Development of a Simple LC-MS Assay for Determination of Regorafenib in Rat Plasma and its Application to a Pharmacokinetic Study

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SUMMARY. Regorafenib is the first small-molecule tyrosine kinase inhibitors (TKI) which exhibits improvement in progression-free survival and overall survival in refractory, heavily pretreated patients with metastatic colorectal cancer. A sensitive and selective LC-MS method for determination of regorafenib in rat plasma was developed. After addition of midazolam as internal standard (IS), protein precipitation by acetonitrile-methanol (9:1, v/v) was used as sample preparation. An electrospray ionization source was applied and operated in positive ion mode; selective ion monitoring (SIM) mode was used for quantification using target fragment ions \( m/z \) 483 for regorafenib and \( m/z \) 326 for the IS. Chromatographic separation was achieved on a Zorbax SB-C18 (2.1 mm x 50 mm, 3.5 μm) column with acetonitrile-0.1% formic acid in water as mobile phase with gradient elution. Calibration plots were linear over the range of 8-5000 ng/mL for regorafenib in rat plasma. Mean recoveries of regorafenib in rat plasma were in the range of 84.4-93.0%. RSD of intra-day and inter-day precision were both < 13%. The method was successfully applied to pharmacokinetic study of regorafenib after oral and intravenous administration in rats. The bioavailability of regorafenib was 92.3% in rats.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the USA, and the third leading cause of cancer death in both genders. The incidence and mortality have been steadily declining over the last decade, which is probably related to the improvements in the awareness, early detection and treatment of CRC. However, CRC-related mortality is continuing to increase in the underdeveloped countries with limited resources and healthcare 1. Combination chemotherapy (e.g. fluorouracil, capetabine and either irinotecan or oxaliplatin) with or without monoclonal antibody agents (e.g. bevacizumab or cetuximab and panitumumab) is the backbone of treatment for metastatic CRC (mCRC) 2-5.
However, drug-related adverse events and drug resistance may limit the potential of such treatments. Especially, many patients with advanced mCRC develop resistance to these agents, leaving very limited options for third-line treatment. Hence, there is continued need to develop new effective multiple signalling pathways agents that overcome this resistance.

Regorafenib (Stivarga, Fig. 1) is a small molecule inhibitor of multiple protein kinases, including a broad range of angiogenic, stromal and oncogenic kinases targeting VEGF receptors 1, 2 and 3, tyrosine kinase with immunoglobulin and EGF homology domain 2 (TIE-2), PDGF receptor-β, c-kit, ret, raf-1 and BRAF. It is recently approved for the treatment of mCRC in patients who have previously received all standard systemic anticancer treatments in the US, EU and Canada, and in patients with unresectable, advanced or recurrent CRC in Japan. Regorafenib is the first small-molecule tyrosine kinase inhibitors (TKI) exhibiting improvement in progression-free survival and overall survival in refractory, heavily pretreated patients with mCRC. The introduction of regorafenib could provide patients with CRC a new therapeutic option and help improve their survival and induce disease control.

There have been several literatures reported for determination of regorafenib in plasma. However, these methods were not full validated and very brief described. In this article, we describe a full validated analytical method for the quantitation of regorafenib in rat plasma in support of providing an attractive rationale of pharmacology features for this anticancer agent. Furthermore, the bioavailability of regorafenib was studied in rats.

**MATERIAL AND METHODS**

**Chemicals and reagents**

Regorafenib (purity > 98%) was a gift from Shanghai Institute of Pharmaceutical Industry (Shanghai, China). Midazolam (IS, purity > 98%) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). LC-grade acetonitrile and methanol were purchased from Merck Company (Darmstadt, Germany). Ultra-pure water was prepared by Millipore Milli-Q purification system (Bedford, MA, USA).

**Instrumentation and conditions**

Bruker Esquire HCT ion-trap mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) controlled by ChemStation software (Version B.01.03 [204], Agilent Technologies, Waldbronn, Germany).

Chromatographic separation was achieved on an Agilent Zorbax SB-C18 (2.1 mm × 50 mm, 3.5 µm) column at 40 °C, with acetonitrile-0.1% formic acid as mobile phase. The flow rate was 0.4 mL/min. A gradient elution programme was conducted for chromatographic separation with mobile phase A (0.1% formic acid), and mobile phase B (acetonitrile) as follows: 0-2.0 min (10-80% B), 2.0-5.0 min (80-80% B), 5.0-6.0 min (80-10% B), and 6.0-10.0 min (10-10% B).

Drying gas flow and nebuliser pressure was set at 6 L/min and 25 psi. Dry gas temperature and capillary voltage of the system were adjusted at 350 °C and 3500 V. LC-MS was performed with SIM mode using target ions at m/z 483 for regorafenib (Fig. 2a) and m/z 326 for midazolam (IS, Fig. 2b), in positive ion electrospray ionization interface, respectively.

**Calibration standards and quality control samples**

The stock solutions of regorafenib (1.0 mg/mL) was prepared in methanol-water (50:50) and midazolam (IS, 100 µg/mL) was prepared in methanol-water (50:50). Working solutions for calibration and controls were prepared from the stock solution by dilution using...
methanol. The 2.0 µg/mL working standard solution of IS was prepared from the IS stock solution by dilution using methanol. All of the solutions were stored at 4 °C and were brought to room temperature before use.

Regorafenib calibration standards were prepared by spiking blank rat plasma with appropriate amounts of the working solutions. Calibration plots were constructed in the range of 8-5000 ng/mL for regorafenib in rat plasma (8, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/mL). Quality-control (QC) samples were prepared by the same way as the calibration standards, three different plasma concentrations (12, 400, and 4000 ng/mL). The analytical standards and QC samples were stored at -20 °C.

**Sample preparation**

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 10 µL of the IS working solution (2.0 µg/mL) was added to 100 µL of collected plasma sample followed by the addition of 200 µL acetonitrile-methanol (9:1, v/v). The tubes were vortex mixed for 1.0 min. After centrifugation at 14900 g for 10 min, the supernatant (2 µL) was injected into the LC-ESI-MS system for analysis.

**Method validation**

The method was validated for selectivity, linearity, accuracy, precision, recovery and stability according to the literatures 15,16. Validation runs were conducted on three consecutive days. Each validation run consisted of one set of calibration standards and six replicates of QC plasma samples.

The selectivity of the method was evaluated by analyzing blank rat plasma, blank plasma spiked regorafenib and IS and a rat plasma sample.

Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of regorafenib to IS were plotted against analyte concentrations, and standard curves were well fitted to the equations by linear regression with a weighting factor of the reciprocal of the concentration (1/x) in the concentration range of 5-5000 ng/mL. The LLOQ was defined as the lowest concentration on the calibration curves.

To evaluate the matrix effect, blank rat plasma were extracted and then spiked with the analyte at 12, 400, and 4000 ng/mL. The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, and this peak area ratio is defined as the matrix effect (ME). The matrix effect of IS was evaluated at the concentration (200 ng/mL) in the same manner.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (12, 400, and 4000 ng/mL) in three validation days. The precision was expressed by RSD.

The recovery of regorafenib was evaluated by comparing peak area of extracted QC samples with those of reference QC solutions reconstituted in blank plasma extracts (n = 6). The recovery of the IS was determined in a similar way.

Carry-over was assessed following injection of a blank plasma sample immediately after 3 repeats of the upper limit of quantification (ULOQ) and the response was checked 17.

The stabilities of regorafenib in rat plasma were evaluated by analyzing three replicates of plasma samples at the concentrations of 12 and 4000 ng/mL, which were exposed to different conditions. These results were compared with those freshly prepared plasma samples. The short-term stability was determined after the ex-
posure of the spiked samples at room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) in the HPLC autosampler at room temperature for 24 h. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (-20 to 25 °C) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at -20 °C for 30 days. The stability of the IS (200 ng/mL) was evaluated in a similar way.

**Pharmacokinetic study**

Male Sprague-Dawley rats (200-220 g) were obtained from Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China) used to study the pharmacokinetics of regorafenib. All eighteen rats were housed at Laboratory Animal Center of Wenzhou Medical University. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical University and were in accordance with the Guide for the Care and Use of Laboratory Animals. Diet was prohibited for 12 h before the experiment but water was freely available. Blood samples (0.3 mL) were collected from the tail vein into heparinized 1.5 mL polythene tubes at 0.0833, 0.25, 0.75, 1.25, 2, 3, 4, 6, 8, 10, 12, 24, 48, 60, and 72 h after oral or intravenous administration of regorafenib. The samples were immediately centrifuged at 3000 g for 10 min. The plasma obtained (100 µL) was stored at -20 °C until analysis. Plasma regorafenib concentration versus time data for each rat was analyzed by DAS (Drug and statistics) software (Version 3.0, Wenzhou Medical University, China).

**RESULTS AND DISCUSSION**

**Method Development**

The liquid chromatographic conditions were developed to separate as many interfering compounds as possible from the analyte and IS. Different columns, such as Zorbax SB-C18 (150 mm x 2.1 mm, 5 µm), Zorbax SB-C18 (100 mm x 2.1 mm, 3.5 µm) and Zorbax SB-C18 (50 mm x 2.1 mm, 3.5 µm) were compared for chromatographic separation. The Zorbax SB-C18 (50 mm x 2.1 mm, 3.5 µm) column demonstrated proper retention time \( t_R = 3.3 \) min for IS, and \( t_R = 4.8 \) min for regorafenib) and better peak shape than other columns. The mobile phase played a critical role in achieving good chromatographic behavior and appropriate ionization. Various combinations of acetonitrile, methanol, water and 0.1% formic acid in with water changed content of each component were investigated and compared to identify the optimal mobile phase. Acetonitrile was chosen as the organic phase because it could provide sharper peak shape and lower pump pressure compared with methanol. Formic acid (0.1%) added into the water could improve the sensitivity, therefore consist of acetonitrile and 0.1% formic acid in water was chosen as mobile phase.

**Selectivity and matrix effect**

Fig. 3 shows the typical chromatograms of a blank plasma sample, and a plasma sample. No interfering endogenous substance was observed at the retention time of the analyte and IS. The ME for regorafenib at concentrations of 12, 400, and 4000 ng/mL were measured to be 89.7 ± 9.6, 87.3 ± 4.4 and 85.4 ± 6.9% (n = 6), respectively. The ME for IS (200 ng/mL) was 95.7 ± 3.5% (n = 6). As a result, the ME from plasma could be acceptable in this method.

**Calibration curve and sensitivity**

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 8-5000 ng/mL for regorafenib in rat plasma. Typical equation of the calibration curve was: \( y = 0.00119x + 0.06038, r = 0.9970, \)
where y represents the ratios of regorafenib peak area to that of IS and x represents the plasma concentration. The LLOQ for the determination of regorafenib in rat plasma was 8 ng/mL. The precision and accuracy at LLOQ were 8.0 and 87.0%, respectively. The LOD, defined as a signal/noise ratio of 3, was 2.5 ng/mL for regorafenib in rat plasma.

**Precision, accuracy and recovery**

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three validation days. Intra-day precision was 12% or less and the inter-day precision was 13% or less at each QC level. The accuracy of the method ranged from 84.2 to 103.7% at each QC level. Mean recoveries of regorafenib were better than 84.4%. The recovery of the IS (200 ng/mL) was 95.5 ± 3.3%. Assay performance data was presented in Table 1.

**Carry-over**

None of the analytes showed any significant peak (≥ 20% of the LLOQ and 5% of the IS) in blank samples injected after the ULOQ samples. Adding 4 extra min to the end of the gradient elution effectively washed the system between samples thereby eliminating carry-over 17.

**Stability**

The auto-sampler, room temperature, freeze-thaw and long-term (30 days) stability results indicated that the analyte was stable under the storage conditions described above since the bias in concentrations were within ± 12% of their nominal values, and the established method was suitable for the pharmacokinetic study.

**Table 1**. Precision, accuracy and recovery for regorafenib of QC sample in rat plasma (n = 6).

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>RSD (%)</th>
<th>Accuracy (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
<td>Intra-day</td>
</tr>
<tr>
<td>8</td>
<td>8.0</td>
<td>12.1</td>
<td>87.0</td>
</tr>
<tr>
<td>12</td>
<td>11.9</td>
<td>10.4</td>
<td>90.0</td>
</tr>
<tr>
<td>400</td>
<td>8.9</td>
<td>9.5</td>
<td>100.6</td>
</tr>
<tr>
<td>4000</td>
<td>8.2</td>
<td>6.9</td>
<td>96.5</td>
</tr>
</tbody>
</table>

**Table 2**. The main pharmacokinetic parameters after oral and intravenous administration regorafenib in eighteen rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dosage</td>
<td></td>
<td>1 mg/kg, iv</td>
<td></td>
<td>5 mg/kg, po</td>
<td></td>
<td>20 mg/kg, po</td>
<td></td>
</tr>
<tr>
<td>AUC(0-1)</td>
<td>ng/mL*h</td>
<td>9432.9</td>
<td>2458.1</td>
<td>43513.4</td>
<td>6514.6</td>
<td>138716.7</td>
<td>50608.1</td>
</tr>
<tr>
<td>AUC(0-24)</td>
<td>ng/mL *h</td>
<td>10215.0</td>
<td>2499.3</td>
<td>43570.6</td>
<td>6559.9</td>
<td>153262.2</td>
<td>55343.3</td>
</tr>
<tr>
<td>MRT(0-1)</td>
<td>h</td>
<td>16.9</td>
<td>1.4</td>
<td>14.1</td>
<td>0.2</td>
<td>22.9</td>
<td>2.5</td>
</tr>
<tr>
<td>MRT(0-24)</td>
<td>h</td>
<td>24.3</td>
<td>4.5</td>
<td>14.2</td>
<td>0.2</td>
<td>31.1</td>
<td>6.6</td>
</tr>
<tr>
<td>t1/2z</td>
<td>h</td>
<td>23.9</td>
<td>9.0</td>
<td>6.5</td>
<td>1.4</td>
<td>22.4</td>
<td>8.7</td>
</tr>
<tr>
<td>T max</td>
<td>h</td>
<td>0.2</td>
<td>0.3</td>
<td>9.0</td>
<td>2.6</td>
<td>10.7</td>
<td>2.1</td>
</tr>
<tr>
<td>C max</td>
<td>ng/mL</td>
<td>669.1</td>
<td>127.0</td>
<td>2254.3</td>
<td>495.3</td>
<td>5172.8</td>
<td>772.1</td>
</tr>
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</table>

Bioavailability 92.3%
Application

The method was applied to a pharmacokinetic study in rats. The mean plasma concentration–time curve after oral (5 and 20 mg/kg) and intravenous (1 mg/kg) administration of regorafenib was shown in Fig. 4. The main pharmacokinetic parameters from non-compartment model analysis were summarized in Table 2. The pharmacokinetic of regorafenib and its two active metabolites in humans has been described in three recent papers 12,14,17, however the bioavailability of regorafenib has not been reported.

CONCLUSION

A sensitive and selective LC-MS method for determination of regorafenib in rat plasma was developed and validated. A time-saving protein precipitation procedure made the method readily applicable in a further clinical study. The LC-MS method successfully applied to a pharmacokinetic study of regorafenib after oral and intravenous administration in rats. The bioavailability of regorafenib was 92.3% in rats.

REFERENCES