalysis performed on 10% of the analyzed samples have met the specification limits. Plasma concentration–time profiles of EZM, SS and SSA were analyzed by the noncompartmental method using WinNonlin Professional Software, Version 5.0.1 (Pharsight Corporation, Mountain View, CA, USA). For all three analytes, the mean plasma concentrations versus time profiles under fed condition are represented in Figure 7. The $C_{\text{max}}$, AUC$_{0-\text{inf}}$ and $T_{\text{max}}$ values obtained during the study were 10.61 ± 5.287, 77.58 ± 29.367 and 1.62 ± 0.436 for EZM, 69.74 ± 45.274, 190.71 ± 107.271 and 1.74 ± 0.480 for SS and 25.36 ± 23.576, 139.24 ± 131.653 and 3.95 ± 0.671 for SSA, respectively.

Discussion

A simple, selective and sensitive bioanalytical method is an essential prerequisite for the successful completion of bioequivalence/pharmacokinetic studies of generic drugs. Indeed, bioequivalence/pharmacokinetic studies are essential for the generic drug approval by various international regulatory authorities like US FDA, EMA etc. So far, no published methods are available to determine SS, SSA and EZM in human plasma with tandem mass detection using the API-4000 (27) configuration in the Indian population (28). The proposed method is specific, sensitive, robust and simple owing to the utilization of an isocratic elution which is easier, and more time- and cost-saving than other reported methods (5, 6, 10, 27, 28, 31). Here, we have developed a method for the determination of SS, SSA and EZM in human plasma, with a range of 0.2–80 ng/mL for SS, 0.1–60 ng/mL for SSA and 0.05–15 ng/mL for EZM. The method uses deuterated ISs with the SPE technique, which provides better sample clean-up for mass spectrometric analysis. To the best of our knowledge, we have for the first time reported such a pharmacokinetic study in the Indian population with a focus on analytical method development and validation.

Conclusion

A rapid, sensitive, selective, reproducible, precise and accurate method was developed for simultaneous determination of SS, SSA and EZM in human plasma using deuterated ISs. The method is meeting the acceptance criteria for bioanalytical method validation defined as per the compendial standards. Under the conditions of the current method, no interconversion was observed between SS and its major metabolite SSA, and between free EZM and its phenoxyl glucuronide metabolite EZMG. The method was successfully applied for pharmacokinetic study following a single oral administration of EZM and SS 10/80 mg tablets conducted in a healthy Indian population under fed conditions.

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References


