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Quantitative determination of rosuvastatin in human plasma by ion pair liquid–liquid extraction using liquid chromatography with electrospray ionization tandem mass spectrometry

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Abstract

A simple and sensitive liquid chromatography/tandem mass spectrometry method was developed and validated for the quantification of rosuvastatin in human plasma. After being treated with acetic acid and tetrabutyl ammonium hydroxide, the analyte was extracted by simple one-step liquid–liquid extraction with the internal standard (IS: estrone). The chromatographic separation was performed on a Phenomenex Luna C_{18} column with a mobile phase consisting of 2% formic acid/methanol (20:90, v/v) at a flow rate of 1.00 mL/min with a split of 200 μL to mass spectrometer. The retention time of rosuvastatin and internal standard was 2.3 and 3.4 min, respectively. Triple–quadrupole MS/MS detection was operated in positive mode by monitoring the transition of *m/z* 482 → 258 for rosuvastatin and *m/z* 271 → 253 for IS. Validation results indicated that the lower limit of quantification (LLOQ) was 0.1 ng mL^{-1} and the assay exhibited a linear range of 0.1–20 ng mL^{-1} and gave a correlation coefficient (*r*) of 0.9990 or better. Inaccuracy was less than 8.4% and imprecision less than 12.8% at all tested concentration levels. 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 was successfully applied to the analysis of rosuvastatin in human plasma samples derived from clinical pre-trials.

Keywords: Rosuvastatin; Ion pair; Extraction; Tetrabutyl ammonium hydroxide; HPLC–MS/MS

1. Introduction

Rosuvastatin (formerly known as ZD4522), a chemically bis [(*E*)-7-[4-(4-fluorophenyl)-6-isopropyl-2-[methyl-(methylsulfonyl)amino]pyrimidin-5-yl](3*R*,5*S*)-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 [6] 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 [6]. This metabolite was formed primarily by CYP 2C9 isozyme with lesser contributions coming from CYP 2C19 and 3A4 isozymes [6].

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 [8]. Peak plasma concentrations of rosuvastatin were reached after 3–5 h following oral administration in humans [7]. 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 [7]. Serum protein binding of rosuvastatin was around 88% [8], 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 [3,9]. But no association was found between

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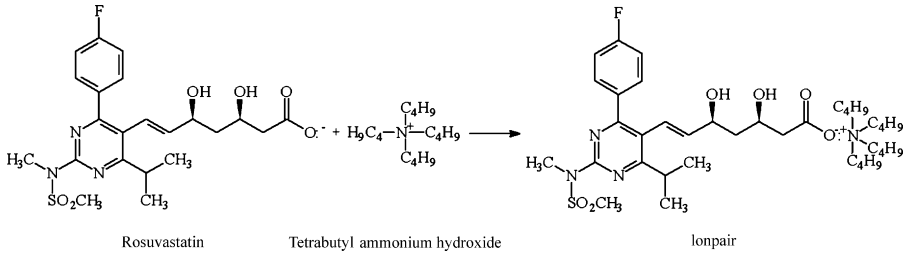

Fig. 1. Formation of ion pair of rosuvastatin with tetrabutyl ammonium hydroxide.

the pharmacokinetics of rosuvastatin and genetic variation in SLCO1B1 in Asians [10]. Further studies demonstrated that BCRP 421C>A polymorphism may play an important role in the pharmacokinetics of rosuvastatin in healthy Chinese males after the exclusion of impact of SLCO1B1 and CYP2C9 genetic polymorphism [11].

Besides an RP-HPLC-UV method for determination of rosuvastatin in rats plasma after a large oral dose [12], several assays for quantification of rosuvastatin in human plasma were documented, as discussed in the review by Pasha et al. [13]. All of these assays employed a tandem mass spectrometric detection due to the ultra-low concentration of rosuvastatin in plasma after oral dosing, but sample purification steps varied. Hull et al. reported an automated solid phase extraction [14] which was successfully applied to a series of clinical trials on bioavailability, pharmacokinetic and metabolic [15] study of rosuvastatin. A similar method employing solid phase extraction and HPLC–MS/MS with a relative higher LLOQ of 1 ng mL^{-1} was recently reported by Singh et al. [16]. Recently, a microbore HPLC in combination with tandem MS which was cross-validated to provide similar information on the concentration of rosuvastatin exhibited an ability of greatly reduced sample consumption [17]. These above assays with automated solid phase extraction were relatively fit for the requirements of routine pharmacokinetic and bioavailability study of rosuvastatin, but more expensive than traditional liquid–liquid extraction. However, it is difficult to develop a liquid–liquid extraction procedure according to the poor solubility of rosuvastatin in water and most organic solvents [18]. An assay employing liquid–liquid extraction with ethyl acetate for simultaneous determination of rosuvastatin and fenofibric acid in human plasma was documented with an extraction recovery of about 74% [19], and another work using ether as the extraction solvent with an average recovery greater than 61% for rosuvastatin was also reported by Xu et al. [20].

It can be seen that although many polar (hydrophilic) groups are comprised in the molecule of rosuvastatin, the solubility of rosuvastatin in pure water is still extremely low and its lopP is 3.135 (http://redpoll.pharmacy.ualberta.ca/drugbank/cgi-bin/getCard.cgi?CARD=APRD00546.txt). But in our preliminary studies, it was found that the extraction recovery of rosuvastatin in many common organic solutions, such as ethyl acetate, ether, dichloromethane, trichloromethane, etc., was less than 20%. However, a carboxyl group in the structure of rosuvastatin forms a salt with calcium ion which indicates that rosuvastatin is apt to ionization. So the application of ion pairing with tetrabutyl ammonium hydroxide will significantly improve the lipophility of rosuvastatin (shown in Fig. 1). From this point of view, a quantification method of rosuvastatin in human plasma employing ion pair liquid–liquid extraction with tetrabutyl ammonium hydroxide followed by tandem mass spectrometric detection is developed and validated according to the FDA guidelines on bioanalytical method validation [21]. The present study provides an alternative with a simpler and cheaper approach for the quantification of rosuvastatin in human plasma.

2. Experimental

2.1. Chemicals and reagents

Rosuvastatin was supplied by Jingxin pharmaceutical (Zhejiang, PR China). Estrone selected as internal standard was obtained from the National institute for the control of pharmaceutical and biological products (Beijing, PR China). Methanol, glacial acetic acid and formic acid were purchased from TEDIA company (Fairfield, IA, USA) and tetrabutyl ammonium hydroxide (TAH) from KeLong chemical (Chengdu, Sichuan, PR China). Ethyl acetate (Analytical pure) was obtained from ARK chemical (Chengdu, PR China). Healthy human plasma was obtained from the Chengdu Center of Blood products (Sichuan, PR China). Water (HPLC grade) was prepared by distillation in glass and passage through a Milli-Q plotwater purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of standard and quality control samples

According to the method reported by Hull et al. [14], stock solutions of rosuvastatin were made up in methanol at approximately 100 μg mL^{-1}. A 10-fold dilution of the stock solution was prepared in methanol (working solution A), refrigerated and protected from light less than 1 month. Working solution A of rosuvastatin was diluted 10-fold with 1 M acetic acid/methanol (50:50, v/v) as working solution B, and working standard solutions of varying concentrations of rosuvastatin were prepared on the day of analysis by diluting working solution B with 1 M acetic acid/methanol (50:50, v/v). Internal standard stock solution was made up in methanol at a concentration of approximately 50 μg mL^{-1}, and was stored refrigerated and protected from light for a maximum period of 1 month. On the day of analysis a dilution of this solution was prepared in methanol to give a working concentration of approximately 1000 ng mL^{-1}.

Each day, before extraction, the calibration curve in human plasma was prepared by spiking known amounts of rosuvastatin into human plasma (1000 μL). The standard curve in human

plasma was 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng mL^{-1}. The concentration of internal standard in plasma was 100 ng mL^{-1}.

Quality control (QC) samples were prepared fresh on the day of analysis and in bulk at four concentrations: 0.1, 0.5, 1 and 10 ng mL^{-1} rosuvastatin. All QC samples were stored frozen at −20 °C until required.

2.3. Sample extraction

Before extraction, control plasmas for calibration and QC samples, were removed from the freezer and thawed at room temperature. Control plasma with a volume at 1100 μL (1.0 mL of blank plasma spiked with 100 μL of working standard solutions of varying concentrations) and sample plasma with a volume of 1100 μL [1.0 mL of sample plasma added with 100 μL of 1 M acetic acid/methanol (50:50, v/v)] were made ready for extraction in 7 mL polypropylene tubes. After the plasma were combined with 100 μL of 0.5% acetic acid in water and briefly shaken, 600 μL of 0.5% TAH in water was added to form an ion pair with rosuvastatin. After being briefly shaken, 100 μL of internal standard working solution was added and mixed well. Subsequently, a sample with a total volume of 1900 μL was extracted with 4 mL of ethyl acetate following vortex mixing at 2000 rpm for 5 min (Vortex Genius 3, IKA, Germany). The tube was centrifuged for 5 min at 8000 × *g* (EBA21 table centrifuge, Hettich, Germany), and the upper organic phase was transferred to another 5 mL polypropylene tube and evaporated to dryness under a stream of nitrogen at 40 °C (N-EVAP 11155, Organomation, USA). The residue was reconstituted in 200 μL of 0.5% acetic acid in water/methanol (50:50, v/v) by vortex mixing at 3000 rpm for 3 min. After being centrifuged for 5 min at 10 000 × *g*, the sample was transferred to the glass autosampler vial insert and 50 μL was injected into the chromatographic system.

2.4. Chromatographic and mass spectrometric conditions

An HPLC mobile phase of methanol/2% formic acid in water (80:20, 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 Phenomenex Luna C_{18} (2) 5 μm (4.6 mm i.d. × 150 mm) was maintained at 20 °C [14]. The injection volume was 50 μL and the injector needle was washed in 0.5% acetic acid 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 (API3000 LC–MS/MS system, Applied Biosystems, Foster City, CA, USA). The samples were analyzed employing the transition of *m/z* 482 → 258 amu for rosuvastatin with a dwell time of 200 ms. The mass transition for the internal standard was *m/z* 271 → 253 amu, with the same dwell time. The ionspray voltage was set at 5000 V, the decluster potential was set at 120, 60 V and the collision energy at 50, 25 V for rosuvastatin and internal standard, respectively. The entrance potential was set at 10.0 V, and the focusing potential at 400 V. The nebulizer gas (nitrogen) pressure was set at 8 (arbitrary units). The curtain gas (nitrogen) was set at 7 (arbitrary units).

3. Results and discussion

3.1. Method development and mass spectrometry

The MS/MS spectrum of rosuvastatin has been well demonstrated in some previous papers. The Q1 and MS2 mass spectra of rosuvastatin obtained in the present study were in accordance with previous reports [14,17,19,20], and the same transition of *m/z* 482 → 258 amu for rosuvastatin, which was documented in several articles, was employed for detection of rosuvastatin. The decluster potential, collision energy, collision cell exit energy, entrance potential and focusing potential were automatically optimized by needle pump mode (Harvard apparatus, Holliston, MA, USA), and the TurboIonspray heater, ionspray voltage, nebulizer gas and curtain gas were automatically optimized by FIA mode without chromatography column. The parameters presented in the experimental section are the result of these optimizations.

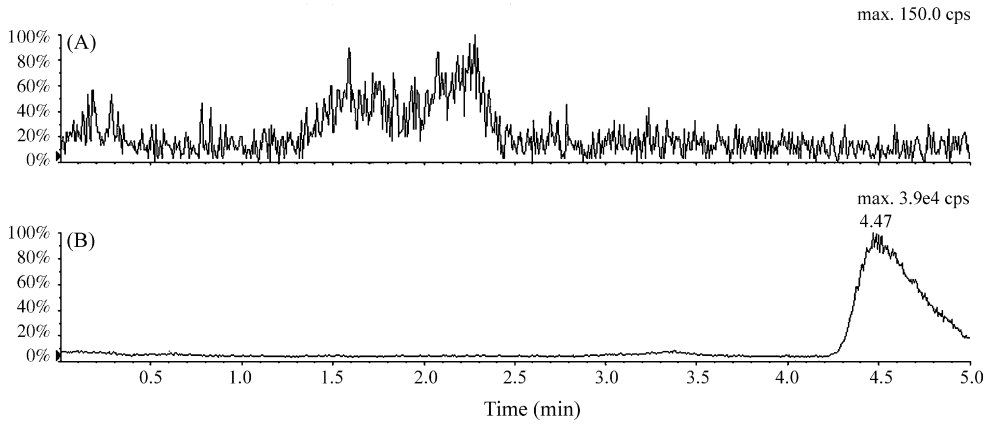

Fig. 2. Ion chromatogram of a double blank plasma sample (without rosuvastatin and IS): (A) rosuvastatin channel and (B) internal standard channel. *X*-scale represents retention time and *Y*-scale expressed as relative abundance (%).

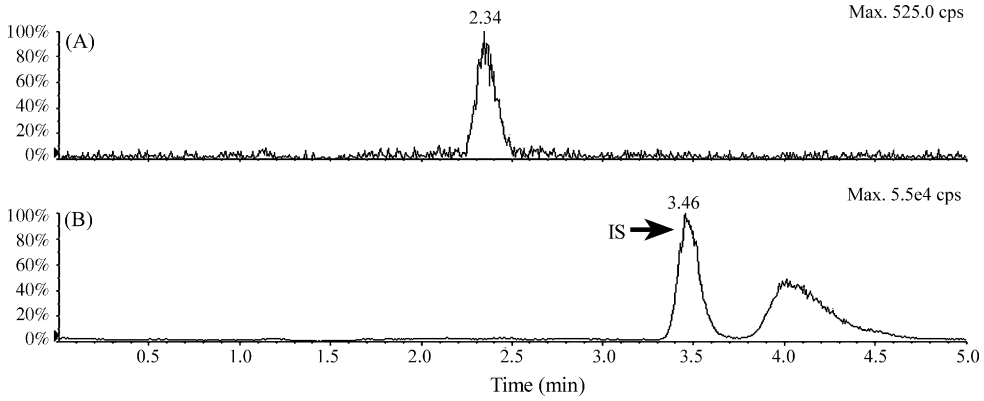

Fig. 3. Ion chromatogram of a rosuvastatin spiked plasma (0.1 ng mL^{-1}): (A) rosuvastatin channel and (B) internal standard channel. *X*-scale represents retention time and *Y*-scale expressed as relative abundance (%).

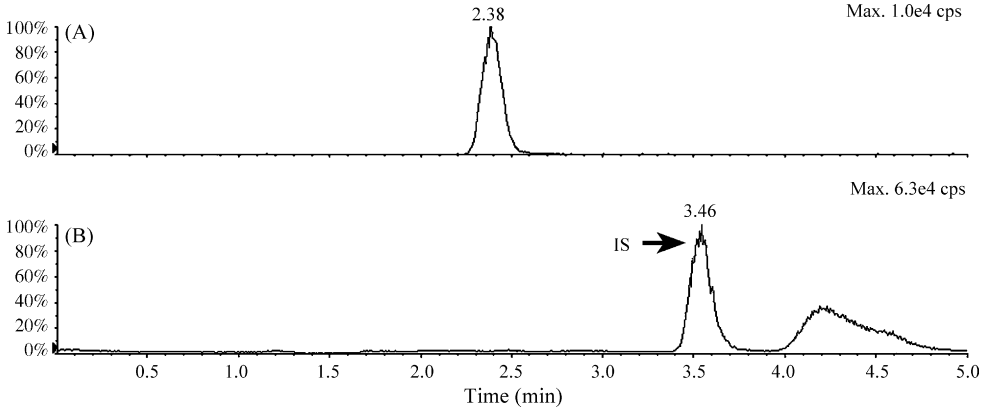

Fig. 4. Ion chromatogram of a rosuvastatin spiked plasma (5 ng mL^{-1}): (A) rosuvastatin channel and (B) internal standard channel. *X*-scale represents retention time and *Y*-scale expressed as relative abundance (%).

Similar HPLC conditions were optimized according to the report of Hull et al. [14] to assure high throughput with a relatively short retention time. The typical chromatogram of a double blank plasma (without rosuvastatin and internal standard) was shown in Fig. 2. The chromatogram of a spiked plasma sample with rosuvastatin (approximately 0.1 and 5 ng mL^{-1}) and internal standard was shown in Figs. 3 and 4, respectively. And a plasma sample of a volunteer collected at 2.0 h after administration of 5 mg of rosuvastatin was shown in Fig. 5, in which the concentration of rosuvastatin was 6.34 ng mL^{-1}.

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 200 μL of 0.5% acetic acid in water/methanol (50:50, v/v) by vortex mixing at 3000 rpm for 3 min to ensure

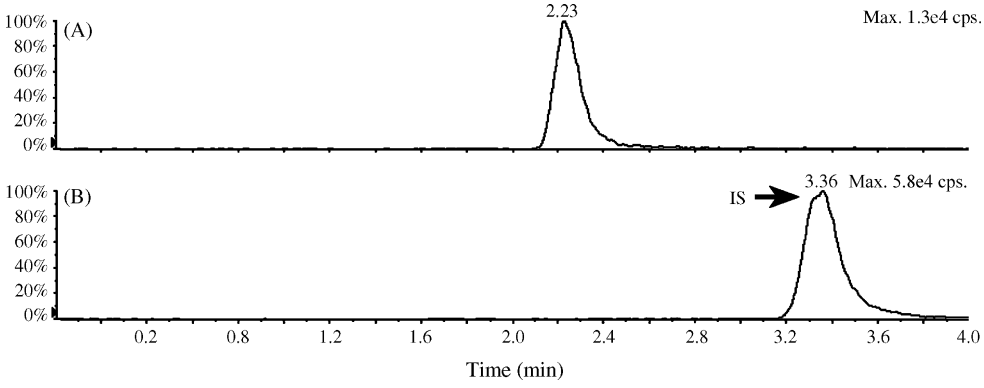

Fig. 5. Ion chromatogram of a study sample of a volunteer collected at 2.0 h after administration of 5 mg of rosuvastatin: (A) rosuvastatin channel and (B) internal standard channel. *X*-scale represents retention time and *Y*-scale expressed as relative abundance (%).

Table 1
TAH concentration dependent recovery of rosuvastatin with and without treat with TAH solution

Amount of acetic acid[a] (μL)	Amount of TAH[b] (μL)	Plasma pH	Recovery ($n=6$) (%)			
			0.1 ng mL^{-1}	0.5 ng mL^{-1}	1 ng mL^{-1}	10 ng mL^{-1}
0	0	7.38	10.5 ± 4.2	9.6 ± 2.3	12.7 ± 3.0	9.9 ± 4.2
100	0	6.72	9.8 ± 3.1	7.4 ± 2.1	10.5 ± 3.5	11.5 ± 4.3
100	200	6.84	10.4 ± 3.7	8.5 ± 2.1	10.5 ± 5.9	8.4 ± 4.0
100	400	6.97	12.3 ± 5.6	19.2 ± 7.1	11.4 ± 2.1	9.2 ± 3.4
100	500	7.05	57.4 ± 5.9	59.1 ± 9.0	53.2 ± 7.8	55.4 ± 6.6
100	600	7.12	61.3 ± 4.5	62.2 ± 8.7	47.5 ± 5.5	53.2 ± 5.3

[a] 0.5% acetic acid in water.
[b] 0.5% tetrabutyl ammonium hydroxide in water.

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.

3.2. Extraction recovery

The extraction recoveries of rosuvastatin from plasma were determined at four concentrations (0.1, 0.5, 1 and 10 ng mL^{-1}, $n=6$), and for the internal standard at the concentration used in the assay (500 ng mL^{-1}, $n=24$), by comparing the areas of extracted samples with none-extracted samples (pure standard solutions of rosuvastatin or internal standard diluted with 0.5% acetic acid in water/methanol (50:50, v/v) at the test concentrations). The mean extraction recoveries and standard deviation were 61.3 ± 4.5%, 62.2 ± 8.7%, 47.5 ± 5.5% and 53.2 ± 5.3% for 0.1, 0.5, 1 and 10 ng mL^{-1} of rosuvastatin; and 54.3 ± 7.5% for the internal standard. These results indicated that the sample procedure of ion pair liquid–liquid extraction with ethyl acetate is efficient for the extraction of trace rosuvastatin in plasma. The assay has been proven to be robust in high throughput bioanalysis.

In the report of Trivedi et al. [19], it was documented that the recovery of rosuvastatin with ethyl acetate was about 74.61%. Similarly, an absolute recovery of about 60% was obtained by simple one-step liquid–liquid extraction with ether [20]. In spite of the hydrophilicity of rosuvastatin compared with other statins, rosuvastatin was sparingly soluble in water and its lopP was 3.135 (http://redpoll.pharmacy.ualberta.ca/drugbank/cgi-bin/getCard.cgi?CARD=APRD00546.txt). It seemed that rosuvastatin might be more soluble in organic solutions than in water. But in the preliminary study of extraction solution screening, it was found that the extraction recovery of rosuvastatin in most common organic solutions, such as ethyl acetate, ether, dichloromethane, trichloromethane, etc., was less than 20%, resulting in an unstable extraction recovery, and imprecise and inaccurate assay. It could be seen in Table 1 that the extraction recovery of rosuvastatin in pure ethyl acetate was less than 15%. These results were not consistent with the previous reports. The difference might derive from the preparation of non-extracted samples, which was not well described in the previous reports [19,20]. In order to improve the extraction recovery of rosuvastatin, especially at low concentrations, it was necessary to improve the liposoluble characteristic of rosuvastatin. Ion pairing techniques were widely used in the mobile phase of RP-HPLC to maintain the retention time of analytes and optimize the peak shape of analytes. The simple employment of an ion pair regent can significantly improve the lipophilic characteristic of analytes and subsequently stabilize the analytes and maintain a good peak shape and appropriate retention time. In the case of this study, ion pair regent-TAH was added to form an ion pair with rosuvastatin.

Human plasma normally exhibits an alkalescence pH of 7.35–7.45. In the physiological pH of plasma, the carboxyl group of rosuvastatin may exist as an ionic type. Therefore, in the sample procedure, an aliquot of 100 μL of 0.5% (v/v) acetic acid in water was added first to make the carboxyl group of rosuvastatin maintain a carboxylic acid structure and adjust the pH of plasma solution to approximately 6.5. The solution of 0.5% TAH in water used in experiments exhibited alkaline. The extraction recoveries of rosuvastatin from plasma solution and the pH of plasma solution were investigated by adding different amounts of TAH. It was demonstrated that the pH was maintained at 6.5–7.0 when 400 μL or less TAH solution was added and the extraction recoveries of rosuvastatin remained less than 15% under these conditions, which is approximately equal to the one employing pure ethyl acetate extraction. However, once the amount of TAH solution was adjusted to more than 500 μL, the recovery of rosuvastatin from plasma with ethyl acetate suddenly showed a significant increase to more than 50%, indicating that an ion pair of rosuvastatin with TAH was formed under these conditions. The TAH concentration dependent recoveries of rosuvastatin with and without treat with TAH solution were listed in Table 1. Six hundred microliters of TAH solution was chosen in the sample procedure, and this assay was proven to be robust and stable by repeating more than three batch tests.

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

Table 2
Inaccuracy and imprecision of the method as measured by the performance of samples analyzed on three different days at four concentrations

Concentration (ng mL^{-1})	n	Intra-batch		n	Inter-batch	
		Inaccuracy	Imprecision		Inaccuracy	Imprecision
0.1	6	1.2	10.5	6	−2.6	12.8
0.5	6	2.6	5.0	6	8.4	6.2
1	6	−7.7	3.9	6	5.0	9.8
10	6	−3.5	4.9	6	6.5	8.0

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.

3.4. Linearity, precision, accuracy and limit of quantification

The assay was linear over the range 0.1–20 ng mL^{-1} for rosuvastatin. The standard curve fitted to a 1/c weighted linear regression which was calculated by the quantitative module of Analyst software. The mean equation (curve coefficients ± S.D.) of the calibration curve ($n = 8$) obtained from three single batches in method validation was $y = 2.8815(\pm 0.1011)\,x + 0.0064(\pm 0.0049)$ (correlation coefficient $r = 0.9984 \pm 0.012$) for rosuvastatin, where y represents the rosuvastatin peak area to estrone 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 ng mL^{-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 2. 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 ng mL^{-1} with the inter-batch inaccuracy <20% and imprecision <±20%.

3.5. Stability

Rosuvastatin spiked plasma at LLOQ, low, medium and high concentrations (0.1, 0.5, 1.0 and 10.0 ng mL^{-1}) were analyzed at fresh preparing and left in the autosampler 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 °C, 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 °C 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 °C. The concentration determined showed that the plasma samples were stable at room temperature for at least 24 h and at −20 °C for at least 8 weeks.

4. Conclusions

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 ng mL^{-1}. Rosuvastatin was shown to be stable in routine analysis conditions and in human plasma for up to 6 months when stored at −20 °C. The method has been used to analyze human plasma samples from a clinical pre-studies of rosuvastatin in the Chinese volunteers.

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