Development and validation of LC-MS method for the determination of Rosuvastatin Hydrochloride in human plasma

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ABSTRACT
A simple and sensitive liquid chromatography/tandem mass spectrometry method was developed and validated for determining rosuvastatin in human plasma, a new synthetic hydroxyl methyl glutaryl-coenzyme a reductase inhibitor. The analyte and internal standard (IS: Fluconazole) were extracted by simple one-step liquid/liquid extraction with Methyl-tert-Butyl Ether. The organic layer was separated and evaporated under a gentle stream of nitrogen at 40± 5°C. The chromatographic separation was performed on an Kromosil, 5µ, 100×4.6mm column with a mobile phase consisting of 5mM Ammonium acetate pH 3.5 : Acetonitrile (10:90v/v) at a flow rate of 0.800ml/min. The retention time of rosuvastatin and internal standard was 1.22 and 1.23 min, respectively. Triple–quadrupole MS/MS detection was operated in positive mode by multiple reaction monitoring (MRM) using the precursor-to-product combinations of Drug: 482.20/288.20 (m/z) and ISTD: 307.20/220.10 (m/z) the areas of peaks from the analyte and the IS were used for quantification of rosuvastatin. The method was validated according to the FDA guidelines on bioanalytical method validation. Validation results indicated that the lower limit of quantification (LLOQ) was 0.1 ng/mL and the assay exhibited a linear range of 24.979 - 5003.808ng/mL and gave a correlation coefficient (r) of 0.999 or better. Quality control samples (0.5, 9, 24 and 46 ng/mL) in six replicates from three different runs of analysis demonstrated an intra-assay precision (RSD) 7.97-15.94%, an inter-assay precision 3.19-15.27%, and an overall accuracy (relative error) of < 3.7%. The analyte was stable in human plasma following three freeze/thaw cycles and for up to 8 weeks following storage at −20 °C. The assay can be applied to the analysis of rosuvastatin in human plasma samples derived from clinical trials.

Keywords: Rosuvastatin, Fluconazole, Method validation, LC–MS/MS, Human plasma, Methyl-tert-Butyl Ether
INTRODUCTION

Rosuvastatin (Fig. 1) (formerly known as ZD4522), a chemically bis [(E)-7-[4-(4-fluorophenyl)-6-isopropyl-2-[methyl-(methylsulfonyl) amino]pyrimidin-5-yl](3R,5S)-3,5-dihydroxyhept-6-enoicacid] calcium salt, is a new, synthetic, orally active and competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase with significant and specific low-density lipoprotein (LDL) cholesterol-lowering activity in vitro and in vivo [1,2].

Rosuvastatin is a hepato-selective drug, with selectivity achieved through active transport processes into the liver [3–5]. Compared with several other HMG-CoA reductase inhibitors, rosuvastatin does not appear to be metabolized significantly by cytochrome P450 3A4 and, therefore, may not possess the same potential for drug interactions as seen for some other statins. In spite of the metabolism of rosuvastatin not being extensive, N-desmethyl rosuvastatin was identified as the primary metabolite. This metabolite was formed primarily by CYP 2C9 isozyme with lesser contributions coming from CYP 2C19 and 3A4 isozymes. [6] In order to quantify plasma concentrations of rosuvastatin in clinical trials, it was necessary to develop and validate a simple, sensitivity, selectivity, accuracy and precise assay method.

Assays for other statins (and their metabolites) have employed a number of different techniques and approaches. An enzyme-linked immunosorbent assay for pravastatin alone [7], and gas chromatography– mass spectrometry (GC–MS) [8] and HPLC–MS– MS [9] assays for pravastatin and metabolites have performed with reference to the guidance of Shah et al. [10].
The pharmacokinetic, metabolic and drug–drug interactive profiles of rosuvastatin have been extensively studied. Pharmacokinetic studies in humans using oral doses (5–80 mg) showed that maximum plasma concentrations and areas under the concentration–time curve were approximately linear with dose [11]. Peak plasma concentrations of rosuvastatin were reached after 3–5 h following oral administration in humans. The elimination half-life was found to be approximately 19 h and steady-state concentration was reached within 4–5 days after dosing. Repeated dosing of rosuvastatin was found to have little or no effect on accumulation of drug in plasma [12]. Serum protein binding of rosuvastatin was around 88%, and the absolute oral bioavailability of rosuvastatin was around 20% [8]. It was found that organic anion transporting polypeptide 1B1 (SLCO1B1) contributes to the hepatic uptake of rosuvastatin [13, 14].

From this point of view, a quantification method of rosuvastatin in human plasma employing liquid–liquid extraction with Methyl-tert-Butyl Ether (t-BME) followed by tandem mass spectrometric detection is developed and validated according to the FDA guidelines on bioanalytical method validation [15]. The present study provides an alternative with a simpler and cheaper approach for the quantification of rosuvastatin in human plasma.

**EXPERIMENTAL SECTION**

2.1. **Chemicals and reagents:**
Rosuvastatin was obtained from Matrix Laboratories Limited, India and Fluconazole used as the internal standard, was supplied by Sigma Aldrich. Methanol, Acetonitrile, and formic acid were purchased from Merck, Methyl-tert-Butyl Ether (t-BME) was purchased from Spectrochem and Water (HPLC grade) was prepared by distillation in glass and passage through a Milli-Q plotwater purification system (Millipore, Bedford, MA, USA). Analytes free healthy human plasma was procured from the Navajeevan Pathological Laboratory at Hyderabad from different individual sources.

2.2. **Equipment:**
HPLC was carried out with a Shimadzu LC-10A pump, a Hewlett-Packard Series 1100 pump with an EVA-1 Rheodyne Model 7000 switching valve (Jones Chromatography, Mid Glamorgan, UK) and a CTC Analytics PAL auto sampler (Herefordshire, UK). The analytical column employed was a Kromosil (C18, 5µ, 100 X 4.6mm). A Sciex API 4000 (Applied Biosystems, Warrington, Cheshire, UK) mass spectrometer, equipped with a Turboionspray interface, was used for detection. The data capturing system was an Apple Macintosh Power Macintosh 9500/132. The Eppendorf’s centrifuge, the Spinix’s vortex mixer and the tube rotator a Stuart TR-2 was used. Gilson and Anachem autopipettes were used for dispensing plasma and stock solutions. Polypropylene sample tubes (4 ml) from Anachem (Bedfordshire, UK) and Chromacol (Hertfordshire, UK) 250-ml autosampler vials were used throughout.

2.3. **Preparation of standard and quality control samples:**
Stock solutions of rosuvastatin were made up in methanol at approximately 1 mg/ ml. A 50-fold dilution of the stocks was prepared in methanol, refrigerated and protected from light for up to 1 month. Working standard solutions of varying concentrations of rosuvastatin were prepared on the day of analysis by diluting the stocks with 1 M acetic acid /methanol (50:50 v/v). Each day, before ex- traction, the calibration curve in human plasma was prepared by spiking known amounts of rosuvastatin into human plasma (500 µl), internal standard (50 µl), 5mM ammonium acetate buffer pH 3.5 (500 µl) and formic acid (1 %, 750 µl) was added to give a final volume of 1800 µl.
The standard curve in human plasma was 0.1, 0.2, 0.5, 1, 5, 10, 15 and 30 ng/ml. The concentration of internal standard in plasma was 15 ng/ml. Quality control (QC) samples were prepared fresh on the day of analysis and in bulk at four concentrations: 0.1, 0.3, 15 and 25 ng/ml rosuvastatin. Dilution QC samples were prepared at 250 ng/ml to confirm that samples could be diluted to within the working range of the assay. Bulk QC samples were stored frozen at -70/80 °C until required.

2.4. Sample extraction:
Before extraction, control plasma for calibration and QC samples and bulk spiked QC samples, were removed from the freezer and thawed at room temperature. Calibration standards, fresh QC samples and bulk spiked QC samples were then made ready for extraction in 4 ml polypropylene tubes. All calibration standards were prepared in duplicate at each concentration.

Exactly 250 µl of plasma was pipette out into prelabelled polypropylene tubes, to this 50 µl of ISTD (1µg/ml) was added and vortex (Vortex Genius 3, IKA, Germany) for 20 seconds, to this add 2.5 ml of TBME, again vortex for 10 minutes, after this The tubes was centrifuged for 5 min (EBA21 table centrifuge, Hettich, Germany) at 4500 rpm, and the upper Organic phase was transferred to an other 5mL polypropylene Tube and evaporated to dryness under a stream of nitrogen at 40°C (N-EVAP11155, Organomation, USA), for 25 minutes The residue was Reconstituted in 150 µl of mobile phase by vortex mixing at 3000 rpm for 3 min. The reconstituted sample was transferred to the glass auto sampler vial insert and 5 µl was injected into the chromatographic system.

2.5. Chromatographic and mass spectrometric conditions:
An HPLC mobile phase of Ammonium Formate, 10 mM pH 4.5: Acetonitrile (10:90 v/v) was delivered at a rate of 1.0 mL/min using Agilent 1100 series HPLC system, with a split of 200 µL to mass spectrometer and 800 µL to waste. The column of kromosil, C18, 5 µ, 50 X 4.6 mm was maintained at 20 °C [16]. The injection volume was 5 µL and the injector needle was washed in water/methanol (50:50 v/v). Peaks of the HPLC–MS/MS chromatograms were evaluated using an Analyst workstation (2003 editions, Applied Biosystem/MDS SCIEX and POET Software Corporation, USA) and a Mass spectrometry Toolkit (version3.3, 1998–2000 Sierra Analytics, USA).

The mass spectrometer was operated in the positive ion mode with the TurboIonspray heater set at 450 °C (API 4000 LC–MS/MS system, Applied Biosystems, Foster City, CA, USA). The samples were analyzed employing the transition of Drug: 482.20/288.20 (m/z) for rosuvastatin with a dwell time of 200 milli sec. The mass transition for the internal standard (Fluconazole) was 307.20/220.10 m/z amu, with the same dwell time. The ionspray voltage was set at 5500.00 the decluster potential was set at 32.00, 40.00 V and the collision energy at 32.00, 25.00 for rosuvastatin and internal standard, respectively. The entrance potential was set at 10.0V, and the focusing potential at 400V. The nebulizer gas (nitrogen) pressure was set at 8 (arbitrary units). The curtain gas (nitrogen) was set at 15.00 (arbitrary units).

RESULTS AND DISCUSSION

3.1. Mass spectrometry:
In order to develop a method with the desired sensitivity (0.1 ng/ml), it was necessary to use MS–MS detection, as the compound did not possess the UV absorbance or fluorescence properties needed to achieve this limit. The inherent selectivity of MS–MS detection was also expected to be beneficial in developing a selective and sensitive method. The most sensitive
mass transition was from \( m/z \) 482 to \( m/z \) 288, which relates to the production of the product ion illustrated in Fig. 1. Essentially the same mass transition was used for the Fluconazole (\( m/z \) 307.20/220.10). This fragment ion was the most sensitive ion detected and because it was specific to rosuvastatin and internal standard was considered to be the most appropriate choice for a specific and sensitive method. The ring voltage, orifice voltage and collision energy were optimized to deliver effective fragmentation of the [M+H]^+ without excessive fragmentation, which would have reduced sensitivity. The parameters presented in the methods section are the result of this optimisation.

Fig. 2. Positive ion TurboIonspray Q1 mass spectra (\( m/z \) 110–550) of rosuvastatin

Solvents of rosuvastatin throughout this assay contained acid. Due to the chromatographic separation of ionic type and molecular type, a double-peaked chromatogram was apt to be formed without enough acidic environments. In an acidic mobile phase and solution, rosuvastatin existed as ionic type. The residue was reconstituted in the mobile phase by vortex mixing at 2500 rpm for 3 min to ensure that the residue adhered to the wall of tubes could be entirely dissolved.
Because trace plasma protein remained in the reconstituted solution, a relatively high centrifugal speed (10 000×g) must be applied to precipitate the protein and other undissolvable substance.

Fig. 3. Positive ion TurboIonspray product ion mass spectra (m/z 110–550) of rosuvastatin

3.2. Method development:

The HPLC conditions were optimised such that the retention time was kept for rosuvastatin at 1.22 minutes and fluconazole at 1.23 minutes in order to assure high throughput. Some retention of the compound on the HPLC column was employed with the eluent from the first 45 sec of the run going to waste. This limited the amount of endogenous material entering the mass spectrometer and thereby reduced the amount of system maintenance required. The kromosil HPLC column was chosen based on positive experience in the chromatography of acid compounds and because it demonstrates good stability at the low pH of the mobile phase. The composition of the mobile phase with Ammonium Formate, 10mM pH 4.5: Acetonitrile (10:90v/v) was chosen for its compatibility with mass spectrometric detection. The pH of
ammonium formate buffer was kept at 4.5 because it was found to be necessary in order to lower
the pH to protonate the acidic rosuvastatin and thus deliver good peak shape. The percentage of
ammonium formate was optimised to maintain this peak shape whilst being consistent with good
ionisation and fragmentation in the mass spectrometer. The typical chromatogram of double
blank plasma (without rosuvastatin and internal standard) and a spiked plasma sample with
rosuvastatin (approximately 1 ngmL$^{-1}$) was shown in Figs. 3 and Ion chromatogram of a
rosuvastatin spiked plasma (0.1 ngmL$^{-1}$): (A) rosuvastatin channel and (B) internal standard
channel was shown in Fig 4.

3.3. Specificity, selectivity and matrix effects:
The standard curve in biological fluids was compared with standard in buffer to detect matrix
effects. Besides, parallelism of diluted study samples were evaluated with diluted standards to
detect matrix effects. The results showed that precision, selectivity, and sensitivity was not
compromised. The specificity/selectivity of the method was investigated by screening several
separate human plasma samples and looking for endogenous peaks which accounted for more
than 20% of the peak area of rosuvastatin or the internal standard in the LLOQ of calibration
samples. Using these criteria, no endogenous substances were detected which significantly
interfered with the quantification of rosuvastatin or the internal standard. Pre-dose samples
analyzed from preliminary clinical studies have confirmed that there were no other endogenous
plasma components, which would have led to significant interference in the assay.
The mean equation (curve coefficients± S.D.) of the calibration curve (eq. 8) obtained from three single batches in method validation was $y = 2.8815 \pm 0.0049$ (correlation coefficient $r = 0.9982 \pm 0.012$) for rosuvastatin, where $y$ represents the rosuvastatin peak area to fluconazole peak area ratio and $x$ represents the corresponding rosuvastatin concentration to internal standard concentration ratio.

Intra-batch inaccuracy and imprecision were assessed by running a single batch of samples containing a calibration curve and six replicates of test samples at each of the four concentrations (0.1, 0.5, 1, and 10 ngmL−1). For inter-batch inaccuracy and imprecision three batches of samples were analyzed. Each batch contained a calibration curve and duplicate test samples at each of the four concentrations. The inter- and intra-batch CV and accuracy of the method, as measured by the performance of the test samples for rosuvastatin at all four levels of concentration, were shown in Table 1. The imprecision and inaccuracy were within the pre-specified acceptable limits of <±15% and <15%, respectively, across the calibration range. The LLOQ of rosuvastatin in this assay was verified as 0.1 ngmL−1 with the inter-batch inaccuracy <20% and imprecision <±20%.
Table 1: Inaccuracy and imprecision of the method as measured by the performance of samples analyzed on three different days at four concentrations

<table>
<thead>
<tr>
<th>Concentration (ngmL−1)</th>
<th>n</th>
<th>Intra – batch</th>
<th>Inter - batch</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Inaccuracy</td>
<td>Imprecision</td>
</tr>
<tr>
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<td>1.4</td>
<td>10.5</td>
</tr>
<tr>
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</tr>
<tr>
<td>10</td>
<td>6</td>
<td>-4.6</td>
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3.5. Extraction recovery:
The extraction recoveries of rosuvastatin from plasma were determined at four concentrations (0.1, 0.5, 1 and 10 ngmL−1, n = 6), and for the internal standard at the concentration used in the assay (500 ngmL−1, n = 24), by comparing the areas of extracted samples with none-extracted samples (pure standard solutions of rosuvastatin). The mean extraction recoveries and standard deviation were 65.3±4.5%, 72.2±8.7%, 57.5±5.5% and 63.2±5.3% for 0.1, 0.5, 1 and 10 ngmL−1 of rosuvastatin; and 65.3±7.5% for the internal standard. These results indicated that the sample procedure of ion pair liquid–liquid extraction with ter-butyl-methyl ether is efficient for the extraction of trace rosuvastatin in plasma. The assay has been proven to be robust in high throughput bioanalysis.

3.6. Stability:
Rosuvastatin spiked plasma at LLOQ, low, medium and high concentrations (0.1, 0.5, 1.0 and 10.0 ngmL−1) were analyzed at fresh preparing and left in the auto sampler at room temperature for 24 h to investigate the processed sample stability. The results indicated that the processed samples were stable at room temperature for at least 24 h. Similarly, four different concentrations of spiked plasma were analyzed at fresh preparing and stored at −20 ºC, then subjected to three freeze and thaw (12 h) cycles to investigate freeze and thaw stability. The concentrations found were within the allowed limit ±15% of nominal concentration, revealing no significant substance loss during repeated freezing and thawing. The plasma samples remained stable after freezing and thawing for at least three times. Four sets of samples were likewise prepared and stored at room temperature for 24 h and at −20 ºC for 8 weeks. After first analyzing the samples were analyzed using freshly prepared calibration samples 24 h later under the circumstances of room temperature and in 2 and 4 weeks later under the circumstances of −20 ºC. The concentration determined showed that the plasma samples were stable at room temperature for at least 24 h and at −20 ºC for at least 8 weeks.

CONCLUSION

A sensitive, specific, accurate and reproducible LC–MS/MS method employing ion pair liquid–liquid extraction for the quantification of rosuvastatin in human plasma was developed and validated. The desired sensitivity for rosuvastatin was achieved with an LLOQ of 0.1 ngmL−1. Rosuvastatin was shown to be stable in routine analysis conditions and in human plasma for up to 6 months when stored at −20 ºC. The method has been used to analyze human plasma samples from clinical pre-studies of rosuvastatin in the Indian volunteers.

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