Simultaneous determination of rivaroxaban and sitagliptin in rat plasma by LC–MS/MS and its application to pharmacokinetic drug-drug interaction study

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ABSTRACT

A sensitive and accurate LC-MS/MS method was developed and validated for the simultaneous quantification of rivaroxaban (RIV) and sitagliptin (SIT) in rat plasma using apixaban as internal standard (IS). An Agilent Eclipse plus C18 column (2.1 × 100 mm, 3.5 μm, Agilent) was used for chromatographic separation with isocratic elution. Multiple reaction monitoring (MRM) using positive-ion ESI mode to monitor ion transitions of m/z 436.8 → 144.9 for RIV, m/z 407.7 → 173.8 for SIT, m/z 459.8 → 442.8 for IS. The procedure of method validation included selectivity, linearity, precision, accuracy, matrix effect, extraction recovery and stability were conducted according to the guidelines of EMA and FDA. The results indicated that no obvious drug-drug interactions occurred might be owing to their differences in metabolic pathways.

KEYWORDS

LC-MS/MS, rivaroxaban, sitagliptin, drug-drug interaction

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycemia which on progression leads to many secondary complications [1]. The function of vascular endothelial cells in diabetic patients is impaired to form a hypercoagulable state, which changes blood rheology and promotes platelet activation and many other ways, thereby causing thrombosis and thromboembolic diseases [2–4]. The basis of the treatment of diabetes mellitus with thrombosis or thromboembolic complications is to control blood sugar. For the treatment of thrombosis, arterial thrombosis emphasizes antiplatelet therapy, and venous thrombosis emphasizes anticoagulant therapy [5]. Therefore, the treatment of such patients often requires taking anticoagulants and hypoglycemic drugs at the same time.

Traditional anticoagulants warfarin has some limitations such as bleeding and need of frequent monitoring, which make clinical administration difficulty [6]. Rivaroxaban (RIV, Fig. 1), is a small molecule, orally active factor Xa direct inhibitor with predictable
pharmacokinetics and pharmacodynamics [7, 8]. It is used as a novel anticoagulant taken as once tablet, once-daily, for the prevention of venous thromboembolism (VTE) in adult patients undergoing elective (planned) hip or knee replacement surgery [9]. RIV is a kind of small-molecular inhibitors with good oral bioavailability but also no cross resistant, weak toxicity and little side effect [10]. RIV gradually becomes the new oral intake of anticoagulant for the aged diabetes mellitus patients after the orthopedics operation because of many advantages such as convenient application, no requirement of determining coagulation function, lower weak toxicity and little side effect [10]. RIV is a kind of small-molecular inhibitors with good oral bioavailability but also no cross resistant, weak toxicity and little side effect [10].

SIT (SIT, B), apixaban (IS, C)

Fig. 1. The chemical structures of rivaroxaban (RIV, A), sitagliptin (SIT, B), apixaban (IS, C)

SITaglin (SIT, Fig. 1), a dipeptidyl peptidase-4 (DPP-4) inhibitor, selectively inhibits DPP-4, the enzyme responsible for the degradation of Glucagon-like peptide-1 (GLP-1), thereby increasing endogenous GLP-1 and leading to increased insulin secretion [11]. As a novel oral hypoglycemic medicine, not only decrease the Level of HbA1C, fasting glucose and postprandial glycemia, but also improve the dysfunction of pancreatic cells by increasing the secretion of insulin and inhibiting the secretion of glucagon, without increasing the body weight and the incidence of hyperglycemia [12]. SIT is approved as a monotherapy and in combination with other anti-hyperglycemic drugs in more efficacy and safety [15].

However, there is no report on the pharmacokinetic DDI of these two drugs. Carrying out this research requires the development of a suitable bioanalytical method to simultaneous determination of the two drugs in plasma, which can be used for therapeutic drug monitoring.

Experimental

Chemicals and reagents

The chemical standards of RIV, SIT and apixaban (IS) with purity of more than 99% were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile of LC-MS grade were purchased from Fisher (Fisher Corp., Fair lawn, NJ, USA). Ammonium acetate of HPLC grade was purchased from Sigma-Aldrich (St. Louis, MO, USA). All experimental water was prepared by Millipore Milli-Q ultrapure water preparation device (Millipore Sigma, Bedford, MA, USA).

Instruments and analytical conditions

The LC-MS/MS system consisted of an Agilent liquid chromatography 1,260, equipped with a quaternary pump, flow-through needle autosampler, temperature controlled column compartment (Agilent Technologies, Santa Clara, CA, US), and an Agilent 6460c triple quadrupole mass spectrometer equipped with an ESI (electrospray ionization) source (Agilent Technologies, Santa Clara, CA, US). An Agilent Eclipse plus C18 column (2.1 × 100 mm, 3.5 μm, Agilent) was used for chromatographic separation. Mobile phase A consisted of water with 5 mM ammonium formate and 0.1% formic acid; mobile phase B was consisted of 0.1% formic acid in acetonitrile. Isocratic elution was applied at a flow rate of 0.4 mL min⁻¹ and operation time of 6.0 min (A: B = 40: 60% v/v). The injection volume was 10 μL, whereas the column temperature and autosampler temperature was at 40 °C and 4 °C.

We selected the parent and daughter ions were as follows: RIV-m/z 436.8→442.8, SIT-m/z 407.7→173.8, IS-m/z 449.8→428.8. The mass spectra of RIV, SIT and IS are shown in Fig. 1. The fragmentor (Frag) values set for RIV, SIT and IS were 130 V, 100 V, 120 V, respectively. The collision energy (CE) values set for RIV, SIT and IS were 32 eV, 26 eV and 13 eV, respectively. The collision energy (CE) values set for RIV, SIT and IS were 32 eV, 26 eV and 13 eV, respectively. The sample ionization parameters were as follows: 360 °C (gas temperature); 12.0 L min⁻¹ (gas flow); 350 °C (sheath gas temperature); 12.0 L min⁻¹ (sheath gas flow); 3500 V (capillary); 0.27 μA (chamber current); 45 psi (nebulizer). The target compounds were analyzed by the multiple reaction monitoring (MRM) using positive-ion ESI mode.

Stock solutions and working solutions

The standard stock solutions of RIV (1.0 mg mL⁻¹), SIT (1.0 mg mL⁻¹) and internal standard (1.0 mg mL⁻¹) were prepared in methanol and were stored at −20 °C. Stock solutions are used for quality control (QC) and calibration standards samples, respectively. Quality control (QC) and Calibration standards samples were prepared in batches in the same manner before the beginning of verification. Calibration standards of seven spiking levels by diluting two individual stock solutions of RIV and SIT. Quality control (QC) samples were prepared by spiking six rat plasma samples at low (2 ng mL⁻¹ of RIV and 5 ng mL⁻¹ of SIT),

Fig. 1. The chemical structures of rivaroxaban (RIV, A), sitagliptin (SIT, B), apixaban (IS, C)
medium (55 ng mL\(^{-1}\) of RIV, 100 ng mL\(^{-1}\) of SIT) and high level (250 ng mL\(^{-1}\) of RIV, 1,500 ng mL\(^{-1}\) of SIT). Calibrating solutions were prepared at 1, 5.5, 10, 55, 100, 200, 300 ng mL\(^{-1}\) for RIV and 2, 10, 55, 100, 550, 1,000, 2,000 ng mL\(^{-1}\) for SIT. The IS stock solution was diluted with acetonitrile to obtain a working solution of 50 ng mL\(^{-1}\).

**Sample preparation**

To an aliquot of 100 μL of rat plasma, 10 μL of IS solution (50 ng mL\(^{-1}\)) was added, and vortexed for 30 s; then 1.0 mL of acetonitrile was used. The mixture was vortexed for 3 min and centrifuged at 10,000 \( \times \) g for 15 min at 4°C. 800 mL of organic phase was transferred to another test tube and evaporated under the stream of nitrogen at 35°C. The residue was dissolved with 100 μL of acetonitrile, vortex for 3 min, and centrifuge at 10,000 \( \times \) g for 15 min at 4°C. Finally, 10 μL of the obtained supernatant was injected into LC-MS/MS system.

**METHOD VALIDATION**

According to the Bioanalytical Method Validation Guidance of FDA and EMA, the method validation procedure was

![Fig. 2 Typical MRM chromatograms of RIV, SIT and IS. (A) blank plasma; (B) blank plasma spiked with RIV and SIT at the LLOQ level; (C) Pharmacokinetic samples](image)

**Table 1. Precision and accuracy for RIV and SIT in rat plasma. (n = 6, Mean ± SD)**

<table>
<thead>
<tr>
<th>Spiked conc. (ng mL(^{-1}))</th>
<th>Intra-day (n = 6)</th>
<th>Inter-day (n = 3 × 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured conc. (Mean ± SD)</td>
<td>Precision (% RSD)</td>
</tr>
<tr>
<td>RIV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.05 ± 0.08</td>
<td>7.6</td>
</tr>
<tr>
<td>2</td>
<td>2.23 ± 0.12</td>
<td>5.3</td>
</tr>
<tr>
<td>55</td>
<td>52.64 ± 3.57</td>
<td>6.7</td>
</tr>
<tr>
<td>250</td>
<td>274.25 ± 9.32</td>
<td>3.4</td>
</tr>
<tr>
<td>SIT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.25 ± 0.16</td>
<td>6.9</td>
</tr>
<tr>
<td>5</td>
<td>5.39 ± 0.67</td>
<td>12.5</td>
</tr>
<tr>
<td>100</td>
<td>91.86 ± 8.18</td>
<td>8.9</td>
</tr>
<tr>
<td>1,500</td>
<td>1557.41 ± 101.23</td>
<td>6.5</td>
</tr>
</tbody>
</table>

**Table 2. Recovery and matrix effect of RIV and SIT in rat plasma. (n = 6, Mean ± SD)**

<table>
<thead>
<tr>
<th>Analyses</th>
<th>Spiked conc. (ng mL(^{-1}))</th>
<th>Recovery</th>
<th>Matrix effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± SD)</td>
<td>RSD (%)</td>
<td>(Mean ± SD)</td>
</tr>
<tr>
<td>RIV</td>
<td>2</td>
<td>91.45 ± 4.54</td>
<td>4.81</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>97.67 ± 5.26</td>
<td>5.38</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>96.57 ± 6.21</td>
<td>6.43</td>
</tr>
<tr>
<td>SIT</td>
<td>5</td>
<td>93.87 ± 5.15</td>
<td>5.49</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>93.17 ± 4.47</td>
<td>4.81</td>
</tr>
<tr>
<td></td>
<td>1,500</td>
<td>95.37 ± 3.79</td>
<td>3.98</td>
</tr>
<tr>
<td>IS</td>
<td>50</td>
<td>102.68 ± 3.37</td>
<td>3.29</td>
</tr>
</tbody>
</table>

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conducted including the selectivity, linearity, accuracy, precision, matrix effect, extraction recovery, and stability.

The selectivity was assessed to determine whether substances present in the plasma would affect the determination of the analyte. It was evaluated with retention time of analytes by comparing chromatograms of blank plasma samples, blank rat plasma samples with a concentration of LLOQ, and pharmacokinetic samples.

Carry-over effects were assessed, with relevant criteria, by injecting blank samples following the calibration standard at the highest concentration. Carry-over in the blank should not exceed 20% of the LLOQ and 5% for the IS.

The linearity of RIV and SIT with at least seven different spiked plasma samples were used to obtain the standard curves, which were set up by a linear regression function taking the peak area ratios ($y$) of the analyte to IS versus the concentrations of the analytes ($x$) using a $1/x^2$ weighted with three occasions. The lower limit of quantification (LLOQ) was determined as the lowest concentration point of the calibration curve, where acceptable accuracy and precision were obtained should less than 20%.

Precision and accuracy were assessed by analyzing four QC levels (LLOQ, QL, QM, QH) with at least six replicates in a single day (intra-day) and four batches in three day in a row (inter-day), respectively. The precision assessed by the relative standard deviation (RSD) and the accuracy defined as the relative error (RE) should be within ±15% for QC samples, and 20% for LLOQ.

The matrix effects were assessed by analyzing three QC levels (QL, QM, QH) in the matrix from six separate sources, with at least six replicates at each level, to verify the influence on the assay. The recoveries were calculated by comparing the ratio of the responses of analytes with IS spiked before the extracted process to those in post-extraction spiked plasma.

Stability analysis of QC samples (low, medium, and high concentration levels) was investigated by measuring under different conditions: at room temperature after preparation for 12 h, extracted samples was kept for 24 h at 4 °C, the samples have gone through 3 freeze-thaw cycles, the samples stored at −80 °C for 1 month, and standard stock solutions stored for 1 week at −20 °C.

### Application to DDI pharmacokinetic study in rats

Sprague–Dawley rats, male/female, weighing from 250 ± 20 g, were purchased from the Experimental Animal Center of the Air Force Military Medical University (Xi’an, China). Rats were maintained at 50 ± 10% relative humidity under specific pathogen free conditions (12 h light/dark cycle) and suitable temperature (25 ± 3 °C). Before the experiment, all rats were fasted for 12 h and free access to water.

For pharmacokinetic study, the rats were randomly divided into three groups ($n = 8$). After overnight fasting, the first group was orally administrated with RIV (1 mg kg⁻¹), the second group was orally administrated with SIT (10 mg kg⁻¹) and the third group was orally administrated with RIV (1 mg kg⁻¹) plus SIT (10 mg kg⁻¹). Blood samples of 0.3 mL
from the suborbital venous plexus under ether anesthesia at 0, 5, 15, 30, 60, 120, 240, 360, 480, 720, 1,440 min, post dose. The samples were centrifuged at 12,000×g under 4 °C for 15 min to obtain plasma. The resulting plasma samples were transferred and kept frozen at −80 °C prior to analysis. A non-compartmental pharmacokinetic analysis was evaluated through Drug and Statistics version 3.0 (DAS 3.0, Mathematical Pharmacology Professional Committee of China, Shanghai, China). All data were imported into Prism software (GraphPad Software Inc. Version 7, San Diego, CA) for statistical comparison between different groups.

RESULTS & DISCUSSION

Method validation
The LC-MS/MS method was validated by determining the selectivity, linearity, precision, accuracy, extraction recovery, matrix effect and stability of the analytes.

Best resolution in terms of peak shape and strong signals were obtained with ammonium formate and formic acid containing mobile phase. The best chromatographic analysis was achieved on an Agilent Eclipse plus C18 column (2.1 × 100 mm, 3.5 μm) using 5 mM ammonium formate and 0.1% formic acid in water and 0.1% formic acid in acetonitrile (40:60, v/v) as mobile phase at a flow rate of 0.4 mL min⁻¹. The precursor [M + H]⁺ of RIV, SIT and IS were m/z 436.8, 407.7 and 459.8, respectively. Then the ion m/z 144.9, 173.8 and 442.8 were selected as product ions of RIV, SIT and IS in the product ion mode, respectively.

Representative chromatograms of blank plasma sample, blank plasma sample spiked with RIV and SIT (LLOQs) and IS, and pharmacokinetic samples were represented in Fig. 2. The retention times of analyte in LLOQ concentration level were variably within the acceptable limits of ±5% RSD. The result demonstrated that there was no interference with the determination of the RIV and SIT, granting good method selectivity.

Carry-over evaluation was performed in each analytical run to ensure that the accuracy and the precision of the proposed method were not affected. No enhancement in the response was observed in the double blank after subsequent injection of the highest calibration standard at the retention time of analyte.

The typical linear regression equation and r² values of the calibration curves can be expressed as follows: $y = 0.0516x + 0.1366$, $r^2 = 0.9992$ (RIV, $n = 7$), $y = 0.0841x + 1.5651$, $r^2 = 0.9923$ (SIT, $n = 7$). The LLOQ of RIV and SIT were 1 ng mL⁻¹ and 2 ng mL⁻¹, respectively. The accuracy and precision of LLOQs were presented in Table 1.

The precision (% RSD) of intra-day and inter-day were ranged from 3.4%–11.9% (RIV) and 5.9%–2.5% (SIT). The accuracy (% RE) ranged from −8.9% to 11.6% and −8.3%–12.3% as represented in Table 1. The accuracy and precision valued for all QC samples of analyte were within the acceptable criteria for bioanalytical guidelines.

The results of matrix effect and extraction recovery are shown in Table 2. The results indicating that the matrix effect of analytes was stable during the processing and detection procedure and the extraction recovery of this method was reproducible and consistent.

Three QC levels of RIV and SIT were used to evaluated the stability of the analytes under different storage conditions in rat plasma. As shown in Table 3, processed samples were placed at room temperature for 12 h, extracted samples were left at 4 °C for 24 h, after three freeze-thaw cycles and samples kept at −80 °C for one month were evaluated. In addition, standard solutions remained stable for at least 1 week when kept at −20 °C.

Pharmacokinetic DDI study in rats
The possible DDI between RIV and SIT were evaluated through comparative pharmacokinetic parameters in rats with separated and co-administration. After method development and validation, the method has been successfully applied to the pharmacokinetic study of the RIV (1 mg kg⁻¹), SIT (10 mg kg⁻¹) and their combination (RIV: 1 mg kg⁻¹, SIT: 10 mg kg⁻¹) after oral administration to rats. By using non-compartmental analysis, the key pharmacokinetic parameters and the mean plasma concentration-time curves were presented in Table 4 and Fig. 3. The results demonstrated that compared to the alone administration group, no significant statistical differences ($P > 0.05$) in plasma pharmacokinetic parameters were observed after the co-administration. Most of drugs are metabolized by cytochrome P450 enzymes in the intestine or liver. The inhibition or induction of P450 enzymes is the main cause of pharmacokinetic DDI interactions, drug transporters are also an important factor in the production of DDI interactions. RIV

Table 4. Main pharmacokinetic parameters of SIT and API after oral administration of RIV (1 mg kg⁻¹) and SIT (10 mg kg⁻¹) alone and their combination in SD rats ($n = 8$, Mean ± SD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RIV</th>
<th>RIV + SIT</th>
<th>SIT</th>
<th>RIV + SIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC₀→t (ng mL⁻¹·min⁻¹)</td>
<td>40453.8 ± 6394.1</td>
<td>41650.6 ± 8446.9</td>
<td>224323.3 ± 53196.5</td>
<td>247582.7 ± 21674.1</td>
</tr>
<tr>
<td>AUC₀→∞ (ng mL⁻¹·min⁻¹)</td>
<td>41844.4 ± 6000.6</td>
<td>47890.4 ± 13336.8</td>
<td>235380.6 ± 59124.2</td>
<td>251786.6 ± 19608.5</td>
</tr>
<tr>
<td>tₘax (min)</td>
<td>67.5 ± 21.2</td>
<td>60 ± 27.7</td>
<td>47.1 ± 16.1</td>
<td>63.7 ± 25.1</td>
</tr>
<tr>
<td>t₁/₂ (min)</td>
<td>290.8 ± 104.3</td>
<td>325.7 ± 80.1</td>
<td>337.4 ± 208.1</td>
<td>235.3 ± 139.5</td>
</tr>
<tr>
<td>Clz/F (L min⁻¹ kg⁻¹)</td>
<td>0.024 ± 0.003</td>
<td>0.022 ± 0.005</td>
<td>0.045 ± 0.011</td>
<td>0.04 ± 0.003</td>
</tr>
<tr>
<td>Cmax (ng mL⁻¹)</td>
<td>148.5 ± 23.5</td>
<td>134.8 ± 24.9</td>
<td>1313.4 ± 345.6</td>
<td>1445.3 ± 219.9</td>
</tr>
</tbody>
</table>
is mainly metabolized via CYP 3A4 or P-gp pathways. The main enzymes involved in a small amount of SIT metabolism are CYP3A4 and CYP2C8, at the same time, it will not inhibit or induce the CYP3A4, 2C8 or 2C9. The lack of DDI interactions between RIV and SIT might be attributed mainly to their differences in metabolic pathways and almost no inhibition or induction of CYP enzymes.

CONCLUSION

A simple, sensitive and accurate LC-MS/MS method was developed and validated for the simultaneous quantification of RIV and SIT in rat plasma. The pharmacokinetic DDI interactions of RIV and SIT in rats were reported for the first time. No significant DDI interactions between the two drugs have been observed in rats might be owing to their differences in metabolic pathways. The results of this study might be of certain reference value for the research of therapeutic drug monitoring and DDI interactions.

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