Determination and Pharmacokinetic Study of Apatinib in Rat Plasma by UPLC

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Abstract

In this study, a simple, sensitive, and robust analytical method based on ultra-performance liquid chromatography (UPLC) has been developed for the determination of apatinib in rat plasma using carbamazepine as internal standard (IS). After sample preparation by a simple liquid–liquid extraction, chromatography was performed on an Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm particle size) and total run time was 2.0 min. The method was linear over the concentration range 5–1000 ng/mL with a lower limit of quantification of 5 ng/mL. Inter- and intraday precision were all within 6.5% and the accuracy was ≤3.5%. Recoveries of apatinib and IS were >80%. Stability studies showed that apatinib was stable under a variety of storage conditions. The method was successfully applied to a pharmacokinetic study involving oral administration of apatinib to rats.

Introduction

Apatinib, (N-[4-(1-cyano-cyclopentyl)phenyl]-2-(4-pyridylmethyl) amino-3-pyridine carboxamide) Figure 1, is a novel tyrosine kinase inhibitor that selectively inhibits the vascular endothelial growth factor receptor-2 with an IC₅₀ of ~ 1 nM (1). It is an orally bioavailable, small molecule agent which is thought to inhibit angiogenesis in cancer cells; specifically, apatinib inhibits VEGF-mediated endothelial cell migration and proliferation thus blocking new blood vessel formation in tumor tissue (2, 3). This agent also mildly inhibits c-Kit and c-SRC tyrosine kinases (3, 4). Apatinib was first synthesized by Advenchen Laboratories in California, USA and is currently being developed by Jiangsu Hengrui Medicine (China) (1). It is an investigational cancer drug currently undergoing phase II/III clinical trials in China as a potential targeted treatment for metastatic gastric carcinoma, metastatic breast cancer and advanced hepatocellular carcinoma (1, 5–8). These clinical trials demonstrate that apatinib has potential antitumor activity across a broad range of advanced solid tumors.

Now, several analytical methods exist for quantifying apatinib in biological samples. The majority of them involve liquid chromatography–tandem mass spectrometry (LC–MS–MS) (9) and UPLC-UV/Q-TOF/MS (2). However, in pharmacokinetic studies, the proposed method should be simple and able to process hundreds of samples in a limited time. While some of these methods are successful in the determination of apatinib, these methods have several limitations. These include a long chromatographic running time, complicated, time-consuming sample pretreatment procedures and expensive instruments. Therefore, to characterize the pharmacokinetic properties of apatinib, it is very necessary to develop an accurate and selective bioanalytical method for the determination of apatinib in plasma.

As a result of recent advances in analysis techniques, ultra-performance liquid chromatography (UPLC) shows a dramatic enhancement in speed, resolution as well as the sensitivity of analysis by using particle size <2 µm and the system is operational at higher pressure, while the mobile phase could be able to run at greater linear velocities when compared with HPLC. This technique is considered as a new focal point in field of liquid chromatographic studies (10–14). Thus, in the present work a highly rugged, selective and rapid UPLC method has been developed and fully validated as per the USFDA guidelines for measurement of apatinib in rat plasma using carbamazepine as internal standard (IS). The method offered a small turnaround time for analysis and high sensitivity for the analyte, and utilized only 200 µL rat plasma for sample processing using a simple one-step liquid–liquid extraction by ethyl acetate. The method was free from endogenous matrix interference and was successfully applied to a pharmacokinetic study in rats.
A

B

Figure 1. The chemical structures of apatinib and IS in the present study: (A) apatinib; (B) carbamazepine (IS).

Experimental

Chemicals and reagents

Apatinib (purity >98%) was purchased from Shanghai Canspec Scientific Instruments Co., Ltd (Shanghai, China). Carbamazepine (IS purity >98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol, trifluoroacetic acid (TFA) and ethyl acetate were of LC grade and were purchased from Merck Company (Darmstadt, Germany). Ultrapure water, prepared by a Milli-Q Reagent system (Millipore, MA, USA), was used throughout the study.

UPLC conditions

UPLC analysis was performed with a Waters (Milford, MA, USA) Acquity UPLC system equipped with a binary solvent manager, sample manager, column-heating compartment and tunable UV detector. This system was controlled by Waters Empower software. The analytes were separated on a 2.1 × 50 mm, 1.7 µm particle size, Acquity UPLC BEH C18 column. The analysis was achieved with a mobile phase of solvent A (water) : solvent B (0.1% TFA in water) : solvent C (acetonitrile) = 45 : 20 : 35. The flow rate was 0.30 mL/min and total run time was 2.0 min. The column temperature was set at 30°C. UV absorbance detection was performed at 286 nm (t1 = 2.0 min). The sample manager was operated at 8°C and the sample injection volume was 5 µL in full-loop mode.

Standard solutions, calibration standards and quality control sample

Standard stock solutions of apatinib and carbamazepine (IS) were prepared in methanol at 1 mg/mL. Then the stock solutions were diluted with methanol to obtain fresh standard working solution. Calibration standards were prepared by adding corresponding working solutions in drug-free plasma. The final concentrations of apatinib in plasma were 5, 10, 20, 50, 100, 200, 500, 1000 ng/mL, and IS was 20 ng/mL, respectively.

Low-, mid- and high-level quality control (QC) samples containing 10, 250 and 800 ng/mL of apatinib were prepared in a manner similar to that used for the preparation of calibration samples. All stock solutions, working solutions, calibration standards and QC samples were stored at −20°C and were brought to room temperature before analysis.

Sample preparation

Before analysis, the plasma samples were thawed to room temperature. In a 1.5-mL centrifuge tube, an aliquot of 20 µL of the IS working solution (20 ng/mL) and 100 µL of NaOH (1 mol/L) were added to 200 µL of collected plasma sample followed by the addition of 1.0 mL ethyl acetate. The tubes were vortex mixed for 2.0 min. After centrifugation at 13,000 × g for 10 min, the supernatant organic layer was transferred into a 1.5-mL centrifuge tube and dried under nitrogen stream at 40°C. The dried residue was reconstituted in 75 µL of mobile phase and a 5 µL aliquot of this was injected into UPLC system for the analysis.

Method validation

The method was validated for specificity, linearity, accuracy, precision, recovery and stability according to the guidelines set by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) (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 specificity of the method was tested by analyzing six different drug-free plasma samples from six rats. Each blank sample was handled by the procedure described in Section 2.4 and confirmed that endogenous substances did not interfere with the analyte and the IS.

Calibration curves (y = a + bx) were generated by plotting the peak area ratio (y) of the analyte to IS versus the nominal concentration (x) of the analyte with weighted (1/x2) least square linear regression. The LLOQ was defined as the lowest concentration on the calibration curve where a signal to noise (S/N) was at least 10. The acceptance criteria for accuracy and precision of calibration curve data were 80–120% of the nominal concentrations and relative standard deviation (RSD) of ±20% of the nominal concentration at the LLOQ, respectively.

The precision and accuracy of the method were assessed by assaying QC samples at three different concentrations (10, 250 and 800 ng/mL). Six replicates of each level were analyzed in 1 day or over three consecutive days. Accuracy was expressed as the percentage of observed value to true value, and precision was expressed as the percentage RSD, %.

The recovery showed an ability to extract the analyte from the test biological samples. Recovery of apatinib by ethyl acetate extraction was determined at three different levels (10, 250 and 800 ng/mL) (n = 6). The recovery of apatinib was evaluated by comparing the peak area of extracted QC samples with those of reference QC solutions reconstituted in blank plasma extracts. The recovery of IS was determined similarly.

The robustness was evaluated when QC samples were analyzed through altered chromatography parameters. These alterations correspond to mobile phase flux (0.30–0.40 mL/min) and column temperature (30°C–35°C). The extraction parameters were checked during the method development in accordance with % recovery, and then the best condition was chosen.

Carry-over was assessed following injection of a blank plasma sample immediately after three repeats of the upper limit of quantification (ULOQ), after which the response was checked for accuracy.

To ensure the reliability of the results with regard to handling and storing of the plasma samples, stability studies were carried out on QC samples at concentrations of 10, 250 and 800 ng/mL. The protocol for the stability assay comprised freeze–thaw stability, short- and long-term stability. During the freeze–thaw stability assay, the samples were thawed at the ambient temperature without any assistance, and then kept in the freezer (−20°C) again for minimum of 12 h before carrying out the next thawing, until accomplished three freeze and thaw cycles. The QC samples stored at room temperature for 12 h were evaluated for short-term stability. The long-term stability was determined by analyzing the QC plasma samples after 42 days of storage of −20°C. The resulted stabilities for these samples were then compared with those of the freshly prepared samples.

Application to a pharmacokinetic study

Male Sprague–Dawley rats (180–220 g) were obtained from Laboratory Animal Center of Wenzhou Medical University (Wenzhou,
used to study the pharmacokinetics of apatinib. All six rats were housed at Wenzhou Medical University Laboratory Animal Research Center. 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 (0.5 mL) were collected from the tail vein into heparinized 1.5 mL polythene tubes at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 h after oral administration of apatinib (40 mg/kg). The samples were immediately centrifuged at 4000 \( \times g \) for 8 min. The plasma obtained (200 µL) was stored at \(-20^\circ C\) until analysis. Plasma apatinib concentration versus time data for each rat was analyzed by DAS (Drug and statistics) software (Version 2.0, Wenzhou Medical University, China).

Results

Assay validation

Figure 2 shows the representative chromatograms of blank plasma, a plasma sample spiked with apatinib and IS and a plasma sample obtained at 0.5 h after oral administration of apatinib (40 mg/kg). No interference was found in the chromatograms of plasma samples at the retention times of apatinib or IS, which were 1.284 and 1.478 min, respectively, and the total running time for each sample was 2.0 min.

The assay was linear over the concentration range of 5–1000 ng/mL with a typical calibration curve equation of \( y = 0.179x - 0.00361 \) and correlation coefficient of \( r^2 = 0.999 \). The LLOQ of apatinib in rat plasma was found to be 5 ng/mL, which are sufficient for the pharmacokinetics study. The precision and accuracy at LLOQ were 9.7 and 104.9%, respectively.

The intra- and interassay variations were found to be within the accepted limits. The intra- and interday precision of apatinib at three QC concentrations, as presented in Table I, was <6.5%. Assay accuracy was found to be within ±3.5%. The results indicated that the present method was reliable and reproducible for the quantitative determination of apatinib.

Mean recoveries of apatinib at concentrations of 10, 250 and 800 ng/mL were measured to be 80.6 ± 4.1, 81.4 ± 2.9 and 82.1 ± 1.6% (\( n = 6 \)), respectively. The recovery of the IS (20 ng/mL) was measured to be 82.6 ± 3.2%. The results indicate reasonable recoveries with no obvious suppression or enhancement of ionization of either apatinib or IS. In addition, the results of robustness portray that the method possesses adequate chromatography robustness. None of the analytes showed any significant peak in blank samples injected after the ULOQ samples.

The autosampler, room temperature, freeze–thaw and long-term (42 days) stability results indicated that the analyte was stable under the storage conditions described above since the bias in concentration was within ±15% of nominal values, and the established method was suitable for the pharmacokinetic study (Table II). This result is within the FDA acceptance criteria.

Application of the method in a pharmacokinetic study

The method was applied to a pharmacokinetic study in rats. The mean plasma concentration–time curve after oral administration of 40 mg/kg apatinib was shown in Figure 3. The main pharmacokinetic parameters from non-compartment model analysis were summarized in Table III.

Discussion

Method development and optimization

In the present study, several columns (Acquity BEH C18, Ultimate XB-C8, Ultimate XB-C18, Thermo Accucore XL C18, Hanbon Dubhe C18, and Shiseido Proteonavi C4) were compared on the basis of the peak shape and retention times. Finally, the Acquity BEH C18 column was chosen to determine apatinib in rat plasma for good peak shape and acceptable retention times. The formulation of the mobile phase is another important factor for separating apatinib from IS and endogenous components. In order to achieve a suitable mobile phase, several solvent mixtures were tested, including acetonitrile, methanol and water of various ratios. Under these conditions, the

Table I. Precision and Accuracy of Method for the Determination of Apatinib in Rat Plasma (\( n = 6 \))

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Concentration added (ng/mL)</th>
<th>Intraday precision</th>
<th>Interday precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Apatinib</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>9.9 ± 0.6</td>
<td>6.5</td>
</tr>
<tr>
<td>250</td>
<td></td>
<td>257.2 ± 12.8</td>
<td>5.0</td>
</tr>
<tr>
<td>800</td>
<td></td>
<td>815.0 ± 22.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>
An UPLC method for the determination of apatinib in rat plasma was developed and validated. To the best of our knowledge, this is the first report of the determination of apatinib level in rat plasma using an UPLC method. The method offered sample preparation with a simple liquid–liquid extraction of plasma protein by ethyl acetate and shorter run time of 2.0 min. The method meets the requirement of high sample throughput in bioanalysis and has been successfully applied to the pharmacokinetic study of apatinib in rats.

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Conflict of interest statement. None declared.

**References**

and derivatives in vegetable oils, margarines and supplement capsules using pentafluorophenyl column; *Talanta*, (2014); 130: 299–306.