

Method Development and Validation of Pravastatin Sodium in Human Plasma by Using LCMS/MS

Kumud Sampath, Ramesh N*, Suresh Kumar, Sasi jith SL and James D Terish

Bioanalytical Department, SeQuent Research Limited, 120 A & B Industrial Area, Baikampady, New Mangalore-575011, India

Abstract

An Ultra Flow liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for estimation of pravastatin in human plasma. Pravastatin and omperazole (internal standard) were extracted from human plasma using a solid phase extraction procedure with Strata X cartridges. Samples were chromatographed on Hypurity Advance C18, 50 x 4.6 mm, 5µm column using a mobile phase consisting of (80:20, v/v), acetonitrile and 2 mM ammonium formate. Pravastatin and the internal standard were ionised using the electro spray interface operating in negative ion mode. The characteristic ion dissociation transitions m/z 423.1→321.2 and m/z 344→193.8 was monitored for pravastatin and internal standard respectively. The limit of quantitation was 5.078 ng/mL using 250 µl of plasma. Inter and intra batch precision expressed by relative standard deviation was less than 9%. The assay was robust, sensitive, and highly specific and there was no interference from human plasma observed. With a total run-time of 2 minutes, the method was suitable for supporting clinical studies and applied to the analysis of samples from a bioequivalence study.

Keywords: Human plasma; Pravastatin; Solid-phase extraction; LCMS/MS

Introduction

Pravastatin sodium is designated chemically as 1-Naphthalene-heptanoic acid, 1,2,6,7,8,8a-hexahydro-β,δ,6-trihydroxy-2-methyl-8-(2-methyl-1-oxobutoxy)-, monosodium salt, [1S-[1α(βS*,δS*),2α,6α,8β(R*),8αα]]. Pravastatin sodium is an anti-hypercholesterolemic agent having an inhibitory activity against 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-determining enzyme in the cholesterol synthesis and conversion of HMG-CoA to mevalonate [1,2]. Pravastatin is characterized as one of the best, due to the hydroxyl group attached to its decalin ring, which results in a greater hydrophilicity than other HMG-CoA reductase inhibitors. Many pharmacokinetic studies have been performed and different HMG-CoA reductase inhibitors have been compared.

Numerous methods have been developed and validated for detection/estimation of Pravastatin in plasma including high-performance liquid chromatography with UV detection [3-5], liquid chromatography/ tandem mass spectrometry (LC/ MS/MS) [6-12]. Calibration curve range has been selected based on the in-vivo concentration profile of drug. Pravastatin is available in different dosages, for 20 mg C_{max} is 20.3 - 26.3 ng/mL, since we have selected highest concentration 2 times of the expected C_{max} of the maximum dose and the lowest concentration to less than 1/10th of C_{max} of minimum dose. Since the requirement for Pravastatin 80 mg is 106 ng/mL. Hence the selected calibration curve range is 5.078 ng/mL to 210.534 ng/mL. UFLC offers advantage in shortening of analysis time through ultra fast analysis reduces delay risks. Omeprazole internal standard has been selected based on extraction efficiency which was equivalent to analyte and shorter retention time on column.

The goal of this study was to develop and validate a sensitive, specific and reproducible LC-MS/MS method of Pravastatin detection/estimation in human plasma by using Omeprazole as an internal standard. At the same time, method was efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of Pravastatin.

Experimental

Chemicals and reagents

Acetonitrile and methanol of HPLC grade was procured from JT Becker. Water HPLC grade was obtained from a Milli-Q water purification system. Ammonium Formate was procured from CDH. A reference standard of Pravastatin sodium & Omeprazole internal standard was provided by Strides Arcolab Bangalore India.

Instrumentation and chromatographic conditions

Ultra flow liquid chromatography Tandem Mass Spectrometry was used for the method development and validation. Mass Spectrometry Model API 4000, UFLC model UFLC XR equipped with a model LC-20ADXR a binary pump, SIL-20ACXR auto sampler was used to keep temperature at 5°C, CTO-20AC column oven used to keep temperature at 35° C and CBM-20Alite system controller. Detection was made at m/z 423.1/321.2 for pravastatin and 344/193.8 for internal standard using ESI Negative ion spray ionization mode. Analyst 1.5.1 software was used for the quantification. The stationary phase was a Hypurity Advance C18, 50 X 4.6 mm, 5µm column.

Optimization of LCMS method

The LCMS procedures were optimized with a view to develop a method of estimation for Pravastatin. The mixed standard stock solution was diluted in methanol to a concentration containing 5 ng/mL of pravastatin. Then, the stock solution is injected into the Hypurity Advance C18 column. Different ratio of acetonitrile and 2mM

*Corresponding author: Mr. Ramesh N, Bioanalytical Department, SeQuent Research Limited, 120 A & B Industrial Area, Baikampady, New Mangalore-575011, India, Fax: + 91 8242402256; E-mail: ramesh.narayanasamy@srl.sequent.in

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ammonium formate buffer was tried. The optimum mobile phase was found to be acetonitrile: 2mM ammonium formate (80:20, v/v). The separation was carried out at ambient temperature with a flow rate of 0.5 mL. The injection volume was 10 μ l and run time was 2 minutes. The RT of analyte and internal standard was 1.33 and 1.39 minutes.

Preparation of standard and quality control samples

Stock solutions of pravastatin were made up in methanol at approximately 1 mg/ml and these stock solutions were refrigerated and protected from light. Working standard solutions of varying concentrations of Pravastatin were prepared on the day of analysis by diluting the stocks with dilution solution. Each day, before extraction, the calibration curve in human plasma was prepared by spiking known amounts of pravastatin into human plasma (250 μ l) and internal standard (25 μ l).

Calibration curve standards consisting of a set of eight non-zero concentrations ranging from 5.078 ng/mL to 210.534 ng/mL for Pravastatin were prepared. Prepared quality control samples consisted of Pravastatin concentrations of 5.089 ng/mL (QCLLQ), 13.123 ng/mL (QCL), 90.224ng/mL(QCM) and 164.044 ng/mL (QCH). These samples were stored below -50°C until used. Twelve sets of QCL and QCH were stored to below -20°C freezer for generation of below -20°C stability.

Preparation of internal standard stock solution

Stock solutions of Omeprazole were made up in methanol at approximately 1 mg/ml and these stock solutions were refrigerated and protected from light. Appropriate dilutions were made with dilution solution to make a IS working dilution of 350 ng/mL.

Sample extraction

Before extraction, bulk spiked CC and QC samples, were removed from the deep freezer and thawed at room temperature. Calibration standards and QC samples were then made ready for extraction in 4 ml polypropylene tubes. Exactly 250 μ l of plasma was pipette out into prelabelled polypropylene tubes, to this 25 μ l of internal standard dilution (350 ng/ml) was added and vortex for 20 seconds. The cartridges {Strata X (30 mg/1cc)} were conditioned with 1.0 mL methanol, equilibrated with the 1.0 ml of milli-Q water and samples were loaded, cartridges were washed with 1.0 mL of washing solution and were eluted with 0.5 + 0.5 mL of mobile phase and these sample was transferred to the auto sampler vial and 10 μ l was injected into the chromatographic system.

Preparation of mobile phase, dilution and washing solution

The mobile phase was prepared by mixing acetonitrile: 2mM ammonium formate (80:20, v/v), was filtered using a 0.45 μ m membrane filter (Millipore). The dilution solution was prepared by mixing milli-Q water and acetonitrile in the ratio of 20:80, v/v. The washing solution was prepared by mixing milli-Q water and methanol in the ratio of 90:80, v/v.

Method validation parameters

The validation was performed as per the US FDA and ANVISA guidelines and in-house operating procedures [13,14]. The optimized LCMS method was validated with respect to the following parameters:

System suitability

The system suitability was performed before starting each day's activity according to in-house.

Specificity / selectivity

Specificity and Selectivity was performed in six different lots of plasma having K3EDTA as anticoagulant.

Matrix effect

Blank samples (plasma) from six independent sources of matrix were processed and then spiked with analyte at QCL level and internal standard at the concentration used in the method being validated just before injection into the LC-MS/MS. An aqueous solution of analyte was prepared at QCL level with internal standard in mobile phase.

Carry over

Processed and injected two blank, two LLOQ, two ULOQ samples followed by re-injection of first two blank samples.

Linearity

A regression equation with a weighting factor of $1/x^2$ of drug to IS concentration was judged to produce the best fit for the concentration-detector response relationship for Pravastatin in human plasma. The representative calibration curves for regression analysis are illustrated in Figure 3 (included as supplementary data).

Precision and accuracy

The precision of the assay was measured by the percent coefficient of variation over the concentration range of QCLLQ, QCL, QCM and QCH samples respectively during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ, low, middle and high quality control samples to their respective nominal values, expressed in percentage.

Ruggedness

One complete precision and accuracy batch was processed and analyzed by different analyst using different column.

Recovery

Six sets of aqueous recovery comparison samples were prepared and injected. The recovery comparison samples of Pravastatin were compared against extracted samples of QCL, QCM and QCH samples.

Dilution integrity

Six sets of dilution integrity samples (approximately 1.7 times of highest standard concentration) were processed by diluting them twice and another six sets by diluting them four times using pooled plasma. These quality control samples were analyzed along with a freshly spiked and processed calibration curve standards. The quality control sample concentrations were calculated using appropriate dilution factor.

Stabilities

Bench top stability: Bench top stability was determined for 16 hours, using six sets each of QCL and QCH. The quality control samples were quantified against the freshly spiked calibration curve standards.

Freeze-thaw stability three cycles

The stability in human plasma was determined for three freeze-thaw cycles. Six replicates of QCL and QCH were analyzed after undergoing three freeze-thaw cycles. The freeze-thaw quality control samples were quantified against the freshly spiked calibration curve standards.

Long term stability at below -20°C and -50°C

Six replicates of QCL and QCH were stored below -20°C and below -50° C in the freezer and deep freezer respectively for 30 days. These samples were quantified against the freshly spiked calibration curve standards. The stability of the analytes was evaluated by comparing each of the back calculated concentrations of stability QCs with the mean concentrations of the respective QCs analyzed in the first accepted precision and accuracy batch (PA-1).

Auto sampler stability

Six replicates of QCL and QCH were analyzed and stored in autosampler to prove stability. These samples were injected after a period of 50 hours and were quantified against freshly spiked calibration curve standards.

Re-injection reproducibility

Six replicates of QCL and QCH of the precision and accuracy batch PA-3 were retained in the autosampler at 5°C for 50 hours to test the re-injection reproducibility of the method. Reinjection reproducibility concentrations were compared against the PA-3 batch concentrations.

Stock dilution stability

The stability of stock dilutions of analytes and the internal standard was evaluated at room temperature. Aqueous stock dilutions of the analytes and the internal standard were prepared. One portion of the stock dilution was placed in the refrigerator between 2-8°C, while the other portion was placed at room temperature for 23 hours.

Stock solution stability

Stock solution stability was carried out for 30 days by injecting six replicates of stock dilution of stability standards (analyte and internal standard which prepared and stored in the refrigerator between 2 - 8°C) and freshly prepared stock dilutions of Comparison standard (analyte and internal standard). The response of stability sample was corrected by multiplying with correction factor.

Chromatography

Representative chromatograms of aqueous mixture, blank plasma, QCM & calibration curve of pravastatin are given in Figure 1-3 (included as supplementary data).

Data processing

The chromatograms were acquired and were processed by peak area ratio method using the Analyst Version 1.5.1 Software. The concentration of the unknown was calculated from the following equation using regression analysis of spiked standard with the reciprocal of the ratio of the (drug concentration)² to internal standard concentration as a weighing factor (1/x²):

$$y = mx + c$$

where, y = peak area ratio of Pravastatin to internal standard

m = slope of calibration curve

x = concentration of Pravastatin

c = y-axis intercept of the calibration curve

Results and Discussion

The method was validated in terms of limit of quantification, Recovery, Selectivity, Precision, accuracy and stability.

Specificity/selectivity

No significant interference was observed at the RT and m/z of Pravastatin and internal standard in all the batches screened.

Matrix effect

The IS-normalized matrix factor was found to be 0.943 (close to unity) for six different matrix lots for Pravastatin and the % CV was 1.66.

Carry over

The % carry over was found to be 0.00 for analyte and 0.00 for internal standard.

Linearity

The method was validated over the range of 5.078–210.534 ng/ml. The correlation coefficient found to be greater than 0.9986.

Recovery

The recovery was determined by comparing the aqueous solution and the spiked drug. The percentage recovery of the drug and the internal standard was calculated and it was 87.50% and 93.55 % respectively.

Precision and accuracy

The accuracy, precision and intraday precision were carried out by preparing six individual samples of QCH, QCM and QCL. The % CV and % nominal was calculated. Refer Table 1 for the results of Within-Batch Precision and Accuracy, Intraday Batch Precision and Accuracy and Between Batch Precision and Accuracy.

Ruggedness

The mean accuracy ranged from 92.83 % (QCL) to 101.51 % (QCLLQ) and the precision ranged from 0.50 % (QCH) to 3.43 % (QCLLQ).

Within Batch Precision	PA1	1.23 % - 3.65 % (QCM-QCL)
	PA2	0.99 % - 3.33 % (QCL-QCLLQ)
	PA3	1.34 % - 2.19 % (QCLLQ-QCM)
Within Batch Accuracy	PA1	90.63 % - 100.03 % (QCL-QCH)
	PA2	89.64 % - 100.89 % (QCL-QCM)
	PA3	91.49 % - 100.15 % (QCL-QCH)
Intraday Batch Precision	Day-1	1.49 % - 2.68 % (QCH - QCLLQ)
	Day-2	1.34 % - 2.19 % (QCLLQ - QCM)
Intraday Batch Accuracy	Day-1	90.14 % - 100.46% (QCL - QCM)
	Day-2	94.20 % - 100.02 % (QCL - QCLLQ)
Between Batch Precision		1.48 % - 3.19 % (QCH - QCL)
Between Batch Accuracy		91.49 % - 100.15 % (QCL - QCH)

Table 1: Precision and Accuracy.

Dilution integrity

The within batch precision and accuracy, for a dilution factor of 2 was 5.19 % and 99.16 %. The within batch precision and accuracy, for a dilution factor of 4 was 0.97 % and 107.90 %.

Stability

Stability of the method was carried out by performing short term and long term stock stability. The percentage mean ratio of the drug and internal standard were calculated. Stability of the plasma samples was carried out bench top, freeze thaw, below -20°C and below -50°C, auto sampler, re-injection reproducibility, stock dilution and solution. The results for stability studies as mentioned below:

Bench top

The percent nominal ranged from 101.32 % (QCH) to 107.81 % (QCL) and the precision ranged from 4.10 % (QCH) to 11.14 % (QCL).

Freeze Thaw (FT3)

The percent nominal ranged from 97.50 % (QCL) to 97.99 % (QCH) and precision ranged from 2.86 % (QCH) to 4.40 % (QCL) respectively.

Long Term below -20°C

The percent nominal ranged from 105.21 % (QCH) to 112.75 % (QCL) and precision ranged from 2.84 % (QCH) to 9.40 % (QCL) respectively.

Long Term below -50°C

The percent nominal ranged from 98.41 % (QCH) to 101.49 % (QCL) and precision ranged from 1.72 % (QCH) to 1.89 % (QCL) respectively.

Auto sampler

The percent nominal at around 50 hours ranged from 91.26 % (QCL) to 96.76 (QCH) and precision ranged from 1.94 % (QCH) to 3.57 % (QCL) respectively. The percent change observed for the internal standard (Omeprazole) was 2.31%.

Re-injection reproducibility

The % change is 3.13 (QCL) and 3.15 (QCH).

Stock dilution

The percent change for Pravastatin was 0.07 % and for Omeprazole is 0.51 %, respectively.

Stock solution

The percent change for Pravastatin was 0.04 % and for Omeprazole is 0.11 %, respectively.

Conclusion

The bioanalytical method developed is simple, accurate, precise, sensitive and reproducible. It can be used for the estimation of Pravastatin in biological fluids. The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of pravastatin without any interference. The developed method could be applied in bioequivalence, pharmacokinetic and toxicokinetic studies.

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