BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **20**: 924–936 (2006)
Published online 7 February 2006 in Wiley InterScience (www.interscience.wiley.com)
DOI: 10.1002/bmc.622

ORIGINAL RESEARCH

Simultaneous quantification of atorvastatin and active metabolites in human plasma by liquid chromatography–tandem mass spectrometry using rosuvastatin as internal standard

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Received 17 October 2005; accepted 15 November 2005

ABSTRACT: A simple, sensitive, selective and rapid liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed and validated for the quantification of atorvastatin and its active metabolites *ortho*-hydroxyatorvastatin and *para*-hydroxyatorvastatin in human plasma using rosuvastatin as internal standard (IS). Following simple liquid–liquid extraction, the analytes were separated using an isocratic mobile phase on a reversed-phase C_{18} column and analyzed by MS in the multiple reaction monitoring mode using the respective $[M+H]^+$ ions, *m/z* 559/440 for atorvastatin, *m/z* 575/466 for *ortho*-hydroxyatorvastatin, *m/z* 575/440 for para-hydroxyatorvastatin and *m/z* 482/258 for the IS. The assay exhibited a linear dynamic range of 0.1–20 ng/mL for atorvastatin and its two metabolites in human plasma. The lower limit of quantification was 100 pg/mL with a relative standard deviation of less than 8%. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. The average absolute recoveries of atorvastatin, *ortho*-hydroxyatorvastatin, para-hydroxyatorvastatin and the IS from spiked plasma samples were 54.2 ± 3.2, 50.1 ± 3.8, 65.2 ± 3.6 and 71.7 ± 2.7%, respectively. A run time of 2.5 min for each sample made it possible to analyze more than 300 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: atorvastatin; metabolites; LC-MS/MS; pharmacokinetic study

INTRODUCTION

Atorvastatin is a HMG-CoA reductase inhibitor that efficiently and dose-dependently lowers both cholesterol (Nawrocki *et al.*, 1995) and triglyceride (Bakker-Arkema *et al.*, 1996) levels in hyperlipidaemic patients. Atorvastatin produces larger reduction of cholesterol and triglycerides compared with other drugs in this class (Jones *et al.*, 1998). It has also been shown to reduce the levels of small, dense LDL and IDL (Guerin *et al.*, 2002). Recently the US FDA has approved atorvastatin for use to reduce the risk of stroke and heart attack in people with type 2 diabetes without evidence of heart disease.

Atorvastatin is administered in its active acid form and undergoes extensive first-pass metabolism (Chong *et al.*, 2001) that results in very low plasma concentrations (ng/mL levels). Liver metabolism produces two active hydroxy metabolites, *ortho*-hydroxyatorvastatin and *para*-hydroxyatorvastatin, and three corresponding inactive lactone metabolites (Jacobsen *et al.*, 2000). About 70% of the total plasma HMG-CoA reductase inhibitory activity is accounted for by active metabolites (Parke-Davis, 2004). The plasma concentration of active metabolites is very low (pg/mL levels). Therefore, the quantification of atorvastatin and active metabolite concentrations in plasma requires a bioanalytical method with high sensitivity.

The actual plasma concentrations of both parent compound and metabolites are of major interest in pharmacokinetic studies. The bioanalytical component of a pharmacokinetic study requires a drug assay with simplicity, high sensitivity, selectivity, small volume requirements and rapid turnaround time. Several methods for the quantification of atorvastatin in plasma have been reported, such as enzyme inhibition assay (Cilla *et al.*, 1996; Gibson *et al.*, 1996; Shumm *et al.*, 1998; Siedlik *et al.*, 1999), radioimmunoassay (Posvar *et al.*, 1996; Radulovic *et al.*, 1995) and liquid chromatography

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Abbreviations used: IS, internal standard.

with ultraviolet detection (Zarghi *et al.*, 2005). Methods involving immunoassay techniques have become attractive for routine clinical monitoring because of their ease of performance and sensitivity. However, immunoassay methods lack specificity and in some cases are subject to problems with cross-reactive interferences. Quantification of atorvastatin by chromatographic methods is preferred due to the sensitivity and specificity. Recently a liquid chromatography method with ultraviolet detection was reported for the quantification of atorvastatin over the concentration range 20–800 ng/mL with a lower limit of quantification (LLOQ) of 20 ng/mL.

Quantification of drugs in biological matrices by liquid chromatography–tandem mass spectrometry (LC-MS/MS) is becoming more common, owing to the improved sensitivity and selectivity of this technique (Jemal, 2000; Niessen, 2003). In recent years, we have reported a number of bioanalytical procedures using LC-MS/MS with high sample throughput capacity (Ramakrishna *et al.*, 2004a–e, 2005a–f; Nirogi *et al.*, 2005a,b). Several LC-MS/MS methods were reported for the quantification of atorvastatin and its metabolites in human plasma/serum. Bullen *et al.* (1999) reported a validated LC-MS/MS assay for the quantification of atorvastatin and its active metabolites *ortho*-hydroxyatorvastatin and *para*-hydroxyatorvastatin in human, dog and rat plasma using deuterated internal standards, which are seldom commercially available. The LLOQ for all analytes was 250 pg/mL and the total run time was longer (6 min). The assay involves a back extraction step that is tedious and time-consuming. Jemal *et al.* (1999) reported an LC-MS/MS method for simultaneous quantification of both the acid and lactone forms of atorvastatin, *ortho*- and *para*-hydroxyatorvastatin, in human serum using a respective deuterated internal standards, which are seldom commercially available. The LLOQ for all six analytes was 500 pg/mL and the total run time was 3.5 min. Recently an LC-MS/MS method was reported for the quantification of atorvastatin and its metabolites in human plasma based on solid-phase extraction (Hermann *et al.*, 2005). The LLOQ was 200 pg/mL for atorvastatin and *para*-hydroxyatorvastatin and 500 pg/mL for *ortho*-hydroxyatorvastatin and the total run time was longer (21 min). A gradient HPLC mobile phase system was used in the above latter two methods. Koytchev *et al.* (2004) measured the plasma concentration of atorvastatin using an LC-MS/MS method with an LLOQ of 400 pg/mL. All the above three methods except Hermann *et al.*'s method, have been applied (with minor modifications) in clinical studies, with atorvastatin doses ranging between 40 and 80 mg (Kantola *et al.*, 1998; Lins *et al.*, 2003). Herman *et al.*'s method, the most sensitive among the above methods, has been applied in a clinical study after the administration of atorvastatin at the lower registered dose (10 mg).

The purpose of this investigation was to explore the high selectivity and sensitivity of a triple-quadrupole MS system with an electrospray interface for the development and validation of a robust reversed-phase LC-MS/MS method in multiple reaction monitoring (MRM) mode for the quantification of atorvastatin and its active metabolites *ortho*- and *para*-hydroxyatorvastatin in human plasma using a commercially available compound rosuvastatin as an internal standard. It was essential to establish an assay capable of quantifying atorvastatin and its active metabolites *ortho*- and *para*-hydroxyatorvastatin at concentrations down to 100 pg/mL. At the same time, it was expected that this method would be efficient in analyzing large numbers of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of atorvastatin.

EXPERIMENTAL

Chemicals. Atorvastatin calcium, *ortho*-hydroxyatorvastatin calcium and *para*-hydroxyatorvastatin sodium were obtained from Ranbaxy Laboratories Ltd (Gurgaon, India) and rosuvastatin (internal standard, IS) was from the R&D department of this Institute (Hyderabad, India). Chemical structures are presented in Fig. 1. Drug-free human plasma was obtained from the Usha Mullapudi Cardiac Center (Hyderabad, India). HPLC-grade LiChrosolv methanol and LiChrosolv acetonitrile were purchased from Merck (Darmstadt, Germany). Formic acid, *ortho*-phosphoric acid and *N*,*N*-dimethylacetamide were purchased from Merck (Worli, Mumbai, India). HPLC-grade water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

LC-MS/MS instrument and conditions. The Agilent 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) is equipped with a G1312A binary pump, a G1379A degasser, a G1367A autosampler equipped with a G1330B thermostat, a G1316A thermostatted column compartment and a G1323B control module. The chromatographic separation was on Waters symmetry® C_{18} column (5.0 µm, 100 × 4.6 mm i.d.) at 30°C. The isocratic mobile phase composition was a mixture of 0.03% formic acid–acetonitrile (30:70, v/v), which was pumped at a flow-rate of 1.0 mL/min with a split ratio of 10:90.

Mass spectrometric detection was performed using an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. A turbo-electrospray interface in positive ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed using the Analyst 1.4.1 software package (SCIEX).

Sample preparation. A plasma sample (1 mL) was transferred to a 15 mL glass test tube, then 25 µL of IS working

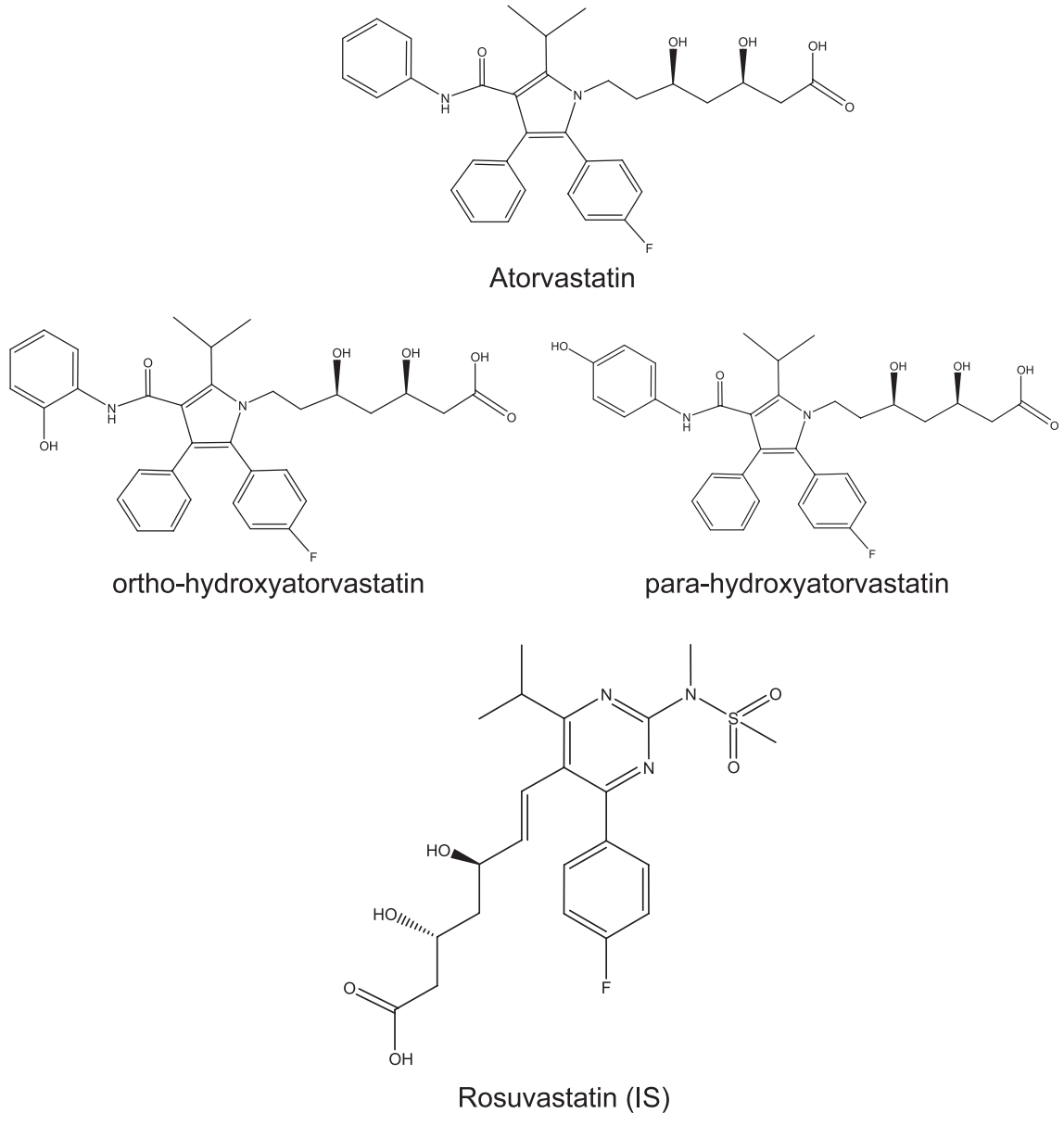


Figure 1. Chemical structures for atorvastatin and *ortho*- and *para*-hydroxyatorvastatin and IS (rosuvastatin).

solution (1 μg/mL) and 100 μL of 10% *ortho*-phosphoric acid were added. After vortex mixing for 30 s, 5 mL aliquot of the extraction mixture, diethyl ether–dichloromethane (7:3, v/v), were added and the sample was vortex-mixed for 4 min. The organic layer (4 mL) was transferred to a 5 mL glass tube and evaporated to dryness using an evaporator at 40°C under a stream of nitrogen. Then the dried extract was reconstituted in 250 μL mobile phase and a 10 μL aliquot was injected into the chromatographic system.

Bioanalytical method validation. Standard stock solutions of atorvastatin (0.5 mg/mL), *ortho*-hydroxyatorvastatin (0.5 mg/mL) and *para*-hydroxyatorvastatin (0.5 mg/mL) were separately prepared by dissolving appropriate amounts in 1 mL *N*,*N*-dimethylacetamide and the final volume was made up to 10 mL with methanol. Stock solution of IS (0.5 mg/mL) was prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water–methanol (50:50, v/v; diluent). The IS working solution (1 μg/mL) was prepared by diluting its stock solution with diluent. Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) as a bulk, to obtain atorvastatin, *ortho*-hydroxyatorvastatin and *para*-hydroxyatorvastatin concentration levels of 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng/mL as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent weighing of drug and its metabolites, at concentrations of 0.1 (LLOQ), 0.3 (low), 8 (medium) and 16 ng/mL (high) as a single batch at each concentration for each analyte. The calibration and control bulk samples were divided into aliquots in micro-centrifuge tubes (Tarson, 2 mL) and stored in the freezer at below –50°C until analysis.

Table 1. Tandem mass spectrometer main working parameters

Parameter	Value
Source temperature, °C	250
Dwell time per transition, msec	200
Ion Source gas 1, psi	20
Ion Source gas 2, psi	20
Curtain gas, psi	25
Collision gas, psi	5
Ion spray voltage, V	5500
Entrance potential, V	10
Declustering potential, V	80 (atorvastatin) 80 (*ortho*-hydroxyatorvastatin) 80 (*para*-hydroxyatorvastatin) and 100 (IS)
Collision energy, V	30 (atorvastatin) 18 (*ortho*-hydroxyatorvastatin) 30 (*para*-hydroxyatorvastatin) and 40 (IS)
Collision cell exit potential, V	13 (atorvastatin) 15 (*ortho*-hydroxyatorvastatin) 15 (*para*-hydroxyatorvastatin) and 18 (IS)
Resolution	Unit
Mode of analysis	Positive
Ion transition for atorvastatin, *m/z*	559.2 ± 0.5/440.2 ± 0.5
ortho-hydroxyatorvastatin, *m/z*	575.2 ± 0.5/466.4 ± 0.5
para-hydroxyatorvastatin, *m/z*	575.2 ± 0.5/440.2 ± 0.5
IS (rosuvastatin), *m/z*	482.1 ± 0.5/258.3 ± 0.5

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and eight non-zero samples covering the range 0.1–20 ng/mL, including the LLOQ for each analyte. The calibration curves were generated using the analyte to IS peak area ratios by weighted ($1/x^2$) least-squares linear regression on five consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that for each back-calculated standard concentration was 15% deviation from the nominal value except at the LLOQ, which was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification.

The within-batch precision and accuracy were determined by analyzing five sets of QC samples in a batch. The between-batch precision and accuracy were determined by analyzing five sets of QC samples on three different batches. The QC samples were randomized daily, processed and analyzed in a position either (a) immediately following the standard curve, (b) in the middle of the batch or (c) at the end of the batch. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the other concentrations.

Recovery of the analytes from the extraction procedure was determined by a comparison of the peak area of atorvastatin and *ortho*- and *para*-hydroxyatorvastatin in spiked plasma samples (five each of low, medium and high QCs) to the peak area of atorvastatin and *ortho*- and *para*-hydroxyatorvastatin in samples prepared by spiking extracted drug-free plasma samples with the same amounts of atorvastatin and *ortho*- and *para*-hydroxyatorvastatin, respectively, at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples (n = 5) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

RESULTS AND DISCUSSION

Mass spectrometry

In order to develop a method with the desired LLOQ (100 pg/mL), it was necessary to use MS-MS detection, as MS-MS methods provide improved limit of detection for trace-mixture analysis (Jemal, 2000). The inherent selectivity of MS-MS detection was also expected to be beneficial in developing a selective and sensitive method. The product ion mass spectrum of atorvastatin, *ortho*-hydroxyatorvastatin, *para*-hydroxyatorvastatin and the IS are shown in Fig. 2. $[M+H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain the product ion spectra. Even though the analytes have a carboxylic acid moiety, electrospray in negative ion mode was less sensitive than the positive mode. Compared with other statins, such as lovastatin, simvastatin and pravastatin, the formation of the $[M+H]^+$ ion is expected to be more facile with atorvastatin since this compound contains two nitrogen groups (Jemal *et al.*, 1999). The most sensitive mass transition was from *m/z* 559 to 440 for atorvastatin, from *m/z* 575 to 466 for

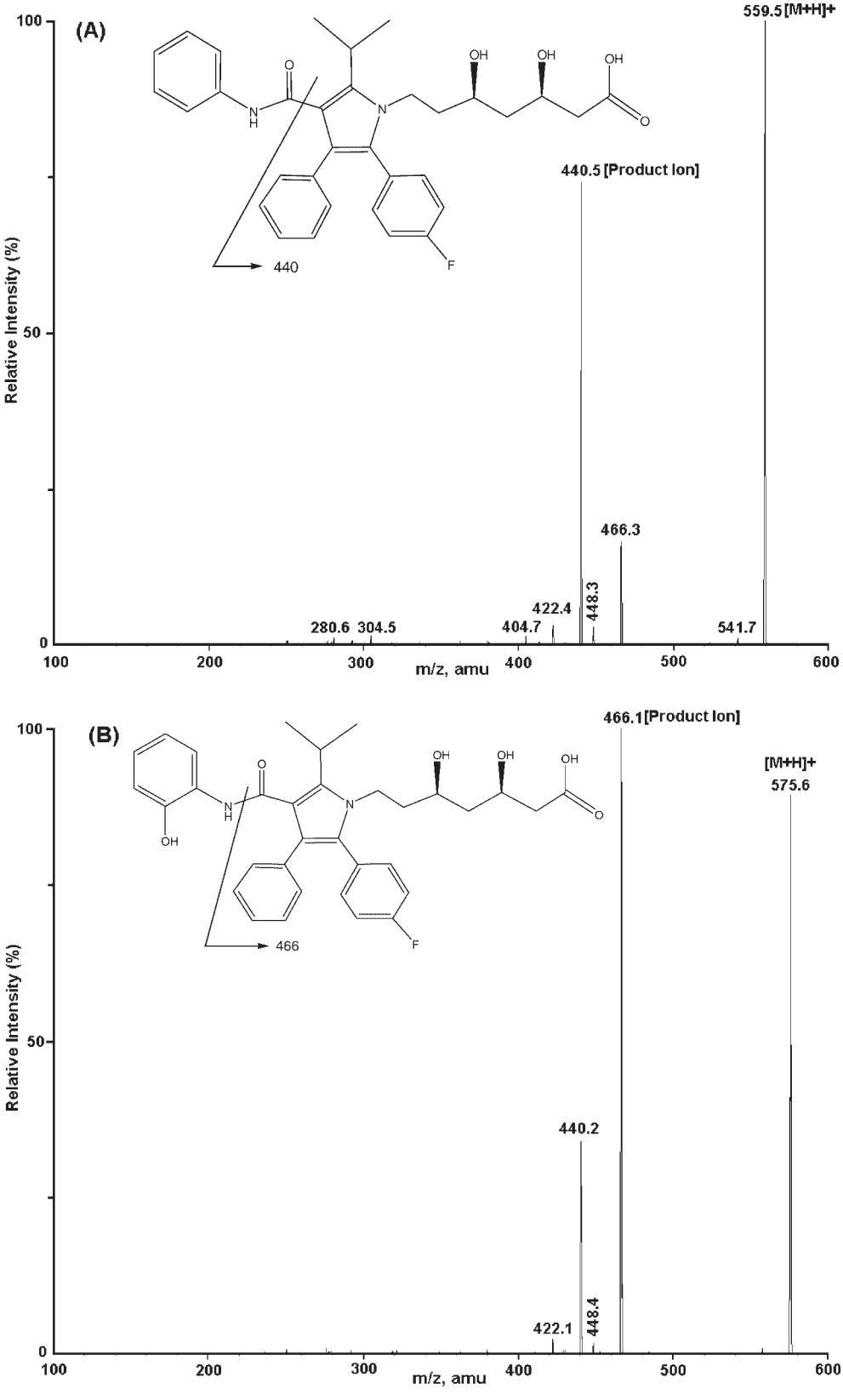


Figure 2. Full scan positive ion turboionspray product ion mass spectra and the proposed patterns of fragmentation of (A) atorvastatin, (B) *ortho*-hydroxyatorvastatin, (C) *para*-hydroxyatorvastatin and (D) rosuvastatin (internal standard). The protonated molecules were used as precursor ions for MS/MS.

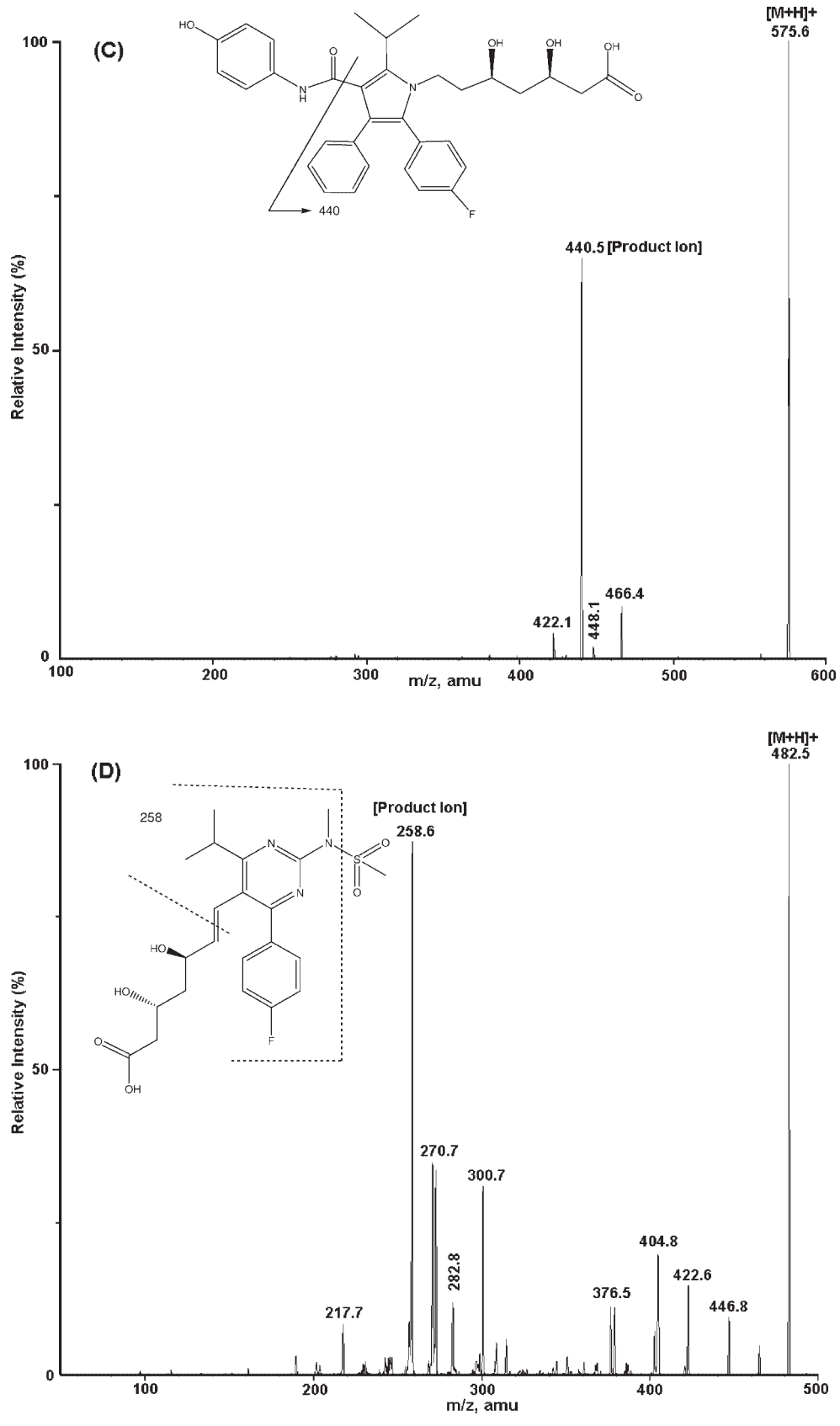


Figure 2. (*continued*)

ortho-hydroxyatorvastatin, from *m/z* 575 to 440 for *para*-hydroxyatorvastatin and from *m/z* 482 to 258 for the IS. A proposed fragmentation pattern is also shown in Fig. 2. For atorvastatin and *para*-hydroxyatorvastatin, the major product ion (*m/z* 440) is formed by the neutral loss of the phenylaminocarbonyl group, whereas for *ortho*-hydroxyatorvastatin, the major product ion (*m/z* 466) is by the neutral loss of phenylamino group. This may be due to the intramolecular hydrogen bonding with the NH group in the *ortho* position. This product ion (*m/z* 466) was specific to *ortho*-hydroxyatorvastatin and was considered to be the most appropriate choice for a specific and sensitive method.

LC-MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. Separate MRMs were preferred for atorvastatin and *ortho*- and *para*-hydroxyatorvastatin to provide a most sensitive and selective method. The MRM state file parameters were optimized to maximize the response for the analytes. The parameters presented in Table 1 are the result of this optimization.

Selection of mobile phase

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analytes and IS, as well as a short run time. It was found that a mixture of 0.03% formic acid–acetonitrile (30:70, v/v) could achieve this purpose and was finally adopted as the mobile phase. The formic acid was found to be necessary in order to lower the pH to protonate the analytes and thus deliver good peak shape. The percentage of formic acid was optimized to maintain this peak shape while being consistent with good ionization and fragmentation in the MS. The high proportion of organic solvent eluted atorvastatin and *ortho*- and *para*-hydroxyatorvastatin and the IS at retention time of 1.8, 1.6, 1.1 and 1.3 min, respectively. The retention time of *para*-hydroxyatorvastatin was shorter than *ortho*-hydroxyatorvastatin due to the difference in polarity. The polarity of *ortho*-hydroxyatorvastatin is diminished due to the intramolecular hydrogen bonding with the NH group in the *ortho* position, which is not possible in *para*-hydroxyatorvastatin (Jemal *et al.*, 1999). Therefore *para*-hydroxyatorvastatin is expected to be the more polar of the two. A flow rate of 1 mL/min produced good peak shapes and permitted a run time of 2.5 min.

Internal standard

For an LC-MS/MS analysis, utilization of stable isotope-labeled drugs as internal standards proves to be helpful when a significant matrix effect is possible. However, isotope-labeled analyte was not obtainable commercially and the cost of custom synthesis is prohibitive to its use. Rosuvastatin was found to be best for the present study. Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. These potential matrix effects were evaluated by spiking blank plasma extracts (after LLE treatment as described above) at the low and high QC levels. The resulting chromatograms were compared with those obtained for clean standard solutions at the same concentrations. Five independent plasma lots were used, with five samples from each lot. The results (data not shown) showed that there was no significant difference between peak responses for spiked plasma extracts and clean solutions. This result most likely reflects the efficacy of the sample clean-up with LLE. In any event, the use of matrix-matched calibration standards would have minimized any such effects on the quantification.

Calibration curves

The eight-point calibration curve was linear over the concentration range 0.1–20 ng/mL for atorvastatin and *ortho*- and *para*-hydroxyatorvastatin. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and $1/\sqrt{x}$). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor. The mean correlation coefficient of the weighted calibration curves generated during the validation was more than 0.999; Table 2 summarizes the calibration curve results.

Selectivity

The selectivity of the method was examined by analyzing blank human plasma extract [Fig. 3(A)] and an extract spiked only with the internal standard [Fig. 3(B)]. As shown in Fig. 3(A), no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analytes. Similarly, Fig. 3(B) shows the absence of direct interference from the internal standard in the MRM channel of the analytes. Figure 3(C) depicts a representative ion-chromatogram for the LLOQ (0.1 ng/mL). Excellent sensitivity was observed for a 10 µL injection volume with a split ratio of 10:90; the LLOQ corresponds to ca. 1 fg on-column.

The MRM chromatograms obtained for an extracted plasma sample of a healthy subject who participated in a bioequivalence study conducted on 24 subjects is

Table 2. Mean calibration curve results for the validation of atorvastatin and *ortho*- and *para*-hydroxyatorvastatin in human plasma (n = 5)

Analyte	Slope	Intercept	Coefficient of correlation (r)
Atorvastatin	0.0003	0.0018	0.9996
Ortho-hydroxyatorvastatin	0.0002	0.0010	0.9997
Para-hydroxyatorvastatin	0.0002	0.0008	0.9996

depicted in Fig. 4. Atorvastatin and *ortho*- and *para*-hydroxyatorvastatin were identified and quantified as 6.6, 1.1 and 0.2 ng/mL respectively.

Extraction efficiency

Liquid–liquid extraction (LLE) was used for the sample preparation in this work. LLE can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS/MS analyses. A mixture of diethyl ether and dichloromethane (7:3, v/v) was found to be optimal, which can produce a clean chromatogram for a blank plasma sample. Plasma samples were mixed with *ortho*-phosphoric acid (10%) in order to insure that the analytes were in the unionized form prior to extraction. The average absolute recoveries of atorvastatin and *ortho*- and *para*-hydroxyatorvastatin from spiked plasma samples were 54.2 ± 3.2, 50.1 ± 3.8 and 65.2 ± 3.6%, respectively and the recovery of the IS was 71.7 ± 2.7% at the concentration used in the assay (1 μg/mL). Table 3 summarizes the recovery results. Recoveries of the analytes and IS were good, and they were consistent, precise and reproducible. Therefore, the assay has proved to be robust in high-throughput bioanalysis.

Table 3. Absolute recoveries of atorvastatin, *ortho*-hydroxyatorvastatin and *para*-hydroxyatorvastatin from human plasma

Sample concentration (ng/mL)	Absolute recovery (%) (mean ± SD, n = 5)
Atorvastatin	
0.3	57.3 ± 1.2
3	54.2 ± 1.6
8	51.0 ± 0.8
Ortho-hydroxyatorvastatin	
0.3	54.3 ± 2.5
3	49.1 ± 1.7
8	46.8 ± 1.5
Para-hydroxyatorvastatin	
0.3	68.8 ± 3.3
3	65.1 ± 2.4
8	61.6 ± 2.1

Validation parameters at the LLOQ

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and was found to be 0.1 ng/mL in human plasma. The mean response for the analytes peak at the assay sensitivity limit (0.1 ng/mL) was ~10-fold greater than the mean response for the peak in five blank human plasma samples at the retention time of the analytes. The between-batch precision at the LLOQ was 4.4, 8.0 and 3.6%, and the between-batch accuracy was 101.4, 98.5 and 101.3% for atorvastatin and *ortho*- and *para*-hydroxyatorvastatin, respectively (Tables 4–6). The within-batch precision was 2.3, 6.5 and 7.1% and the accuracy was 106.0, 92.0 and 99.2% for atorvastatin and *ortho*- and *para*-hydroxyatorvastatin respectively.

Validation parameters at the middle and upper concentrations

The middle and upper quantification levels of the analytes ranged from 0.3 to 16 ng/mL in human plasma. For the between-batch experiments the precision ranged from 0.3 to 4.3, 0.7 to 4.7 and 0.4 to 5.4% and the accuracy from 98.8 to 105.2, 97.6 to 105.7 and 96.7 to 102.4% for atorvastatin and *ortho*- and *para*-hydroxyatorvastatin, respectively (Tables 4–6). For the within-batch experiments the precision and accuracy for the analytes met the acceptance criteria (<±15%).

The upper concentration limits can be extended with acceptable precision and accuracy to 80 ng/mL by a 10-fold dilution with control human plasma. These results suggest that sample with concentrations greater than the upper limit of the calibration curve can in this way be assayed to obtain acceptable data.

Stability studies

The stability of the analytes and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated as follows. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (–50°C), and to freeze–thaw stability studies. All the stability studies were conducted at two

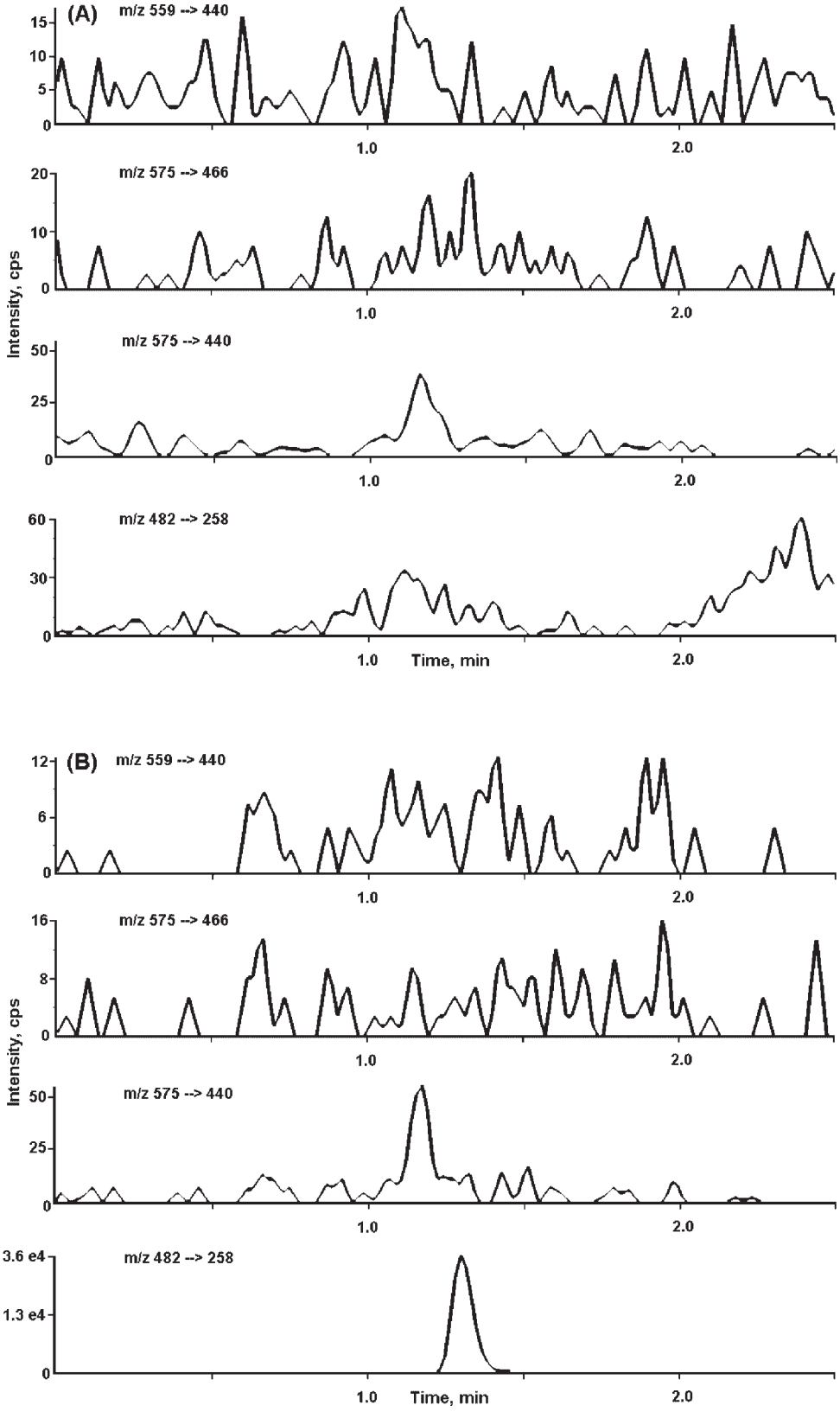
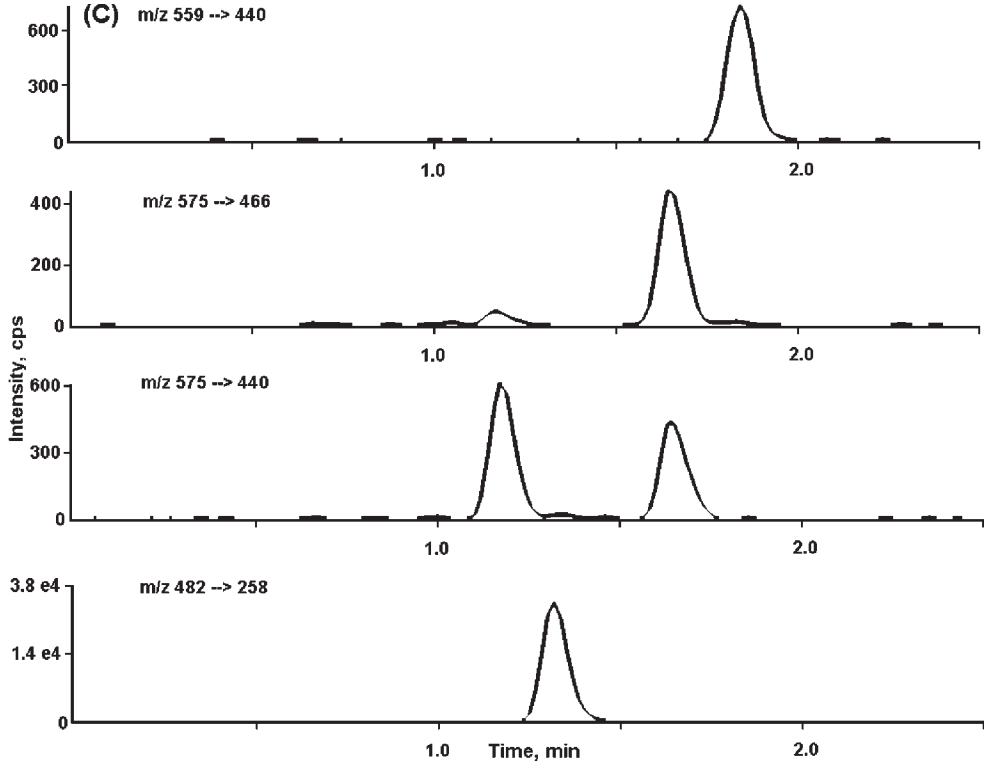


Figure 3. MRM chromatograms for atorvastatin, *ortho*- and *para*-hydroxyatorvastatin and the IS resulting from analysis of: (A) blank (drug- and IS-free) human plasma; (B) blank (drug-free spiked with IS) human plasma; (C) 100 pg/mL (LLOQ) of atorvastatin and *ortho*- and *para*-hydroxyatorvastatin spiked with the IS.



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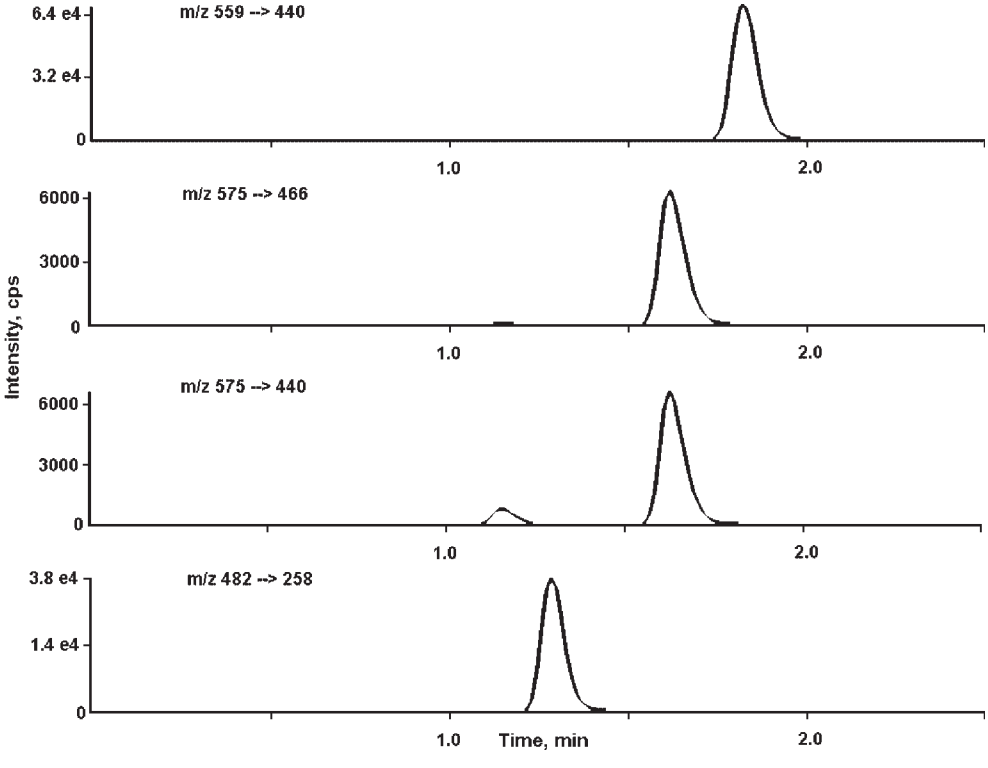

Figure 4. MRM-chromatograms resulting from the analysis of a volunteer plasma sample after the administration of 10 mg oral single dose of atorvastatin. The sample concentration was determined to be 6.6, 1.1 and 0.2 ng/mL for atorvastatin and *ortho*- and *para*-hydroxy atorvastatin, respectively.

Table 4. Precision and accuracy of the method for determining atorvastatin concentrations in plasma samples

	Within-batch precision (n = 5)			Between-batch precision (n = 3)		
Concentration added (ng/mL)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
0.1	0.11 ± 0.0	2.3	106.0	0.10 ± 0.0	4.4	101.4
0.3	0.31 ± 0.01	2.2	102.1	0.30 ± 0.01	4.3	98.8
8	8.12 ± 0.17	2.1	101.5	8.41 ± 0.27	3.2	105.2
16	15.84 ± 0.35	2.2	99.0	15.81 ± 0.04	0.3	98.8

Table 5. Precision and accuracy of the method for determining *ortho*-hydroxyatorvastatin concentrations in plasma samples

	Within-batch precision (n = 5)			Between-batch precision (n = 3)		
Concentration added (ng/mL)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
0.1	0.09 ± 0.01	6.5	92.0	0.10 ± 0.01	8.0	98.5
0.3	0.28 ± 0.01	3.7	94.7	0.29 ± 0.01	4.6	97.6
8	8.2 ± 0.2	2.4	102.5	8.45 ± 0.40	4.7	105.7
16	15.74 ± 0.15	0.9	98.4	15.69 ± 0.12	0.7	98.1

Table 6. Precision and accuracy of the method for determining *para*-hydroxyatorvastatin concentrations in plasma samples

	Within-batch precision (n = 5)			Between-batch precision (n = 3)		
Concentration added (ng/mL)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
0.1	0.10 ± 0.01	7.1	99.2	0.10 ± 0.00	3.6	101.3
0.3	0.30 ± 0.01	3.6	98.8	0.30 ± 0.00	0.4	98.6
8	8.18 ± 0.11	1.4	102.2	8.19 ± 0.44	5.4	102.4
16	15.97 ± 0.26	1.6	99.8	15.48 ± 0.58	3.7	96.7

concentration levels (0.3 and 16 ng/mL as low and high values) with five determinations for each.

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples were extracted and analyzed as described above and the results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the autosampler for 25 h was also assessed. The results indicate that solutions of the analytes and the IS can remain in the autosampler for at least 25 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time.

The stability data of the analytes in plasma over three freeze–thaw cycles indicate that the analytes are stable in human plasma for three freeze–thaw cycles, when stored at below –50°C and thawed to room temperature.

The long-term stability data of the analytes in human plasma stored for a period of 21 days at below –50°C showed reliable stability behavior, as the mean of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of the analytes in plasma samples at below –50°C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 2 and 24 h, and under refrigeration (~4°C) for 3 months. The recoveries for atorvastatin, *ortho*- and *para*-hydroxyatorvastatin and IS were 100.3 [coefficient of variance (CV) 1.2%], 99.5 (CV 0.5%), 100.9 (CV 1.8%), 103.3 (CV 2.9%), 101.7 (CV 1.1%), 101.6 (CV 1.0%), 99.2 (CV 0.9%), 102.7 (CV 1.3%), 100.2 (CV 0.8%) and 95.3 (CV 1.7%), 104.7 (CV 1.2%) and 101.6 (CV 1.5%) respectively. The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

Application

The validated method has been successfully used to quantify atorvastatin and *ortho*- and *para*-hydroxyatorvastatin concentrations in human plasma samples after the administration of a single 10 mg oral

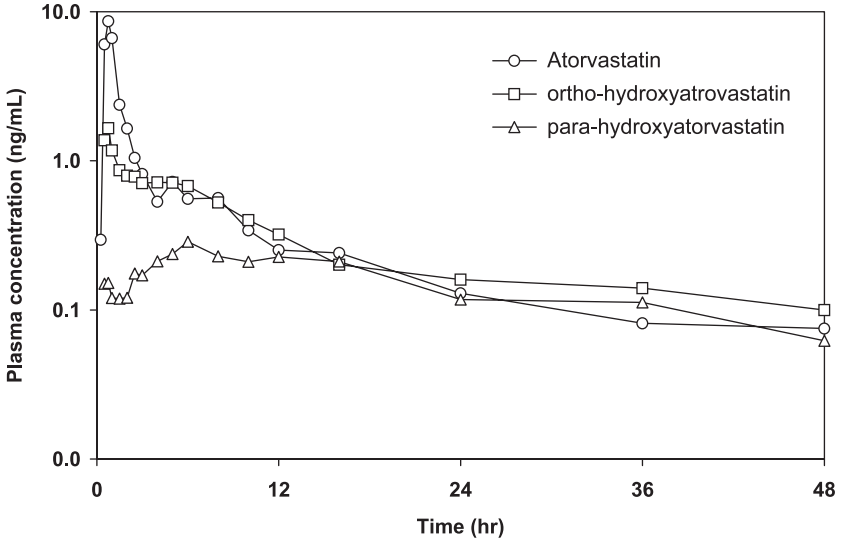

Figure 5. Representative data showing plasma concentration–time profile of a healthy subject after the administration of an oral single dose of 10 mg of atorvastatin.

dose of atorvastatin. The representative concentration vs time profile of a subject, receiving a single dose, is presented in Fig. 5.

CONCLUSIONS

In summary, a method has been described for the quantification of atorvastatin and *ortho*- and *para*-hydroxyatorvastatin in human plasma by LC-MS/MS in positive electrospray ionization mode using multiple reaction monitoring, and fully validated according to commonly accepted criteria (Shah *et al.*, 1991). This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity, faster run time (2.5 min) and rapid extraction. With dilution integrity up to 10-fold, we have established that the upper limit of quantification is extendable up to 80 ng/mL. Hence, this method is useful for single and multiple ascending dose studies in human subjects. The current method has shown acceptable precision and adequate sensitivity for the quantification of atorvastatin and *ortho*- and *para*-hydroxyatorvastatin in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. The desired sensitivity of atorvastatin and *ortho*- and *para*-hydroxyatorvastatin was achieved with an LLOQ of 100 pg/mL, which has a within- and between-batch CV of ≤7.1% and ≤8.0%, respectively. Many variables related to the electrospray reproducibility were optimized for both precision and sensitivity to obtain these results. The simplicity of the assay, using rapid liquid–liquid extraction with a commercially available IS and sample turnover rate of 2.5 min per sample, make it an attractive procedure in high-throughput bioanalysis of atorvastatin. The validated method allows quantification of atorvastatin and *ortho*- and *para*-hydroxyatorvastatin in the 0.1–20 ng/mL range.

Acknowledgments

The authors wish to acknowledge the support received from Mr Venkateswarlu Jasti, Managing Director, Suven Life Sciences Ltd, Hyderabad.

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