Short Communication

LC–MS/MS assay for pitavastatin in human plasma and subsequent application to a clinical study in healthy Chinese volunteers

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A B S T R A C T

A rapid, selective and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the determination of pitavastatin in human plasma. Following a liquid–liquid extraction, both the analyte and internal standard telmisartan were separated on a Luna C18 column with a mobile phase consisted of acetonitrile–methanol–1% formic acid in water (50:25:25, v/v/v). Mass spectrometric detection involved electrospray ionization in the positive ion mode followed by multiple reaction monitoring (MRM) of the transitions at m/z 421.9 → 290.1 for pitavastatin and m/z 515.2 → 276.2 for the IS. The assay for pitavastatin showed good linearity (r ≥ 0.99) over the ranges 0.2—400 ng/ml, with a lower limit of quantitation of 0.2 ng/ml. 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 (RSD) of less than 15% for both analytes. The mean extraction recovery of pitavastatin and IS were both above 70%. Matrix effect hasn’t been found in this method. The method has been successfully applied to a clinic pharmacokinetic study of pitavastatin administered.

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1. Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) inhibit the synthesis of mevalonate, a rate limiting step in cholesterol level. Competitive inhibition of HMG-CoA reductase by the statins decreases hepatocyte cholesterol synthesis, which results in increased extraction of LDL-C from the blood and decrease circulating LDL-C concentrations [1]. High plasma LDL-cholesterol is a risk factor of cardiovascular diseases and, therefore, cholesterol-lowering drugs are used to prevent them. Some randomized controlled trials have shown that statins have potent cholesterol-lowering effects.
effects and reduce the risk of cardiovascular diseases in everyday medical practice [2–5].

Pitavastatin (NK-104, previously called itavastatin or nisvastatin) is a novel, fully synthetic statin with the chemical name of C\textsubscript{50}H\textsubscript{46}CaF\textsubscript{2}N\textsubscript{2}O\textsubscript{8} [6,7]. It can reduce plasma level of LDL-cholesterol by 40% in hypercholesterolaemic patients [8]. Pitavastatin has been launched in Japan, and is becoming available in Europe, the US, and Asia. Normal-phase HPLC with UV detection was described by Kojima et al., with a LLOQ of 0.5 ng/ml achieved [9]. However, the chromatographic run time for one sample was relatively long (25 min). Hui et al. [10] used above method and achieved a LLOQ of 1.0 ng/ml. Lv et al. [11] established an HPLC–MS/MS method with liquid–liquid extraction (LLE) to determine pitavastatin in human plasma, with a LLOQ of 0.2 ng/ml. The need to better characterize the clinical pharmacokinetic properties of pitavastatin compelled us to set up and validate a simple, specific and sensitive analytical method. In this study, a technique based on LC–MS/MS intended for the determination of pitavastatin was developed and validated, with a LLOQ of 0.2 ng/ml and shorter chromatographic cycle time (2.1 min). This method was successfully applied to clinical pharmacokinetic study among Chinese people.

2. Materials and methods

2.1. Chemicals and reagents

Pitavastatin calcium were supplied by Qidu Company Ltd., and telmisartan (internal standard, IS) were from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of the two chemicals were 99.7%, 99.3%, respectively. HPLC grade methanol, acetonitrile, amino acid and ammonium acetate (purity: C21 99.5%) were all obtained from Dikma (Beijing, China). All other chemicals and reagents were of analytical grade and were used without any further purification.

2.2. Instrumentation

An Agilent 1100 system (Agilent, USA) consisting of a vacuum degasser (G1379A), a quaternary pump (G1311A), a column oven (G1316A) and an autosampler (G1313A) was used for solvent and sample delivery. An Applied Biosystems API 2000 triple-quadrupole mass spectrometer equipped with a TurboIonSpray ionization (ESI) source was used for mass spectral analysis and the system was operated in positive mode. Data acquisition was performed with Analyst 1.4.1 software (Applied Biosystems, USA). Peak integration and calibration were carried out using SPSS 11.5 software (SPSS Inc., USA).

2.3. Operating conditions

Separations of analytes were achieved using a Luna C\textsubscript{18} column (50 mm × 2.0 mm, 5 μm) protected by a Security guard (Waters, Inc.), eluting isocratically at 0.25 ml/min with a mobile phase of acetonitrile–methanol–0.1% formic acid (50:25:25, v/v/v). The run time of one sample was about 2.1 min. In order to assure the repeatability of retention time, the column temperature was controlled at 20 °C. The optimized TurbolonSpray voltage and temperature were set at 5000 V and 400 °C, respectively. The source parameters, viz. the nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 60, 20, 70 and 5 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 60, 42, 8, 6 eV and 80, 10, 5 eV for the IS. 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 421.9 → m/z 290.1 for pitavastatin and m/z 515.2 → m/z 276.2 for IS, respectively. The production scan of pitavastatin and telmisartan was shown in Fig. 1. The scan time for each analyte was set to 0.2 s.

2.4. Drug administration and sample collection

An open-label, randomized study was conducted in Phase I Clinical Research Institute of the Beijing Military General
Hospital (Beijing, P.R.China). The Independent Ethics Committee (IEC) of Beijing Military General Hospital approved the protocol and the volunteers were provided with informed written consent. Thirty healthy Chinese volunteers (15 females and 15 males) were chosen to attend the study. The volunteers aged between 23 to 33 y and their IBM figures were between 19 and 24. 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. Blood samples were collected at pre-dose and 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 8, 12, 24 and 48 h, in heparin vacutainer collection tubes. The tubes were centrifuged at 3000 r/min for 10 min and the plasma was collected. The collected plasma samples were stored at −70 °C till their use. The 2 mg group then completed a 7-day washout period. Beginning on the morning of day 8, subjects received 2 mg of pitavastatin once daily for a total of 7 doses.

2.5. Preparation of stock solutions of analytes and IS

The stock solutions of pitavastatin (100 μg/ml) and the IS (100 ng/ml) were separately prepared in methanol. Serial dilutions were prepared with methanol at concentrations of 2000, 1000, 500, 100, 50, 10, 2.5 and 1 ng/ml for pitavastatin, respectively. Working solutions of IS were prepared by further diluting the IS stock solutions with methanol at concentrations of 1 μg/ml and 50 ng/ml. All the working standard solutions were stored at 4 °C; they were found to be stable for 8 d.

2.6. Preparation of calibration curves and quality control samples

Calibration samples were prepared by spiking 250 μl of control human plasma with 50 μl working standard solution of the analyte, to produce the matrix-matched calibration standards at concentrations of 0.2, 1.0, 2.0, 10.0, 20.0, 100.0, 200.0, 400.0 ng/ml of pitavastatin. Each sample also contained 50 μl IS solutions (50 ng/ml). In each calibration run a plasma blank sample (no IS) was also analyzed.

The QC samples of three different concentration levels (0.5, 20, 320 ng/ml) were selected to cover the entire range of calibrations. They were prepared daily by spiking 250 μl plasma samples with appropriate volumes of a standard solution to produce the stated final concentration of pitavastatin and 50 ng/ml of the IS.

2.7. Sample preparation

QC samples, calibration standards, and clinical plasma samples were extracted employing a LLE technique. To each tube containing 250 μl plasma, 50 μl of the 50 ng/ml of the IS, and 100 μl of 0.05 mol/l phosphoric acid, vortexed for 1 min, then 2 ml of a mixture of ether: dichloromethane (4:1, v/v) were added, and the mixture was then vortexed for 10 min. The samples were then centrifuged for 5 min at 10,000 r/min. 1.5 ml of the organic layer was transferred to another clean tube and was evaporated to dryness at 38 °C under a gentle stream of nitrogen. The residue was dissolved in 100 μl of the mobile phase, and a 15 μl aliquot was injected onto the LC–MS/MS system for analysis.

2.8. Method validation

The analytical procedure was validated in terms of specificity, sensitivity, linearity, precision and accuracy, extraction recovery, matrix effect and stability. Specificity of the method was assessed by analyzing six blank human plasma matrix samples. The responses of the interfering substances or background noises at the retention time of the pitavastatin are acceptable if they are less than 20% of the response of the lowest standard curve point or LLOQ. The responses of the interfering substances or background noises at the retention time of the internal standard are acceptable if they are less than 5% of the response of the working internal standard.

Calibration curves for each analyte were individually constructed by least-squares linear regression analysis of an eight-point calibration curve by plotting analyte-to-IS peak area ratio versus its nominal concentration, using 1/x² as a weighting factor. Sensitivity was established from the background noise or response from six spiked LLOQ samples. The six replicates should have a precision of ≤20% and an accuracy of ±20%.

The intra-day accuracy and precision were evaluated by analyzing six replicates at three QC concentration levels for human plasma samples on the same day. The inter-day assay accuracy and precision were determined by analyzing the three-level QC samples on three consecutive d. The criteria for acceptability of the data included accuracy within ±15% deviation from the nominal values and a precision of within 15% relative standard deviation (RSD), except at the LLOQ, where it should not exceed ±20%.

Extraction recovery and matrix effect were evaluated at three concentration levels (LQC, MQC and HQC) for each analyte and at the working concentration for internal standards. Post-spiked QCs used in recovery and matrix effect test, were prepared by spiking the extracted blank matrix with analyte and IS to ensure that concentrations are equivalent to those in the LQC, MQC and HQC extracted samples. Extraction recovery was calculated by comparing the signal of analyte or IS of regular QCs with those of post-spiked QCs. Matrix effect was evaluated by comparing the signal of post-spiked QCs with those of the neat solutions at LQC, MQC and HQC levels.

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at refrigerated conditions (4 °C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (2 h), processed samples stability (room temperature for 8 h), freeze-thaw stability (two cycles), and long-term stability (20 d) were performed at LQC, MQC and HQC levels using three replicates at each level. Samples were considered to be stable if assay values were within...
Fig. 2 – Typical MRM chromatograms of pitavastatin (left panel) and IS (right panel) in (A) human blank plasma, and (B) human plasma spiked with IS, and (C) a plasma sample from a volunteer 3.0 h after oral administration of 1 mg pitavastatin.
the acceptable limits of accuracy (±15% SD) and precision (≤15% RSD).

### 3. Results and discussion

#### 3.1. Mass spectrometry and chromatography conditions

The objective of the present work was to develop and fully validate a LC–MS/MS method for the determination of pitavastatin in human plasma. To realize this aim, the extraction procedure, mass spectrometry and chromatographic conditions were optimized. Due to the alkaline nitrogen atom on the pyridine cycle in the molecular structure, the positive ionization mode should be more appropriate for pitavastatin than the negative mode. The mass spectrometry of pitavastatin and the internal standard telmisartan exhibited a protonated molecular ion at m/z 421.9 and 515.2, respectively. The high collision energy gave the most abundant product ion than the negative mode. The mass spectrograms of pitavastatin and telmisartan exhibited a protonated molecular ion at m/z 421.9 and 515.2, respectively. Therefore, the precursor to product transition was assigned in the selected reaction monitor mode as follows: m/z 421.9 → m/z 290.1 for pitavastatin and m/z 515.2 → m/z 276.2 for telmisartan. In order to obtain the highest sensitivity, the mass spectrometry parameters were optimized by the auto-tuning. To attain an ideal Taylor cone shortens the chromatographic cycle time and the acid modifier, formic acid, improved the signal intensity greatly. Therefore, the mixture of acetonitrile, methanol and 1% formic acid (50:25:25, v/v/v) was finally used as mobile phase. A flow rate of 0.25 ml/min permitted a run time of 2.1 min.

#### 3.2. Method validation

##### 3.2.1. Selectivity

The degree of interference by endogenous plasma constituents with the analytes and IS was assessed by inspection of chromatograms derived from processed blank plasma sample. As shown in Fig. 2, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free plasma at the retention time of the analytes.

##### 3.2.2. Linearity, sensitivity, accuracy and precision

The method exhibited good linear response for the concentration range 0.2–400 ng/ml with correlation coefficients of 0.9974. The linear equation for the concentration vs. the peak area was \( y = 0.0641x + 0.00826 \) where \( y \) is analyze/IS peak area ratio and \( x \) is analyze concentration. The LLOQ for pitavastatin was found to be 0.2 ng/ml. Three concentrations of QC samples were analyzed to determine the accuracy and precision of the assay. Precision and accuracy data for intra- and inter-day plasma samples were presented in Table 1. The accuracy expressed as bias and precision expressed as RSD were all found to be within the criteria limit.

##### 3.2.3. Recovery and matrix effects

The recoveries, matrix effects for pitavastatin and IS are presented in Table 2. The mean extraction recoveries for pitavastatin were 79.9 ± 8.23%, 71.5 ± 13.9% and 74.4 ± 6.35% at concentrations of 0.5, 20 and 320 ng/ml, respectively. The mean extraction recoveries of the IS were 76.5 ± 12.8% at concentrations of 50 ng/ml. Matrix interferences caused by plasma were evaluated by comparing the peak areas of the post-spiked standards with those of the neat standard at the QC concentrations. In this study, the matrix effects of the analytes were 104.3 ± 11.4%, 102.8 ± 7.73% and 100.7 ± 8.41% at

| Table 1 – Intra-day and inter-day precision and accuracy for pitavastatin. |
|-------------------|-------------------|-------------------|-------------------|
| Analyte           | QC(spiked concentration ng/ml) | Intra-day (n = 18) | Inter-day (n = 18) |
|                   | Mean concentration (ng/ml) | Precision (RSD,%) | Accuracy (%)   | Mean concentration (ng/ml) | Precision (RSD,%) | Accuracy (%)   |
| Pitavastatin      | 0.5 (LQC)          | 0.51 ± 0.84      | 8.84            | 102.0          | 0.51 ± 0.13      | 5.13          | 102.0          |
|                   | 20.0 (MQC)         | 19.4 ± 5.41      | 5.41            | 97.2           | 20.5 ± 11.8      | 11.8          | 102.6          |
|                   | 320.0 (HQC)        | 314.5 ± 7.08     | 7.08            | 98.3           | 321 ± 8.41       | 8.41          | 100.4          |

| Table 2 – Absolute recoveries and matrix effect of pitavastatin and telmisartan (IS) in human plasma. |
|-------------------|-------------------|-------------------|-------------------|
| Analyte           | QC(spiked concentration, ng/ml) | Recovery (n = 18) | Matrix effect (n = 9) |
|                   | Mean value (%)     | Mean value (%)    | Mean value (%)    |
| Pitavastatin      | 0.5 (LQC)          | 79.9 ± 8.23       | 104.3 ± 11.4      |
|                   | 20.0 (MQC)         | 71.5 ± 13.9       | 102.8 ± 7.73      |
|                   | 320.0 (HQC)        | 74.4 ± 6.35       | 100.7 ± 8.41      |
| Telmisartan       | 50 (IS)            | 76.5 ± 12.8       | 98.9 ± 4.57       |
concentrations of 0.5, 20 and 320 ng/ml, respectively. The matrix effect of the IS were 98.9 ± 4.57% at concentrations of 50 ng/ml. The results indicates that the plasma extract did not cause significant ionization suppression or enhancement for both analytes and IS in different lots of plasma.

3.2.4. Stability
QC samples were subjected to short-term room temperature conditions, long-term storage conditions (−20 °C), and two freeze-thaw recycls. All the stability studies were conducted at three concentration levels with three determinations for each. The stability experiment confirmed that samples were stable over 20 d when stored at −20 °C and through 2 freeze-thaw cycles. The plasma samples were stable for at least 2 h pre-preparation and 8 h post-preparation at room temperature. Detail stability data are shown in Table 3.

### Table 3 - Stability samples result for pitavastatin in human plasma.

<table>
<thead>
<tr>
<th>Stability test</th>
<th>QC (spiked concentration, ng/ml)</th>
<th>Mean concentration found (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-preparative stability 2 h at room temperature</td>
<td>0.5 (LQC) 20.0 (MQC) 320.0 (HQC)</td>
<td>0.47 ± 8.69 18.8 ± 7.25 303 ± 10.8</td>
</tr>
<tr>
<td>Post-preparative stability 8 h at room temperature</td>
<td>0.5 (LQC) 20.0 (MQC) 320.0 (HQC)</td>
<td>0.54 ± 3.47 19.4 ± 1.19 344 ± 4.80</td>
</tr>
<tr>
<td>Freeze and thaw stability 2 cycles at −20 °C</td>
<td>0.5 (LQC) 20.0 (MQC) 320.0 (HQC)</td>
<td>0.52 ± 3.69 18.6 ± 4.99 343 ± 3.65</td>
</tr>
<tr>
<td>Long-term stability 20 d at −20 °C</td>
<td>0.5 (LQC) 20.0 (MQC) 320.0 (HQC)</td>
<td>0.49 ± 8.29 20.1 ± 1.79 301 ± 2.34</td>
</tr>
</tbody>
</table>

3.3. PK study
The validated LC/MS/MS method was successfully applied in Phase I clinical pharmacokinetic study to quantify the pitavastatin concentration in plasma which were collected from Chinese volunteers after the administration of a single oral dose of 1, 2 and 4 mg pitavastatin and a multiple dose of 2 mg pitavastatin followed. Profiles of the mean plasma concentration–time curves after administration at three single dosage levels and multiple dosage level of 2 mg are presented in Figs. 3 and 4. The main pharmacokinetic parameters are listed in Table 4.

The pharmacokinetics results showed that plasma C\(_{\text{max}}\) values of pitavastatin at three single dosage levels were 27.8 ± 9.11, 51.5 ± 15.0 and 108 ± 45.5 ng/ml, respectively. The absorption of pitavastatin was rapid, with t\(_{1/2}\) occurring 0.65 ± 0.17, 0.81 ± 0.34 and 0.88 ± 0.18 h, respectively. The area under the curve (AUC) were found to be 82.5 ± 32.2, 146 ± 39.2 and 272 ± 102 ng h/ml, respectively. After the administration of a single dose 2 mg pitavastatin, the C\(_{\text{max}}\) and AUC were 62.5 ng/ml and 207.4 ng h/ml in another study in Chinese [12], which is similar with the results of this study. However, the results of this study were different from another study in Japanese volunteers, in which C\(_{\text{max}}\) and AUC were found to be 26.1 ng/ml and 58.8 ng h/ml, respectively [9]. We assume that this is caused by the individual difference among the subjects who participated in the trials. In this study, Comparing by single-factor analysis of variance, no significant differences were found among the following pharmacokinetic parameters: t\(_{1/2}\), MRT\(_{0-48\text{h}}\), MRT\(_{0-\infty}\), CL/F and Vd/F after a single oral administration of pitavastatin at three dosage levels. The linearities between pharmacokinetic parameters (C\(_{\text{max}}\), AUC\(_{0-48\text{h}}\) and AUC\(_{0-\infty}\)) and dosage levels were good (correlation coefficient r > 0.99), suggesting linear pharmacokinetics.

![Fig. 3](image_url) – Mean plasma concentration–time curve of pitavastatin at different single dosage (1, 2 and 4 mg) of administration (n = 10).
The pharmacokinetics results of the 2 mg group which were administered a multiple dosage for seven d (Table 5) showed that the $t_{1/2}$, AUC$_{0-48h}$, AUC$_{0-\infty}$, $C_{max}$, MRT$_{0-48h}$, MRT$_{0-\infty}$, CL/F and Vd/F values of single dosage of 2 mg and multiple dosage of 2 mg have no difference ($P > 0.05$). The accumulation factor (Rac) of pitavastatin was $0.98 \pm 0.30$ showing that no drug accumulation was observed in the body.

4. Conclusion

A sensitive, specific, rapid and costless LC–MS/MS method for analysis of pitavastatin in human plasma has been developed and validated. Simple liquid–liquid extraction sample preparation procedure and chromatography analysis were used in the method. The method has a lower limit of quantitation of 0.2 ng/ml and the chromatographic cycle time (2.1 min) is shorter than the reported method. Our method allowed high-throughput analysis with minimal matrix interference and has been shown to be successfully applied to phase I pharmacokinetic studies in healthy subjects. In this work we have studied the effect of multiple dosage (2 mg) of pitavastatin in human bodies which showed there was no significant accumulation. The sensitive and specific method was suitable for analysis of the large batches of sample.

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### Table 5 – Pharmacokinetic parameters of pitavastatin following single dose and multiple dose of 2 mg (Mean ± SD, n = 10).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>2 mg (single dose)</th>
<th>2 mg (multiple dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{max}$ (h)</td>
<td>$0.81 \pm 0.34$</td>
<td>$0.80 \pm 0.48$</td>
</tr>
<tr>
<td>$C_{max}$ (ng/ml)</td>
<td>$51.5 \pm 15.0$</td>
<td>$48.5 \pm 23.2$</td>
</tr>
<tr>
<td>$C_{min}$ (ng/ml)</td>
<td>$–$</td>
<td>$1.17 \pm 0.43$</td>
</tr>
<tr>
<td>$C_{av}$ (ng/ml)</td>
<td>$5.05 \pm 1.05$</td>
<td>$–$</td>
</tr>
<tr>
<td>$K_{e}$ (h$^{-1}$)</td>
<td>$0.068 \pm 0.043$</td>
<td>$0.075 \pm 0.038$</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>$12.8 \pm 5.40$</td>
<td>$11.2 \pm 4.93$</td>
</tr>
<tr>
<td>AUC$_{0-48h}$ (ng/ml h)</td>
<td>$146 \pm 39.2$</td>
<td>$139 \pm 31.4$</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (ng/ml h)</td>
<td>$155 \pm 41.9$</td>
<td>$146 \pm 31.3$</td>
</tr>
<tr>
<td>MRT$_{0-48h}$ (h)</td>
<td>$7.80 \pm 2.29$</td>
<td>$8.52 \pm 2.32$</td>
</tr>
<tr>
<td>MRT$_{0-\infty}$ (h)</td>
<td>$11.7 \pm 4.05$</td>
<td>$12.3 \pm 4.12$</td>
</tr>
<tr>
<td>CL/F (l/h)</td>
<td>$13.6 \pm 2.97$</td>
<td>$14.3 \pm 3.06$</td>
</tr>
<tr>
<td>Vd/F (l)</td>
<td>$253 \pm 124$</td>
<td>$232 \pm 116$</td>
</tr>
<tr>
<td>DF</td>
<td>$–$</td>
<td>$9.19 \pm 3.05$</td>
</tr>
<tr>
<td>Rac</td>
<td>$–$</td>
<td>$0.98 \pm 0.30$</td>
</tr>
</tbody>
</table>
Acknowledgments

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REFERENCES