

Development and Validation of LC-MS/MS Method for the Estimation of Lovastatin and its Metabolite (β -Hydroxy Acid) in Pharmaceutical Dosage Form

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ABSTRACT

The objective of this study was to validate a simple, specific, accurate and precise Liquid - Liquid extraction high performance liquid chromatographic method with Tandem Mass Spectrometry-Thermo Scientific TSQ Quantum Ultra method for the determination of Lovastatin and Lovastatin acid in human plasma using Atorvastatin as Internal Standard (IS). The precision and accuracy data have to fulfill the requirements for quantification of the analytes in biological matrices to generate data for bioequivalence, bioavailability investigations. A Luna C18, 5 μ m column having 4.6 x 150 mm internal diameter in isocratic elution mode with flow rate 1.0 mL/min of mobile phase containing and Methanol, 5mM ammonium formate in 0.1% formic acid (80:20v/v) were used. The chromatographic separation was achieved by using elution solution consisting of Methanol and 5mM ammonium formate in 0.1% formic acid (80:20%v/v), diluent solution of methanol and water (50:50%,v/v) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) mode. The method was validated for both Lovastatin and Lovastatin acid over the concentration range of 0.05-5.00 ng/mL using 300 μ L plasma samples. Limit of quantification was found 0.05 ng/mL. The retention time for Lovastatin acid and Lovastatin min 2.84 and 3.45 respectively and Internal Standard were 2.06 min and overall chromatography run time was 5.50 minutes. The mean recovery of Lovastatin (71.00%) and Lovastatin acid (81.20) and IS (79.84%) from spiked plasma samples was consistent and reproducible. The method was validated for linearity, accuracy, precision, specificity, limit of quantification and robustness. The intra- and inter-day precision and accuracy values were found to be within the assay variability limits as per the FDA guidelines¹. The developed assay method was applied to a clinical pharmacokinetic study in human volunteers.

Keywords: Lovastatin, Lovastatin acid, Atorvastatin, LC-MS/MS, Linearity, Validation.

INTRODUCTION

Lovastatin is a cholesterol-lowering agent isolated from a strain of *Aspergillus terreus*. After oral ingestion, Lovastatin, which is an inactive lactone, is hydrolyzed to the corresponding β -hydroxy acid form. This is a principal metabolite and inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, which is an early and rate limiting step in the biosynthesis of cholesterol and was the first of this class of agents approved for clinical use². Lovastatin is [1 S - [1 α (R*),3 α ,7 β ,8 β (2 S*,4 S*), 8 α]]-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)ethyl]-1-naphthalenyl 2-methylbutanoate. The empirical formula of Lovastatin is C₂₄H₃₆O₅ and its molecular weight is 404.55 g/mol. Lovastatin acid is (3R,5R)-7-[(1S,2S,6R,8S,8aR)-2,6-dimethyl-8-[(2S)-2-methylbutanoyl]oxy-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoic acid. The empirical formula of Lovastatin acid is C₂₄H₃₈O₆ and its molecular weight is 422.562 g/mol. The molecular

formula of Atorvastatin (Internal Standard) is C₃₃H₃₅FN₂O₅ and molecular weight 558.64 g/mol. The molecular structure of Lovastatin, Lovastatin acid and Atorvastatin were shown in figure 1, 2 and figure 3 respectively.

Typical LC-MS/MS spectra of Lovastatin, Lovastatin acid and Atorvastatin were shown in figure 4, 5 and figure 6 respectively.

Lovastatin and Lovastatin acid are soluble in acetone, methanol, ethanol, ethyl acetate and water. Lovastatin pKa values are 2.8 and 14.91 at 37°C and the partition coefficient (log P) in n-octanol is 4.26. Lovastatin levels in plasma after therapeutic oral doses are reported to be very low compared with levels observed after intravenous dosing. The results in the existing literature show that only 5 % of the drug is bioavailable when given orally. This explains the need for sensitive and selective methods for the determination of Lovastatin in plasma for therapeutic monitoring of drug levels.

Among chromatographic techniques; the reversed-phase (RP) HPLC was widely used for the analysis. Present study

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involves development of LC-MS/MS method using simple mobile phase which was sensitive and rapid for quantification of Lovastatin and Lovastatin acid in plasma samples as well as subsequent validation of developed method according to ICH guide lines.

MATERIALS AND METHODS

Instrument

The liquid chromatographic system consisted of Thermo Scientific TSQ Quantum Ultra mass detector containing LCQUAN version 2.5 with Xcalibur 1.4 chromatographic data system, auto sampler and column oven. Chromatographic analysis was performed using Luna C18, 5 μ m column having 4.6 x 150 mm internal diameter in isocratic elution mode. Sartorius electronic balance was used for weighing purpose.

Reagents and Materials

Methanol of HPLC grade was purchased from Merck, India. HPLC grade water was obtained by double distillation and purification through Milli-Q water purification system. Ammonium formate and Formic acid of HPLC grade were procured from Sigma-Aldrich, India.

Preparation of Solutions

The buffer solution consisting of ammonium formate diluted to 1000 mL Milli Q-water and added 1mL of formic acid and filtered through 0.22 μ m membrane filter. The elution solution consisting of methanol and water (80:20%, v/v), diluent solution of methanol and water (50:50%, v/v) and mobile phase of 5mM ammonium formate and methanol (20:80% v/v in 0.1% formic acid) were prepared and filtered through Nylon (0.22 μ m) membrane sample filter paper and degas. The all solutions were stored at ambient temperature.

Preparation of Drug Stock Solution

A stock solution of Lovastatin, Lovastatin acid and internal standard were prepared by accurately weighing 5 mg of drug/internal standard, transferring to 10 mL of volumetric flask, containing 10 mL of methanol and water (50:50%, v/v) dissolving it to obtain final standard solution of 0.5 mg/mL of Lovastatin, Lovastatin acid and internal standard. Resultant solution was stored in refrigerator between 2-8 °C.

Preparation of Standard Blank and Zero Standard Samples

The standard blank samples were prepared by transferring 0.3 mL screened blank plasma into pre-labeled vials. The zero standard was prepared by adding 20 μ L of internal standard solution (1.0 μ g/mL) into 0.3 mL screened blank plasma into a pre-labeled vial and vortex for 15 seconds.

Preparation of Calibration Curve Solutions and Samples

The calibration curve standards spiking solutions were prepared by adding 20 μ L of drug stock solution (0.5 mg/mL) with diluent (Methanol: Water, 50:50%v/v) in different vials and add 20 μ L internal standard solution (1.0 μ g/mL) and vortex for 15 seconds. Prepare the calibration curve standards by spiking the respective calibration curve standards spiking solutions in screened blank plasma in different vials to obtain final concentration of 0.054, 0.148, 0.643, 1.286, 2.144, 3.107, 4.142 and 5.178 ng/mL of both Lovastatin and Lovastatin acid.

Preparation of Quality Control Solutions and Samples

The quality control spiking solutions were prepared by adding 20 μ L of drug stock solution (0.5 mg/mL) with diluent (Methanol: Water, 50:50%v/v) in different vials and add 20 μ L internal standard solution (1.0 μ g/mL) and vortex for 15 seconds. Prepare the quality control samples by spiking the respective quality control spiking solutions in screened blank plasma in different vials to obtain lower limit of quantification quality control (LLOQ QC), lower quality control (LQC), middle quality control-2 (MQC2), middle quality control-1 (MQC1), higher quality control (HQC) and upper limit of quantification quality control (ULOQ QC) samples of concentrations of 0.054, 0.151, 1.252, 2.845, 3.951 and 5.0ng/mL of both Lovastatin and Lovastatin acid respectively. The LLOQ QC and ULLOQ QC solution were prepared only for method validation and screening of blank plasma samples.

Preparation of Working Solutions for Stability Testing

The working spiking solutions were prepared for long-term solution stability and short-term solution stability by adding drug stock solution (0.5 mg/mL) with diluent (Methanol: Water, 50:50%v/v) in different vials and vortex for 15 seconds.

Chromatographic Conditions

The high performance liquid chromatography method with Tandem Mass Spectrometry- Thermo Scientific TSQ Quantum Ultra was developed for analysis of Lovastatin and Lovastatin acid in human plasma matrix. The mobile phase solution pumped into a Luna C₁₈, 5 μ column having 4.6 x 50 mm internal diameters in isocratic elution mode was maintained at 1.0 mL/min flow rate. The injection volume was 10 μ L and acquisition time 5.50 minutes was equilibrated with mobile phase flowing through the system. The auto sampler temperature was 5°C.

Calibration Curve

The calibrated curve and quality control samples were injected into liquid chromatographic system, chromatograms were obtained and peak area ratio was determined for each concentrations of Lovastatin and Lovastatin acid. Calibration curves of Lovastatin and Lovastatin acid were constructed by plotting peak area ratio versus applied concentrations of Lovastatin and Lovastatin acid using linear calibration function fit curve and weighting method with offset 1/X².

Method Validation

The validation was performed to evaluate the method in terms of the parameters e.g., linearity response, sensitivity, selectivity, precision and accuracy (within-batch and between-batch/inter-day), stabilities (freeze-thaw, bench top, short-term and long-term stock solutions, working solutions and long term stability in matrix), carryover effects, recovery, dilution integrity, matrix effect, matrix factor, auto sampler re-injection reproducibility and ruggedness experiment.

System suitability

System suitability experiment was performed by injecting six consecutive injections at least once in a day with using aqueous MQC1 solution. System performance experiment was performed by injecting sequence of injections at the beginning of analytical batch or before any re-injection.

The Signal to Noise ratio should be more than or equal to 10.0 for LLOQ QC sample.

Carryover effect

The carryover effect of the auto sampler was performed by injecting sequence of injections during the start of the method validation, new equipment, change in configuration or any major malfunction of auto sampler.

Specificity/selectivity and sensitivity

Specificity and sensitivity of the analytical method were assessed in relation to interference peaks from endogenous plasma constituents by comparing their retention times with those of Lovastatin, Lovastatin acid and Internal standard. The specificity of the intended method was established by screening the standard blank plasma (without spiking with drug or internal standard). Ten different batches of plasma out of which, seven normal, one lipidemic and one hemolyzed plasma having anticoagulant and one heparinized plasma as an anticoagulant was screened. The specificity was evaluated by comparing the responses of interfering peak at the retention time of Lovastatin, Lovastatin acid and internal standards in the standard blank against the response of the respective extracted LLOQ and aqueous LLOQ. The sensitivity was evaluated by calculating the precision and accuracy of LLOQ (concentration should be equivalent to LLOQ) sample in each of the at least three acceptable precision and accuracy batches individually and in total (between batches).

Matrix effects

The matrix effect for the intended method was assessed by using concentrations equivalent to LQC and HQC prepared with seven different plasma batches/lots. Process and analyze the samples with freshly spiked calibration curve standards and at least two sets of batch qualifying quality control samples were prepared in previously screened biological matrix.

Matrix factors

The matrix factor was given as the ratio of analysis of the analytical response obtained from analysis of extracted blank matrix samples spiked after extraction with the Lovastatin and Lovastatin acid, at four concentrations (low and high) and Internal Standards (at the working concentrations) relative to the analytical response obtained from reference solutions. Six batches of human plasma (including hemolytic and lipidemic lot) were processed and after extraction of the blank plasma samples, was spiked with Lovastatin and Lovastatin acid at concentration equivalent to those in the low and high Quality control extracted samples and internal standard at its working concentration.

Calibration of standard curve (Linearity and range)

The linearity of the method was determined by using standard plots associated with 8point standard curve including LLOQ and ULOQ. Concentration of calibration curve standards was calculated against the calibration curve and the linearity of the method was evaluated by ensuring the acceptance of precision and accuracy of calibration curve standards. Two consecutive calibration curve standards should not be beyond the acceptance criteria. The lower limit of quantification (LLOQ) was the lowest concentration at which the precision expressed by

relative standard deviations (RSD, CV%) is better than 20% and the accuracy (bias) expressed by relative difference of the measured and true value was also lower than 20%.

Precision and accuracy

The within-run and between-run percentage mean of precision and accuracy of the Lovastatin and Lovastatin acid were measured by the percent coefficient by using 6 replicate samples of variation over the concentration range of LLOQ, low, middle 1&2 and high quality control samples for the three precision and accuracy batches to their nominal values. The acceptable % coefficient of precision and accuracy should be less than 15%. The between and within batch % mean precision and accuracy for LQC, MQC1, MQC2 and HQC samples were within the range of 85.00-115.00% and for the LLOQ QC within the range of 80.00-120.00% respectively.

Recoveries

The % mean Lovastatin and Lovastatin acid recoveries was determined by comparing the mean peak area of the 6 replicates of extracted plasma quality control samples at high, middle 1&2 and low concentrations against respective mean peak area of the 6 replicates of un-extracted quality control samples at high, middle 1&2 and low concentrations⁴. A good recovery should be more than 90%. The % mean Internal Standard recovery was determined by comparing the mean peak area of internal standard in the extracted plasma quality control samples at MQC1 and MQC2 concentration against the mean peak area of internal standard in the un-extracted quality control samples at MQC1 and MQC2 concentrations.

Dilution integrity

The dilution integrity of the method was evaluated by diluting the stock solution prepared as spiked quality control sample at concentration 1.5-2 times above the concentration of the highest standard in the calibration curve in the screened biological matrix⁵. Conduct dilution integrity experiment by using six replicates each of diluted quality control (1/5) and diluted quality control (1/10) samples. Process and analyze the diluted quality control samples along with freshly spiked calibration curve standards and at least two sets of batch qualifying quality controls (at lower and higher).

Ruggedness

Ruggedness of the method was evaluated by using different analyst and different column of the same make and model or different equipment of the same make and model. The ruggedness experiment should meet the acceptance criteria for linearity and intra-batch accuracy & precision.

Stability of Lovastatin & Lovastatin acid and Atorvastatin (IS)

Short term stock solution stability

Short term stock solution stability for Lovastatin, Lovastatin acid and internal standard were performed at the stock concentration by six consecutive injections of aqueous standard equivalent to ULOQ concentration and working concentration respectively after storage of 06 hours at ambient temperature. Stability was assessed by comparing the stock dilutions of Lovastatin, Lovastatin

acid and Internal Standard prepared from the freshly prepared stock solutions (comparison) against stock dilutions of Internal Standard prepared from the stock solutions stored at ambient temperature (stability). Short term stock solution stability was evaluated by comparing the mean response of stability samples against mean response of comparison samples.

Long term stock solution stability

Long term stock solution stability for Lovastatin, Lovastatin acid and internal standard were performed at the stock concentration by using six consecutive injections of aqueous standard equivalent to ULOQ concentration and working concentration respectively after storage of 07 days by considering the expected duration of the usage of the stock solution in the refrigerator at 2-8°C. Stability was assessed by comparing the stock dilutions of Lovastatin, Lovastatin acid and Internal Standard prepared from the freshly prepared stock solutions (comparison) against stock dilutions of Lovastatin, Lovastatin acid and internal standard prepared from the stock solutions stored at 2-8°C (stability)⁶. Long term stock solution stability was evaluated by comparing the mean response of stability samples against mean response ratios of comparison samples.

Working solution stability

Short term stability (06 hours at ambient temperature) and long term stability (07 days at 2-8°C) for working solutions of drug (stock solution ULOQ and LLOQ) and Internal Standard were performed by using six consecutive injections of equivalent aqueous standards prepared from fresh and stored solutions. Short term stability and long term stability of working solution were evaluated by comparing the mean response of stability samples against mean response of comparison samples.

Stability of Drug in Biological Matrix

Perform the matrix stability experiment by using freshly prepared calibration curve standard and two replicates of freshly prepared batch qualifying quality control samples at HQC and LQC levels⁷. Stability studies in biological matrix were conducted in the various conditions at LQC and HQC levels as described below:

Freeze thaw stability

Freeze thaw stability of the spiked quality control samples were determined after 1st and 5th freeze thaw cycles stored at -20±5°C. Six replicates of each HQC and LQC samples were used for assessing each freeze thaw experiment (for first and fifth cycle at both the freezing temperatures). The first freeze-thaw cycle was of at least 24 hours followed by minimum of 12 hours for subsequent cycles. Process and analyze freeze thaw stability samples along with freshly spiked calibration curve and comparison samples (6 replicates of each LQC and HQC) in screened biological matrix. Evaluate the freeze thaw stability on the basis of % change of LQC and HQC samples⁸. The % Change and % CV of LQC and HQC should be within ± 15.0 and □ 15.0 respectively.

Bench top stability

Spiked quality controlled samples (6 replicates of each LQC and HQC) were stored in deep freezer at temperature -20±5°C, which was retrieved after minimum 12 hours of

freezing and was kept at ambient temperature on working bench for recommended period of 06 hours. Six replicates of each HQC and LQC samples were used for assessing the bench top stability experiment. Upon the completion of recommended period, processed and analyzed bench top stability samples along with freshly spiked calibration curve and comparison samples (6 replicates of each LQC and HQC) in screened biological matrix⁹. Evaluated the bench top stability on the basis of % change of LQC and HQC samples.

Auto sampler re-injection reproducibility

Auto sampler re-injection reproducibility was evaluated by re-injecting accepted precision & accuracy batch, which were stored in auto sampler for 24 hours or as per requirement.

RESULTS

Stock Solution stability Verification

The stock solution verification was conducted by preparing the stock solution each of Lovastatin, Lovastatin acid and internal standard diluted by spiking 20 µL of working calibration standard and 20 µL of working internal standard level in 960 µL of elution solution. The CV% of response ratio of six replicate injections from stock solutions of Lovastatin, Lovastatin acid and internal standard was 4.6, 3.2 and 2.6, respectively.

System Suitability

System performance experiment was performed by injecting six consecutive injections at the beginning of analytical batch or before any re-injection and signal to noise ratio was 29.5 for LLOQ QC sample and % CV of area ratio was 3.86, respectively. Injector carryover test was performed and there was no significant injector carryover observed.

Specificity/Selectivity and Sensitivity

Selected blank human plasma from six different sources and were carried through the extraction procedure and chromatographed to determine the extent to which endogenous human plasma components may contribute to chromatographic interference with the Lovastatin, Lovastatin acid and internal standard. One hemolyzed and one lipemic plasma were also checked for specificity along with this run¹⁰. The retention time of Lovastatin acid, Lovastatin and internal standard were approximately 2.84, 3.45 and 2.06 respectively. No significant interference was observed in six different lots of human plasma, hemolyzed plasma and lipemic plasma samples. The peaks were completely separated and there was no interference peaks from endogenous substances in plasma that was co-eluted with Lovastatin, Lovastatin acid as well as the internal standard. The lower limit of quantitation for both Lovastatin, Lovastatin acid, signal to noise ratio and %CV of area ratio were 0.05ng/mL, 29.5 and 19.4% respectively. The overall chromatography run time was 5.50 minutes.

Matrix Factor

Samples of the relevant biological matrix from six different sources were collected. The lower calibration standard samples from each source were prepared and injected along with the six replicates of aqueous lower

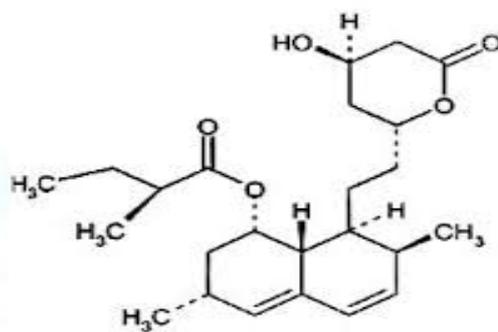


Figure 1: Molecular structure of Lovastatin.

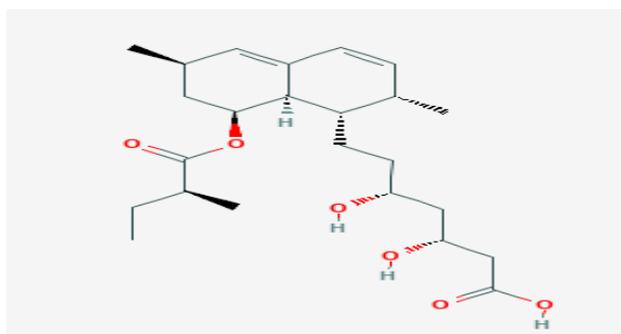


Figure 2: Molecular structure of Lovastatin Acid.

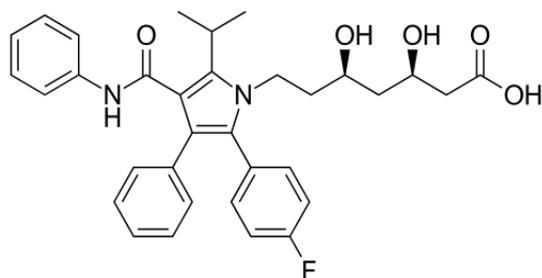


Figure 3: Molecular structure of Atorvastatin (Internal Standard).

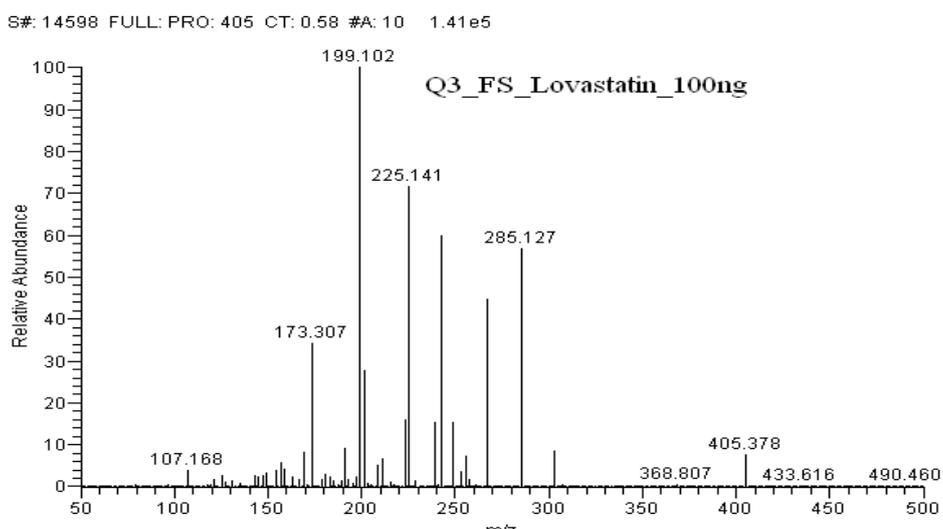


Figure 4: Typical Spectra of Lovastatin analyzed by LC-MS/MS.

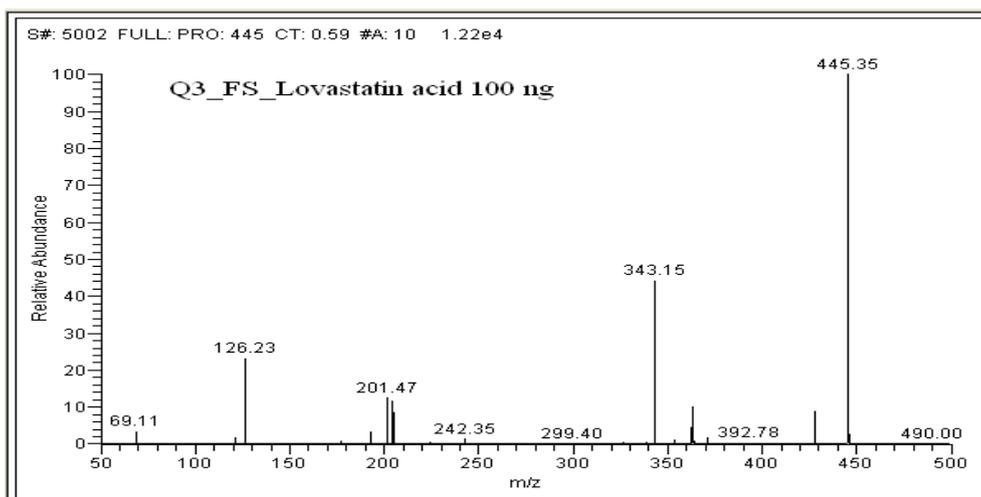


Figure 5: Typical Spectra of Lovastatin acid analyzed by LC-MS/MS.

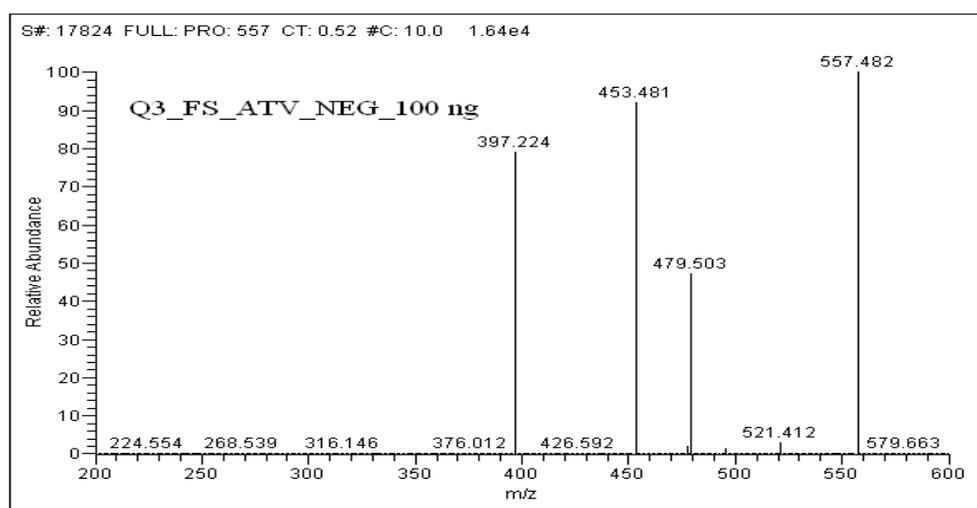


Figure 6: Typical Spectra of Atorvastatin analyzed by LC-MS/MS.

Table 1: Recovery of Lovastatin and Lovastatin acid in spiked plasma.

Spiked concentration of Lovastatin and Lovastatin acid (ng/mL)	Peak Area (mean \pm SD), n=6				% Recovery	
	Extracted Lovastatin response	Extracted Lovastatin Acid response	Un-extracted Lovastatin response	Un-extracted Lovastatin Acid response	Lovastatin	Lovastatin acid
HQC (3.695)	2770486.3 \pm 150721.54	1094121.5 \pm 24517.83	3923268.0 \pm 392975.72	1369672.8 \pm 71299.38	70.6	79.9
MQC1 (2.217)	1732945.8 \pm 72153.93	1102768.2 \pm 44394.84	2376766.7 \pm 187574.81	1329638.5 \pm 41019.42	72.9	82.9
MQC2 (1.109)	891713.0 \pm 87774.95	1121005.7 \pm 61746.52	1248422.5 \pm 162395.82	1357105.7 \pm 31838.66	71.4	82.6
LQC (0.133)	103576.5 \pm 14600.44	1146454.7 \pm 25432.28	149699.2 \pm 22349.18	1441114.2 \pm 59423.39	69.2	79.6

calibration standard level concentrations¹¹. The %CV of matrix factor for Lovastatin and Lovastatin acid were 5.4, and 4.2, respectively.

Calibration of Standard Curve (Linearity and Range)

The calibration curve was constructed between peak height ratios of Lovastatin, Lovastatin acid to the internal standard against Lovastatin, Lovastatin acid concentrations as given in Figure 07&08. Linearity was

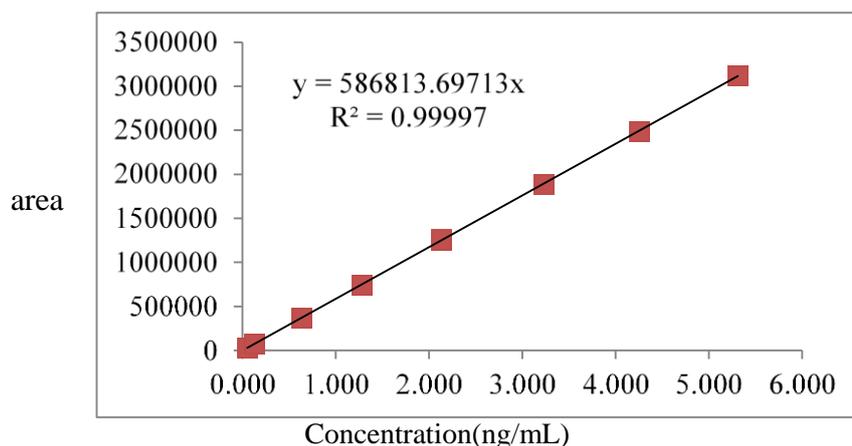


Figure 7: Typical calibration curve of Lovastatin in plasma.

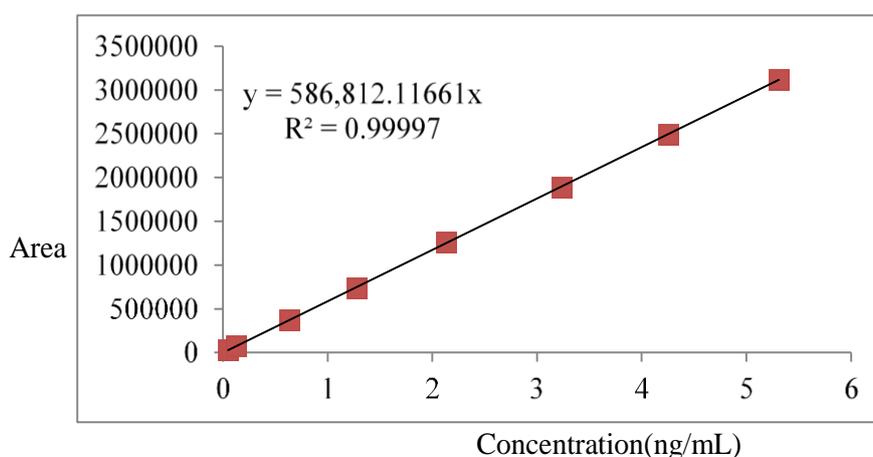


Figure 8: Typical calibration curve of Lovastatin acid in plasma

demonstrated by multiple analysis of spiked plasma sample containing both Lovastatin and Lovastatin acid between 0.051-5.309 ng/mL calibration ranges. The regression equation of Lovastatin and Lovastatin acid concentrations over its peak area ratio was found to be $y=586813.69713x$ and $y=586,812.11661x$, where x is the concentration of Lovastatin and Lovastatin acid and y is the respective peak area. The regression coefficient (r^2) is 0.9997 for both Lovastatin and Lovastatin acid. A good linear relationship with the coefficient of determination (r^2) of more than 0.99 was employed for determining of Lovastatin and Lovastatin acid concentrations in plasma. Back calculations were made from the calibration curves to determine Lovastatin and Lovastatin acid of each calibration standard. The lower limit of quantification (LLOQ) was established at 0.05 ng/mL with the coefficient of variation of 11.7% indicates the sensitivity of the method. Analyzed plasma Lovastatin and Lovastatin acid concentrations below the quantification limit were defined as zero ng/mL.

Recovery

The % recovery was calculated from ratio of area of extracted to un-extracted samples at each level are shown in Table 1. The six un-extracted samples each of low, medium 1&2 and high quality control samples were prepared by spiking of 20 μ L drug and 20 μ L internal standards in

extracted blank plasma were eluted. The recovery results were based on a comparison of the LC-MS/MS response from plasma (un-extracted) to those from extracted plasma blank. The mean % recovery for LQC, MQC2, MQC1 and HQC of Lovastatin and Lovastatin acid are 69.2, 71.4, 79.2 & 70.6 and 79.6, 82.6, 82.9, 79.9 respectively. The mean % recovery for Atorvastatin (IS) is 89.9.

Precision and Accuracy

Between-run accuracy and precision

The between-run accuracy and precision evaluation were assessed by the repeated analysis of human plasma samples containing different concentrations of Lovastatin and Lovastatin acid on separate occasions¹². A single run consisted of a calibration curve, six replicates of lower limit of quantitation (LLOQ), low, medium (1&2) and high quality control samples and results are expressed as the percentage of accuracy of the analytical method presented in Table 2. The between-run % coefficient of variation for Lovastatin and Lovastatin acid ranged from 1.9 to 11.5 and 1.5 to 14.9 respectively. The between-run % of accuracy value for Lovastatin and Lovastatin acid were ranged from 98.9 to 106.0 and 97.4 to 106.2 respectively.

Within-run accuracy and precision

Analyzing replicate concentrations of Lovastatin and Lovastatin acid in human plasma performed within-run accuracy and precision evaluations. The run consisted of a calibration curve plus a total of 30 spiked samples,

Table 2: Between-run precision and accuracy of the analytical method for Lovastatin and Lovastatin acid in plasma concentrations (ng/mL)

Amount Added		Measured (mean ± SD)	Measured (mean ± SD)	Lovastatin		Lovastatin acid	
Lovastatin	Lovastatin acid	Lovastatin	Lovastatin acid	% CV	% Accuracy	% CV	% Accuracy
HQC 3.695	HQC 3.734	3.77003± 0.070826	3.73615 ± 0.056074	1.9	102.0	1.5	101.1
MQC-1 2.217	MQC-1 2.225	2.19990 ± 0.075686	2.15910 ± 0.038325	3.4	99.2	1.8	97.4
MQC-2 1.109	MQC-2 1.152	1.17507 ± 0.072758	1.17735 ± 0.102743	6.2	106.0	8.7	106.2
LQC 0.133	LQC 0.130	0.13157 ± 0.011055	0.13220 ± 0.015556	8.4	98.9	11.8	99.4
LOQ QC 0.053	LOQ QC 0.051	0.05473 ± 0.006301	0.05590 ± 0.008344	11.5	103.3	14.9	105.5

including six replicates each of the lower limit of quantitation (LLOQ), low, medium (1&2) and high quality control samples¹³. The between-run (inter-day) precision and accuracy of the assay procedure are shown in Table 3. The within-run % coefficient of variation for Lovastatin and Lovastatin acid ranged from 6.1 to 18.8 and 6.7 to 19.7 respectively. The within-run % of accuracy for Lovastatin and Lovastatin acid were ranged 98.0 to 105.5 and 91.1 to 116.6 respectively.

Dilution Integrity

Dilution quality control was diluted fifth and tenth times in human plasma. Prior to extraction, six samples each of fifth and tenth diluted samples were processed and analyzed with freshly processed calibration samples. The calculated concentrations, including the dilution factor for Lovastatin and Lovastatin acid of 1/5th and 1/10th yielded coefficient of variation of 4.3 & 2.9 and 4.3 & 2.9 respectively. Percentage of nominal values for dilution factor for Lovastatin and Lovastatin acid of 1/5th and 1/10th were 97.0 102.1 and 97.6&100.7, respectively.

Ruggedness

Different analyst with different column defines ruggedness. The run consisted of a calibration curve and a total of 18 spiked samples, including 6 replicate each of the low, medium and high quality control samples. The % coefficient of variation for Lovastatin and Lovastatin acid were ranged from 6.9 to 14.9 and 7.1 to 10.8. The percentage of nominal values for Lovastatin and Lovastatin acid were ranged from 90.0 to 108.9 and 90.9 to 108.9, respectively.

Short Term Stock Solution Stability

Short term stock solution stability at room temperature

Stock solution each of Lovastatin and Lovastatin acid and internal standard were stable after 06 Hrs and 30 Min at room temperature. For Lovastatin and Lovastatin acid and internal standard the % change was -2.5 & 3.2 and -1.2 respectively.

Short term stock solution stability at refrigerator (2-8 °C)

Stock solution each of Lovastatin and Lovastatin acid and internal standard were stable after 08 Hrs and 30 Min at refrigerated temperature 2-8 °C. For Lovastatin and Lovastatin acid and internal standard the % change was -

1.1 & 1.2 and -0.8 respectively.

Short Term Working Solution Stability

Short term working solution stability at room temperature

One solution each of Lovastatin and Lovastatin acid and internal standard solution were stable after 06 Hrs and 30 Min at room temperature. For Lovastatin and Lovastatin acid and internal standard the % change found is -4.2&1.5 and 1.1.

Short term working solution stability at refrigerator (2-8 °C)

One solution each of Lovastatin and Lovastatin acid at working calibration standard level and internal standard solution at working internal standard solution level were stable after approximately 06 Hrs and 30 Min at refrigerated temperature 2-8 °C. For Lovastatin and Lovastatin acid and internal standard the % change found was 0.7&1.3 and 1.3.

Long Term Stock and Working Solution Stability

The long-term stock and working solution stability experiment were completed after completion of the study sample analysis.

Long term stock solution stability in refrigerator between 2-8 °C

Solution each of Lovastatin and Lovastatin acid at working curve standard level and internal standard solution at working internal standard level were stable for 07 days. For Lovastatin and Lovastatin acid and internal standard, the % change was 2.2& 1.3 and 1.6 respectively.

Long term working solution stability in refrigerator between 2-8 °C

Working solution each of Lovastatin and Lovastatin acid at working curve standard level and internal standard solution at working internal standard level were stable for 07 days. For Lovastatin and Lovastatin acid and internal standard, the % change found is 2.3& 1.5 and -0.1, respectively.

Bench Top Stability

The bench top stability samples each of low and high QC (stability samples) was kept on bench at room temperature was found stable for 12 Hrs and 30 Min. The % change for Lovastatin and Lovastatin acid of LQC and HQC were 1.6 & 1.8 and 1.9 & 1.2 respectively.

Freeze and Thaw stability (at -20 ± 5 °C)

Table 3: Within-run precision and accuracy of the analytical method for Lovastatin and Lovastatin acid in plasma. concentrations (ng/mL)

Amount Added		Measured (mean ± SD)	Measured (mean ± SD)	Lovastatin		Lovastatin acid	
Lovastatin	Lovastatin acid	Lovastatin	Lovastatin acid	% CV	% Accuracy	% CV	% Accuracy
HQC	HQC	3.8378 ± 3.695	3.7758 ± 0.49982	6.1	103.9	13.2	102.2
MQC-1	MQC-1	2.2815 ± 2.217	2.1320 ± 0.22462	6.6	102.9	10.5	96.2
MQC-2	MQC-2	1.1705 ± 1.109	1.1047 ± 0.07366	5.9	105.5	6.7	99.6
LQC	LQC	0.1303 ± 0.133	0.1212 ± 0.01391	7.0	98.0	11.5	91.1
LOQ QC	LOQ QC	0.0527 ± 0.053	0.0618 ± 0.01217	18.8	99.4	19.7	116.6

The freeze and thaw stability samples each of LQC and HQC were found to be stable in human plasma after four freeze and thaw cycles (at -20 ± 5 °C). The % change for Lovastatin and Lovastatin acid of LQC and HQC were 1.8 & 1.5 and 1.0 & 1.5 respectively.

Auto sampler Stability

The stability samples each of LQC and HQC was found to be stable for approximately 72 Hrs in auto sampler (at 5 ± 3 °C). The % change for Lovastatin and Lovastatin acid of LQC and HQC were -0.7 & 0.8 and 0.2 & 0.9 respectively.

DISCUSSION

A rapid, sensitive and rugged liquid-liquid extraction high performance liquid chromatography tandem mass spectrometry (LC- MS/MS) method was developed for determination of Lovastatin and Lovastatin acid in human plasma. The analysis was performed on a triple-quadrupole tandem mass spectrometer by multiple reactions monitoring mode via electro spray ionization. An optimization mobile phase was performed based on asymmetric factor and peak area obtained. Different mobile phases were tried but satisfactory separation, well resolved and good symmetrical peaks were obtained with the mobile phase 5mM ammonium formate and methanol (20:80%, v/v). The retention time of Lovastatin, Lovastatin acid and Atorvastatin (IS) were found to be 2.84, 3.45 and 2.06 minutes and overall run times 5.50 minutes indicates a good baseline. The calibration curves for Lovastatin and Lovastatin acid obtained by plotting peak area ratio versus concentrations was found to be constituency accurate and precise over the 0.050-5.00 ng/mL calibration ranges with regression coefficient (r^2) ≥ 0.99 . The coefficient of variation of response ratio of Lovastatin, Lovastatin acid and Atorvastatin (IS) stock solutions were $< 5\%$ respectively and no significant injector carry-over effects were observed. No significance interference was observed in system performance experiment determined the good selectivity and specificity of the Lovastatin, Lovastatin acid and internal standard during the extraction process and plasma matrix. The percentage of CV of matrix factor of Lovastatin, Lovastatin acid and internal standard was less than 15% shows the absence of additional peaks indicates no interference of the endogenous substances in

plasma matrix. The high percentage of recovery of Lovastatin, Lovastatin acid was found to be 72.9 & 82.9% indicates that the proposed method is highly accurate. The percentage accuracy and precision studies obtained were less than 15% for QC sample and less than 20% for LOQ QC samples revealed that developed method was accurate and precise as per the FDA guideline. The limit of quantitation for Lovastatin, Lovastatin acid was found to be 0.050ng/mL and 0.050ng/mL, indicates the sensitivity of the method and was highly reproducible with excellent chromatography properties. Hence this developed method can be applied to estimation of Lovastatin, Lovastatin acid in different bioavailability and bioequivalence studies.

CONCLUSION

Proposed study describes new LC-MS/MS method for the estimation of Lovastatin and its metabolite (β -hydroxy acid) (Lovastatin acid) in pharmaceutical formulation. The method was validated and found to be simple, sensitive, accurate and precise. Percentage of recovery shows that the method is free from interference of the excipients used in the formulation. Therefore, the proposed method can be used for routine analysis of estimation of Lovastatin and Lovastatin acid to a clinical pharmacokinetic study in human volunteers.

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