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# Quantification of rosuvastatin in human plasma by automated solid-phase extraction using tandem mass spectrometric detection

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**Abstract**

An assay employing automated solid-phase extraction (SPE) followed by high-performance liquid chromatography with positive ion TurboIonspray tandem mass spectrometry (LC–MS–MS) was developed and validated for the quantification of rosuvastatin (Crestor™) in human plasma. Rosuvastatin is a hydroxy-methyl glutaryl coenzyme A reductase inhibitor currently under development by AstraZeneca. The standard curve range in human plasma was 0.1–30 ng/ml with a lower limit of quantification (LLOQ) verified at 0.1 ng/ml. Inaccuracy was less than 8% and imprecision less than ±15% at all concentration levels. There was no interference from endogenous substances. The analyte was stable in human plasma following three freeze/thaw cycles and for up to 6 months following storage at both −20 and −70 °C. The assay was successfully applied to the analysis of rosuvastatin in human plasma samples derived from clinical trials, allowing the pharmacokinetics of the compound to be determined. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Rosuvastatin; HMG-CoA reductase inhibitor

## 1. Introduction

Rosuvastatin (Crestor™) (Fig. 1) is a new synthetic HMG-CoA reductase inhibitor [1] licensed for development by AstraZeneca. Rosuvastatin exhibits a high degree of specificity for uptake into the liver and is a potent in vitro and in vivo competitive inhibitor of HMG-CoA reductase. Compared with several other HMG-CoA reductase inhibitors, rosuvastatin does not appear to be metabolised significantly by cytochrome P450 3A4 [2] and, therefore, may not possess the same potential for drug interactions as seen for some other statins e.g. lovastatin [3] and simvastatin [4].

In order to quantify plasma concentrations of rosuvastatin in clinical trials, it was necessary to develop and validate an assay with appropriate sensitivity, selectivity, accuracy and precision. Assays for other statins (and their metabolites) have employed a number of different techniques and approaches. An enzyme-linked immunosorbent assay for pravastatin alone [5], and gas chromatography–mass spectrometry (GC–MS) [6] and HPLC–MS–MS [7] assays for pravastatin and metabolites have

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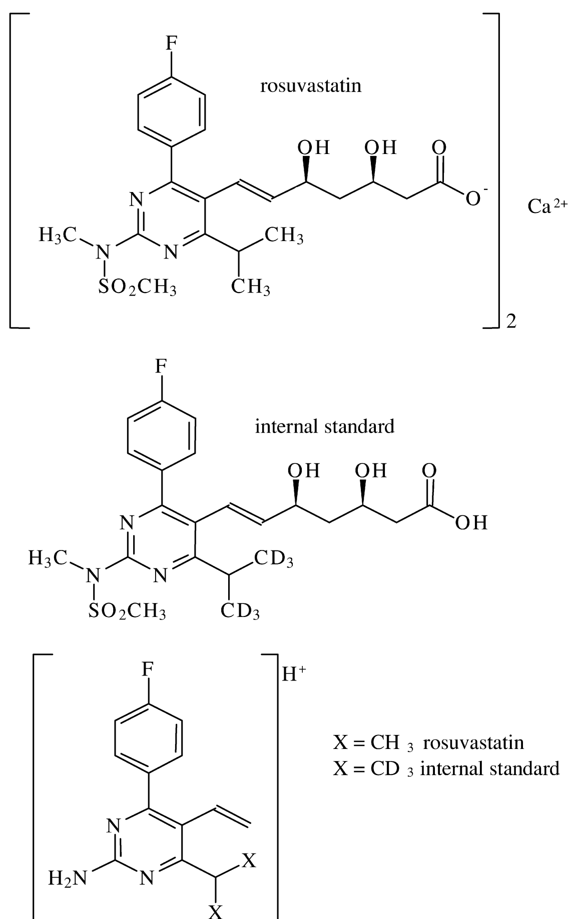


Fig. 1. The structures of rosuvastatin, deuterated rosuvastatin and the fragment ion of rosuvastatin monitored by MS.

been successfully validated. A specific HPLC–MS–MS assay was developed for atorvastatin and its two primary active metabolites [8]. An enzyme inhibition assay (HMG-CoA reductase inhibition assay) [9] has also been developed and applied to the assay of atorvastatin and active metabolites. The most attractive approach for an assay, which would be robust, sensitive, selective and would allow high throughput, was a HPLC–MS–MS-based method. We now report for the first time an LC–MS–MS method developed and validated for the quantitative determination of rosuvastatin in human plasma. This work was carried out at Quintiles Limited, Edinburgh, UK and was performed with reference to the guidance of Shah et al. [10].

## 2. Experimental

### *2.1. Chemicals and reagents*

Rosuvastatin and 5-*S*-Lactone were obtained from AstraZeneca (Cheshire, UK). Deuterated $D_6$ rosuvastatin, used as the internal standard, was supplied by Shionogi Pharmaceutical Company (Osaka, Japan). Methanol, glacial acetic acid and formic acid were purchased from Fisher Scientific (Loughborough, UK) and sodium acetate trihydrate from Sigma–Aldrich (Dorset, UK). Water was distilled in-house at Quintiles Limited. Drug-free human plasma was supplied in-house at Quintiles Limited.

### *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 autosampler (Herefordshire, UK). The analytical column employed was a Luna $C_{18}$(2) 5 μm (4.6 mm I.D.×150 mm). A Sciex API 365 (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 centrifuge was a Jouan MR 18.12, the vortex mixer a Fisher Whirlimixer (Fisher, Leicestershire, UK) and the tube rotator a Stuart TR-2. Samples were extracted automatically using a Tecan Genesis RSP 100 automated solid-phase extraction system (Tecan, Reading, UK) with Oasis HLB, 30 mg, 96-well blocks (Waters, Watford, UK). Extracts were dried down using a Micro DS96 drying block (Porvair, Middlesex, UK). 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-μl 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 extraction, the calibration curve in human plasma was prepared by spiking known amounts of rosuvastatin into human plasma (500 μl). Acetate buffer (0.1 *M*, pH 4, 500 μl) was added, internal standard (50 μl) and acetic acid (1 *M*, 750 μl) 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.

Internal standard stock solution was made up in methanol/0.1 *M* acetate buffer pH 4.0 (50:50 v/v) at a concentration of approximately 100 μg/ml, 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 1 *M* acetic acid/methanol (50:50 v/v) to give a working concentration of approximately 150 ng/ml.

### 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.

Extraction was performed on a Tecan Genesis RSP100 fitted with a Porvair vacuum manifold. Prior to addition to the extraction blocks, all test samples were vortexed for approximately 2 s followed by centrifugation at 738 *g* for 7 min. SPE sorbent Oasis HLB (30 mg, in 96-well plates) was conditioned, with 1000 μl methanol, followed by 1000 μl 0.5% acetic acid in water. Samples (1700 μl) were then transferred to the extraction blocks and extracted. A wash solvent (1000 μl) of methanol/0.5% acetic acid in water (30:70 v/v) was then applied. The analyte was eluted with 1000 μl acetic acid (0.5%) in methanol. The drawing of solvents through the extraction plate was carried out, by applying a vacuum to the manifold. Following elution, samples were evaporated to dryness at 40 °C under oxygen free nitrogen in a Micro-DS96 drying block and re-suspended in 0.5% acetic acid in water (130 μl). Prior to analysis, samples were either centrifuged in 96-well collection plates (2 ml) at 1700 *g* for 10 min or transferred to autosampler vials and centrifuged at 9500 *g* for 10 min.

### 2.5. Chromatographic and mass spectrometric conditions

An HPLC mobile phase of methanol/0.2% formic acid in water (70:30 v/v) was used. The Luna $C_{18}$(2) 5 μm (4.6 mm I.D.×150 mm) was maintained at room temperature with column switching times of the first 2 min to waste, then 3 min to the mass spectrometer. The flow-rate was 1.0 ml/min with a split of 200 μl to mass spectrometer/800 μl to waste. The injection volume was 100 μl and the injector needle wash 0.5% acetic acid in methanol.

The mass spectrometer was operated in the positive ion mode with the TurboIonspray heater set at 450 °C. The samples were analysed employing the transitions of $m/z$ 482.2–258.2 for rosuvastatin, with a dwell time of 250 ms. The mass transition for the internal standard was $m/z$ 488.2–264.2, with a dwell time of 100 ms. The ionspray voltage was set at +3 kV, the ring voltage at 160 V, and the orifice voltage at 44 V. The nebuliser gas (nitrogen) pressure was set at 14 (arbitrary units) and the TurboIonSpray gas flow-rate at 7.0 l/min (nitrogen). The collision gas (nitrogen) was set at 4 (arbitrary units), and the curtain gas (nitrogen) at 8 (arbitrary units). The

deflector was set at −250 V and the electron multiplier at 2900 V. Collision energy ($RO_2$-Q0) was −47.5 V.

## 3. 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 positive ion TurboIonspray Q1 mass spectrum and product ion mass spectrum of rosuvastatin are shown in Figs. 2 and 3, respectively. $[M+H]^+$ was the predominant ion in the Q1 spectrum, and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition was from $m/z$ 482 to $m/z$ 258, which relates to the production of the product ion illustrated in Fig. 1. Essentially the same mass transition was used for the deuterated internal standard ($m/z$ 488–264). 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,

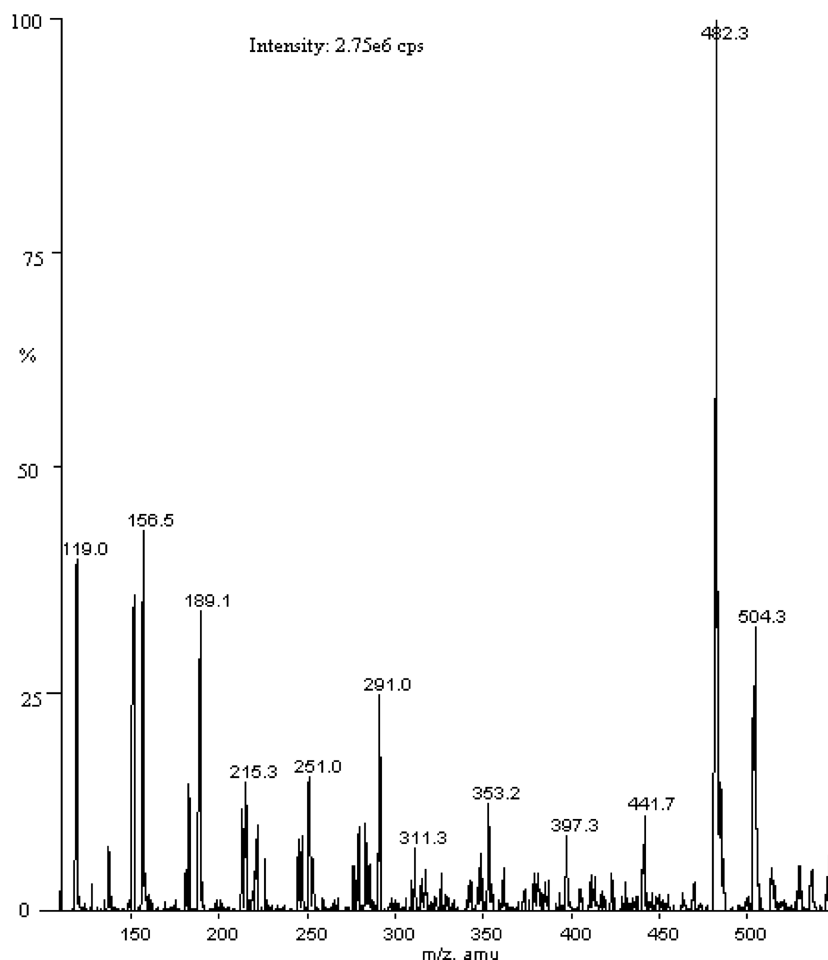


Fig. 2. Positive ion TurboIonspray Q1 mass spectra ($m/z$ 110–550) of rosuvastatin.

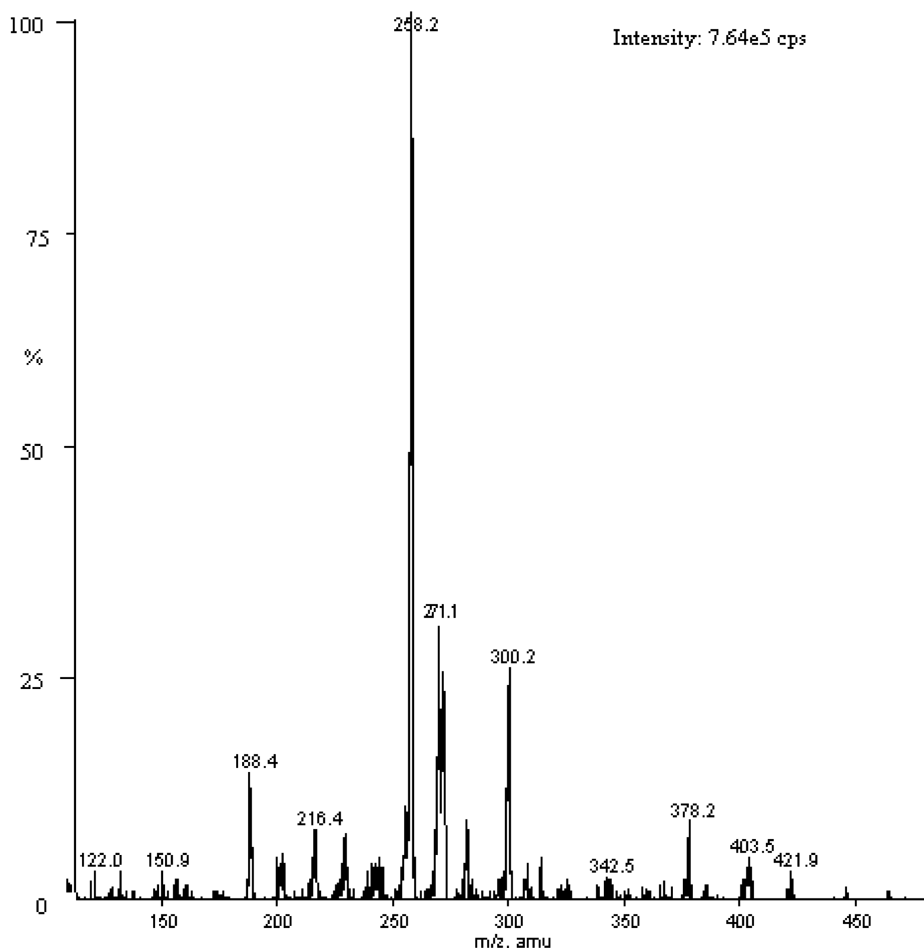


Fig. 3. Positive ion TurboIonspray product ion mass spectra ($m/z$ 110–550) of rosuvastatin.

orifice voltage and collision energy were optimised 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.

### 3.2. Method development

The HPLC conditions were optimised such that the retention time was kept short at 3.5 min in order to assure high throughput. Some retention of the compound on the HPLC column was employed with the eluent from the first 2 min 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 Luna $C_{18}$ 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 methanol/water/formic acid was chosen for its compatibility with mass spectrometric detection. The formic acid 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 formic acid was optimised to maintain this peak shape whilst being consistent with good ionisation and fragmentation in the mass spectrometer. Example chromatograms from a double blank (without rosuvastatin and IS)

and from a dosed volunteer sample at 2.10 ng/ml are shown in Figs. 4 and 5, respectively.

The extraction method used in this assay employed the polymer OASIS. Rosuvastatin is an acid molecule and its retention onto a reversed-phase sorbent would be expected to be enhanced at low pH with the compound unionised. However, due to the requirement to keep the compound at approximately pH 4 (to stabilise the compound and prevent conversion to the 5*S*-lactone) this manipulation of pH was not an option. OASIS is a reversed-phase polymer-based sorbent and is marketed as a phase that functions whether wet or dry. Therefore it was judged that this phase was suitable to extract the acidic compound which would not be fully ionised. The fact that the phase performs well, wet and dry, would be helpful in an assay that was planned to be run in a 96-well plate format.

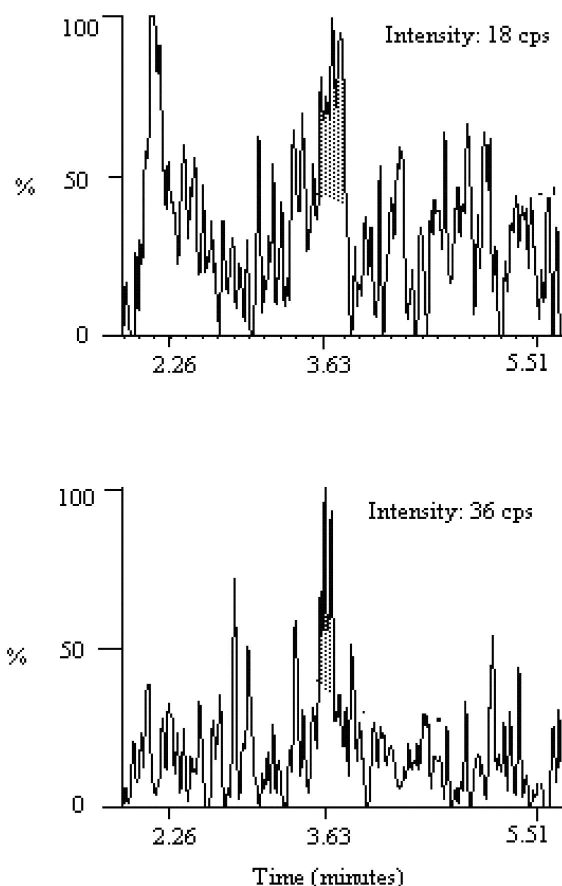


Fig. 4. Ion chromatogram of a double blank extract (rosuvastatin (top), internal standard $D_6$-rosuvastatin (bottom)).

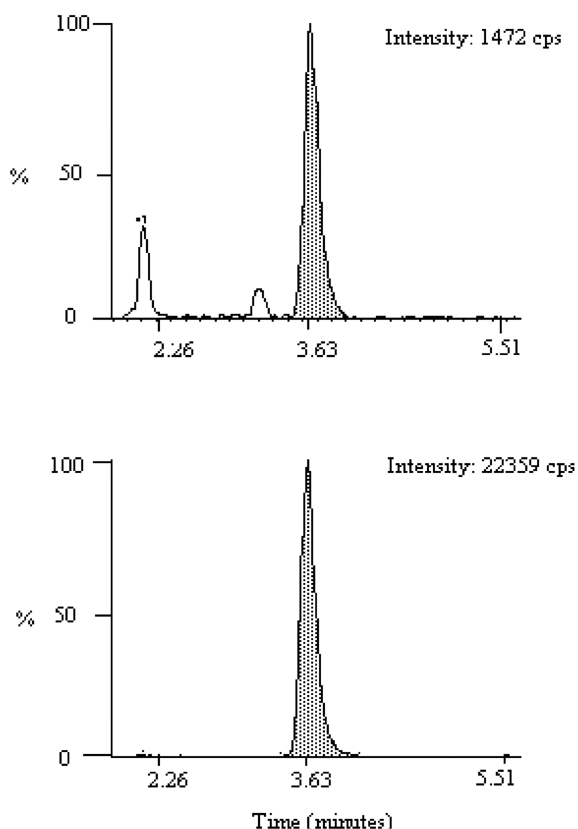


Fig. 5. Ion chromatogram of a study volunteer at 2.10 ng/ml rosuvastatin (top) and internal standard $D_6$-rosuvastatin (bottom) in human plasma.

Due to the known chemical instability and possible inter-conversion of the 5*S*-lactone of rosuvastatin to rosuvastatin at neutral pH, plasma samples were stored and processed after dilution with acetate buffer (0.1 *M*, ph 4.0) in order to limit the conversion of 5*S*-lactone. In order to assess the stability of the 5*S*-lactone during the assay extraction, a single stock solution of the 5*S*-lactone of rosuvastatin was prepared in methanol at approximately 325 μg/ml and further diluted to 250 ng/ml with 50% methanol/50% 1 *M* acetic acid. This was spiked into human control plasma at a concentration of 25 ng/ml under acid or neutral conditions and the samples extracted and analysed for rosuvastatin (Table 1). When the 5*S*-lactone was spiked into blank plasma

Table 1
Interconversion of lactone to rosuvastatin

| 5S-lactone concentration (ng/ml) | Found concentration of rosuvastatin (ng/ml) | | | | | | $n$ | Mean |
|---|---|---|---|---|---|---|---|---|
| 25.0 (acid) | NR | 2.31 | 1.34 | 1.27 | 1.77 | 1.47 | 5 | 1.63 |
| 25.0 (neutral) | 3.44 | 3.56 | 3.68 | 4.50 | 4.52 | 4.64 | 6 | 4.06 |

NR, no result obtained.

under neutral conditions, this returned higher concentrations of rosuvastatin than when prepared under acid conditions as for the normal test samples. The level of conversion observed did not cause any concern as it would cause negligible changes to the rosuvastatin concentrations quantified. Clinical studies have demonstrated that the 5*S*-lactone circulates at low concentrations (relative to rosuvastatin) in human plasma and is stable under the storage and process conditions used for the rosuvastatin assay.

### 3.3. Extraction recovery

The extraction recoveries of rosuvastatin from plasma were determined at two concentrations (0.1 and 25 ng/ml, $n=3$) and for the internal standard at the concentration used in the assay (15 ng/ml), by comparing the areas of extracted samples with blank plasma extracts fortified with drug post extraction. The mean extraction recovery was between 88.2 and 97.6% for rosuvastatin and 98.7% for the internal standard. Similar extraction recoveries were observed for rosuvastatin at the low and high concentrations.

With the high extraction recovery of rosuvastatin and with the deuterated internal standard (to compensate for extraction variability and MS sensitivity), the assay has proved to be robust in high throughput bioanalysis.

### 3.4. Specificity and selectivity

The specificity/selectivity of the method was investigated by screening six 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 low standard. Using these criteria, no endogenous substances were detected which significantly interfered with the quantification of rosuvastatin. Pre-dose samples analysed from a number of clinical studies have confirmed that there are no endogenous plasma components, which lead to significant interference in the assay.

### 3.5. Linearity, precision, accuracy and limit of quantification

The assay was linear over the range 0.1–30 ng/ml. The standard curve was fitted to a $1/x^2$ weighted linear regression (where $x$ was the concentration of the analyte) as this was judged to be the weighting which made the assay most robust. The mean regression coefficient and intercept determined during validation were 0.998 and 0.008 with a slope of 0.128.

Intra-batch inaccuracy and imprecision was assessed by running a single batch of samples containing a calibration curve and six replicates of test samples at each of four concentrations. For inter-batch inaccuracy and imprecision three batches of samples were analysed. Each batch contained a calibration curve and duplicate test samples at each of four concentrations. The inter-batch 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, are shown in Table 2. The imprecision and inaccuracy were within the pre-specified acceptable limits of $<\pm 15$ and $<15\%$, respectively, across the calibration range and were in keeping with the recommendations of Shah et al. [10].

The LLOQ of rosuvastatin was verified as 0.1 ng/ml as this was the lowest concentration assessed at which the inter-batch inaccuracy was $<15\%$ and imprecision $<\pm 15\%$.

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

| Concentration (ng/ml) | $n$ | Intra-batch | | $n$ | Inter-batch | |
|---|---|---|---|---|---|---|
| | | Inaccuracy (%) | Imprecision (%) | | Inaccuracy (%) | Imprecision(%) |
| 0.1 | 6 | 1.8 | 15 | 5[a] | 7.0 | 12 |
| 0.3 | 6 | −6.9 | 7.1 | 6 | 0.4 | 6.0 |
| 15 | 6 | 1.6 | 2.7 | 6 | 0.1 | 3.5 |
| 25 | 6 | −2.1 | 2.9 | 6 | −0.3 | 4.8 |

[a] One sample lost during extraction.

### *3.6. Carryover and sample dilution*

Carryover was assessed, by injecting in increasing concentration, a series of calibration samples interspersed with blank human plasma extracts over the calibration range. Carryover for rosuvastatin at the top calibration point of 30 ng/ml was found to be 0.143%. Carryover for the internal standard at the top calibration point of equivalent to 15 ng/ml of internal standard was found to be 0.299%.

The effect of dilution was investigated by diluting a set of samples spiked at 250 ng/ml, 10-fold (to within the calibration range) with control human plasma/buffer prior to extraction. The diluted samples produced results within ±15% (actual inaccuracy +2.1%) of the target concentration with acceptable precision (<±15% (actual imprecision <±2%)) and therefore it was demonstrated that samples could be diluted at least 10-fold.

### *3.7. Stability*

To examine the batch size that could be analysed, two batches were prepared containing 86 samples and a system suitability sample (87 in total) and analysed consecutively. The run was then examined for changes in retention time and sensitivity throughout. The retention time for rosuvastatin ranged from 3 min 45 s to 3 min 48 s. There was no noticeable change in sensitivity for rosuvastatin or the internal standard. With the run time of 5 min and a batch size of approximately 90 samples, it was evident that the desired high throughput for the assay would be achievable.

The stability of rosuvastatin in human plasma was assessed during routine assay procedure conditions and also for long-term frozen storage. Stability was assessed under a variety of conditions with the maximum period of confirmed stability presented in Table 3. Stored samples were quantified against a freshly prepared standard curve and the accuracy and precision of the results determined with reference to the spiked concentration. Samples from the batch of samples to be used to determine stability were run prior to storage to confirm that the samples had been prepared at the correct strength. In order to confirm stability the inaccuracy of the stored samples (mean) was required to be within ±15% of the spiked

Table 3
Summary of stability of rosuvastatin during long-term frozen sample storage and during routine assay procedure

| Matrix | Stability condition | Maximum period over which stability confirmed |
|---|---|---|
| Human plasma | −70 °C | 6 months |
| Human plasma | −20 °C | 6 months |
| Human plasma/buffer[a] | −70 °C | 6 months |
| Human plasma/buffer[a] | −20 °C | 6 months |
| Human plasma/buffer[a] | Room temperature | 24 h |
| Human plasma/buffer[a] | 4 °C | 24 h |
| Human plasma/buffer[a] | Freeze/thaw | Three cycles |
| Stock solution rosuvastatin | 4 °C | 3 months |

[a] Human plasma samples diluted 1:1 with 0.1 *M*, pH 4.0 sodium acetate buffer.

concentration with imprecision required to be less than 15%. The proposed method of sample handling/storage for rosuvastatin is to dilute the plasma samples 1:1 with 0.1 *M* pH 4 acetate buffer as soon as possible after collection (to minimise any conversion of the 5*S*-lactone) and to store samples at −70 °C prior to analysis. Additional stability experiments were carried out to support situations where it was not possible to buffer samples soon after plasma production or where −70 °C freezers were not available.

Stability samples were made up in bulk by directly spiking aliquots of various rosuvastatin working solutions into suitable aliquots of control plasma and buffered plasma. They were prepared at three concentration levels to provide samples at 0.3, 15 and 25 ng/ml.

Using the criteria stated above, the results of the stability assessments confirmed that rosuvastatin was stable in neat plasma or in buffered plasma for up to 24 h at room temperature and at 4 °C. It was also stable to three freeze/thaw cycles. Long term frozen storage stability was confirmed for up to 6 months at −20 and −70 °C in neat human plasma and in plasma diluted with acetate buffer.

The stability of rosuvastatin in stock solution (methanol) was examined by storing three stock solutions (10 μg/ml) at 4 °C and analysing by LC–MS at appropriate intervals following preparation. The results showed that rosuvastatin was stable in stock solution for a period of up to 3 months, as inaccuracy was within acceptable limits (relative error less than 10%) following this period of storage.

The short-term stability of the compound on the bench and at 4 °C in plasma and the proven long-term stability as neat plasma and buffer-diluted plasma, allow for efficient running of the assay and mean that the data derived are not compromised by instability at any stage.

### 3.8. Method application

The method has been used successfully to analyse samples from a number of clinical trials. The performance of the assay can be illustrated by reference to the data obtained from study IL/0004 [11], which was a randomised, open-label, two-way, crossover study to assess the pharmacodynamic effects of rosuvastatin after administration in the morning or evening. During analysis of this study, 17 analytical runs were performed with mean (SD) calibration parameters for intercept, slope and correlation coefficient being 0.003 (0.003), 0.067 (0.004) and 0.999 (0.001), respectively. A summary of the assay performance data for this study are presented in Table 4. The average $C_{max}$ concentration in this study following a 10-mg dose was approximately 5 ng/ml. The sensitivity of the method allowed the pharmacokinetics of rosuvastatin to be successfully determined in the study.

Table 4
Mean assay performance figures from accepted batches from study IL/0004

| | Target concentration (ng/ml) | | | |
|---|---|---|---|---|
| | 0.30 | 15.0 | 25.0 | 250 |
| *n* | 30 | 32 | 32 | 48 |
| Mean (ng/ml) | 0.32 | 15.6 | 25.9 | 255 |
| SD (ng/ml) | 0.024 | 0.82 | 1.39 | 14.2 |
| Imprecision (%) | 7.5 | 5.3 | 5.4 | 5.6 |
| Inaccuracy (%) | 7.0 | 4.0 | 3.6 | 2.0 |

## 4. Conclusions

A sensitive, specific, accurate and reproducible LC–MS–MS method 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. Rosuvastatin was shown to be stable in routine analysis conditions and in human plasma and buffered plasma for up to 6 months when stored at −20 and −70 °C. The method has been successfully used to analyse many thousands of human plasma samples, from a series of clinical studies of rosuvastatin in humans.

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