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Original article

Development and validation of simultaneous assay of simvastatin, *beta*-hydroxy simvastatin as metabolite in human plasma using liquid chromatography-tandem mass spectrometry

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Abstract: ***Introduction***: Several generic products containing simvastatin are circulating on the Vietnamese market at a more inexpensive price than that of a brand-name one. These formulations, however, have not been assessed for *in vivo* bioequivalence to the reference product. After oral administration, simvastatin (SIM) is extensively converted into an active metabolite, *beta*-hydroxy simvastatin acid (SIM-A) and a very low concentration of simvastatin can be found in plasma. Therefore, a method for quantification of simvastatin and its metabolite needs to be developed with a high specificity and sensitivity to detect these analytes in human plasma at such low concentrations. Our purpose was to develop a reliable LC-MS/MS (liquid chromatography-tandem mass spectrometry) method for simultaneous determination of simvastatin and metabolite of simvastatin, *beta*-hydroxy simvastatin acid, in human plasma and to apply this method to evaluate the bioequivalence of a test product in comparison with the reference product. ***Methods***: Mass spectrometry, internal standard (IS), and chromatographic conditions were investigated to find out the suitable IS and conditions. Human plasma samples were treated by liquid-liquid extraction (LLE). The assay was validated in compliance with US-FDA (United States-Food and Drug Administration), and EMA (European Medicines Agency) guidelines. ***Results***: LC-MS/MS with electrospray ionization interface in positive (for SIM and lovastatin as IS) and negative (for SIM-A) ionization mode performed under the multiple reaction monitoring mode was used for detection of the analytes. The transition of *m/z* is 436.00 → 285.15, 435.10 → 319.15, and 404.95 → 199.10 for SIM, SIM-A, and IS, respectively. *Tert*-buthyl methyl ether was used for extraction of analytes from human plasma by a simple LLE followed by addition of an ammonium acetate buffer. The developed method was fully validated with acceptable selectivity, linearity and linear range, matrix effect, lower limit of quantitation (LLOQ), carryover, dilution integrity, and intra- and inter-day accuracy and precision, free-thaw stability. ***Conclusions***: The method can be applied for quantification of these compounds in human plasma for *in vivo* bioavailability and bioequivalence studies.

Keywords: Simvastatin; beta-hydroxy simvastatin; LC-MS/MS; human plasma; bioequivalence.

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1. INTRODUCTION

Simvastatin (SIM) is one of the pharmaceutical substances belonging to a statin group and is widely used in treatment of dyslipidemia to prevent cardiovascular diseases by LDL-C (low density lipoprotein-cholesterol) and triglyceride lowering, HDL-C (high density lipoprotein-cholesterol) increasing, atherosclerosis stabilizing, and anti-inflammation… SIM is a lipid-lowering agent that inhibits the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonate, a step in the cholesterol biosynthesis process. Inhibition of this enzyme leads to decrease of LDL-C and thus has an important role in preventing atherosclerosis. SIM is extensively metabolized to *beta*-hydroxylated derivatives through cytochrome P450 3A4 metabolism, and around 70% of the HMG-CoA reductase inhibition associated with its *beta*-hydroxylated metabolites [1-3].

Several generic products containing simvastatin are circulating on the Vietnamese market at a more inexpensive price than that of a brand-name one. These formulations, however, have not been assessed for *in vivo* bioequivalence to the reference product. After oral administration, simvastatin is extensively converted into an active metabolite, *beta*-hydroxy simvastatin acid (SIM-A) and a very low concentration of simvastatin can be found in plasma [1-3]. Therefore, a method for quantification of simvastatin and its metabolite needs to be developed with high specificity and sensitivity to detect these analytes in human plasma at such low concentrations. However, to the best of our knowledge, there is only one LC-MS/MS method that have been reported for the simultaneous determination of SIM and its hydroxylated metabolite in human plasma [4-6], [9-13] and no study has been published in Vietnam so far. Our purpose was to develop a reliable LC-MS/MS method for simultaneous determination of simvastatin and its metabolite, *beta*-hydroxy simvastatin acid, in human plasma and to apply this method to evaluate the bioequivalence of a test product in comparison with the reference product.

The chemical structures of SIM, SIM-A, and internal standard (IS) are shown in Figure 1.

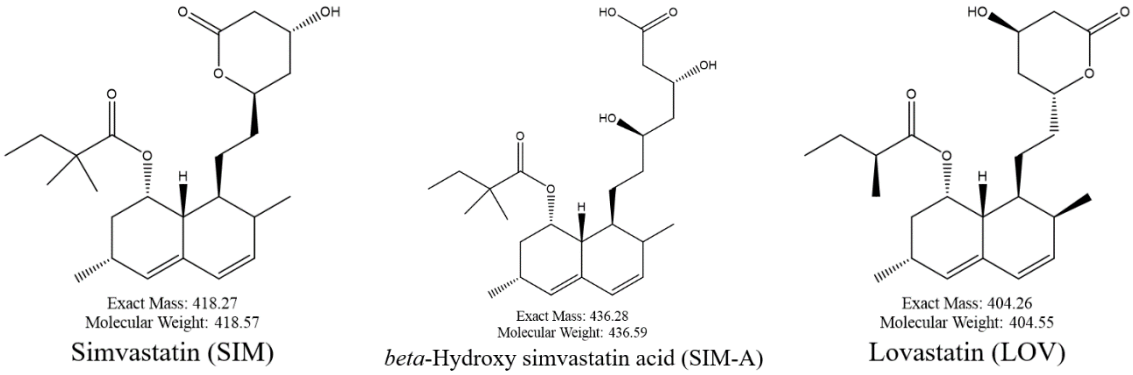

Figure 1. Chemical structures of simvastatin (SIM), beta-hydroxy simvastatin acid (SIM-A) and lovastatin as internal standard (IS)

2. MATERIALS AND METHOD

2.1. Chemicals and reagents

The following reference standards were obtained from the Institute of Drug Quality Control Ho Chi Minh City, Vietnam: simvastatin (99.1%) and lovastatin (100.0%) as internal standard. The hydroxyl metabolite of simvastatin, *beta*-hydroxy simvastatin ammonium salt (98.0%), was purchased from Toronto Research Chemicals (Canada). LCMS-grade acetonitrile and ethyl acetate were obtained from J.T. Baker (USA). LCMS-grade ammonium acetate and methyl *tert*-buthyl ether (MTBE) were supplied by Fisher Scientific. Acetic acid was of analytical grade and obtained from Prolabo. Blank human plasma sources were supplied by Blood Transfusion Hematology Hospital Ho Chi Minh City, Vietnam and stored at below -20^{o}C prior to use.

2.2. Preparation of stock solutions, standards and quality controls

Stock solutions of 100 µg/mL (for SIM and SIM-A) were prepared by dissolving a requisite amount in methanol. From stock standard solutions, dilute working standard solutions containing analyte in same solvent at exact concentrations about 1, 2, 20, 80, 240, 400, 600 and 1000 ng/mL for SIM and 1, 2, 4, 16, 48, 80, 120 and 200 ng/mL for SIM-A. Dilute working standard solutions in plasma to obtain calibration standard samples at approximately exact concentrations: 0.05, 0.10, 1, 4, 12, 20, 30 and 50 ng/mL for SIM; 0.05, 0.10, 0.20, 0.80, 2.40, 4, 6 and 10 ng/mL for SIM-A. The concentrations of the low, medium and high quality controls (QCs) in blank human plasma were 0.15, 25 and 37.50 ng/mL for SIM; 0.15, 5 and 7.50 ng/mL for SIM-A. The QCs were prepared separately from the stock standard solutions and working standard solutions. Fresh calibration standards and QCs were prepared on each day of analysis during the validation. A stock of internal standard (IS) solution at a concentration of 100 µg/mL was prepared by dissolving an appropriate amount of lovastatin in methanol. On each day of analysis, an aliquot of the IS stock solution was diluted in same solvent to obtain the IS working solution (1000 ng/mL). Stock solutions of analytes and IS were stored at -20±2^{o}C, while calibration standards and quality control samples in plasma were kept at below -70^{o}C.

2.3. Sample treatment

Prior to analysis, all frozen plasma were thawed and allowed to equilibrate at room temperature. Spiked exactly 1 mL of plasma containing the analytes into a test tube, add 200 µL of 100 mM ammonium acetate buffer pH 4.5, vortexed for

10 seconds. Add 50 µL of 1000 ng/mL lovastatin internal standard solution in the sample and vortexed for 10 seconds. Extract twice with methyl *tert*-butyl ether, 2 mL each, vortexed for 1 minute, shake 300 rpm for 5 minutes, centrifuge 3000 rpm for 5 minutes at 4°C, take the supernatant solution, and combine the solutions and then evaporating the solvent to obtain the residue with a vacuum centrifuge. Dissolve the residue in 150 µL of 100 mM ammonium acetate buffer pH 4.5 solvent, vortexed for 1 minute, ultrasonic for 5 minutes, centrifuge 3000 rpm for 5 minutes at 0°C, filtered through 0.22 µm membrane and an aliquot of 5 µL was injected into the LC-MS/MS system.

2.4. LC-MS/MS conditions

The Shimadzu UHPLC Nexera X2 coupled with Triple Quadrupole Mass Spectrometer LCMS-8040 was used for setting the reverse-phase liquid chromatographic conditions. The Shimadzu system composed of an autosampler (SIL-30AC), two pumps (LC-30AD), a column oven (CTO-20A) and a controller (CBM 20A). The autosampler and oven temperature were maintained at 5ºC and 40ºC, respectively. HPLC separation of analytes and internal standard was performed using an Eclipse XDB-C8 column (100 × 4.6 mm; 3.5 µm). A gradient mobile phase composing of 1 mM ammonium acetate pH 4.5 and acetonitrile was delivered 0' (60:40), 6' (80:20), 8.5' (80:20), 9 '(60:40), 11' (60:40) at a flow rate of 0.3 mL/min and injection volume was 5 µL.

The MS analysis was operated in positive and negative ionization mode utilizing electrospray ionization (ESI). The interface voltage was set to 4500 V, heat block temperature was 400ºC, desolvation line temperature was 250ºC, nebulizer gas flow rate was 3 L/min, drying gas flow rate was 15 mL/min and dwell time per transition was 100 ms. The multiple reaction monitoring (MRM) transitions for each analyte and IS, as well as their respective optimum MS parameters, including voltage potential (Q1, Q3) and collision energy (CE), are summarized in Table 1. The quantification was applied via peak area. Data acquisition and processing were performed using LabSolutions software for LCMS-8040 system.

Table 1. Tandem mass-spectrometer main parameters

Analytes	MRM transitions m/z	Q1 (V)	CE (V)	Q3 (V)	Acquisition time (min)	Precursor ion	Quantified fragment ion	Identifed fragment ion
SIM	436.00 → 285.15	-30	-16	9	7.51	436.00	285.15	303.19
SIM-A	435.10 → 319.15	16	18	13	4.33	435.10	319.15	115.06
Lovastatin	404.95 → 199.10	-30	-14	-20	6.56	404.95	199.10	225.16

2.5. Method validation

Validation of method was done according to US-FDA and EMA bioanalytical method validation guidance [7, 8].

Selectivity

One blank plasma sample with internal standard phase and six lower limit of quantification (LLOQ) individual blank plasma samples from six lots of different plasma treated, were analyzed to investigate interference peaks at the retention time of each analytes. Selectivity was accepted if the blank response was less than 20% the LLOQ response for each analyte and less than 5% of IS response for the IS.

Carryover

Inject the upper limit of quantification (ULOQ) sample into the chromatographic system. After the analysis is complete, the blank plasma sample is immediately injected into the system. Perform the above procedure 6 times. Inject 6 times of LLOQ sample to evaluate the results. Carryover was accepted if it was less than 20% of the LLOQ for each analyte, and less than 5% of IS response for the IS.

Linearity, lower limit of quantification

The calibration standard curve includes 01 blank plasma sample, 01 blank plasma sample with internal standard such as lovastatin (zero) and 8 plasma samples with analyte standard. The investigated concentration ranges from 0.05 to 50 ng/mL for SIM and 0.05 to 10 ng/mL for SIM-A. Perform analysis of three calibration standard curves repeatedly. The calibration curves were described by the linear equation: $y = ax + b$, where y is the ratio of the analyte peaks and the corresponding IS peaks and x is the concentration of the analyte (µg/mL) and, then, the regression coefficient, slope and y-intercept of the resulting calibration curves were determined by least squared weighted ($1/x^2$) regression.

Accuracy and precision

Prepare a sample lot consisting of 4 concentrations of LLOQ, low level quality control (LQC), medium level quality control (MQC) and high level quality control (HQC), each with 6 concentrations. Processing and analyzing according to the developed method. Repeat for another 2 days. Intra-day (n = 6) and inter-day (n = 18) accuracy and precision was determined. Accuracy was accepted if the back calculated concentration was deviation 15% from the nominal concentration for each QC, and 20% for the LLOQ. Precision was accepted if the coefficient of variation (CV) did not exceed 15% for each QC, and 20% for LLOQ.

Matrix effect

Prepared six individuals plasma from six lots of different plasma, each batch two samples, and conduct sample extraction according to the sample treatment process. After treatment, the working standard solution containing analytes is added in the sample at two concentrations of LQC and HQC to each batch, to calculate the influence of sample matrix. Plasma were tested at high and low QC concentrations. The matrix effect for each analyte and IS were determined separately in each sample by determining the ratio of the peak area in the post-extraction spiked plasma to the peak area in spiked methanol.

Recovery

Three series of six samples with concentrations from LQC, MQC and HQC of the standard curve were prepared separately and analyzed. Then, the ratio of the recorded peak area to the peak area resulting from the direct injection of the solutions in methanol of the analytes with the same concentrations were determined as percentage in each case.

Dilution integrity

Prepared samples in plasma at concentrations of exactly 75 ng/mL for SIM and 15 ng/mL for SIM-A. Dilute this sample 2 times with blank plasma, then process and analyze six samples. Accuracy and precision of the diluted samples within 15% deviation was considered acceptable.

Stability

Freeze and thaw stability: Low and high levels of QC plasma samples were kept at -70ºC for 24 hours and thawed unassisted at room temperature (one cycle). The cycle was replicated three times and, then, analyzed the samples.

Short-term temperature stability: Low and high levels of QC plasma samples were kept at room temperature for 6 hours before analyzed.

Long-term stability: Low and high levels of QC plasma samples kept at -70ºC were measured for a period of 95 days.

Post-preparative stability: The autosampler steadiness was evaluated by reanalyzing the extracted low and high QC samples kept under the autosampler situations (4ºC) for 40 hours.

3. RESULTS

3.1. Optimization of LC-MS/MS conditions

During the method development, electrospray ionization was operated in the positive and negative ionization modes. The Q1 MS full scan spectra for SIM, SIM-A and lovastatin (IS) predominantly contained protonated precursor $[M+NH_4]^+$, $[M-H]^-$, $[M+H]^+$ ions at *m/z* 436.00, 435.10 and 404.90, respectively.

The most abundant product ions in Q3 MS spectra for SIM, SIM-A and lovastatin (IS) were observed at 285.15, 319.15 and 199.10, respectively. Figure 2 shows the Q3 MS spectra *m/z* of the analytes and IS. The MS/MS parameters were systematically optimized for each analyte and IS in order to obtain a consistent and adequate response. A dwell time of 100 ms was sufficient.

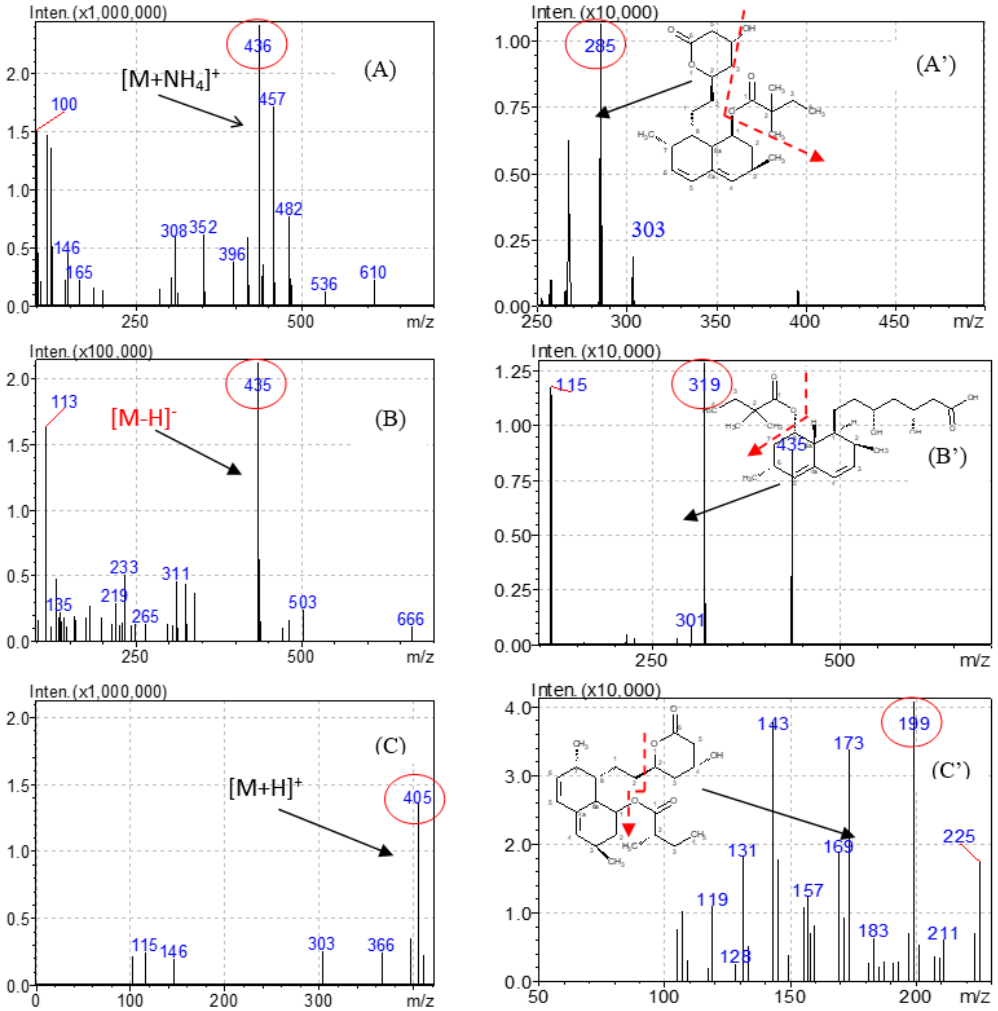

Figure 2. Precursor ion spectrum m/z for (A) simvastatin, (B) *beta*-hydroxy simvastatin acid, (C) lovastatin as internal standard (IS) in positive ionization and negative ionization modes

The chromatographic conditions were investigated by considering the column type, mobile phase component, pH of buffer and strength, column oven temperature, flow rate and injection volume. Thus, these parameters were initially changed in order to obtain a symmetric peak shape, a short run time, minimum matrix interference and solvent consumption. Based on the outcome of various trials, the Eclipse XDB-C8 column (100 × 4.6 mm; 3.5 µm) was selected. The mobile phase consisting of acetonitrile and 1 mM ammonium acetate buffer pH 4.5 adjusted with acetic acid, at a flow rate of 0.3 mL/min ensured separation of SIM, SIM-A and IS at the retention times of 7.51 min, 4.33 min, and 6.56 min, respectively in a total run time of 9 min. Figure 3 shows the chromatograms of the blank plasma and analytes at MQC level with IS. Blank plasma chromatogram was obviously clean with no endogenous interfering peak at the retention times of analytes and IS.

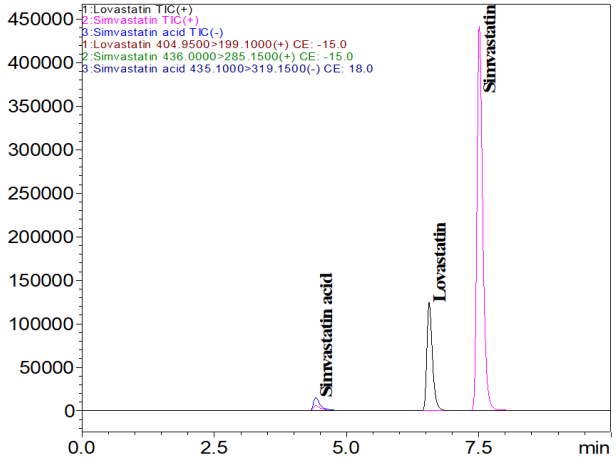

Figure 3. Chromatograms of SIM, SIM-A, IS at optimum MS and chromatographic conditions

3.2. Extraction procedure

Protein precipitation (PP) and liquid-liquid extraction (LLE) are routine sample pretreatment strategies. During the initial stages of method development, PP method was carried out using methanol and acetonitrile as agents; but it failed to achieve a very clean extract and produced a higher background noise with poor sensitivity. Therefore, LLE was desired because this technique can not only purify but also concentrate the sample. LLE was initiated with MTBE, ethyl acetate and a mixture of MTBE and ethyl acetate (1:1, v/v). MTBE was chosen because it showed better recovery than other solvents. Furthermore, the extraction was investigated at various strengths (0-150 mM) and pH (3.5-5.5) of ammonium acetate buffer and volumes were tested. It was demonstrated that 100 mM ammonium acetate buffer pH 4.5 generated higher recoveries and good stability for all analytes. Based on the investigation, MTBE in the presence of 200 µL of 100 mM ammonium acetate buffer pH 4.5 was finally assumed in the present work.

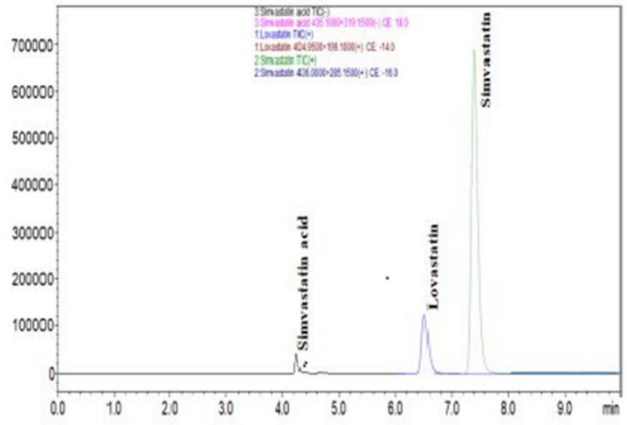

Figure 4. Chromatograms of SIM, SIM-A, IS at optimum extraction procedure

3.3. Method validation

The bioanalytical method described here met full validation criteria for linearity, selectivity, carryover, accuracy, precision, matrix effect, stability, and dilution integrity in accordance with US-FDA and EMEA guidelines. The results of method validation are provided in Table 2.

Carryover evaluation was carried out so as to ensure that it does not affect the accuracy and the precision of the method. The carryover for the analytes and IS was within the acceptance limit. There was no interfering peaks at the retention time of analytes and IS after subsequent injection of ULOQ.

The calibration curves were validated for all analytes over the following ranges: 0.05-50 ng/mL for SIM and 0.05-10 ng/mL for SIM-A. The calibration lines were drawn to give the linear regression equations: y = 0.3645x+ 0.1136 and y = 0.0479x + 0.0006 for SIM and SIM-A, respectively, where y is the peak area ratio of the analyte and IS and x is the concentration of the analyte by least squared weighted ($1/x^2$) regression. The correlation coefficient square (R^2) was more than 0.99, while the accuracies for the calibration curve standards were in the range of 90.21%-106.57% and 90.94%-114.90% for SIM and SIM-A, respectively, which met the requirements (85%-115%). The LLOQ in the standard curve that can be measured with acceptable accuracy and precision, was 0.05 ng/mL for both SIM and SIM-A, at a signal-to-noise ratio (S/N) of more than five.

The intra- and inter-day accuracy and precision of the LLOQ and QCs for all analytes were within the acceptable range (85%-115% for accuracy and CV ≤ 15% for precision). The intra-day precision (CV) varied from 1.48%-9.18% and the accuracy was within 87.56%-114.13% for all analytes. Similarly, the inter-day (CV) varied from 3.80% to 12.40% and the accuracy was within 94.58%-107.08%.

Following a 2-fold dilution, for all analytes the accuracy was within 92.79%-105.59% and the precision (CV) ranged from 2.48% to 4.62%, which was within the acceptance limit; therefore, dilution up to 2 times for volunteer samples higher than the ULOQ was acceptable.

Table 2. Summary of validation results

Property		QC	SIM	SIM-A	IS
Selectivity	Analyte (% LLOQ)	-	0.00	0.00	
	Internal standard (% IS)	-	0.00	0.00	
Carryover	Analyte (% LLOQ)	-	0.00	0.00	
	Internal standard (% IS)	-	0.00	0.00	0.00
Linearity	R square (R^2)	-	0.9984	0.9988	
Intra-day accuracy and precision	Accuracy (%)	LLOQ	102.64	95.68	
		LQC	107.15	87.56	
		MQC	107.67	104.38	
		HQC	114.13	109.54	
	Precision, CV (%)	LLOQ	9.18	12.40	
		LQC	5.18	8.47	
		MQC	3.50	7.39	
		HQC	3.69	6.46	
Inter-day accuracy and precision	Accuracy (%)	LLOQ	102.16	99.54	
		LQC	105.22	94.58	
		MQC	103.19	100.15	
		HQC	107.08	104.10	
	Precision, CV (%)	LLOQ	11.16	12.40	
		LQC	6.29	8.47	
		MQC	6.20	7.39	
		HQC	6.90	6.46	
Matrix effect	Precision, CV (%)	LQC	3.47	4.44	
		HQC	1.94	0.78	
Recovery	Mean	LQC	89.51	84.01	84.08
		MQC	83.61	92.46	82.36
		HQC	81.41	88.86	76.46
	Precision, CV (%)	LQC	2.50	3.66	2.74
		MQC	2.57	4.39	4.68
		HQC	2.05	2.95	3.89
Dilution integrity	Accuracy (%)	-	97.52	100.69	
	Precision, CV (%)	-	2.78	4.62	
Free-thaw stability	% of nominal concentration	LQC	98.56	97.18	
		HQC	107.64	99.88	
At room temperature for 6 hours	% of nominal concentration	LQC	110.42	90.83	
		HQC	93.51	104.27	
In autosampler for 40 hours at 4ºC	% of nominal concentration	LQC	107.86	90.40	
		HQC	105.76	105.02	
Long-term stability (after 95 days)	% of nominal concentration	LQC	92.59	91.00	
		HQC	104.44	105.61	

The stabilities of the analytes and IS in human plasma and stock solutions were investigated under various conditions. Analytes were stable for up to 6 hours at room temperature and for three freeze and thaw cycles at below -70ºC. Spiked plasma samples were found stable for a period of up to 95 days after long-term stability experiment. Autosampler stability was carried out and found that the spiked samples were stable up to 40 hours without significant loss of the analytes at 4ºC.

Analyte responses were stable in plasma on the benchtop (room temperature for 6 hours), after three free-thaw cycles, after 95 days of storage (at -70ºC), and in the autosampler (40 hours, set at 4ºC). Moreover, analyte responses in stored stock were within 15% deviation of the fresh solution responses, which were considered acceptable.

4. DISCUSSION

The ionization efficiency of SIM and SIM-A was quite different. SIM contain methoxy carbonyl groups, which can accept protons, be charged and display responses in (+)ESI mode. However, SIM lacks the functional group that could release protons, thus possessing no signal in (−)ESI mode. SIM-A contains the methoxy carbonyl group that can receive protons and aliphatic carboxylic and hydroxyl groups that can release protons. Hence, SIM-A showed responses in both (+)ESI or (−)ESI mode. Nevertheless, SIM-A displayed higher signal intensities in (−)ESI mode than in (+)ESI mode because of its readiness to lose protons from the carboxylic group. In this context, negative/positive ionization switching was chosen, with SIM-A being monitored in (−)ESI mode and SIM being acquired in (+)ESI mode. The Q1 (−)ESI spectra of SIM-A showed the intense $[M–H]^-$ ion at m/z 435.1

whereas the Q1 (+)ESI spectra of SIM and LOV showed intense $[M+NH_4]^+$ ion of SIM at m/z 436.3 and intense $[M+H]^+$ ion of LOV at m/z 404.90. In this context the $[M+NH_4]^+$ ion of SIM was employed to achieve the desired sensitivity. The ammonium ion was from the mobile phase. Quantitation was performed by multiple reaction monitoring (MRM) mode of precursor–product ion transitions at m/z 436.00 → 285.15 for SIM, m/z 435.10 → 319.15 for SIM-A and m/z 404.95 → 199.10 for LOV, with dwell time set at 100 ms per transition. These fragment ions were selected as quantified ions for SIM, SIM-A and LOV because they had the highest signal among daughter ions for each analyte. The current CE optimization step was critical for the selection of the final MRM transitions for SIM and SIM-A. The fragment ions selected as quantified ions for SIM, and LOV (IS), conform to Euro massBank.

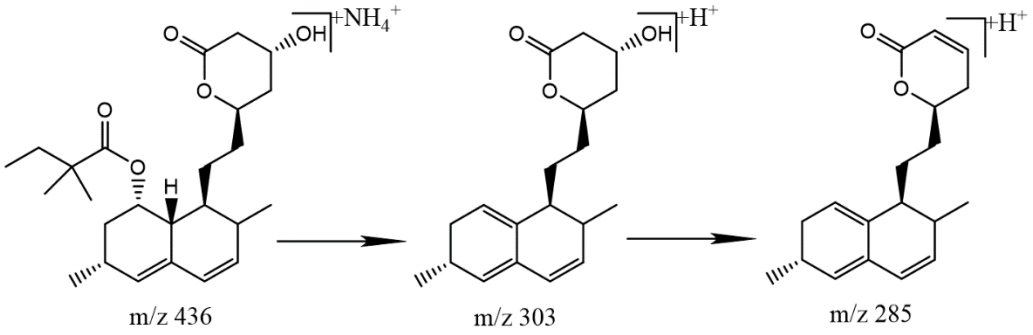

Figure 5. Proposed CE fragmentation pathways for the $[M+NH_4]^+$ ion of SIM (m/z 436) to produce the prominent product ions

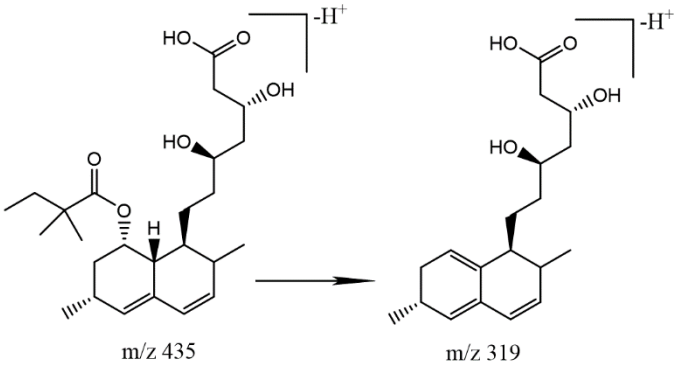

Figure 6. Proposed CE fragmentation pathways for the [M-H]- ion of SIM-A (m/z 435) to produce the prominent product ions

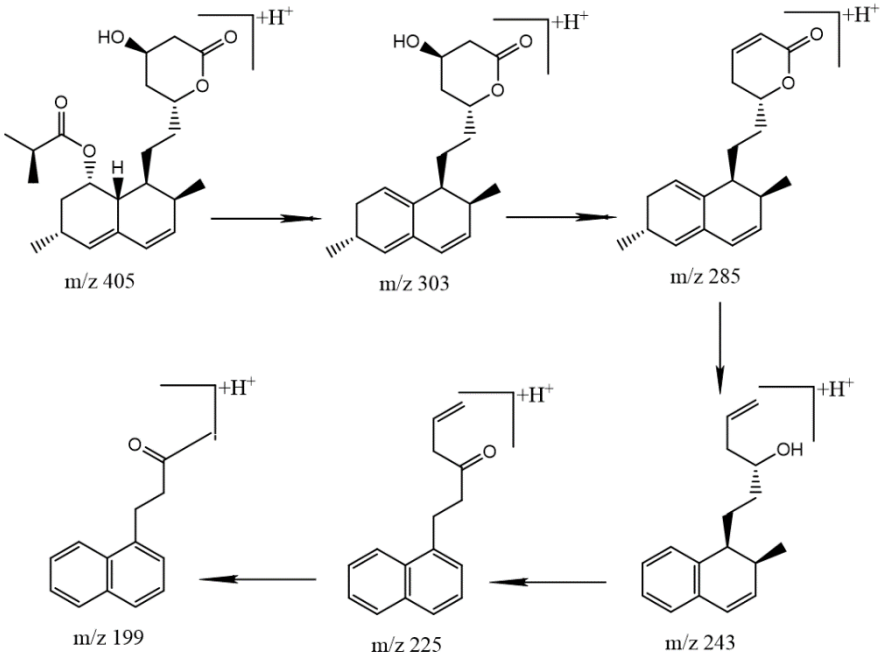

Figure 7. Proposed CE fragmentation pathways for the $[M+H]^+$ ion of LOV (m/z 405) to produce the prominent product ions

Based on the previous literature [4-6], [9-13], we selected the starting conditions toward optimizing the LC parameters. In most of these studies, C18 and C8 analytical columns were employed to achieve good resolution with satisfying peak shape and peak symmetry such as Kromasil C18, Discovery C18, YMC ODS-A C18, Aquasil C18, X-Terra C18, Ascentis Express C18, Symmetry C18, Phenomenex Luna C18, Oyster C8, Supelco Discovery C8. In this study, based on the available conditions of our laboratory, Eclipse XDB-C8 (100 x 4.6 mm; 3.5 µm) column was chosen because it offered a good separation efficiency with short analysis time and popularity. Analytical results showed that the SIM and SIM-A analytes were eluted early, completely separated, short analysis time and low operating pressure. Afterwards, an experimental design was carried out in order to optimize the method found in the previous development. The optimization procedure was focused on the mobile phase composition as well as pH of buffer and strength, column oven temperature, flow rate and injection volume. Ammonium acetate buffer and ammonium formate buffer were selected for investigation and the pH of the buffer solution was also investigated from 3.5 to 5.5. The mobile phase of acetonitrile-ammonium formate buffer, acetonitrile-ammonium acetate buffer at different concentrations, pH and ratios were investigated. When using the acetonitrile mobile phase - 1 mM ammonium acetate buffer pH 4.5 (gradient program), the SIM and SIM-A analytes were completely separated, short analysis time, low column pressure and high response signal. Once the method was optimized to select suitable chromatographic conditions and was fully validated to demonstrate that the final method was suitable for our purpose.

Previous publications [11, 13] have described several sample preparation methods for determination of SIM or SIM along with its metabolite in plasma. Methods of simultaneous determination of SIM and SIM-A concentrations in animal and human plasma by LC-MS/MS utilizing solid phase extraction (SPE), liquid–liquid cartridge extraction and liquid-liquid extraction (LLE) were reported and became well used. Development of sample preparation methods that are compatible to LC-MS/MS has become more demanding at this stage. In this research, we developed a sample treatment process with these methods. The SPE is simple, efficient and easy to automate but it is expensive, so it was not preferred with condition of our laboratory. Two methods of protein precipitation (PP) and LLE were investigated to optimize sample cleaning to reduce matrix influence, increase extraction recovery and prolong the life of the analytical column. Initially, the PP was chosen due to the advantages of easy operation, less time consuming, less solvent; however, the sample after treatment was less pure and mixed with many impurities, leading to low extraction recovery (below 30 % for SIM and LOV), and CV > 15%. The LLE is a traditional but effective method to extract drugs from biological samples. LLE is compatible with the electrospray ionization source because it desalts samples well. The LLE provided further simplified sample preparation steps, samples after treatment were cleaner, the dilution was less, so the sample was concentrated to increase the signal of analytes, suitable for low analytes concentration in human plasma and better extraction recovery. The use of buffer solutions according to the references to break protein bonds to stabilize the analytes in the plasma sample matrix increased the extraction efficiency of the analytes. In this study, ammonium acetate buffer was chosen because it had similar properties to SIM-A and was not a strong oxidizing agent. Extraction efficiency was significantly different at different concentrations, so 100 mM ammonium acetate buffer pH 4.5 was selected for further investigation. Experiments showed that the extraction efficiency of the analytes was high and stable, ensuring that the LLE method can be applied to simultaneous determination of SIM and SIM-A concentrations in the plasma samples of volunteers later in bioequivalence studies.

For simultaneous quantification of SIM and SIM-A in human plasma, most of published articles [4, 9, 10] involved a relatively expensive solid phase extraction (SPE) and LC-MS/MS with LLOQ within the range of 0.10-0.50 mg/mL for both SIM and SIM-A. There are only two studies [11, 13] with LLOQ of 0.05 ng/mL for SIM and its metabolite SIM-A using LC-MS/MS. It was, however, challenging to deal with several complex steps in automatic 96-well liquid-liquid extraction plate [11, 12] or liquid-solid extraction/liquid-liquid cartridge extraction (LSE/LLCE) [13]. The stable isotope-labeled internal standards $^{13}CD_3$-SIM and $^{13}CD_3$-SIM-A, LOV, SIM-D_6 and SIM-A-D_6, SIM-D_6 and SIM-A-D_4 used in these studies are also relatively expensive. Additionally, consumption of high amounts of solvents, state-of-the-art instruments and equipment and extraction procedure time are drawbacks of these studies. In our study, lovastatin, atorvastatin, and rosuvastatin were selected to investigate the internal standard due to its high purity, ease of finding and low cost than other substances, structurally similar to the analyte. The results showed that lovastatin was selected as the internal standard for quantification because lovastatin gave higher signal intensity than atorvastatin and rosuvastatin.

In this study we developed and optimized another novel quantitative method using a simple, rapid, economic and easy-to-apply liquid-liquid extraction (LLE) technique for simultaneous quantification of SIM and SIM-A in human plasma. The method revealed a prodigious and reliable LLOQ at 0.05 ng/mL for both analytes with accuracy and precision within the acceptable range as per FDA and EMA guidelines. Besides, we developed linearity ranges of 0.05-50 ng/mL and 0.05-10 ng/mL for SIM and SIM-A respectively, providing a novel improved method with considerable advantages. The new method is highly applicable and appropriate for quantification human plasma samples obtained from bioequivalence studies of generic drugs containing simvastatin at various strengths in pharmaceutical market. The method can be applied at a small to medium sized laboratory at a low-operating cost. Comparing to other researches [4-6], [9-13] on the requirements for method validation such as the time analysis, LLOQ, linearity, accuracy and precision, the free-thaw stability, and recovery, our method has a low LLOQ.

Conclusion

We have developed and validated a novel, sensitive assay for the quantification of simvastatin and its metabolite in human plasma. The described method offered several advantages such as a simple extraction procedure, and a short chromatographic run time, which makes the method suitable for the analysis of large sample batches resulting from study of pharmaceutical products containing simvastatin.

AUTHORS' CONTRIBUTION

TDN, NNC developed the idea of the study. TTL, TTT performed the analysis. SVP, HVT check the results. TDN, NNC drafted the manuscript, revised the manuscript. All authors were responsible for the final content.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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