Gradient Elution LC-MS/MS Determination of Gefitinib in Rat Plasma and its Pharmacokinetic Study

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SUMMARY. A sensitive and simple liquid chromatography/electrospray mass spectrometry (LC-MS/MS) method for determination of gefitinib in rat plasma using one-step protein precipitation was developed. After addition of estazolam as internal standard (IS), protein precipitation by acetonitrile was used as sample preparation. Chromatographic separation was achieved on an SB-C18 (2.1 mm × 50 mm, 3.5 μm) column with methanol-0.1% formic acid as mobile phase with gradient elution. Electrospray ionization (ESI) source was applied and operated in positive ion mode; multiple reaction monitoring (MRM) mode was used to quantification using target fragment ions m/z 447.0→127.7 for gefitinib and m/z 294.7→266.8 for the IS. Calibration plots were linear over the range of 5-2000 ng/mL for gefitinib in rat plasma. Lower limit of quantification (LLOQ) for gefitinib was 5 ng/mL. Mean recovery of gefitinib from plasma was in the range 78.6-93.0%. RSD of intra-day and inter-day precision were both less than 15%. This developed method is successfully used in pharmacokinetic study of gefitinib in rats.

INTRODUCTION

Gefitinib, 4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy) quinazoline (ZD1839, Iressa®) (Fig. 1) is an orally active, selective EGFR-TK inhibitor (EGFR-TKI) that causes complete inhibition of EGF-stimulated EGFR autophosphorylation in cell lines. In preclinical studies, gefitinib has demonstrated antitumor activity against a variety of human cancer cell lines expressing EGFR, including lung, ovarian, breast, and colon 1,2. EGFRs are frequently overexpressed in non-small cell lung cancer (NSCLC). Lung cancer is a major cause of morbidity as the overall 5-year survival is at a dismal 15% 3. Consequently, EGFR inhibitors such as gefitinib are attractive therapeutic agents as they provide a targeted treatment approach by interfering with the signal transduction pathway implicated in cancer cell proliferation 4.

There have several high performance liquid chromatography-mass spectrometry (LC-MS) methods been published for the determination of gefitinib and its metabolites. A sensitive and selective method was performed using HPLC with ultraviolet detection (UV) 5. A simple and rapid method was developed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and fluorescence detection (FD) 6. A rapid and simple LC-ESI-MS/MS method for the determination of gefitinib and its metabolites in rat plasma was achieved by gradient elution 7. Another report described a sensitive and rapid LC-MS/MS method for the determination of gefitinib in rat plasma using one-step protein precipitation 8.

KEY WORDS: Gefitinib, Gradient elution, LC-ESI-MS, Rat plasma.

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of gefitinib in different biological matrices. Liquid chromatography in combination with mass spectrometry (LC-MS) is a well established analytical tool in many fields of application. However, the high selectivity of LC-MS does not guarantee the effective elimination of interferences from endogenous impurities. An operational strategy is to modify the chromatographic conditions to shift the retention time of the target analytes far away from the area affected by signal suppression or enhancement. In this paper, an easy and effective way to do this adjustment is to modify gradient conditions. A simple and sensitive LC-ESI-MS method for the determination of gefitinib in rat plasma using one-step protein precipitation with gradient elution was developed and validated. The developed method was successfully applied to pharmacokinetic studies of gefitinib in rats following oral administration.

**MATERIAL AND METHODS**

**Chemicals and Reagents**

Gefitinib (purity > 98.0 %) was purchased from Cayman Chemical Company (Michigan, USA) and estazolam (purity > 98.0 %) was purchased from Institute of Forensic Science under the Ministry of Justice (Shanghai, China). LC-grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). While LC-grade formic acid was Tedia Company (Cincinnati, USA). Ultra-pure water was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA).

**Instrumentation and Conditions**

All analysis was performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment, and a Bruker Esquire HCT ion-trap mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software (Version B.01.03 [204], Agilent Technologies, Waldbronn, Germany).

<table>
<thead>
<tr>
<th>Load time (min)</th>
<th>Pump flow (µL/min)</th>
<th>Formic acid %</th>
<th>Methanol %</th>
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</thead>
<tbody>
<tr>
<td>0.0</td>
<td>300</td>
<td>90</td>
<td>10</td>
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<tr>
<td>1.5</td>
<td>300</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>6.0</td>
<td>300</td>
<td>20</td>
<td>80</td>
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<td>10</td>
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<tr>
<td>10.0</td>
<td>300</td>
<td>90</td>
<td>10</td>
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</table>

*Table 1. HPLC gradient for detection of gefitinib in rat plasma.*

Chromatographic separation was achieved on an Agilent Zorbax SB-C18 (2.1 mm × 50 mm, 3.5 µm) column at 30 °C, with methanol-0.1 % formic acid as mobile phase. The flow rate was 0.3 mL/min. The HPLC gradient profile can be seen in Table 1.

Drying gas flow and nebuliser pressure was set at 6 L/min and 20 psi. Dry gas temperature and capillary voltage of the system were adjusted at 350 °C and 3,500 V, respectively. LC-MS was performed with MRM mode was used to quantification using target fragment ions m/z 447.0→127.7 for gefitinib with the fragmentation energy of 0.26 v and m/z 294.7→266.8 for estazolam (IS) with the fragmentation energy of 0.30 v, in positive ion electrospary ionization interface.

**Calibration Standards and Quality Control Samples**

Individual stock solutions of gefitinib (1.0 mg/mL) and estazolam (internal standard, IS) (100 µg/mL) were prepared in methanol. Working solutions for calibration and controls were prepared from the stock solution by dilution using methanol. 2.0 µg/mL working standard solution of IS was prepared by dilution of the IS stock solution with methanol. All of the solutions were stored at 4 °C and were brought to room temperature before use.

Gefitinib calibration standards were prepared by spiking blank rat plasma with appropriate amounts of the working solutions. Calibration plots were constructed in the range 5-2000 ng/mL for gefitinib in rat plasma (concentrations 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL). Quality-control (QC) samples were prepared by the same way as the calibration standards, three different plasma concentrations (10, 100, and 1000 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 internal standard working solution (2.0 µg/mL) was
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added to 100 µL of collected plasma sample followed by the addition of 200 µL acetonitrile. The tubes were vortex mixed for 0.5 min. After centrifugation at 14900 g for 10 min, the supernatant (5 µL) was injected into the LC-MS/MS system for analysis.

Method Validation

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

Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of gefitinib 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 5-2000 ng/mL.

To evaluate the matrix effect, blank rat plasma were protein precipitated and then spiked with the analyte at 10, 100, and 1000 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 working 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 (10, 100, and 1000 ng/mL) in three validation days. The precision was expressed by coefficient of variation (RSD) and the accuracy by relative error (RE).

The recoveries of gefitinib at three QC levels (n = 6) were determined by comparing peak area of the analytes in QC samples to which the analytes were added post-protein precipitation at equivalent concentrations. The recovery of the IS was determined in a similar way.

Stability of gefitinib in rat plasma was evaluated by analyzing three replicates of plasma samples at the concentrations of 10, 100, and 1000 ng/mL, which were exposed to different conditions. The short-term stability was determined after the exposure 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.

Pharmacokinetic study

Male Sprague-Dawley rats (200–220 g) were obtained from Laboratory Animal Center of Wenzhou Medical College (Wenzhou, China) were used to study the pharmacokinetics of gefitinib. All six rats were housed at Wenzhou Medical College Laboratory Animal Research Center. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals. Animals were housed under controlled conditions (25 ± 1 °C, RH 55 ± 10 %) with a natural light–dark cycle. They were allowed to adapt to the housing environment for at least 1 week before the study. 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, 0.16667, 0.33333, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36, and 48 h after oral administration of gefitinib (30 mg/kg). The samples were immediately centrifuged at 2,500 g for 5 min. The plasma obtained (100 µL) was stored at -20 °C until analysis. Plasma gefitinib concentration versus time data for each rat was analyzed by DAS software (Version 2.0, Wenzhou Medical College, China).

RESULTS AND DISCUSSION

Method Development

The feasibility of electrospray in positive and negative ion modes of detection was evaluated during the early stages of assay development. It was found that electrospray ionization with positive ion detection resulted in the better signal-to-noise ratio. The MS detector parameters were assessed by infusion of a standard solution directly into the ESI source. In order to optimize MS-MS conditions, the daughter ion spectrum of the [M + H]+ ion was recorded by ramping the capillary voltage and the fragmentation energy. The daughter ion spectrum is shown in Fig. 1. The most abundant fragment was detected at m/z 127.7 with the capillary voltage of 3,500 v and the fragmentation energy of 0.26 v for gefitinib. Therefore, the m/z 447.0→127.7 transition was selected for further LC-MS-MS analysis in MRM mode.

Selectivity and Matrix Effect

Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with gefitinib and IS, and a plasma sam-
ple. No interfering endogenous substances were observed at the retention times of the analyte and IS.

The ME for Gefitinib at concentrations of 10, 100, and 1000 ng/mL were measured to be 102.2 ± 9.4, 85.5 ± 5.9, and 88.7 ± 2.6 % (n = 6), respectively. The ME for IS (200 ng/mL) was 92.3 ± 6.8 % (n = 6). As a result, ME from plasma was negligible in this method.

**Calibration Curve and Sensitivity**

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 5-2000 ng/mL for gefitinib in rat plasma. Typical equation of the calibration curve was: 

$$ y = (0.0012 ± 0.0001) x - (0.0139 ± 0.0129), \quad r = 0.9963 ± 0.0040 \quad (n = 3), $$

where y represents the ratios of gefitinib peak area to that of IS and x represents the plasma concen-

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<tr>
<th>Concentration (ng/mL)</th>
<th>RSD(%)</th>
<th>RE(%)</th>
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<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>10</td>
<td>14.2</td>
<td>12.4</td>
</tr>
<tr>
<td>100</td>
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</tr>
<tr>
<td>1000</td>
<td>2.2</td>
<td>1.8</td>
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</table>

Table 2. Precision and accuracy for gefitinib of quality control sample in rat plasma (n=6).
A sensitive, simple and specific LC-MS/MS method with gradient elution for the determination of gefitinib in rat plasma was developed and validated over the concentration range of 5-2000 ng/mL. This chromatographic conditions of gradient elution is successfully shift the retention time of the target analytes far away from matrix effect. The method was validated to meet the requirements for pharmacokinetic determination of the gefitinib in rat plasma.

**REFERENCES**