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Journal of Chromatography B, 832 (2006) 191–196
JOURNAL OF CHROMATOGRAPHY B
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Application of microbore HPLC in combination with tandem MS for the quantification of rosuvastatin in human plasma

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Received 31 May 2005; accepted 26 December 2005
Available online 31 January 2006

Abstract

The potential of microbore high-performance liquid chromatography (HPLC) in combination with tandem mass spectrometry (MS/MS) for the sensitive detection of rosuvastatin (Crestor™) in human plasma was investigated. Three microbore HPLC columns with internal diameters (i.d.) of 0.5, 1.0 and 2.0 mm were evaluated for column efficiency and mass sensitivity, and compared to a conventional 4.6 mm i.d. column. The 2.0 and 1.0 mm i.d. columns performed very well while the 0.5 mm i.d. column was slightly less efficient, this is probably due to a lower packing density. Good results with respect to gains in mass sensitivity compared to the conventional analytical column were achieved with the 2.0 and 1.0 mm columns. Thus, the 2.0 mm i.d. column had an improved signal-to-noise (S/N) ratio of 16 whilst the 1.0 mm i.d. column had an improved S/N ratio of greater than 70. Experiments with the 1.0 mm i.d. HPLC column were performed to determine the robustness of the microbore method for human plasma extracts after sample preparation using solid-phase extraction (SPE). A number of problems were encountered with extracts including high backgrounds, the blocking of the column and a rapid deterioration in column performance. The blocking of the column by particulates was solved by off-line filtration of the sample extracts. Peak tailing of the analytes and high background, both of which were due to endogenous interferences in the extracts, were eliminated using gradient elution. Using these approaches over 500 injections of plasma extracts were achieved without significant deterioration in assay performance. Quantities of rosuvastatin of 0.3 pg on-column could be detected and cross-validation experiments demonstrated that the conventional and the microbore HPLC-MS/MS methods provided similar information on the concentration of rosuvastatin but with greatly reduced sample consumption using the microbore method.

Keywords: Microbore HPLC; Rosuvastatin; Human plasma; Bioanalysis

1. Introduction

The determination of drugs in biological samples such as urine, plasma and tissues is an essential part of drug discovery and drug development providing the pharmacokinetic information that defines safety margins and treatment regimens.

Currently, high-performance liquid chromatography (HPLC) in combination with tandem mass spectrometry (MS/MS) is the analytical technique of choice for the quantification of drugs in biological samples [1]. However, since samples from rodents, particularly mice, are often only available in limited amounts, it is usually the case that assays with high sensitivity are required (pg on column). In order to maintain the concentration detection limit of analytical assays, but reduce the required sample volumes, the most obvious strategy is to use miniaturized separation systems ('microbore') and thereby enhance the mass sensitivity of the methodology. The development of such ultra-sensitive assays could greatly benefit the drug development process by enabling complete pharmacokinetic profiles to be obtained from single animals, improving both the quality of the resulting data and reducing the numbers of animals required for such studies.

HPLC columns with internal diameters (i.d.) of 0.5–3 mm are classified as microbore columns. Such columns should, in theory, provide a gain in mass sensitivity over a conventional 4.6 mm HPLC column that is proportional to the square of the reduction factor of the internal diameter of the column employed. Experiments performed with columns of different dimensions have demonstrated an increase in mass sensitivity when using 1.0 mm i.d. columns compared to 4.6 mm i.d. columns of 17-fold [2] and 16–18-fold [3]. The gains in mass

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doi:10.1016/j.jchromb.2005.12.040

sensitivity were slightly lower than the 21-fold predicted, possibly as a result of extra column effects such as band broadening.

However, despite the advantage of the higher mass sensitivity of microbore columns, and therefore the possibility of reduced sample volumes, their applications in drug bioanalysis, as evidenced by the published literature, remain limited. Probably the main reason for this is that in high throughput environments such as bioanalytical laboratories reliability is at a premium and conventional 4.6 mm i.d. columns are robust, easy to handle and widely available. Recently, several groups have reported HPLC-MS/MS assays using 2 or 3 mm i.d. columns, after either liquid–liquid extraction or solid-phase extraction (SPE), to determine drug compounds in plasma samples at low concentrations [4–15]. With all of these published methods, the lower limit of quantification of the drug in plasma was $<1\ ng\ ml^{-1}$ and the on-column mass sensitivity was ≤ 5 pg. To achieve these low concentration and mass sensitivities, plasma samples were generally concentrated two to five times off-line and injections of 5–25 μl of the extracts were applied. From this it can be concluded that these analytical methods needed initial plasma sample volumes of at least 50 μl. In order to quantify drugs in smaller sample aliquots (e.g. 1–10 μl) analytical columns with smaller dimensions are required. There are few reports on the separation of drugs in plasma extracts using columns of 1 mm i.d. [16–18]. None of them describe better results regarding sample volumes, detection limits or on-column sensitivities than the reports on 2 and 3 mm i.d. columns.

Here, the use of a microbore HPLC method in combination with MS/MS detection for the determination of the rosuvastatin was compared with the existing conventional HPLC-MS/MS method [19].

2. Experimental

2.1. Chemicals

Rosuvastatin and the internal standard D_6-rosuvastatin and control human plasma (containing $10\ IU\ ml^{-1}$ heparin) were provided by AstraZeneca (Alderley Park, Macclesfield, UK). Methanol (HPLC grade), glacial acetic acid and formic acid were purchased from Fisher Scientific (Loughborough, UK) and sodium acetate trihydrate was obtained from Sigma–Aldrich (Dorset, UK). Water was deionised with a water purification system (Elgastat Maxima, Elga, High Wycombe, UK).

2.2. HPLC-MS/MS system

The HPLC system was a HTC PAL auto sampler (CTC Analytics, Switzerland), PE series 200 vacuum degasser, series 200 micro pumps and series 200 solvent mixer (Perkin Elmer Inc., Norwalk, CT, USA). The conventional analytical column was a Luna C_{18} (2) 5 μm of 15 cm × 4.6 mm i.d. (Phenomenex, Torrance, CA, USA). The microbore columns of 5 cm × 2.0, 1.0 and 0.5 mm i.d. were packed on custom order with 3 μm Luna C_{18} (2) packing material. The isocratic mobile phase was a mixture of methanol–water (7:3, v/v) with 0.2% (v/v) formic acid. Gradient conditions, given in Table 1, were based on phase A of water and phase B a mixture of methanol–water (95:5, v/v). Both phases contained 0.2% (v/v) formic acid. A 4-position injector (Valco International, Schenkon, Switzerland) was used to inject volumes of 0.5 or 1.0 μl, and a 6-position valve with external loops of 20 or 100 μl (Valco International) was used for the injections of 10–100 μl. All samples were injected opposite the flow of the mobile phase. Needle and injector valve washes were carried out using methanol–water (1:1, v/v) with 0.2% (v/v) formic acid and methanol–water (9:1, v/v) with 0.5% (v/v) acetic acid.

Table 1
Mass spectrometry operating conditions for the Sciex API-4000

Source conditions		Compound conditions	
Collision gas	4	Declustering potential (V)	110
Curtain gas	25[a]	Entrance potential (V)	12
Ion source gas 1	25	Collision energy (eV)	42
Ion source gas 2	30	Collision exit potential (V)	8
Ion spray voltage (V)	5000	Dwell time (ms)	100
Temperature (°C)	500		

[a] The curtain gas was 15 for 0.5 and 1.0 mm i.d. columns.

The HPLC columns were connected using approximately 30 cm PEEK tubing of 130 μm i.d. (2.0 and 4.6 mm i.d. columns) or 50 μm i.d. (0.5 and 1.0 mm i.d. columns) to a Sciex API 4000 mass spectrometer (Applied Biosystems, Warrington, UK). The instrument was equipped with a TurboIonspray™ interface operating in the positive ion mode. Source and compound conditions for the MS instrument are shown in Table 2. Rosuvastatin and D_6-rosuvastatin were monitored at the transition of *m/z* 482.2–258.2 and *m/z* 488.2–264.2, respectively. Analyst 1.2 software (Applied Biosystems) was used to control the LC and MS instruments and for data acquisition.

2.3. Sample preparation

Preparation of the standards, spiking of the human plasma and the extraction and concentration procedures were based on a previously published method [19]. A rosuvastatin stock solution was prepared by dissolving 10 mg of the compound in methanol in a 10 ml volumetric flask, followed by dilution with methanol to a concentration of $20\ \mu g\ ml^{-1}$. This stock solution was used to prepare the working and quality control solutions in a mixture of methanol–1 M acetic acid (1:1, v/v) at 20 times higher concentrations than the calibration standards and quality control samples in human plasma. By spiking these solutions into pooled blank human plasma (1:20, v/v) calibration standards at 0.1, 0.2,

Table 2
Gradient conditions

Time (min)	Phase A	Phase B	Slope
0.0	58	42	
0.1	58	42	
1.5	5	95	Linear
3.5	5	95	
3.7	58	42	Linear
5.5	58	42	

Phase A: water + 0.2% (v/v) formic acid. Phase B: methanol: water (95:5) + 0.2% (v/v) formic acid.

0.5, 1, 5, 10, 15 and 30 ng ml^{-1} were prepared. The quality control samples were prepared at 0.3, 15, 25 and 200 ng ml^{-1}. Prior to extraction, the latter was diluted 10 times with blank human plasma to bring the sample concentration within the calibration curve. All human plasma samples were stored at −20 °C.

The internal standard D_6-rosuvastatin was dissolved at 0.1 mg ml^{-1} in a mixture of methanol–0.1 M acetate buffer pH 4 (1:1, v/v), followed by dilution to 300 ng ml^{-1} using a mixture of methanol–1 M acetic acid (1:1, v/v). The latter was used as internal standard spiking solution.

On the day of analysis, the blank plasma, calibration and quality control samples were defrosted at room temperature followed by centrifugation at 3600 rpm for 10 min. The human plasma samples (500 μl) were transferred to disposable glass culture tubes and 25 μl internal standard spiking solution and 475 μl 0.1 M sodium acetate buffer pH 4 were added. Extractions of the plasma mixtures were performed by using 96-well plates containing Oasis HLB (30 mg) SPE phase (Waters, Milford, MA, USA) on a Multiprobe II HT/Ex robot system (Packard Bioscience Company, Downers Grove, IL, USA). Preconditioning of the SPE phase was carried out with 1 ml methanol wash, followed by 1 ml of 0.5% (v/v) acetic acid in water. After this, 900 μl of the samples were loaded and extracted under vacuum. Washing of the cartridges was performed with 1 ml of a mixture of methanol–0.5% (v/v) acetic acid in water (3:7, v/v). The analytes were eluted by 2 × 500 μl of 0.5% (v/v) acetic acid in methanol. After extraction, the samples were filtered using 96-well plates (Captiva 0.45 μm polypropylene filters, Ansys Technologies, Lake Forest, CA, USA) on a Tomtec Quadra 96 model 320 liquid handling system (Tomtec, Hamden, CT, USA). The filtrates were evaporated to dryness under a stream of nitrogen at 40 °C and then re-dissolved in 150 μl of 0.5% (v/v) acetic acid in water. Prior to analysis, the extracts were centrifuged at 3000 rpm for 10 min.

3. Results and discussion

3.1. Conversion of the HPLC and MS system

This study examined the analytical benefits and consequences of reducing column internal diameter from 4.6 to 2.0, 1.0 and 0.5 mm on the analysis of rosuvastatin. On changing from conventional to microbore columns, it rapidly became clear that it was necessary to have extensive modification to the system components, including the syringe, the loop of the injector and the tubing between the column and MS interface, to minimise band broadening. Therefore, a 10 μl syringe and 20 μl external loop were used to inject sample volumes of 10 μl and the injections of smaller volumes (0.5 and 1.0 μl) were carried out using an internal loop injector. In the original HPLC analysis system for this compound, using 4.6 mm i.d. columns, tubing of 130 μm i.d. was used to connect the injector and the column and between the end of the column and the MS interface. With the 2.0 mm i.d. column, the same diameter tubing could still be used, but with the smaller columns (0.5 and 1.0 mm i.d.) unacceptable peak broadening of the analytes was found. Better peak shapes were obtained by using 50 μm i.d. micro tubing between the column and the MS interface. The effect of changing the tubing from the injector to the column was minimal and it was therefore kept the same as in the original method.

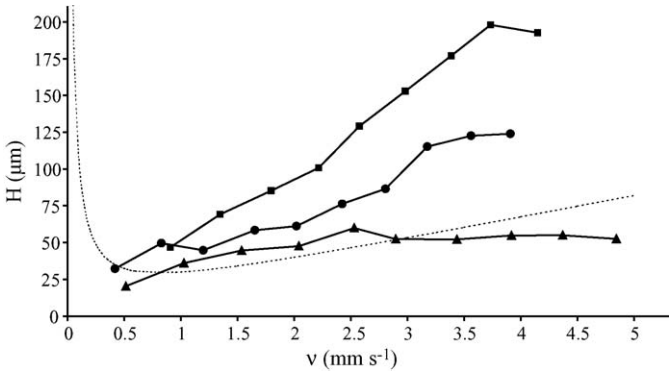

Fig. 1. H–ν curves for rosuvastatin (50 ng ml^{-1}) on 5 cm columns with internal diameters of 2.0, 1.0 and 0.5 mm (from bottom to top). Stationary phase: Luna C18 3 μm; injection volume: 1 μl (2.0 and 1.0 mm i.d. columns) and 0.5 μl (0.5 mm i.d. column); mobile phase: methanol–water (7:3) with 0.2% (v/v) formic acid.

Since optimal conditions for the ionspray MS interface depend on the flow rate of the mobile phase into the source, the sensitivity for rosuvastatin was initially evaluated by flow injection analysis (FIA) at 50 μl min^{-1} using a standard solution at 500 ng ml^{-1} and varying the ionspray conditions. Compared to the original interface settings optimized for a flow rate of 1 ml min^{-1}, it was found that a lower curtain gas improved the sensitivity for the analyte. The other settings were maintained as per the original method.

3.2. Efficiency and sensitivity of microbore columns

The improved efficiency of microbore columns, typically given by theoretical plate numbers, refers to their ability to provide narrow peaks for the analyte. A more efficient column provides higher resolution, improved mass sensitivity and the ability to use short run times. In Fig. 1, the H–ν curves (van Deemter plots) of the 0.5, 1.0 and 2.0 mm i.d. columns obtained with a rosuvastatin standard at 500 ng ml^{-1}, using an isocratic eluent of methanol–water (7:3, v/v) with 0.2% (v/v) formic acid, are shown. Injection volumes were 0.5, 1 and 1 μl, respectively. According to the plate height equation, no significant differences in efficiency were expected with these columns as they were packed with stationary phase from the same batch. The results obtained experimentally demonstrated that the 2.0 mm i.d. column showed the highest efficiency, which was nearly independent of the linear velocity of the mobile phase. The 1.0 mm i.d. column also performed well, and gave results similar to the 2.0 mm i.d. column when using linear velocities <2.5 mm s^{-1} (<60 μl min^{-1}). With higher mobile phase flow rates, the efficiency decreased. The efficiency of the 0.5 mm i.d. column was significantly lower than either the 2.0 or the 1.0 mm i.d. columns and decreased with increasing flow rate. This result was unexpected as theory predicts that with a reduction in column diameter an increase in efficiency will be observed. However, although the columns contained the same packing material, it

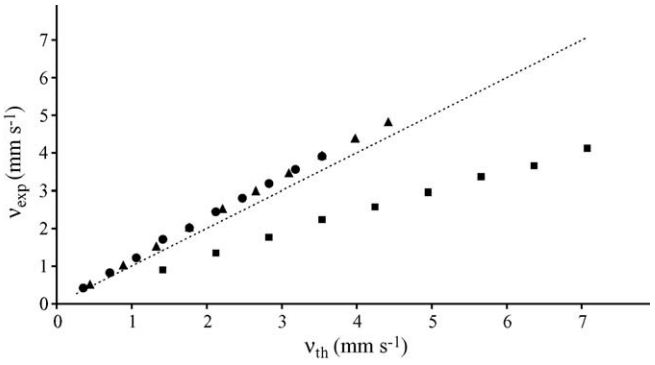

Fig. 2. Relation between the linear velocity of the mobile phase obtained experimentally using (▲) 2.0, (●) 1.0 and (■) 0.5 mm i.d. columns vs. the predicted values calculated with an interparticle porosity of 0.6 (- - -). Experimental conditions as in Fig. 1.

was thought to be possible that the packing density was not as good for the 0.5 mm i.d. columns, providing more opportunity for band broadening, thereby resulting in lower than expected separation efficiencies. To investigate this, the linear velocities at each flow rate of the three columns obtained experimentally were compared with the predicated values calculated using an interparticle porosity of 0.6 (Fig. 2). As can be seen in this figure, the linear velocities obtained for the 0.5 mm i.d. column were significantly lower than both the predicted trend for the 0.5 mm i.d. and the observed linear velocities for the other columns. This indicates that the 0.5 mm i.d. column had a larger void volume, which is consistent with a lower packing density, which may have caused extra peak broadening in the column. A lower capacity factor (k') for rosuvastatin was also obtained with this column, and this is also consistent with a lower packing density. As a result of the relatively poor performance of the 0.5 mm i.d. column, further studies were limited to the 2.0 and 1.0 mm i.d columns.

As indicated in Section 1, the major benefit expected from the use of smaller columns is the expected gain in mass sensitivity, and therefore the possibility to reduce the sample size required. The sensitivity obtained for rosuvastatin by HPLC-MS/MS using the 2.0 and 1.0 mm i.d. columns was compared with a 15 cm × 4.6 mm i.d. column by the analysis of 10 μl of a rosuvastatin solution at 1 ng ml^{-1} under isocratic conditions. The flow rates were 1, 250 and 60 μl min^{-1} for the 4.6, 2.0 and 1.0 mm i.d. columns, respectively. These flow rates used were selected to maximise column performance and were chosen based on the experimentally derived Van Deemter plots (Fig. 1) and although higher than the theoretical values, these generate similar linear velocities while maintaining efficiency and decreased analysis time. The resulting signal-to-noise (S/N) values obtained are given in Table 3 for rosuvastatin together with the noise levels of the background signal of the MS system. As can be seen, for all columns at the appropriate flow rates, the background signals were <100 counts. The S/N ratio for rosuvastatin obtained with the 4.6 mm i.d. column was acceptable (7.5). However, compared to the 4.6 mm i.d. column, a significant improvement in signal intensity was observed for the microbore columns. Thus, the 2.0 mm i.d column gave a 16-fold

Table 3
Signal-to-noise (S/N) of rosuvastatin at 1 ng ml^{-1} and background noise of the MS obtained with columns with different dimensions under isocratic conditions

Column	S/N	Noise (counts)
15 cm × 4.6 mm i.d.	7.5	40
5 cm × 2.0 mm i.d.	120	25
5 cm × 1.0 mm i.d.	550	60

increase in sensitivity and the 1.0 mm i.d. column offered a 70-fold increased in sensitivity compared to the original method. This gain is higher than the theoretical 21-fold result expected for such a column. The reason for this is unclear. A slightly higher gain in sensitivity can be expected since the microbore columns contained a smaller packing material (3 μm) than the 5 μm phase used in the 4.6 mm i.d. column. Indeed, experiments with 5 cm × 4.6 mm i.d. packed with the 3 μm stationary phase showed a slightly higher S/N ratio (8.5) than the 7.5 of the original 4.6 mm i.d column. However, this improvement could not explain the gain in sensitivity of the 1.0 mm i.d. microbore column. A possible explanation is an increase in the mass sensitivity of the mass spectrometer at the reduced flow rates used for the microbore columns.

3.3. Microbore HPLC-MS/MS with human plasma

The 1.0 mm i.d. column was selected for further experiments as it demonstrated the greatest increase in sensitivity. Evaluation of the system was performed with rosuvastatin spiked into human plasma over the range previously used for the validated HPLC-MS/MS method [19]. Prior to injection, the samples were extracted and concentrated by off-line SPE as described in Section 2.

In contrast to the performance of the assay on 4.6 mm i.d. columns, we found that with multiple injections of plasma extracts on to the 1.0 mm i.d. column, a noticeable increase of the backpressure occurred, eventually leading to column failure. To improve the lifetime of the column, it was necessary to filter the human plasma extracts (0.45 μm filters) to remove particulates and with this the problem of the column backpressure was solved. However, again in marked contrast to the conventional HPLC-MS/MS method, it was only possible to separate approximately 50 plasma extracts before the analysis failed because of high background signals and deteriorating peak shape for both drug and its internal standard. After about 500 injections of human, plasma peak splitting occurred and the column needed to be replaced. This problem was solved by changing from an isocratic to a gradient system to wash contaminants off the column at the end of each sample analysis. Using a linear gradient up to 90% (v/v) methanol over 2 min, co-extracted endogenous compounds with longer retention times than the drug and its internal standard were observed as seen in Fig. 3. Fig. 3 shows the separation of a human plasma sample spiked with rosuvastatin at about 0.1 ng ml^{-1}. The plasma-related compounds, which were only detected at the mass transition of rosuvastatin, and not the deuterated internal standard, were completely separated from the drug. It is likely that these compounds caused

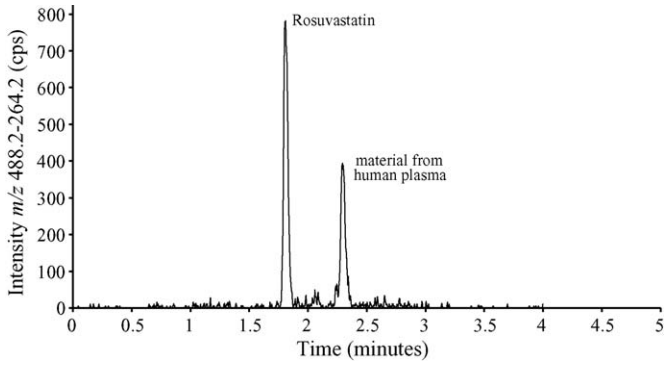

Fig. 3. Separation of rosuvastatin from human plasma-related compounds obtained using gradient elution on a 1.0 mm i.d. column. The figure shows the elution of the human plasma-related compounds at high percentages of organic modifier (cleaning step of the column); these probably caused peak tailing and high background signals under isocratic conditions. Column: 5 cm × 1.0 mm i.d. Luna C18 3 μm; injection volume: 1 μl; flow rate: 60 μl min^{-1}; gradient elution as given in Table 2.

the high background signal and peak broadening seen for the isocratic method. With the gradient system, it was possible to analyze >500 plasma extracts without deterioration in system performance.

A disadvantage of a gradient elution HPLC method is that extra time is required to re-equilibrate the column before the next sample can be injected. A possible way to increase the number of samples per time unit if a gradient is found to be essential, as in the case of the 1.0 mm i.d. columns, is column-switching, where parallel columns are coupled to a switching valve [1]. A short study was undertaken to investigate the use of a two-way switching valve with the microbore HPLC method. This investigation showed that the use of a switching valve had no effect on the chromatography (results not shown) and this option will be examined in more detail in future.

3.4. Cross-validation of HPLC and microbore HPLC-MS/MS methods

The application of the microbore HPLC-MS/MS method to quantify rosuvastatin was examined by the analysis of a standard curve, quality control samples and control blanks prepared in human plasma and the measurements were compared with the results obtained by using the conventional HPLC-MS/MS method. With the microbore system, an injection volume of 1 μl was chosen, while the injection volume of the conventional method was 100 μl. The sensitivity of both methods was adequate for the detection of the bottom standard at 0.1 ng ml^{-1} rosuvastatin, as can be seen in Fig. 4.

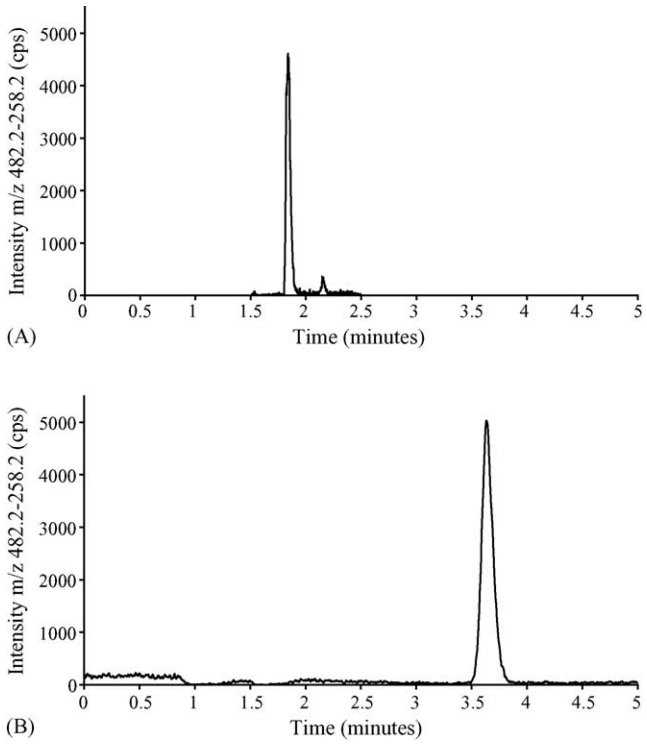

Fig. 4. (A) Typical chromatogram of human plasma at 0.1 ng ml^{-1} rosuvastatin (0.3 pg on-column) obtained by a microbore 5 cm × 1.0 mm i.d. column by gradient elution and compared with (B) the analysis of the same sample extract using the conventional 15 cm × 4.6 mm i.d. column under isocratic conditions (30 pg on-column). Microbore system: 5 cm × 1.0 mm i.d. Luna C18 3 μm column; injection volume: 1 μl; flow rate: 60 μl min^{-1}; gradient elution as given in Table 2. Conventional system: 15 cm × 4.6 mm i.d. Luna C18 5 μm column; injection volume 100 μl; flow rate: 1 ml min^{-1}; isocratic elution with methanol–water (7:3) containing 0.2% (v/v) formic acid.

The analysis of control human plasma extracts demonstrated that the samples did not contain anything that interfered with the determination of rosuvastatin and its internal standard (data not shown). The analysis of blank extracts directly after the separation of the high standards showed that there was no carryover of the analytes and confirmed that the assay can be used to determine rosuvastatin at low concentrations.

The calibration curve contained eight standards over the range of 0.1–30 ng ml^{-1}. Replicates of the standards at 0.1, 0.2 and 30 ng ml^{-1} were extracted and analyzed, while the standards with concentrations in between were measured once. The curve

Table 4
Inter-batch accuracy and precision of the microbore and conventional HPLC-MS/MS method

Concentration (ng ml^{-1})	n	Microbore HPLC-MS/MS method		Conventional HPLC-MS/MS method	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
0.3	6	112	9.0	106	11
15	6	99	2.0	103	1.3
25	6	101	2.1	104	1.0
200[a]	6	85	9.0	86	12

[a] Sample was 10 times diluted with blank human plasma to obtain a concentration within the calibration curve.

was fitted to a $1/x$ weighted linear regression, where x was the concentration of rosuvastatin in the standard solution, with an r-value of 0.9993.

Six replicates of control human plasma samples at 0.3, 15, 25 and 200 ng ml^{-1} rosuvastatin were analyzed to determine the intra-batch accuracy and precision. The results obtained experimentally with the microbore HPLC-MS/MS method and the conventional HPLC-MS/MS method are given in Table 4. The observed inaccuracies and imprecisions of the test samples were within the acceptance limits of ±15%, which confirmed the good repeatability of both methods. With the similar values of the accuracies, it was evident that both methods provided similar information on the concentration of rosuvastatin in the human plasma quality control samples.

4. Conclusions

Microbore HPLC coupled to tandem MS can be used as a highly sensitive method for quantification of rosuvastatin in human plasma samples. The 1.0 mm i.d column gave good efficiency and the maximum gain in mass sensitivity compared to the equivalent 0.5 and 2.0 mm i.d columns. However, for the analysis of human plasma samples on the 1.0 mm i.d. column, it was essential to filter the SPE extracts offline and apply gradient elution up to a high percentage of organic solvent to remove extraneous material from the analytical column. The proposed system was cross-validated with the existing method and enabled the quantification of 0.3 pg on-column of rosuvastatin. This microbore HPLC-MS/MS method should enable the analysis of small sample aliquots of plasma and allow pharmacokinetic profiles to be generated from single rats or mice.

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