Novel and simple LC-ESI-MS-MS method for the bioanalysis of simvastatin and simvastatin acid in rat plasma using ion trap mass spectrometry and its application to pharmacokinetic investigation

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Abstract

A simple, specific, and sensitive liquid chromatography-tandem mass spectrometry method for the simultaneous determination of simvastatin (SV) and simvastatin acid (SVA) has been developed in rat plasma using mewastatin as internal standard (IS). This method is having an advantage of isocratic flow, no polarity switch, no deuterated IS and solid phase extraction procedure over other methods. The analytes were separated on a reversed-phase C18 column and analyzed by MS(Thermo Scientific™ LCQ Fleet) in the multiple reaction-monitoring mode The limit of quantitation for this method was 50 and 10ng/ mL and the linear dynamic range was generally 50-1800 ng /mL and 10-1000 ng/ mL for SV and SVA, respectively. The mobile phase for the analysis consisted of ammonium acetate buffer (1 mM, pH 4.0) and acetonitrile (20:80 v/v) with a flow rate of 500µL/ min. The total chromatographic run time was 4.0 minutes. The method was validated for its precision, accuracy, recovery, stability, specificity and linearity. The method was successfully applied for the estimation of bioavailability of SV and SVA after high dose treatment in a study comprising experimental animals.

Keywords: simvastatin; Simvastatin acid; Liquid chromatography mass spectrometry; Rat plasma

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1. Introduction

Among cholesterol lowering agents, simvastatin ((+)-(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 -butanoate]) is the most widely used statin [Hoffman et al. 1986]. Simvastatin (SV) inhibits 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, an essential enzyme involved in the synthesis of cholesterol [Mauro VF. 1993]. SV is known to be rapidly hydrolyzed in vivo to its corresponding β-hydroxy acid (Fig. 1) called simvastatin acid (SVA). Because of the high first-pass hepatic extraction the concentrations of both SV and SVA are found to be very low in systemic circulation [Vickers et al. 1990]. Oral bioavailability of SV is around 5 % (Mauro et al. 1993). Therefore, to estimate SV and its active metabolite SVA in body fluids needs very sensitive and accurate method of analysis.

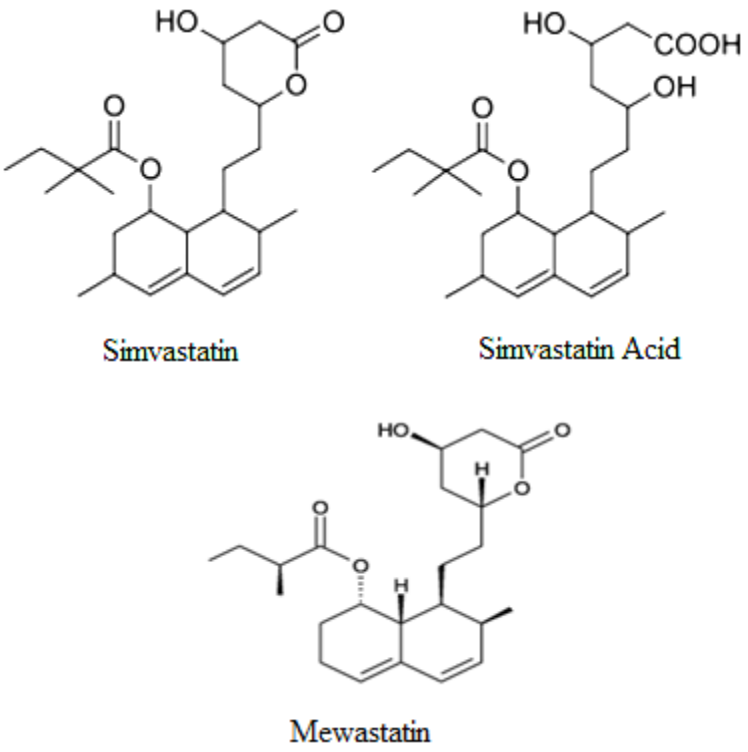

Figure. 1 Chemical structures of simvastatin, simvastatin acid and IS mewastatin.

Several methods have been developed for the analysis of SV in human plasma including liquid chromatographic (LC) coupled to ultra violet (UV) detector [Carlucci et al. 1992; Ochiai et al. 1997; Yang et al. 2003] gas chromatography mass spectrometric (GCMS) [Morris et al. 1993], micellar electrokinetic capillary chromatography (MEKC) [Srinivasu et al. 2002], voltammetry [Coruh et al. 2006], spectrophotometry [Wang et al. 2000] and liquid chromatographic mass spectrometry (LCMS) [Barrett et al. 2006; Zhao et al. 2000; Jemal et al. 2000]. However LC/UV methods are not very sensitive to determine very low SV and SVA concentration levels. GCMS analytical methods include additional derivitization step with a generally complicated and time taking sample preparation procedure. So most often LCMS methods are employed for the determination of SV and SVA in biological fluids, as LCMS methods are more specific, sensitive, accurate and reproducible. Few LCMs methods have been reported using different methods of extraction viz–a-viz liquid–solid extraction [Zhao et al. 2000], direct injection [Jemal et al. 2000] and liquid- liquid extraction [Patel et al. 2008].These methods, although sensitive and accurate, but are limited to very narrow therapeutic plasma concentration range and required very sophisticated and costly work station which is generally not available in all labs. These methods are not applicable to determine higher SV and SVA levels in preclinical studies, resulting from interaction from food and concomitantly administered drugs. Most of these methods have a run time ranging from 5-8 minutes, which is time consuming for pharmacokinetic studies.

Here we developed a rapid LC-ESI-MS-MS method covering a broad range of plasma concentrations of SV (50-1800 ng/ml) and SVA (10-1400 ng/ml) using most simple solid phase extraction procedure and most economical mass analyzer i.e. ion trap mass spectrometer (Holcapek et al. 2012) without gradient flow or polarity switch.

2. Material and Methods

Reference standards SV, SVA and internal standard mewastatin (MW) were obtained from Clearsynth Ltd. Mumbai India. HPLC-MS grade acetonitrile (purity 99.9%) was purchased from Sigma-Aldrich, Germany. Ethyl acetate and were procured from Merck Specialties Pvt. Ltd. MS

grade ammonium acetate and ammonium formate were obtained from Fluka analytical, Sigma-Aldrich, Netherland. Formic acid (purity >98%) was obtained from Fluka analytical, Sigma-Aldrich, Germany. Water used in the entire analysis was prepared in-house with Milli-Q water purification system procured from Millipore (Millipore Corporation, USA). Other chemicals used were of analytical grade from commercial sources.

2.1 Liquid Chromatography conditions

Chromatographic separation was achieved by using a Agilent hypersil ODS column (200x2.1mm, 5-µm particle size), attached to Thermo Scientific Finnigan Surveyor Plus (Serial # 300416, Part # SRVYR-ASP) equipped with a quaternary solvent system, an auto sampler, solvent manager and a MS detector (Thermo Scientific™ LCQ Fleet). The mobile phase for the analysis consisted of ammonium acetate buffer (1 mM, pH 4.0) and acetonitrile (20:80 v/v) in non-gradient elution mode, which was degassed. The flow rate of the mobile phase was at 500µL/ min. A fixed amount of 10 µL of sample solution was injected in each run. The total chromatographic run time was 4.0 min. The column and auto-sampler were maintained at 40±5 and 4±2 ^{0}C, respectively and the pressure of the system was 1500 psi.

2.2 Mass spectrometric conditions

Mass spectrometry was performed on Thermo Scientific LCQ Fleet Ion Trap (Serial# LCF 10356) mass spectrometer (San Jose, CA, USA). The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the Xcaliber software installed for the instrument. Ionization of analytes of interest was carried out using electrospray ionization (ESI) in positive mode. The capillary temperature, sheath gas flow rate, auxiliary gas flow rate, and collision energies were optimized for each analyte separately. The spray voltage used was 5 kV. Analytes and their internal standards were identified and quantified based on their retention times and the presence of parent ion and product ions in selected reaction monitoring mode (SRM).

2.3 Preparation of standard stocks and working solution

Primary stock solutions of SV, SVA and mewastatin (internal standard) were prepared in methanol. Different stock solutions were used to prepare calibration standards (CS) and quality

control samples (QC). A fresh common working solution of IS containing 100 ng/ mL was prepared every day by appropriate dilution of the stock solution in methanol:water (80:20, v/v). Common aqueous CC and QC working solution for SV and SVA were prepared by serially diluting the stock solution with methanol:water (80:20, v/v) and the final concentrations were corrected accounting for potency, molecular weight and the actual weight transferred. All the solutions were protected from light and stored below 5 ^{o}C.

2.4 Preparation of Calibration Curve samples (CC) and Quality Control Samples (QC)

Common CC samples were prepared in blank human plasma by spiking 2%, v/v of the aqueous working solutions for a concentration range of 10-1400 ng/mL and 50–1800 ng/mL for SVA and SV respectively. Similarly common QCs were prepared at lower limits of quantitation (LLOQC) 10, 50 ng/mL, low level quality control (LQC) 20, 100 ng/mL, medium level quality control (MQC) 700, 900 ng/mL and at higher level of quantitation (HQC) 1050, 1350 ng/mL, for SVA and SV respectively. All the spiked samples were stored below -80 ^{o}C.

2.5 Sample extraction protocol

A solid phase extraction method was developed to isolate SVA and SV from plasma. The thawed samples were vortexed to ensure complete mixing of contents. 100 µL of mewastatin working solution was mixed with 400 µL aliquot of each plasma sample in polypropylene tubes. 100 µL of ammonium acetate buffer was added into these polypropylene tubes and vortexed again for 30 seconds (s) to ensure complete mixing. The pretreated samples were then transferred to solid phase extraction (SPE) cartridges (Agilent; Bond Elute Plexa), which had been preconditioned using methanol and water (LC-grade). After centrifugation the analytes were eluted off the cartridges with 20% methanol in water. Eluents were evaporated to dryness at 20 psi and 40 ^{o}C under a stream of dry nitrogen. The residue was reconstituted with 300 µL of mobile phase, transferred into vials and 10 µL was injected into the LCMS–MS system for analysis.

2.6 Methodology for validation

Calibration curves were constructed from blank sample (plasma sample processed without IS), blank+IS samples and eight point calibration standards (lowest and highest standards were used in duplicates) for SVA and SV in plasma. The acceptance criteria for these calibration curves

was a correlation coefficient (r) of 0.9200 or better, and each back-calculated standard concentration must be within 15% deviation from nominal value except for the lower limit of quantitation (LLOQ), for which the maximum acceptable deviation was set at 20%. At least 67% of the non-zero standards were required to meet the acceptance criteria including acceptable lower (LLOQ) and upper limit of quantification (ULOQ). The following parameters were evaluated during validation of the method: selectivity, linearity, precision and accuracy, recovery, stability viz. long term stability, freeze–thaw stability, bench top stability, stock solution stability and auto-sampler stability.

2.6.1 Linearity

The linearity was performed on three batches of spiked samples. Each batch of spiked plasma samples included one complete set of calibration curve standards and six replicates of quality control samples at LLOQC, LQC, MQC and HQC levels. Linearity of the method was determined by plotting the area ratio of analytes/IS against Known concentration of analytes. Calibration measurements were subjected to least squares regression analysis ($1/x^2$) to provide information on the slope, y-intercept, correlation coefficient (r) and the back-calculated concentrations.

2.6.2 Intra and Inter Batch Precision and Accuracy

Intra and inter batch precision and accuracy were determined by analyzing six replicates of QC samples produced at four different concentration levels, i.e., LOQQC, LQC, MQC and HQC, each in a batch and on three different batches, respectively. Precision of the assay was measured by the percent coefficient of variation over different concentration levels. The acceptance criteria for intra and inter batch precision were 20% or better for LLOQ and 15% or better for other non-zero concentrations.

2.6.3 Extraction Efficiency

The extraction efficiency was obtained by comparing the peak area of six replicates of SVA and SV spiked in plasma at three different concentration levels, i.e., LQC, MQC and HQC with the peak area of processed blank (reconstituted with equivalent amounts of LQC, MQC and HQC in mobile phase). The % CV at all levels of QC's should be <20%.

2.6.4 Stability

Stability experiments in stock solution and plasma were performed rigorously to evaluate the stability of SVA, SV and IS. Following experimental conditions which the drugs actually encountered during sample analysis were simulated during method validation to evaluate the various stabilities.

2.6.4.1 Freeze Thaw Stability

The freeze/thaw stability in plasma was evaluated for three consecutive freeze thaw cycles from −80 °C to room temperature. Six replicates of LQC and HQC were analyzed after undergoing three freeze-thaw cycles.

2.6.4.2 Bench Top Stability

Bench top stability was determined for 24 h storage at room temperature, using six sets each of LQC and HQC. The QC samples were quantified against the freshly spiked calibration curve standards.

2.6.4.3 Auto Sampler Stability

In order to assess auto sampler stability, six sets of LQC and HQC samples were kept in an auto sampler in polypropylene container programmed at 1-10°C and were analyzed after 50 h along with freshly spiked samples, and the concentration was calculated against the freshly spiked calibration standards.

2.6.4.4 Injector Stability

Short-term stability was determined after the exposure (of processed samples) at 10 °C for 24 h in auto sampler using six sets each of LQC and HQC. After specified storage conditions, samples were processed and analyzed.

2.6.4.5 Long Term Stability

The long-term stability was assessed after storage of the standard spiked plasma samples at deep freeze (−80 °C) for one month. Six replicates of LQC and HQC were used for analysis.

3. Results and Discussion

For the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacological studies, the development of selective and sensitive bioanalytical methods plays an important role for the quantitative evaluation of drugs and their metabolites (analytes).To develop a method with a desired LLOQ and ULOQ and accuracy the tandem mass spectrometry (MS–MS) detection is best available option as MS–MS methods are capable of discriminating more efficiently as compared to LC or LC–MS between the analyte and matrix signals.

In this method simultaneous extraction of SV and SVA from plasma was very challenging as they undergo rapid interconversion at optimum conditions (Yang et al. 2005; Zhang et al. 2000). This conversion depends on pH, temperature, and storage condition and sample extraction methodology. Here we develop an efficient extraction and quantitative method for the simultaneous estimation of simvastatin and simvastatin acid without polarity switch and gradient flow in rat plasma.

First for both the analytes and IS the tuning was done by using 100 ng/ml solution in both positive and negative ionization modes. The response in positive ionization mode was higher than the negative ionization mode for analytes as well as IS. Previous studied suggest polarity switch between positive to negative for the simvastatin acid and simvastatin respectively. (Yang et al. 2005; Zhang et al. 2000; Barret et al. 2006) The polarity switch from high positive to high negative volts leads to a noisy baseline and variability of results (Chubatyi et al. 2012). Spray voltage was not found to have any significant effect on analyte sensitivity and was maintained at 5kv. The molecular ion and product ion transitions obtained for SV, SVA and IS are 419.00/284.75, 437.00/300.58 and 391.87/185.00 respectively. The flow rates for sheath gas /aux gas were 19/5, 14/5 and 22/5 for SV, SVA and IS respectively. The optimized collision energy for SV was 29%, for SVA 16% and for IS it was 17%.

Much effort was made to make the method more reproducible and robust during method development. Because of good recovery and purity SPE was used for sample extraction rather than liquid–liquid extraction (LLE). This method has advantage over or any other extraction technique as it is effective even when the solutes are present at extremely low concentrations and chances of volatile impurities in the processed sample decreases and gives cleaner sample (Dutta

et al. 2012). Ammonium acetate buffer was added to plasma samples, which helped in keeping both the analytes in unionized condition. Bond Elute Plexa cartridges were used for the extraction, which retained both the unionized analytes. Plexa provides enhanced performance due to a unique polymeric structural design with a non-retentive, hydroxylated, amide-free surface and a non-polar core to retain small molecules. Lipid and protein binding on the polymer surface is reduced which results in cleaner samples and decreased ion suppression.

Various columns and Combinations of the mobile phase were tried to achieve good resolution and symmetric peaks and a low retention time. Acetonitrile and ammonium acetate buffer (1 mM, pH 4.0) in the ratio of 80:20, v/v was found to be a suitable mobile phase. This ratio of the organic phase to the aqueous phase was very critical in eluting both the analytes in their respective ionization windows as SV and SVA have different physicochemical properties. So it was difficult to set chromatographic conditions that produced sharp peak shape and adequate response. Good resolution and good peak shape was achieved using an Agilent hypersil ODS column (200 x 2.1 mm, 5-µm) and the above mentioned mobile phase. The flow rate was optimized to 500µL/min. The RT for SV, SVA and IS were 4.11, 3.35 and 2.89 min respectively, making it possible to quantify both the analytes in a short time and in a single.

3.1 Method Validation

3.1.1 Selectivity

The SPE method employed for the extraction of SV, SVA and IS gave good selectivity for the analysis in the rat plasma. The area observed in all the lots of blank plasma was less than 20% in the LLOQ area, and the area observed at the RT of IS was less than 5%. the selectivity results with the chromatograms of SV, SVA and IS in blank plasma, blank+IS, LLOQ HLOQ are shown in Figures 2 and 3 No interferences were found for SV, SVA and IS. The retention time for all the analytes was less than 4.5 min, which makes it suitable for the analysis large number of sample in short duration.

3.1.2 Linearity

Linearity of SV and SVA was established over a concentration range of 50-1800 ng/ml and 10-1000 ng/ml respectively in spiked rat plasma. Linear coefficient of regression for SV (r^2>0.996) and SVA (r^2>0.994) was obtained using least squares linear regression model using peak area.

The limit of quantification in the present method for SV and SVA was 50 and 10 ng/ml respectively.

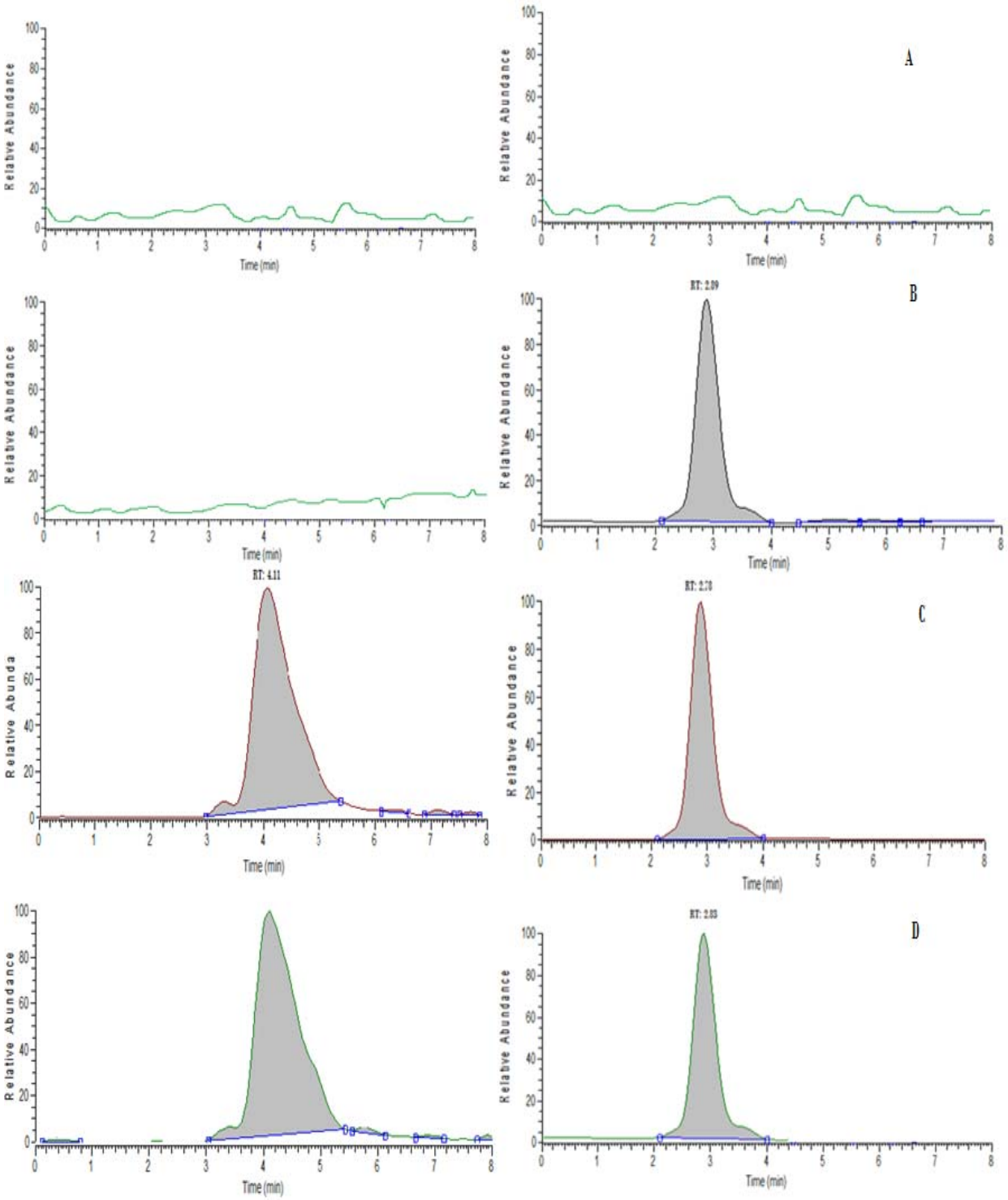

Figure. 2 Simvastatin Chromatograms in double blank plasma (A), blank + internal standard (B), lower LOQ (C) and HQC.

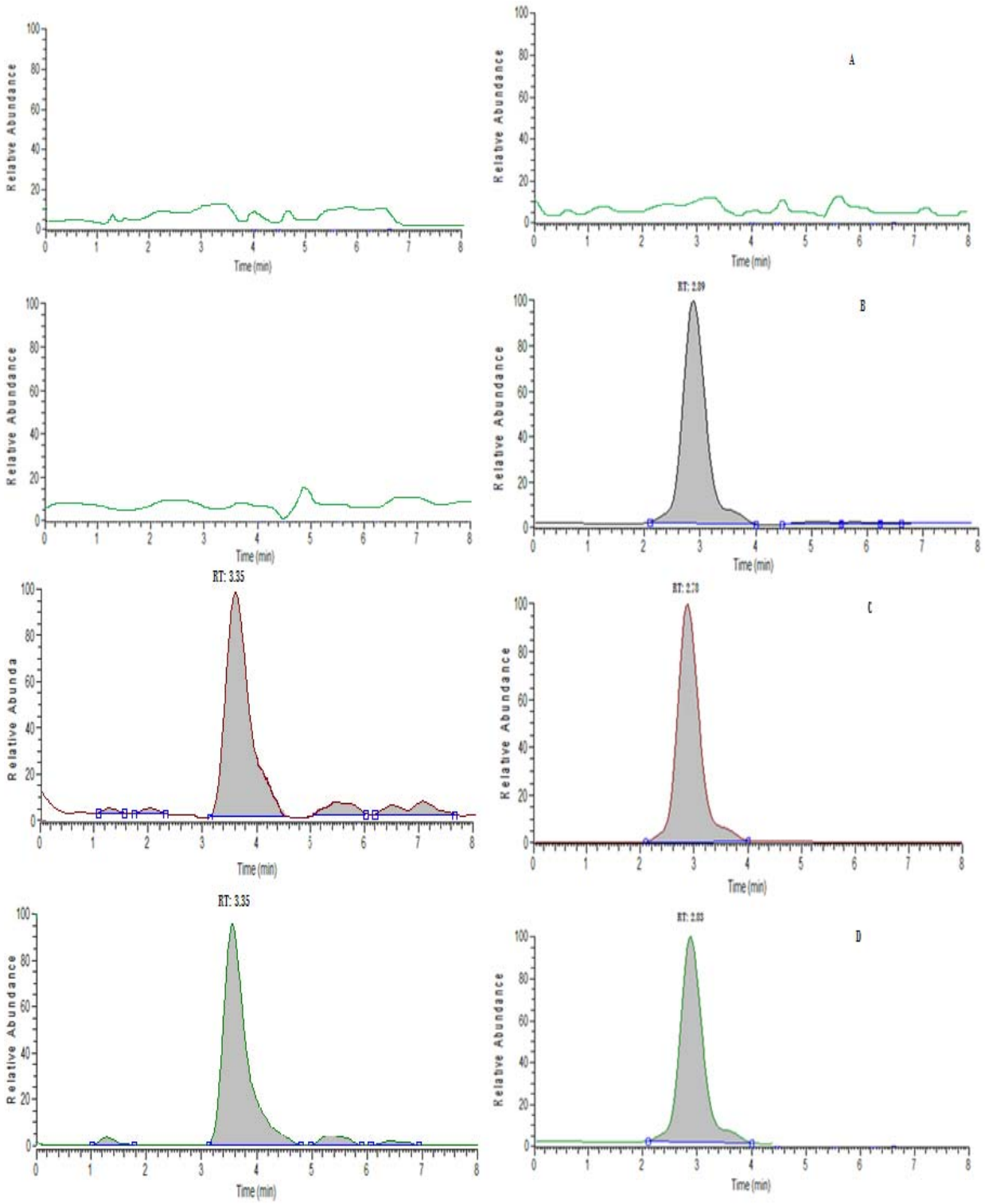

Figure. 3 Simvastatin acid chromatograms in double blank plasma (A), blank + internal standard (B), lower LOQ (C) and HQC.

3.1.3 Intra and inter batch precision and accuracy

Three, batches were run to check intra and interbatch precision and accuracy. The results for precision and accuracy are summarized in Table 1, 2, 3, and 4. Both intra- and inter-CV values ranged from 5.37 to 12.81% for SV and 3.83 to 9.81% for SVA. The accuracy for SV ranged from 91.71 to 106.98% and 99.25 to 104.64% for SVA.

3.1.4 Recovery

The recovery of SV and SVA was calculated by using triplicate samples. The mean recovery of SV and SVA was found to be 79.88 and 74.67%, respectively (Table 5). The CV for all the levels of SV and SVA was below 5%.

3.1.5 Stability

The results of freeze thaw stability, bench top stability, auto sampler stability, injector stability and long term stability are given in Table 6. Freeze–thaw stability was assessed by assaying six replicates of QC samples at low concentrations (LQC), and high concentrations (HQC) previously frozen and thawed at room temperature over three cycles. The comparison was made to freshly spiked calibration standards. The SV and SVA were found to be stable in biological samples for three freeze and thaw cycles. Bench top stability for SV and SVA was assessed for 24 h. Samples were left on bench at room temperature and then processed with freshly spiked sample before analysis. The processed samples were found to be stable in auto sampler for 50 h. The short term injector stability for 10 hrs was ranged from 95.02 to 95.87% for SV and 94.29% to 99.25% for SVA. The plasma samples were found to be stable when stored at −80 °C for a period of 30 days The analytes were found to be stable as the precisions of all the stability samples was <15% and the accuracies was in the range of 100±15%.

3.2 Pharmacokinetic Application

This method was applied to quantitate the SV and SVA in rat plasma samples from a bioavailability study involving vitamin and drug interaction. SV was administered at a dose of 100 mg/kg to experimental animals and blood sampling was done at predefined time points. The mean plasma concentration-time profile is given in Figure 4. The Cmax obtained for SV and SVA was found out to be 1165.69 ng/ml and 748.26 ng/mL, respectively. The AUC_{0-t} for SV

was 3262.52 ng ×h/mL and 1993.49 ng h/mL for SVA.

Table. 1 Intra assay precision and accuracy for simvastatin acid

Batch#	QC samples	Nominal Values (ng/ml)	Statistical Parameters			
			Mean	SD(±)	%CV	% Nominal
1	LOQQC	10	10.30	0.55	5.38	102.97
	LQC	20	20.65	0.79	3.83	103.26
	MQC	700	705.51	43.54	6.17	100.79
	HQC	1050	1042.16	92.07	8.83	99.25
2	LOQQC	10	10.24	1.00	9.81	102.37
	LQC	20	20.93	0.93	4.44	104.64
	MQC	700	711.91	42.88	6.02	101.70
	HQC	1050	1043.93	72.26	6.92	99.42
3	LOQQC	10	10.35	0.87	8.42	103.53
	LQC	20	20.73	0.87	4.17	103.65
	MQC	700	717.65	59.76	8.33	102.52
	HQC	1050	1074.16	102.48	9.54	102.30

Table. 2 Inter assay precision and accuracy for simvastatin acid

QC samples	Nominal Values (ng/ml)	Statistical Parameters			
		Mean	SD(±)	%CV	% Nominal
LOQQC	10	10.30	0.78	7.60	102.96
LQC	20	20.77	0.82	3.95	103.85
MQC	700	711.69	46.64	6.55	101.67
HQC	1050	1053.41	85.71	8.14	100.33

Table. 3 Intra assay precision and accuracy for simvastatin

Batch#	QC samples	Nominal Values (ng/ml)	Statistical Parameters			
			Mean	SD(±)	%CV	% Nominal
1	LOQQC	50	52.96	4.15	7.84	105.92
	LQC	100	102.59	9.16	8.93	102.59
	MQC	900	875.91	50.08	5.72	97.32
	HQC	1350	1238.06	100.30	8.10	91.71
2	LOQQC	50	47.66	4.38	9.20	95.32
	LQC	100	103.51	12.82	12.38	103.51
	MQC	900	906.45	61.86	6.82	100.72
	HQC	1350	1369.14	73.46	5.37	101.42
3	LOQQC	50	49.39	6.33	12.81	98.78
	LQC	100	105.22	13.22	12.56	105.22
	MQC	900	962.80	78.72	8.18	106.98
	HQC	1350	1337.29	158.82	11.88	99.06

Table. 4 Inter assay precision and accuracy for simvastatin

QC samples	Nominal Values (ng/ml)	Statistical Parameters			
		Mean	SD(±)	%CV	% Nominal
LOQQC	50	50.00	5.26	10.52	100.01
LQC	100	103.77	11.21	10.80	103.77
MQC	900	915.05	71.12	7.77	101.67
HQC	1350	1314.83	123.55	9.40	97.39

Table. 5 Percent recovery for simvastatin acid and simvastatin

S.N.	Drugs/Metabolite	% Recovery			Mean	SD (±)	% CV
		LQC	MQC	HQC			
1	Simvastatin Acid	71.25	74.47	78.29	74.67	3.52	4.72
2	Simvastatin	76.58	80.48	82.57	79.88	3.04	3.81

Table 6. Different stability parameters for simvastatin acid and simvastatin

Parameters	Simvastatin Acid				Simvastatin			
	Actual Concentration (ng/ml)	Concentration Found (ng/ml)	Precision %	Accuracy %	Actual Concentration (ng/ml)	Concentration Found (ng/ml)	Precision %	Accuracy %
Freeze Thaw Stability	20	18.46	4.49	92.29	100	96.36	2.68	96.36
	1050	1045.83	8.46	99.60	1350	1297.71	4.40	96.13
Bench Top Stability	20	18.67	6.79	93.35	100	95.14	6.23	95.14
	1050	1065.53	9.50	101.48	1350	1340.66	5.97	99.31
Auto Sampler Stability	20	19.95	5.00	99.73	100	98.10	6.81	98.10
	1050	1097.31	5.54	104.51	1350	1320.63	10.29	97.82
Injector Stability	20	18.86	3.46	94.29	100	95.02	8.21	95.02
	1050	1042.16	8.83	99.25	1350	1294.22	9.20	95.87
Long Term Stability	20	19.30	6.48	96.49	100	101.52	12.06	101.52
	1050	1020.41	6.81	97.18	1350	1369.14	5.37	101.42

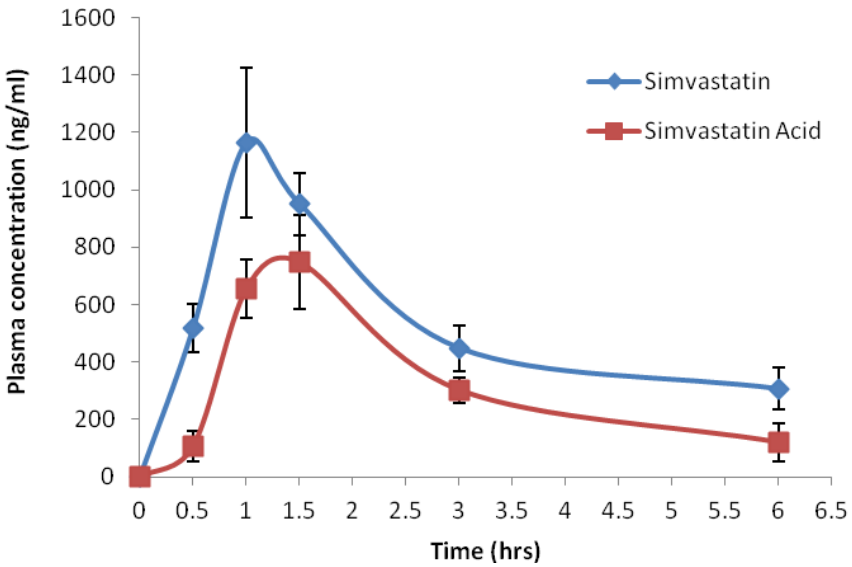

Figure.4 Plasma concentration profile of simvastatin and simvastatin acid (mean values ±SD) in experimental animals after oral administration of 100 mg/kg simvastatin.

4. Conclusion

A simple, reproducible and reliable method has been developed for the simultaneous quantitation of SV and SVA in rat plasma using LC-ESI-MS-MS with positive ionization mode. The method is specific, precise and accu rate in the concentration range of 50-1800 ng/ml and 10-1000 ng/ml SV and SVA respectively in rat plasma. This novel method is able to quantify the SV and SVA simultaneously in a single run without gradient flow and polarity switch and ultimately saves the time and cost both. The specificity and sensitivity of this method make it appropriate for preclinical pharmacokinetics studies of SV and SVA.

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