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Solid-phase extraction and liquid chromatography/tandem mass spectrometry assay for the determination of pitavastatin in human plasma and urine for application to Phase I clinical pharmacokinetic studies

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ABSTRACT

A sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed and evaluated for the determination of pitavastatin in human plasma and urine. Samples were extracted using solid-phase extraction (SPE). The major benefit of the present method was the high sensitivity, with a lower limit of quantification (LLOQ) of 0.08 ng/mL. Pitavastatin and internal standard (IS, rosuvastatin) were separated on a C_{18} column with a mobile phase consisted of methanol/water (75:25, v/v) with 0.05% formic acid. Drug and IS were detected by LC/MS/MS with positive electrospray ionization (ESI). Accuracy and precision for the assay were determined by calculating the intra- and inter-batch variation of quality control (QC) samples at three concentration levels, with relative standard deviations (R.S.D.s) of less than 15%. The developed method was successfully applied to determine pitavastatin in human plasma and urine, and was proved to be suitable for use in Phase I clinical pharmacokinetic study after oral administration of pitavastatin (1, 2 and 4 mg) in healthy Chinese volunteers.

1. Introduction

As the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in the liver is the rate-limiting and key-regulating enzyme of cholesterol biosynthesis, HMG-CoA reductase inhibitors, or statins, have been widely used to decrease cholesterol biosynthesis in patients with hypercholesterolemia. Pitavastatin (NK-104, previously called itavastatin or nisvastatin) is a novel, fully synthetic statin with the chemical name of (+)-monocalcium bis(3*R*,5*S*,6*E*)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5- dihydroxy-6-heptenoate ($C_{50}H_{46}CaF_2N_2O_8$) [1,2]. Clinical trials showed that pitavastatin was an effective lipid-lowering agent at the low dosage, usually 1–2 mg/day [3–5]. At this dosage level, pitavastatin appears to be well tolerated with a safety profile similar to the other statins [6]. Due to its low doses, the concentration of pitavastatin is extremely low in human plasma. So a high-sensitivity method is urgently needed.

So far, an HPLC-UV method has been reported for the estimation of pitavastatin in biological samples, in which column-switching high-performance liquid chromatography was used with an LLOQ of 0.5 ng/mL achieved [7]. However, the chromatographic run time for one sample was 25 min, which is relatively long. Hui et al. [8] used above method in the pharmacokinetics study of pitavastatin in subjects with child-pugh A and B cirrhosis, and achieved an LLOQ of 1.0 ng/mL. Lv et al. [9] developed an HPLC–ESI-MS/MS method with liquid–liquid extraction (LLE) to determine pivastatin in human plasma, with an LLOQ of 0.2 ng/mL. In the study, lovastatin was used as an IS, which is significantly different from pitavastatin in chemical structures. It was more suitable to choose rosuvastatin as an IS because its chemical structure is similar to pitavastatin (Fig. 1), which could ensure the similarity extraction recovery. In this study, an LC–MS/MS method using solid-phase extraction (SPE) technique and rosuvastatin as an IS was developed for the determination of pitavastatin in human plasma and urine samples.

2. Experiment

2.1. Reagents and materials

Pitavastatin calcium (purity: >99% HPLC) and rosuvastatin calcium (purity: >99% HPLC), were supplied by Yabang Pharmaceutical Research Institute Co. Ltd. (Changzhou, China). Pitavastatin lactone was prepared in our laboratory (purity: >98% HPLC). HPLC/Spectrum grade methanol was purchased from Merck

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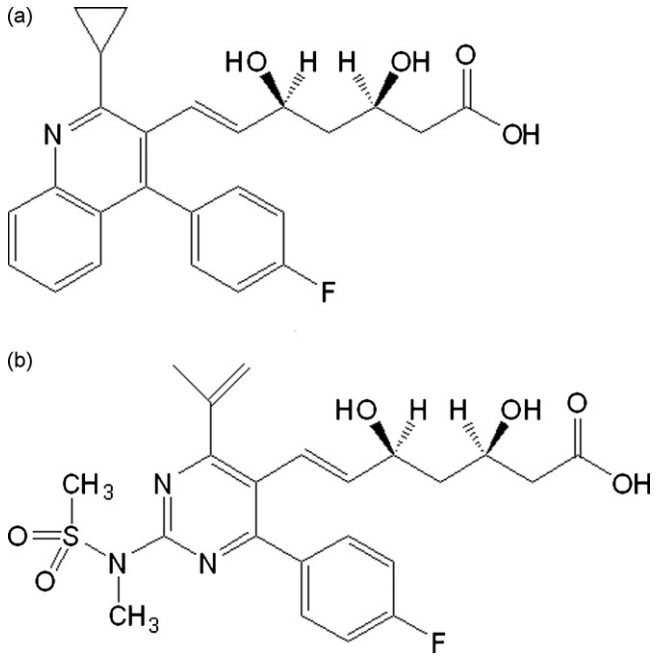

Fig. 1. Chemical structures for pitavastatin (a) and rosuvastatin (b).

Company (Darmstadt, Germany). Other chemicals were of analytical grade and were used as received. Deionized water was purified through PL5242 Purelab Classic UV (PALL Co. Ltd., USA) before use. Blank plasma was supplied by Red Cross Society of China Nanjing Branch. Blank urine was collected in-house from laboratory staff members.

SPE of plasma and urine was carried out using a VISIPREP™ DL system and Supelclean™ LC-18 SPE Tubes (500 mg/3 mL), both from SUPELCO (USA).

2.2. Instrumentation

Thermo-Finnigan TSQ Quantum Ultra tandem mass spectrometer equipped with an electrospray ionization (ESI) source (San Jose, CA, USA), a Finnigan surveyor LC pump and an autosampler were used for the LC–MS/MS analysis. Data acquisition was performed with Xcalibur 1.1 software (Thermo-Finnigan, San Jose, CA, USA). Peak integration and calibration were carried out using LC Quan software (Thermo-Finnigan).

2.3. LC/MS/MS conditions

A Shim-pack VP-ODS column (5 μm, 150 mm × 4.6 mm i.d., Shimadzu, Kyoto, Japan) was used for the chromatographic separations. The mobile phase was a mixture of methanol/water/formic acid (75:25:0.05, v/v/v), which was pumped at a flow-rate of 1.0 mL/min. The run time of one sample was about 6 min. In order to assure the repeatability of retention time, the column temperature was maintained at 25 °C.

Samples were ionized by positive-ion electrospray ionization mode (ESI^+) and were monitored in the selected reaction monitoring (SRM) mode. The MS ionization and acquisition conditions were autotuned using infusion injection at the flow-rate of 20 μL/min, with the resolution of 12.50 ng/mL for both pitavastatin and IS. Nitrogen was used as sheath (49 psi) and auxiliary (5 psi) gases. The capillary temperature was 350 °C, and the spray voltage was 5600 V. Collision induced dissociation (CID) studies were performed and argon was used as collision gas with a collision cell gas pressure of 0.9 mTorr. The collision energy was 10 eV. Based on the full-scan MS and MS/MS spectra of the drug, the most abundant fragment ion was selected and the mass spectrometer was set to monitor the transition m/z 422.0 → 290.1 for pitavastatin and m/z 481.8 → 258.1 for IS, respectively. The scan time for each analyte was set to 0.5 s.

2.4. Drug administration and sample collection

An open-label, randomized study was conducted in Phase I Clinical Research Institute of the Jiangsu Province Hospital (Nanjing, P.R. China). The clinical trial protocol was approved by the Independed Ethics Committee (IEC) of Jiangsu Province Hospital. Thirty healthy Chinese volunteers (15 females and 15 males) were chosen to attend the study. The average age and average body weight (mean ± s) were 22.5 ± 1.7 years of 57.6 ± 6.5 kg, respectively. Subjects were randomized into one of three groups (10 subjects/group) to be given a single dose of 1, 2 and 4 mg pitavastatin per day. The subjects received general care from the physicians and nurses who conducted the clinical trial. The trial was conducted in accordance with the ethical principals in the Declaration of Helsinki and with the requirement in Good Clinical Practice (GCP), including the protocol design and organization, monitoring, audit, record, analysis and report.

Blood samples (3 mL each) were collected at 0 (pre-dose), 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12, 24, 36 and 48 h post-dose. Plasma was separated, decanted, frozen and stored at −20 °C before analysis. Urine samples were collected prior to dosing and over the intervals 0–2, 2–4, 4–6, 6–8, 8–12, 12–24, 24–36 and 36–48 h post-dose. The volume of urine collected in each interval was recorded and 10 mL of it was centrifuged, decanted, frozen and stored at −20 °C before analysis.

2.5. Preparation of pitavastatin standard, IS and QC sample solution

10.00 mg pitavastatin standard was accurately weighed into a 100 mL brown volumetric flask, dissolved and dilute with methanol to 100 mL. The resulting solution was further diluted with methanol to obtain pitavastatin calibration standard solutions with the concentrations as follows: 2.083, 4.167, 12.50, 25.00, 250.0, 1250, 2500, 5000 ng/mL. Stock solutions of IS was prepared in methanol and was further diluted with methanol giving a concentration of 0.975 μg/mL with methanol. A 0.5 M potassium dihydrogenphosphate solution was obtained by dissolving 68 g KH_2PO_4 in 1 L water. The discussed stock solutions were then stored at 4 °C and were brought to room temperature before use.

A quality control (QC) working solution of pitavastatin (100 μg/mL) was prepared separately following the same procedure in the preparation of pitavastatin standards in methanol. The stock solution was further diluted to obtain four levels of QC standard working solutions (2.083, 4.167, 250.0 and 2500 ng/mL in methanol). QC samples, which were used in both the pre-study validation and during the pharmacokinetics study, were prepared by spiking 20 μL of one of the QC standard working solutions in 0.5 mL blank human plasma or urine, giving the pitavastatin concentrations of 0.08 (LLOQ), 0.16 (low), 10.0 (medium), and 100.0 (high) ng/mL of pitavastatin in plasma or urine, respectively. QC samples were stored at −20 °C before being used.

2.6. Sample preparation

Care was taken to keep the sunlight off. SPE was used for sample pretreatment. A cartridge (3 mL) was activated with 3 mL of MeOH followed by 3 mL of H_2O and was then conditioned with 3 mL of 0.5 M potassium dihydrogenphosphate. A plasma or urine

sample of 500 μL was transferred to a 2-mL test tube, into which 20 μL of IS solution (0.975 μg/mL) were spiked. After vortexing for 30 s, the sample mixture was loaded onto the prepared cartridge. The cartridge was washed with 3 mL of 0.5 M potassium dihydrogenphosphate, and then with 3 mL of H_2O. The analyte was eluted with 3 mL of MeOH. The eluted solution was transferred to a 10-mL glass tube and was evaporated to dryness at 50 °C under a stream of nitrogen. Then, the dried extract was dissolved in 100 μL of water/methanol (25:75, v/v) and an aliquot of 20 μL was injected into the chromatographic system.

3. Results and discussion

3.1. MS conditions selection

In order to develop a method with desired sensitivity (0.1 ng/mL), MS/MS detection was used instead of UV detection because the latter does not reach this limit. Another benefit of using MS/MS detection is its inherent selectivity. Pitavastatin was directly introduced into the MS detector using ESI ionization. Operation parameters such as the sheath gas, auxiliary gas, CID and collision energy were adjusted to increase the detection sensitivity of pitavastatin. The optimum MS conditions are listed in Section 2.3.

Pitavastatin and IS were separately scanned under the Q1 MS full-scan mode to determine the parent ion, and under the Q1/Q3 (MS/MS) product ion scan mode to locate the most abundant production. $[M+H]^+$ was the predominant ion in the Q1 spectrum, and was used as the parent ion to obtain the product ion spectra. The most sensitive mass transition was from m/z 422.0 to 290.1 for pitavastatin and from m/z 484.8 to 258.1 for IS, respectively. This is different from what was reported for pitavastatin from m/z 421.90 to 318.12 [9].

Because of the presence of carboxy group in the chemical structures of pitavastatin and rosuvastatin, the negative ion $[M-H]^-$ was also tested. The result showed that the response intensity of pitavastatin was unstable. In addition, it was lower in negative ion mode than that in positive ion mode.

3.2. Chromatographic conditions optimization

It is critical to optimize the chromatographic conditions to achieve symmetrical peak shapes and a short chromatographic analysis time with high sensitivity and selectivity. Several mobile phases were compared and it was found that water–acetonitrile could slightly inhibit the response of pitavastatin in the MS detection. However, when water–methanol was used as a mobile phase, the peak shape of pitavastatin was unsymmetrical. As a result, an isocratic mobile phase consisting of methanol/water/formic acid (75/25/0.05, v/v/v) was chosen because of its compatibility with MS detection and a good peak shape of pitavastatin resulting in. The formic acid was found to be important in lowering the pH to protonate the acidic pitavastatin and thus to give a symmetrical shape. The concentration of formic acid was optimized not only to maintain a symmetrical peak shape in the LC system but also to render good ionization and fragmentation in the MS response. The percentage of methanol was optimized such that the retention time of pitavastatin was kept as short as 6 min.

3.3. Preparation of plasma and urine samples

It was reported that pitavastatin was sensitive to sunlight [7]. Thus, the stability of pitavastatin was evaluated at different pHs (pH 2.5, pH 4.0, pH 5.0, pH 5.8, pH 6.86 and pH 9.12) by sunlight exposure for 2 h. It was found that pitavastatin degradation was much faster at lower pHs that at high pHs. For example, in the buffer of pH 2.5, only 33% pitavastatin remained after sunlight exposure of 2 h. This phenomenon suggested minimizing exposure of plasma and urine samples to sunlight. In addition, because of the presence of formic acid in the mobile phase, the dried extract was dissolved in water/methanol (25:75, v/v) instead of the mobile phase.

The stability of pitavastatin in plasma and urine was also evaluated under indirect sunlight (sunlight in room). It was found that pitavastatin was stable in plasma and urine after the exposure to indirect sunlight for 0.5 h. So, no further restriction was applied in the procedure of sample collection. However, during the procedure of sample preparation and processing, sunlight was free and the infrared light was used only. Furthermore, pitavastatin standards solution were stored in brown volumetric flask and the processed sample solution were stored in brown sample bottles before LC/MS analysis.

Liquid–liquid extraction was described by Kojima et al. [7] and Lv et al. [9], in which used methyl tertbutyl ether (MTBE) [7] and ether [9] was used as the extraction solvent, respectively. In this study, it was attempted to use LLE in the sample preparation. However, the results showed that MTBE and ether performed similarly as the extraction solvents with low extraction recoveries (about 70–80%). Thus, we developed C_{18} SPE cartridges for analyte extraction.

In this study, a pH of the phosphate buffer was used in the SPE because the pH of the buffer is close to pK_a value of the analyte and the IS. Although carboxyl groups are present in the chemical structures of both pitavastatin and IS, the pK_a values were not found in a search of public databases available. Thus, pK_a values were estimated with ACDLabs software, yielding 4.24 (carboxyl) for pitavastatin and 4.25 (carboxyl) for the IS, respectively. Based on these estimates, at pH ≈ 4, most molecules of both chemical have a net neutral charge, which increases their recovery by enhanced the partitioning in reversed-phase SPE. This estimate was experimentally confirmed using 0.5 M potassium dihydrogenphosphate buffers with their pHs adjusted to 3.0, 4.0, 5.0 or 6.0 with phosphoric acid or potassium hydroxide. When a medium concentration (250.0 ng/mL) of QC samples was used, their extraction yields ($n = 5$) were 85.2, 87.8, 75.6 and 68.1% at the pH of 3.0, 4.0, 5.0, and 6.0, respectively. The result suggested that the cartridges could be washed by 0.5 M potassium dihydrogenphosphate buffer (pH 4.0) to maximize the extraction efficiency for pitavastatin. At pH < 4, pitavastatin is protonated, leading to a decrease in its partition in reversed-phase SPE and the recovery. At pH > 4, the carboxylic group in both pitavastatin and the IS undergo ionization, which also results in a decrease in the recovery for the same reason. Thus, 0.5 M potassium dihydrogenphosphate buffer (pH 4.0) was chosen for the sample preparation. This buffer worked reasonably well although it may not be best because there is little orthogonality between extraction and analysis.

At the end of the preparation procedure, a mixture of water/methanol (25:75, v/v) was used to dissolve the residue, in order to minimize the potential effect of solvent on peak shape.

3.4. Method validation

The method was validated for selectivity, accuracy, precision, recovery, calibration curve range, and stability following the guidelines set by the US FDA [10].

3.4.1. Selectivity

The selectivity was investigated by preparing and analyzing six individual human blank plasma and urine samples at the LLOQ. Representative chromatograms of blank human plasma, the LLOQ (0.08 ng/mL) of pitavastatin with IS in plasma and volunteer's plasma sample are shown in Fig. 2. The representative chromatograms of urine samples are shown in Fig. 3. Good selec-

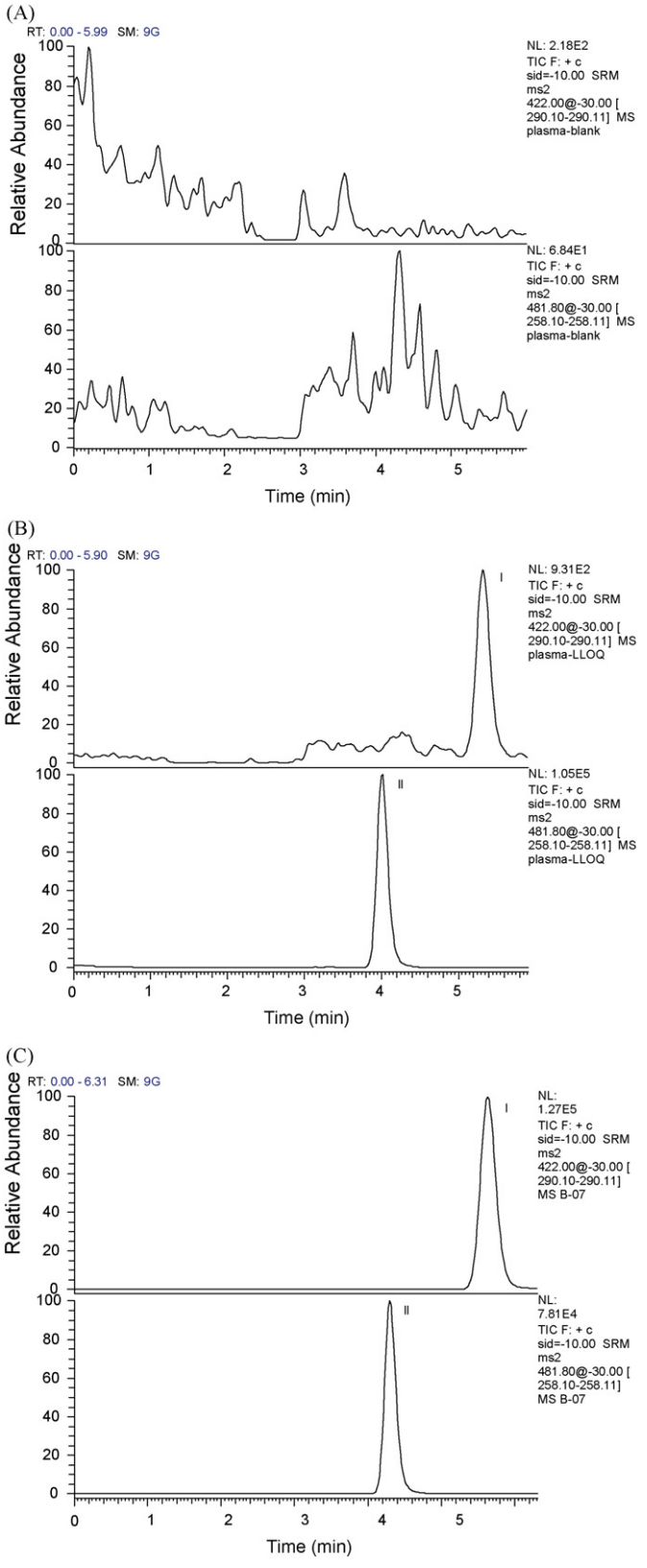

Fig. 2. Chromatograms by selected reaction monitoring (SRM) scan mode: (A) blank plasma (drug and IS free); (B) blank plasma spiked with 0.08 ng/mL (LLOQ) pitavastatin and 0.975 μg/mL IS; (C) plasma sample of a subject 3 h post-oral administration of pitavastatin 2 mg. Peak I: pitavastatin; Peak II: IS.

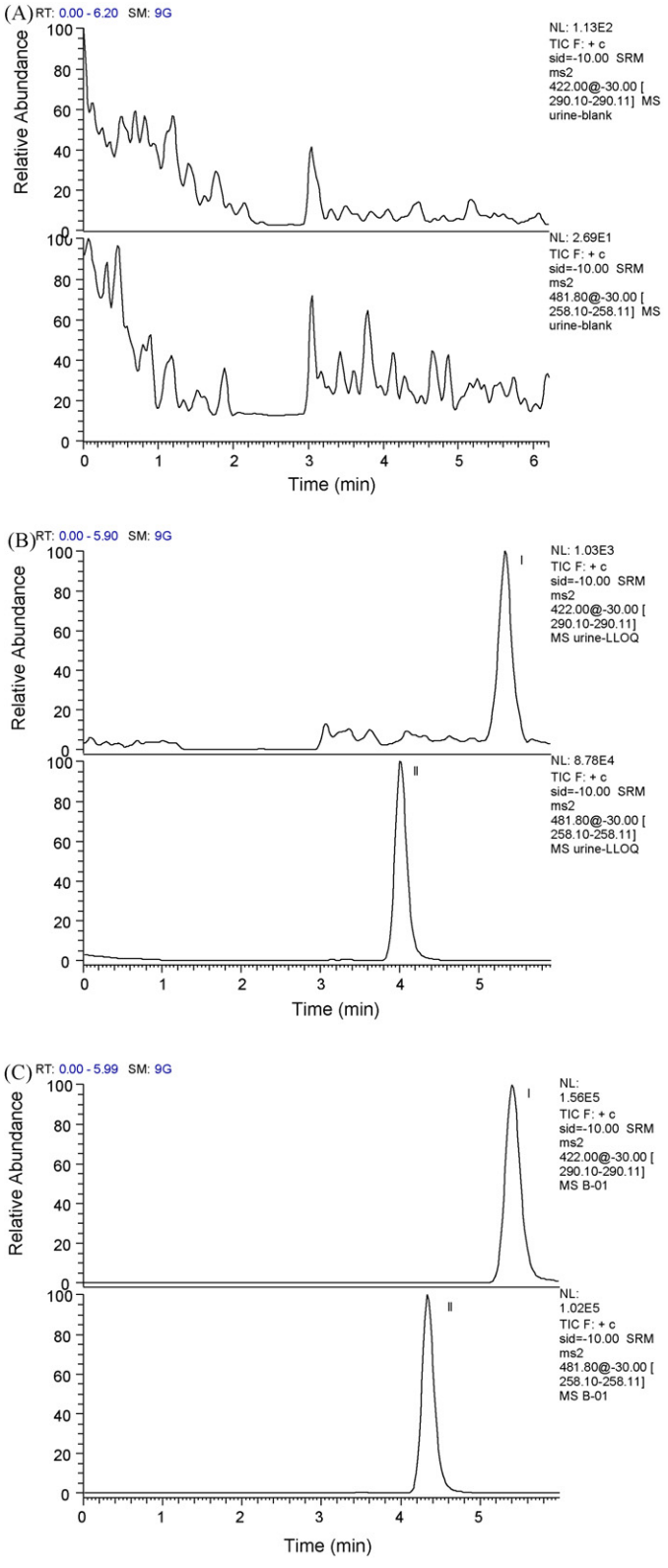

Fig. 3. Chromatograms by selected reaction monitoring (SRM) scan mode: (A) blank urine (drug and IS free); (B) blank urine spiked with 0.08 ng/mL (LLOQ) pitavastatin and 0.975 μg/mL IS; (C) urine sample of a subject 0–2 h post-oral administration of pitavastatin 2 mg. Peak I: pitavastatin; Peak II: IS.

tivities for the analytes are shown by symmetrical resolution of the peaks, with no significant chromatographic interference nearby the retention times of the analytes and IS in the blank human plasma and urine samples. Typical retention times for pitavastatin and IS were 5.3 ± 0.1 and 4.0 ± 0.1 min, respectively. The total run time was about 6 min. Blank human plasma and urine samples collected from six subjects were run up to 10 min. Because of the high selectivity of the SRM mode, no late-eluting interfering peaks were observed up to 10 min. In Figs. 2 and 3, the divert valve was used at 3 min, so the eluant dose not flow into the mass detector during 0–3 min. In addition, for all plasma and urine samples analyzed, there were no peaks with single strength of >20% of the LLOQ for the analyte and there were no signal nearby the retention time of the IS.

3.4.2. Matrix effect

Matuszewski et al. [11] reported that matrix components, which co-elute with analytes, may adversely affect the reproducibility of analyte ionization in the MS. In this study, the matrix effect was evaluated by analyzing at three concentration levels. Firstly, some blank samples (both plasma and urine) from six subjects were processed by the method described in Section 2.6. Secondly, 10 μL of pitavastatin working solution (three concentration levels of 25, 500 and 5000 ng/mL, respectively) and 10 μL of IS solution were transferred to 0.5-mL test tube, into which 80 μL of above-mentioned processed blank solution (from six subjects) was added and mixed well. Reference solutions of were prepared by mixing 10 μL of pitavastatin working solution, 10 μL of IS solution and 80 μL of water/methanol (25:75, v/v). Finally, a 20 μL aliquot of above solutions was injected into the chromatographic system. Compared with those from the reference solutions, the mean peak areas of pitavastatin for the samples from six subjects were 93.4, 84.8 and 86.0% (with the % CV of 4.1, 4.9 and 2.0, respectively) in plasma, 91.8, 93.4 and 90.2% (with the % CV of 5.0, 2.7 and 5.0, respectively) in urine, at the three concentration levels, respectively. This result suggested that endogenous substances slightly suppressed the ionization of the analyte. In addition, the mean peak area of IS was 98.4% (with the CV of 2.7%) of that from the reference solutions.

3.4.3. Calibration curves and LLOQ

Standard solutions for calibration were prepared by spiking 0.5 mL of blank human plasma or urine with 20 μL of standard working solutions (2.083, 4.167, 12.50, 25.00, 250.0, 1250, 2500, 5000 ng/mL, prepared in Section 2.5), resulting in pitavastatin concentrations of 0.08, 0.16, 0.50, 1.0, 10.0, 50.0, 100.0 and 200.0 ng/mL in plasma or urine. Peak area ratios of pitavastatin to IS were used in regression analysis. Calibration curves were generated on three consecutive days. Linearity was assessed by a weighted ($1/\text{concentration}^2$) least squares regression analysis. The result showed that the calibration curve was linear over the concentration range of 0.08–200 ng/mL for the analyte. The 8-level calibration curve gave acceptable results for the analyte and was used. The calibration 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 linear regression equation for the analyte in plasma was $y = 17.706(\pm 0.285)x - 0.116(\pm 0.032)$, and that in urine was $y = 16.041(\pm 0.249)x - 0.033(\pm 0.011)$, where y was the concentration of the analyte and x was the peak area ratio of the analyte to IS. The correlation coefficient was 0.9992 for the plasma and 0.9979 for the urine. The % CV at each level of pitavastatin varied from 2.2 to 11.3 in plasma and from 2.0 to 9.4 in urine, respectively. The mean of the percent deviations (absolute values) from the theoretical value of pitavastatin varied from 0.6 to 6.9 in plasma and from 0.7 to 11.4 in urine, respectively.

The lower limit of quantification (LLOQ) of pitavastatin in human plasma and urine assay was 0.08 ng/mL. LLOQ plasma or urine samples obtained from six-independent subjects were prepared and analyzed. Acceptable mean accuracy of 98.5% for blood (with % CV of 3.6, $n = 6$) and 96.5% for urine (with % CV of 4.3, $n = 6$) were obtained. This LLOQ level was selected because the concentrations of pitavastatin in the samples from the pharmacokinetic study were expected to be close to it.

3.4.4. Precision and accuracy

The intra-batch precision and accuracy were assessed by analyzing QC samples ($n = 5$) at four concentration levels (0.08, 0.16, 10.0 and 100.0 ng/mL) within one batch. The inter-batch precision and accuracy was determined by analyzing QC samples at four concentration levels (15 samples each) on three different batches. As shown in Table 1, the intra-batch precision for plasma ranged between 3.1 and 15.7% over the four concentration levels (0.08, 0.16, 10.0 and 100.0 ng/mL) of pitavastatin, and the corresponding accuracy varied from 97.5 to 107.7%. For urine the intra-batch precision varied from 5.6 to 14.2% and the intra-batch accuracy varied from 92.1 to 101.5%. The inter-batch precision for plasma ranged between 6.7 and 12.9% over the four QC concentration levels (0.08, 0.16, 10.0 and 100.0 ng/mL) of pitavastatin, and the corresponding accuracy varied from 101.1 to 108.8%. The inter-batch precision for urine ranged between 5.5 and 10.5%, the accuracies ranged between 95.5 and 106.5%. These results demonstrate that the method developed has good reproducibilities and accuracies.

3.4.5. Absolute recovery

Recovery of pitavastatin was evaluated by comparing the mean pitavastatin peak areas of 5 extracted (low, medium and high levels of QC samples) with the mean peak areas of direct injection of the pure authentic standard solutions dissolved in the water/methanol (25/75, v/v; diluent). Recovery of IS was evaluated by comparing the IS mean peak areas of eight extracted samples with the mean peak

Table 1
Intra- and inter-batch precision and accuracy of pitavastatin in human plasma and urine

Quality control	Nominal concentration (ng/mL)	Intra-batch assay ($n = 5$)			Inter-batch assay (bath = 3, $n = 5$)		
		Mean (ng/mL)	Precision (CV, %)	Accuracy (%)	Mean (ng/mL)	Precision (CV, %)	Accuracy (%)
Plasma sample							
LLOQ	0.08	0.078	15.7	97.5	0.087	12.9	108.8
QC-low	0.16	0.166	6.8	103.8	0.169	8.5	105.6
QC-medium	10	10.77	4.8	107.7	10.29	7.6	102.9
QC-high	100	102.60	3.1	102.6	101.09	6.7	101.1
Urine sample							
LLOQ	0.08	0.0812	14.2	101.5	0.0805	10.52	100.6
QC-low	0.16	0.160	9.0	100.0	0.171	10.2	106.9
QC-medium	10	10.15	7.8	101.5	10.14	5.5	101.4
QC-high	100	92.09	5.6	92.1	95.52	6.6	95.5

Table 2
Recovery datas for pitavastatin and IS in human plasma and urine

Nominal concentration (ng/mL)	Recovery in plasma (mean ± S.D., %)	Recovery in urine (mean ± S.D., %)
Pitavastatin (n = 5)		
0.16	86.0 ± 7.2	86.1 ± 6.5
10.0	87.8 ± 2.7	95.6 ± 2.8
100.0	84.6 ± 4.3	86.1 ± 2.6
Rosuvastatin (n = 8)		
39.0	88.9 ± 4.1	87.4 ± 3.1

areas of eight neat reference solutions (unprocessed) of the same concentration. The recoveries of pitavastatin and IS from human plasma and urine following solid-phase extraction were shown in Table 2. The absolute recoveries were consistent, precise and reproducible.

3.4.6. Stability studies

The stabilities of pitavastatin and rosuvastatin in stock solutions were determined by placing the stock solutions at room temperature for 6 h and then in a refrigerator (4 °C) for 15 days. Concentrations of pitavastatin and rosuvastatin in the stock solutions did not change at room temperature for 6 h. However, after 15-day storage in the refrigerator, the concentration of pitavastatin decreased from 500.02 to 491.56 ng/mL and the IS concentration decreased from 0.975 to 0.965 μg/mL.

The stability of processed samples (both plasma and urine samples) was evaluated using QC samples of medium concentration. Five samples were prepared following the procedure as described in Section 2.6 and the extracted solutions were well mixed. The mixtures were then placed into a autosampler at 25 °C. After 0, 2, 4, 8 and 12 h, a 20 μL aliquot was injected into the chromatography system, respectively. The stability of processed samples at 25 °C was analyzed by comparing the peak area ratios of pitavastatin to IS. The results demonstrated that extracted samples were relatively stable and could be analyzed after keeping them in the autosampler for at least 12 h without loosing accuracy.

The freeze–thaw, short-term and long-term storage stabilities were determined using QC samples at low, medium and high concentration levels. The freeze–thaw stability was determined over four freeze–thaw cycles within a day. In each of the four freeze–haw cycles, the frozen plasma or urine samples were thawed at room temperature for 1 h and were refrozen for 3 h. The short-term stability was evaluated by keeping QC samples at 25 ± 3 °C for 5 h. The long-term stability of the analyte was determined by placing QC samples at −20 °C for 25 days. Following above treatments, those samples were analyzed against calibration curves obtained from newly prepared standards and the results were compared with those of obtained from the samples immediately processed at 0 h. The freeze–thaw, short-term and long-term storage stabilities were shown in Table 3. The results showed that the concentration of analyte in human plasma and urine decreased slightly after four freeze–thaw cycles, suggesting that human plasma or urine samples cannot be repeatedly thawed and refrozen too many times. It was reported that pitavastatin in human plasma was stable after two freeze/thaw cycles [7]. Thus, the clinical samples should not be subjected to more than two freeze/thaw cycles. The short-term stability results in Table 3 indicate that the analyte was stable in neat plasma or urine for up to 5 h at room temperature (25 ± 3 °C). In addition, the long-term storage stability of pitavastatin suggests that subject samples can be stored at −20 °C for at least 25 days.

3.4.7. Interference experiment

The major metabolic route of pitavastatin is lactonization. Blood samples contain pitavastatin–lactone at a concentration approximately equivalent to that of pitavastatin [7]. Lactone can potentially undergo conversion to the parent drug (pitavastatin) during sample processing, extraction, or ionization in the MS source. To ensure accuracy of the pitavastatin results, an interference test was performed.

9.54 mg of pitavastatin lactone was accurately weighed into a 100-mL brown volumetric flask, dissolved with methanol and diluted to 100 mL. The resulting solution was further diluted with methanol to obtain a pitavastatin lactone working solution of 9.54 μg/mL. Similarly, 9.88 mg of pitavastatin was accurately weighed into a 100-mL brown volumetric flask, dissolved with methanol and diluted with methanol to 100 mL. The resulting solution was further diluted with methanol to obtain QC standard working solutions of two concentration levels (4.94 and 2470 ng/mL in methanol). Stock solution of IS was prepared in methanol and diluted to 1.004 μg/mL with methanol. QC samples were prepared following the procedure described in Section 2.5. For reference samples, 20 μL of QC standard working solutions and 20 μL of IS solution were spiked into 0.5 mL blank human plasma or urine resulting in pitavastatin concentrations of 0.1976 (low) and 98.8 (high) ng/mL in plasma or urine (n = 3). For test samples, 20 μL of QC standard working solutions, 20 μL of IS solution and 20 μL of pitavastatin lactone working solution (9.54 μg/mL) were spiked into 0.5 mL blank human plasma or urine (n = 5), in which the concentration of lactone was 381.6 ng/mL. Those QC samples were prepared following the procedure described in Section 2.6. The results of interference test were shown in Table 4. The percentages of test samples to reference samples for plasma ranged between 93.3 and 113.3% over the pitavastatin concentration range of 0.1976 and 98.8 ng/mL. The percentages of test samples to reference samples for urine was in the range between 85.7 and 114.3% over the same pitavastatin concentration range (0.1976 and 98.8 ng/mL). The results in Table 4 showed that there were no differences of accuracy of pitavastatin whether or not lactone was added to the QC samples. Our newly developed method did not involve the use of any strong acids. The weak acid used in the method, i.e., 0.5 M KH_2PO_4 (pH 4.0), is obviously not strong enough to break the ring in lactone.

3.5. Applied in Phase I clinical pharmacokinetic

The validated LC/MS/MS method was successfully applied to quantify the pitavastatin concentration in plasma and in urine which were collected from Chinese volunteers after the administration of a single oral dose of 1, 2 and 4 mg pitavastatin. The main pharmacokinetic parameters of pitavastatin are presented in

Table 3
Stability datas of freeze–thaw, short-term and long-term stability

Nominal concentration (ng/mL)	Low concentration (0.16 ng/mL)		Medium concentration (10.0 ng/mL)		High concentration (100.0 ng/mL)	
	Plasma	Urine	Plasma	Urine	Plasma	Urine
Processed immediately (0 h)	0.168	0.171	10.07	10.12	90.14	88.04
Short-term for 5 h at 25 ± 3 °C	0.164	0.169	9.98	10.07	90.17	90.22
Freeze–thaw 4 cycles	0.160	0.159	9.53	9.37	79.50	83.40
Long-term at −20 °C for 25 days	0.175	0.161	10.49	10.80	97.41	98.76

Table 4
The results of pitavastatin lactone interference test ($n = 5$)

Nominal concentration (ng/mL)	Low concentration (0.1976 ng/mL)		High concentration (98.8 ng/mL)	
	Plasma	Urine	Plasma	Urine
1	106.67	107.14	101.24	102.81
2	95.04	85.71	96.20	98.76
3	113.33	103.48	97.93	97.36
4	93.33	114.29	98.88	101.52
5	100.00	100.86	103.51	101.00
Mean ± S.D., %	101.67 ± 8.33	102.30 ± 10.56	99.55 ± 2.86	100.29 ± 2.20

Table 5
Main pharmacokinetic parameters of pitavastatin following single dose of 1, 2 and 4 mg, respectively (mean ± S.D., $n = 10$)

Parameters	1 mg-dose	2 mg-dose	4 mg-dose
C_{max} (ng/mL)	29.1 ± 13.8	62.5 ± 15.9	120.8 ± 52.2
T_{max} (h)	0.9 ± 0.2	1.1 ± 0.7	0.8 ± 0.2
$t_{1/2}$ (h)	11.1 ± 3.1	11.2 ± 3.1	11.7 ± 2.0
MRT (h)	7.9 ± 2.0	7.9 ± 1.2	8.7 ± 1.4
AUC_{0-48} (h ng/mL)	99.4 ± 44.0	207.4 ± 50.7	436.3 ± 185.9
$AUC_{0-\infty}$ (h ng/mL)	104.2 ± 45.3	214.7 ± 51.9	453.4 ± 194.6
Cl/F (L/h)	16.6 ± 21.9	9.9 ± 2.6	11.8 ± 9.4
Vd/F (L)	212.3 ± 177.1	160.5 ± 65.1	203.1 ± 178.3
Urinary excretion rate (%)	0.56 ± 0.28	0.52 ± 0.42	0.17 ± 0.16

Table 5. The mean plasma concentration–time curves after administration at three dosage levels are presented in Fig. 4.

The peak plasma level (C_{max}) and the area under the curve (AUC) obtained from this study were 62.5 ng/mL and 207.4 h ng/mL, respectively, which were higher than those previously reported. After the administration of a single oral dose 2 mg pitavastatin, the C_{max} and AUC were 26.11 ng/mL and 58.8 h ng/mL in Japanese volunteers, respectively [7], and were 20.3 ng/mL and 53.8 h ng/mL in Korean volunteers [12]. However, the results from this study were consistent with of those obtained from another study in Chinese was, in which C_{max} and AUC were found to be 65.2 ng/mL and 221.5 h ng/mL, respectively [9]. We assume that this is caused by the age difference among the subjects who participated in the trials. In China, healthy youths from medical universities were often enrolled. The fat of body in youths is lower than that in seniors, leading to the increased pitavastatin distribution in blood of youths. As for $t_{1/2}$, our result ($t_{1/2}$ = 11 h) was the same as that published [13].

In this study, comparing by single-factor analysis of variance, no significant differences were found among the following pharmacokinetic parameters: T_{max}, $t_{1/2}$, MRT, Cl/F and Vd/F after a single oral administration of pitavastatin at three dosage levels. The linearities between pharmacokinetic parameters (C_{max}, AUC_{0-48} and $AUC_{0-\infty}$) and dosage levels were good (correlation coefficient $r > 0.99$), suggesting linear pharmacokinetics. This conclusion was consistent with that published [9]. In a clinical trial conducted by Japanese, six male volunteers were given a single dose of pitavastatin (0.5–8.0 mg/person), it was found that the C_{max} and the AUC of pitavastatin and its lactone were proportional to dose levels, which also suggested linear pharmacokinetics [1].

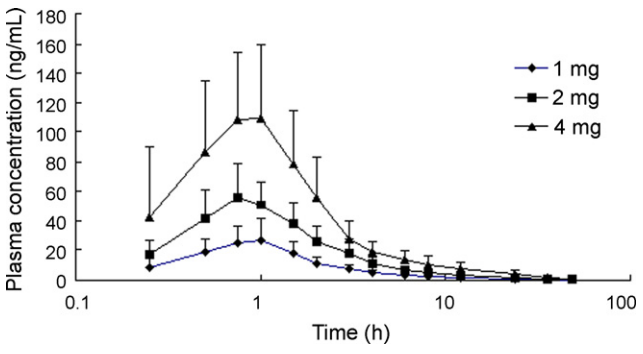

Fig. 4. Mean plasma concentration–time curve of pitavastatin at different dosage (1, 2 and 4 mg) of administration ($n = 10$).

The urinary excretion ratio of pitavastatin accounted for less than 0.6%, which suggested that pitavastatin was not excreted primarily in kidney. It was reported that pitavastatin only undergoes slight metabolism. Pitavastatin and its metabolite are excreted primarily in the faeces via bile. Urinary excretion of pitavastatin and its primary metabolite, lactone, was low (<2%) [13].

Using the method developed in this study, we were able to measure the concentration of pitavastatin in plasma and urine obtained from all subjects up to 48 h (last time point for plasma sample collection) from all subjects after 1 mg dose of pitavastatin.

4. Conclusions

An LC/MS/MS method combined with the solid-phase extraction for the quantification of pitavastatin in human plasma and urine was firstly developed. This method was validated in concentrations ranging from 0.08 to 200 ng/mL with good reproducibility and accuracy. This method was successfully applied to a clinical pharmacokinetic study.

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