ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 609–614

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

www.elsevier.com/locate/jpba

Short communication

Quantification of the *N*-desmethyl metabolite of rosuvastatin in human plasma by automated SPE followed by HPLC with tandem MS detection

Caroline K. Hull [a,*], Paul D. Martin [b], Michael J. Warwick [b], Elizabeth Thomas [b]

[a] *Quintiles Scotland Limited, Research Avenue South, Heriot-Watt University Research Park, Riccarton, Edinburgh EH14 4AP, UK*
[b] *AstraZeneca, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK*

Received 25 June 2003; received in revised form 24 December 2003; accepted 28 December 2003

Available online 12 March 2004

Abstract

A selective, accurate and precise assay was developed for the quantification in human plasma of the *N*-desmethyl metabolite of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor rosuvastatin. The assay—employing automated SPE followed by HPLC with positive ion electrospray tandem MS (HPLC-MS/MS)—was validated. The standard curve range for *N*-desmethyl rosuvastatin in human plasma was 0.5–30 ng/ml with 0.5 ng/ml being the limit of quantification. Plasma samples were mixed 1:1 with sodium acetate buffer (pH 4.0; 0.1 M) soon after separation from red blood cells. *N*-Desmethyl rosuvastatin was stable in plasma:buffer at room temperature for 24 h and at −70 °C for 12 months. The assay was applied successfully to the quantification of *N*-desmethyl rosuvastatin in human plasma following administration of rosuvastatin.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Rosuvastatin; HMG-CoA reductase inhibitor; HPLC-MS/MS; SPE; *N*-desmethyl rosuvastatin

1. Introduction

Rosuvastatin (Crestor®; licensed by AstraZeneca from Shionogi & Co. Ltd., Osaka, Japan) (Fig. 1) is a synthetic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor for the treatment of dyslipidaemia. AstraZeneca has completed Phase-III clinical development of the drug [1].

Rosuvastatin is eliminated mainly via the liver but metabolism is a minor route of clearance: in a human excretion balance trial [2], 90% of a 20 mg oral dose of [^{14}C]rosuvastatin was recovered in faeces primarily as unchanged drug. Analysis of excreta samples from this trial led to the isolation and identification of the *N*-desmethyl metabolite of rosuvastatin (Fig. 1), which is approximately 50% as active against the HMG-CoA reductase enzyme as rosuvastatin.

The aim of this work was to develop and validate an assay with appropriate sensitivity, selectivity, accuracy and precision for the quantification of the *N*-desmethyl metabolite of rosuvastatin in human plasma. Reported here for the first time is a new validated assay, which utilises HPLC with tandem MS (HPLC-MS/MS) and takes into account the guidance of Shah et al. [3].

* Corresponding author. Tel.: +44-131-451-2282; fax: +44-131-451-2585.
E-mail address: caroline.hull@quintiles.com (C.K. Hull).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2003.12.022

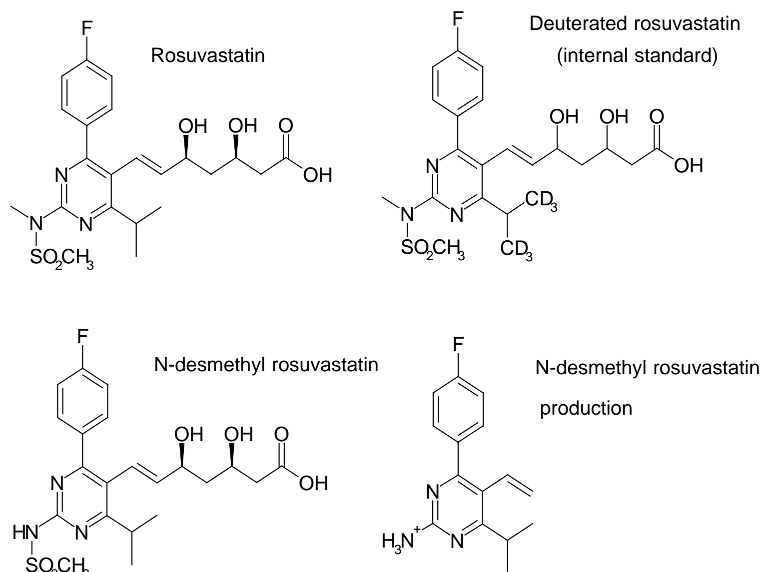

Fig. 1. Structures of rosuvastatin, *N*-desmethyl rosuvastatin and the product ion monitored in the mass spectrometer.

2. Experimental

2.1. Chemicals

AstraZeneca (Cheshire, UK) supplied the *N*-desmethyl metabolite of rosuvastatin (assigned strength 77.3%) and the deuterated $[D_6]$rosuvastatin (assigned strength 99.7% used as the internal standard; Fig. 1). The purity and strength of the test compound and internal standard were taken into account when preparing stock solutions. Acetonitrile, methanol, glacial acetic acid and formic acid were purchased from Fisher Scientific (Leicestershire, UK). Sodium acetate trihydrate was purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK). Quintiles Scotland Limited supplied the drug-free human plasma, and water was distilled in-house. Prior to use, the plasma was diluted 1:1 with sodium acetate buffer (pH 4.0; 0.1 M); this handling procedure was adopted to support analysis of samples from rosuvastatin clinical trials.

2.2. Equipment

Samples were extracted automatically using a Tecan Genesis RSP 100 (Berkshire, UK) automated SPE system with 96-well plates (Waters Ltd., Hertfordshire, UK) containing a hydrophobic-lipophilic balanced copolymer sorbent (Oasis HLB, 30 mg). Extracts were dried down in a Micro DS96 drying block (Porvair, Middlesex, UK).

HPLC was carried out with a Shimadzu LC-10A pump, a Perkin Elmer LC-200 pump with an EVA-1 Rheodyne Model 7000 switching valve (Jones Chromatography, Mid Glamorgan, UK) and a Perkin Elmer AS 200 autosampler (Buckinghamshire, UK).

A Sciex API 365 mass spectrometer (Applied Biosystems, Cheshire, UK), equipped with a TurboIonSpray interface, was used for detection.

Two centrifuges—a Jouan MR 18.12 (Winchester, VA, USA) and an IEC Centra GP8R (Needham Heights, MA, USA)—were used. The vortex mixer was a Fisher Whirlimixer and the tube rotator a Stuart TR-2. Gilson and Anachem (Luton, Bedfordshire, UK) autopipettes were used to dispense plasma and stock solutions. Gilson polypropylene sample tubes (4 ml), Chromacol autosampler vials (250 μl) and a Perkin Elmer Series 200 Peltier cooling tray for HPLC analysis were used throughout.

The data-capture system was an Apple Macintosh System 8.1 equipped with Macquan software (Version 1.6) (PE Applied Biosystems, Warrington, Cheshire, UK).

2.3. Preparation of stock solutions, standards and quality control samples

Stock solutions of *N*-desmethyl rosuvastatin were prepared in 100% methanol (at 1 mg/ml)—with dilutions made up in acetic acid (1 M)–methanol (50:50, v/v)—and stored refrigerated and protected from light for up to 1 month. Stock solutions of [D_6]rosuvastatin were prepared in methanol–acetate buffer (pH 4.0; 0.1 M) (50:50, v/v) and stored refrigerated and protected from light for up to 3 months.

Working standard solutions of *N*-desmethyl rosuvastatin (varying concentrations) and internal standard (150 ng/ml) were prepared on the day of analysis by diluting the stock solutions in acetic acid (1 M)–methanol (50:50, v/v). Each day, before extraction, the standard solutions were used to spike calibration curves in human plasma (500 μl). Acetate buffer (pH 4.0; 0.1 M; 500 μl), internal standard (50 μl) and acetic acid (1 M; 750 μl) were added to the plasma to give a final volume of 1800 μl. Standard curves were prepared at concentrations of 0.5, 1, 2, 5, 10, 15, 25 and 30 ng/ml.

Test samples for the assessment of accuracy and precision were prepared at four concentrations: 0.5, 1.5, 12.5 and 25 ng/ml. A dilution QC sample was also prepared (250 ng/ml); this was used where a run included sample dilution. Samples were prepared on the day of analysis, or in bulk and stored frozen at −70/80 °C until required.

2.4. Sample extraction

Calibration standards and QC samples were prepared for extraction in polypropylene tubes (calibration standards were prepared in duplicate). Prior to extraction, all samples were vortexed briefly (approximately 2 s) and centrifuged at 2000 rpm for 7 min. Samples (1600–1700 μl) were then transferred to the SPE well plates, which had been conditioned with 1000 μl of methanol followed by 1000 μl of acetic acid (0.5%) in water. The extraction phase was washed with 1000 μl of methanol–acetic acid (0.5%) in water (30:70, v/v). The analyte was eluted with 1000 μl of acetic acid (0.5%) in methanol; elution of solvents was achieved by applying a vacuum to the manifold. Following extraction, samples were evaporated to dryness at 25 °C under oxygen-free nitrogen. *N*-desmethyl rosuvastatin was then redissolved in acetic acid (0.5%) in water. Prior to analysis, samples were transferred to autosampler vials and centrifuged at 10,000 rpm (2000 × *g*) for 10 min.

2.5. Chromatographic and mass spectrometric conditions

The following HPLC settings and conditions were employed: the HPLC mobile phase was methanol–formic acid (0.2%) in water (65:35, v/v) at a flow rate of 1 ml/min. The HPLC column was a Luna C_{18} (2) 5 μm (4.6 mm i.d. × 150 mm). Column temperature was ambient. Column switching times were 4.6–6.1 min to MS. The flow rate split was MS:waste 1:4. The injection volume was 100 μl. A needle wash of acetic acid (0.5%) in methanol was used.

The following MS conditions were employed: the MS was operated in positive ion mode. The TurboIonSpray heater setting was 450 °C. The mass transition used was *m/z* 468.2–258.2. The dwell time was 300 ms. Ion spray voltage was +4.5–5 kV. The ring voltage was 230–265 V. Orifice voltage was 36–51 V. Nebuliser gas (nitrogen) pressure 10–13 bar. The TurboIonSpray gas (nitrogen) flow rate was 7 l/min. The collision gas (nitrogen) setting was 4. The curtain gas (nitrogen) setting was 12. The deflector setting was −250 V. The electron multiplier setting was 2800 V. The collision energy setting (RO_2-Q_0) was −46.0 V. The internal standard mass transition was *m/z* 488.1–264.3.

3. Results and discussion

3.1. Mass spectrometry

Positive ion TurboIonSpray Q1 mass spectra revealed that $[M + H]^+$ was the predominant ion in the Q1 spectrum. Therefore, this was used as the precursor ion for obtaining product ion spectra. The structure of the product ion monitored is illustrated in Fig. 1.

3.2. Extraction recoveries and selectivity

The extraction recoveries from human plasma (diluted 1:1 with sodium acetate buffer [pH 4.0; 0.1 M]) were determined at two concentrations (0.5 and

25 ng/ml, $n = 6$) by comparing the peak areas of extracted samples with blank plasma extracts fortified with drug post extraction. The extraction recovery of the internal standard was evaluated at the concentration used in the assay (7.5 ng/ml, $n = 6$). The mean extraction recovery was 77.0% for *N*-desmethyl rosuvastatin and 66.3% for the internal standard. Similar extraction recoveries were observed for *N*-desmethyl rosuvastatin at the high and low concentrations.

The selectivity of the method was investigated by screening six separate human plasma samples and looking for endogenous peaks that accounted for more than 20% of the peak areas of *N*-desmethyl rosuvastatin or the internal standard in the lowest calibration standard. Using these criteria, no endogenous substances that interfered significantly with the quantification of *N*-desmethyl rosuvastatin or the internal standard were detected. Typical chromatograms for double-blank (no compound or internal standard) extracts and for extracts from dosed volunteers are shown in Fig. 2.

3.3. Linearity, accuracy, precision and limit of quantification

The *N*-desmethyl rosuvastatin assay was linear over the range 0.5–30 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 scheme that made the assay most robust. The most suitable fitting was judged by assessment of calibration accuracy across the analytical range.

Table 1 presents a summary of the calibration data obtained for *N*-desmethyl rosuvastatin in a trial to assess the effect of fluconazole on rosuvastatin kinetics [4].

For assessment of the inaccuracy and imprecision of the method, sets consisting of calibration standards and four concentrations of test samples were run on 4 different days (one intra- and three inter-batch). In each set the standards were run in duplicate. The test samples were run in replicates of six for both the

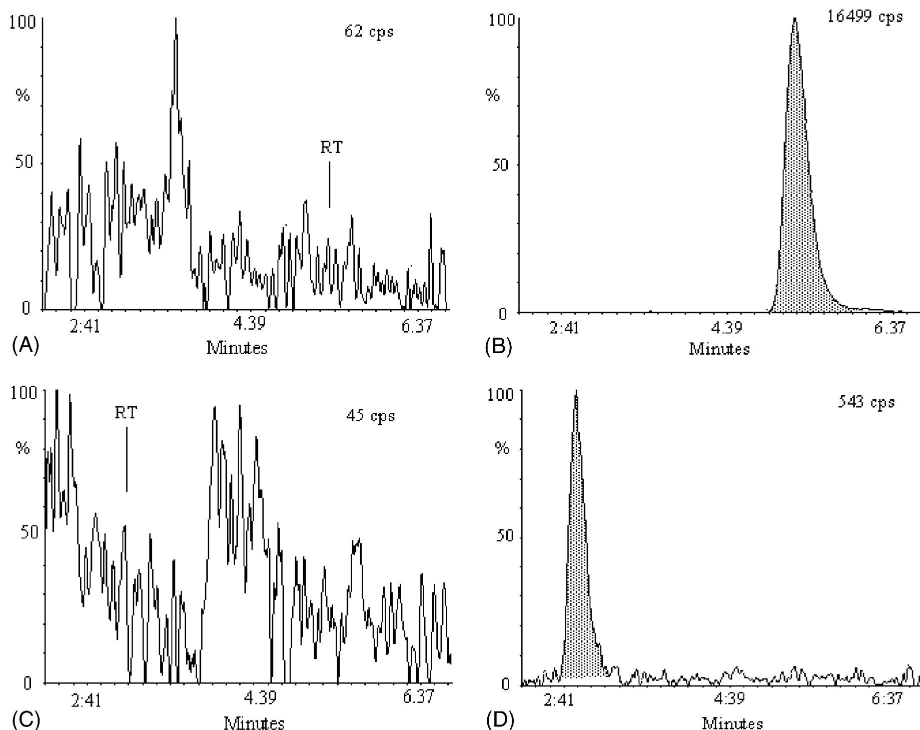

Fig. 2. Ion chromatogram of double-blank human plasma, (A) internal standard channel, (B) *N*-desmethyl rosuvastatin channel (RT represents expected retention time) and plasma extract from the dosed volunteer 009 at 6 h post-dose, (C) internal standard channel and (D) *N*-desmethyl rosuvastatin at 1.48 ng/ml. *Y*-scale expressed as % maximum response; maximum intensity presented as cps value.

Table 1
Summary of mean (standard deviation) calibration characteristics of the *N*-desmethyl rosuvastatin assay method during its application in a clinical trial [4]

Parameter	Value
n	14
Intercept	0.001 (0.004)
Slope	0.053 (0.016)
Regression coefficient	0.997 (0.002)

intra- and inter-batches. The intra- and inter-batch accuracy and coefficients of variation of the method (as measured by the performance of the test samples at all four concentrations) are shown in Table 2. Intra- and inter-batch inaccuracy and imprecision were <20% for *N*-desmethyl rosuvastatin at the limit of quantification, low, mid and high concentrations. The inaccuracy and imprecision of the assay were slightly higher than ideal though still acceptable to produce reliable results. Reasons for this inaccuracy and imprecision could relate to the choice of internal standard and with it the inherent chromatographic separation of the metabolite and IS and enhanced potential for ion suppression effects. The IS was suitable in that it extracted in a similar manner to the test compound and that its ionisation was similar in the MS. Within the selectivity experiment there was no evidence of different response to *N*-desmethyl rosuvastatin or IS between plasma sources so there was no direct evidence of ion suppression effects.

The lower LOQ was verified as 0.5 ng/ml, as this was the lowest assessed concentration at which the inter-batch inaccuracy and imprecision were <20%.

3.4. Carryover and sample dilution

Carryover was assessed by injecting in increasing concentrations a series of calibration samples interspersed with blank human plasma extracts over the calibration range. Carryover was found to be acceptable as it was <20% of the *N*-desmethyl rosuvastatin lower LOQ and internal standard peak area.

The effect of dilution was investigated by diluting high-concentration samples 10-fold (to within the calibration range) with control human plasma prior to extraction. The diluted samples produced results within 10% of the target concentration (actual inaccuracy −6.40%) and with acceptable precision (imprecision <10%), demonstrating that samples could be diluted at least 10-fold.

3.5. Stability

Stability assessments were made to support routine assay procedures and long-term frozen storage of *N*-desmethyl rosuvastatin.

In order to assess stability, plasma:buffer stability samples were spiked with *N*-desmethyl rosuvastatin at specific concentrations. The concentrations chosen assessed the top and bottom end of the calibration range; the number of replicates varied between four and six between experiments. After the storage period, samples were analysed against the respective standard curve. In order to conclude stability, the mean concentration measured in the stability samples was required to be within 20% of the spiked concentration for the low-concentration samples (close to the lower LOQ) and within 15% of the spiked concentration for the higher concentrations assessed. Imprecision for the stability samples was also required to be within the

Table 2
Inaccuracy and imprecision of the method for *N*-desmethyl rosuvastatin as measured by the performance of the QC samples analysed on 4 different days at four concentrations

Concentration (ng/ml)	Intra-batch		Inter-batch	
	Inaccuracy (%)	Imprecision (%)	Inaccuracy (%)	Imprecision (%)
0.5	12.0	17.0	10.0	9.6
1.5	12.0	4.2	−0.7	11.6
12.5	12.0	11.9	−4.0	18.8
25	8.0	7.9	0.0	14.0

Test samples were run in replicates of six for both intra- and inter-batches.

Table 3
Summary of stability of *N*-desmethyl rosuvastatin during routine assay procedures and long-term frozen-sample storage

Matrix	Storage condition	Concentrations assessed (ng/ml)	*n*	Maximum storage period confirmed as stable
Human plasma in sodium acetate buffer (pH 4.0; 0.1 M) (1:1, v/v)	Room temperature	0.5, 25	6	24 h
	−70 °C	2.5, 15, 25	4	12 months
	Freeze/thaw	0.5, 25	4	2 cycles

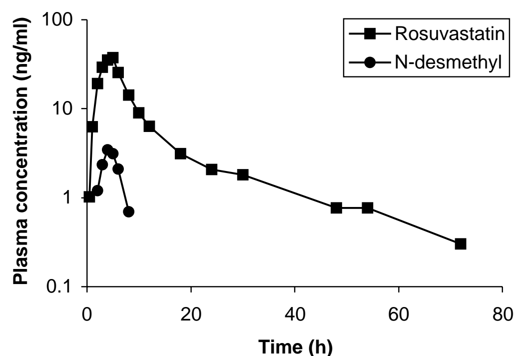

Fig. 3. Geometric mean plasma concentrations of *N*-desmethyl rosuvastatin (and corresponding concentrations of rosuvastatin) following a single oral dose of rosuvastatin 80 mg [4].

ranges set for the validation. *N*-desmethyl rosuvastatin was stable in plasma:buffer at room temperature for 24 h, at −70 °C for 12 months and for two freeze/thaw cycles (Table 3).

3.6. Application of methods

The application of the method is illustrated by the results for *N*-desmethyl rosuvastatin (Fig. 3) from a trial to assess the effect of fluconazole on the kinetics of an 80 mg dose of rosuvastatin [4]. It can be seen from this figure that *N*-desmethyl rosuvastatin circulates at much lower concentrations than parent compound. The sensitivity of the method was sufficient to monitor exposure to the metabolite.

4. Conclusions

A sensitive, specific, accurate and reproducible HPLC-MS/MS method for the quantification of *N*-desmethyl rosuvastatin in human plasma was developed and validated. The desired sensitivity for *N*-desmethyl rosuvastatin was achieved with a lower LOQ of 0.5 ng/ml (from 0.5 ml of plasma). The method has been used successfully to analyse human plasma samples following administration of rosuvastatin in a clinical trial.

Acknowledgements

The authors are grateful to Maria Morrison and Elizabeth Eaton, PhD, for their assistance in the production of the manuscript.

References

[1] A.G. Olsson, F. McTaggart, A. Raza, Cardiovasc. Drug Rev. 20 (2002) 303–328.
[2] P.D. Martin, M.J. Warwick, A.L. Dane, S.J. Hill, P.B. Giles, P.J. Phillips, E. Lenz. Clin. Ther. 25 (2003) 2822–2835.
[3] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309–312.
[4] K.J. Cooper, P.D. Martin, A.L. Dane, M.J. Warwick, D.W. Schneck, M.V. Cantarini, Eur. J. Clin. Pharmacol. 58 (2002) 527–531.