Original Paper

A Rapid and Simple UHPLC-UV Method for Quantitative Determination of Erlotinib and Its Active Metabolite OSI-420 in Human Serum, and Its Application in a Non-Small Cell Lung Cancer Patient

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Abstract
We developed a rapid and simple UHPLC-UV method for quantitative determination of erlotinib and its metabolite, OSI-420, in order to monitor their serum levels in patients with non-small cell lung cancer (NSCLC). Erlotinib and OSI-420 were extracted from 100 μL of human serum by liquid–liquid extraction using t-butyl methyl ether. The analytes were separated on Inertsil ODS-3 (100 mm × 2.1 mm I.D., 2 μm) as an analytical column using 20 mM potassium phosphate buffer (pH 2.5)/acetonitrile (74:26, v/v) as the mobile phase at a flow rate of 0.6 mL/min, and monitored at a UV wavelength of 345 nm. This method covered a linear concentration range of 6–6000 ng/mL for erlotinib and 6–2000 ng/mL for OSI-420, respectively (r > 0.999). The intra- and inter-day precisions of the analysis were < 6.8%, and the accuracy was ± 7.4%. This method has been successfully applied to measure erlotinib and OSI-420 in the serum of an NSCLC patient.

Keywords: Erlotinib; OSI-420; Human serum; UHPLC-UV

1. Introduction
Erlotinib is an oral molecularly targeted drug used for treatment of patients with advanced non-small cell lung cancer (NSCLC) or pancreatic cancer [1,2]. Erlotinib selectively inhibits tyrosine kinase activity of epidermal growth factor receptor (EGFR), which is overexpressed in tumor cells. Because the clinical dose of erlotinib (150 mg once a day) for treatment of NSCLC is the same as the maximum tolerated dose, serious adverse effects such as skin rash, diarrhea, and hepatic dysfunction are observed at high frequency [3,4]. Several factors may contribute to inter-individual variability in erlotinib pharmacokinetics, making development of such serious adverse effects difficult to predict. The bioavailability of erlotinib is influenced by gastric pH and the activities of the multidrug efflux transporter breast cancer resistance protein (ABCG2) and P-glycoprotein (ABCB1) in small intestinal mucosa [5]. Erlotinib is metabolized primarily in the liver, where it undergoes oxidative metabolism, mediated by cytochrome P450 3A4 (CYP3A4), to yield the major active oxidative metabolite OSI-420 [6,7]. In clinical practice, erlotinib is frequently used in combination with other drugs, so adverse effects or therapeutic efficacy of erlotinib treatment could also be influenced by variations in erlotinib.

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pharmacokinetics due to drug–drug interactions [8-10]. Therefore, in order to understand the correlation between the pharmacokinetics of erlotinib and its adverse effects and therapeutic efficacy, it is essential to assess the concentration–time profiles of both erlotinib and OSI-420.

Several analytical methods have been developed using liquid chromatography–tandem mass spectrometry (LC/MS/MS) [11-17] or high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [3,18,19] to determine the concentrations of erlotinib and/or OSI-420 in human biological samples. LC/MS/MS methods are highly selective and sensitive, but due to their cost and complexity, they are not available in most hospital laboratories. On the other hand, HPLC-UV methods are more commonly used. In addition, the use of ultra-high-performance liquid chromatography (UHPLC) results in shorter analysis times, which is important for clinical analysis, allowing easy and reproducible routine analysis. Therefore, we postulated that UHPLC-UV would be an appropriate method for determining erlotinib and OSI-420 concentrations in patient serum.

In this study, we sought to develop and validate an UHPLC-UV method for analysis of erlotinib and OSI-420 in order to monitor their serum concentrations in NSCLC patients. Here, we show that this UHPLC-UV method can be performed within 5 min, which is important for routine analysis. This validated method was applied successfully to analyze the serum from a patient.

2. Materials and methods

2.1. Chemicals

Erlotinib (> 98% purity), OSI-420 (> 98% purity), and 4-methyl erlotinib [OSI-597 (> 98% purity), used as an internal standard (IS) for erlotinib and OSI-420] were purchased from Toronto Research Chemicals (Toronto, Ont., Canada). All other chemicals were analytical grade, and solvents were HPLC or LC/MS grade. Drug-free (blank) human serum was purchased from Biopredic International (Saint-Grégoire, France). Pooled blank serum was prepared by mixing the six blank serum samples and frozen at −80°C until use. Water was purified using a PURELAB Ultra Genetic system (Organo, Tokyo, Japan).

2.2. Equipment and chromatographic conditions

Chromatographic separation was carried out using a Nexera System (Shimadzu, Kyoto, Japan). Aliquots of samples (10 μL) were injected by an auto-sampler, and the analytes were separated on Inertsil ODS-3 (10 mm × 1.5 mm I.D., 3 μm, GL Sciences, Tokyo, Japan) as a guard column and Inertsil ODS-3 (100 mm × 2.1 mm I.D., 2 μm, GL Sciences) as an analytical column. The mobile phase consisted of 20 mM potassium phosphate buffer (pH 2.5)/acetonitrile (74:26, v/v) with a flow rate of 0.6 mL/min.

The column temperature was maintained at 60°C. The absorbance was monitored at a wavelength of 345 nm with a response time of 0.5 sec. Data acquisition was performed with a sampling time of 200 msec. Data were collected and processed using the LabSolutions software (Shimadzu).

2.3. Preparation of stock and working solutions, calibration standards and quality control samples

Stock solutions of erlotinib and OSI-420 at a concentration of 2.0 mg/mL were prepared individually in methanol, and 2.0 mg/mL of IS solution was prepared in acetonitrile. Standard working solutions of erlotinib and OSI-420 were prepared by diluting stock solution with methanol to 6, 20, 60, 200, 600, 2000, or 6000 ng/mL. The IS working solution was prepared by diluting stock solution with acetonitrile to 10 μg/mL. All solutions were stored at −20°C until required.

Quality control (QC) samples at four concentrations, representing the lower limit of quantitation (LLOQ), and low (LQC), medium (MQC), and high (HQC) quality control ranges of the calibration curves, were prepared as follows. In a 1.5 mL polypropylene tube, 100 μL volumes of erlotinib and OSI-420 working solutions were mixed, and the solvents were evaporated to dryness under a nitrogen gas stream at 40°C. Residues were then dissolved in 100 μL of pooled human blank serum to final concentrations of 6, 15, 500, or 5000 ng/mL for erlotinib, and 6, 15, 500, or 1500 ng/mL for OSI-420. LLOQ was defined as the lowest concentration of the calibration curve that could be measured with adequate intra- and inter-assay precision and accuracy.

2.4. Sample preparation

Prior to preparation, frozen serum samples stored at −80°C were thawed at room temperature. In a 1.5 mL polypropylene tube, human serum samples (100 μL) containing erlotinib or OSI-420 were mixed with 10 μL of IS working solution (100 ng IS-containing acetonitrile) and 1 mL of t-butyl methyl ether. Then, the solution was mixed vigorously on a vortex mixer for 30 s and centrifuged at 12,000 × g for 1 min at room temperature. The resultant supernatant was transferred to another 1.5 mL polypropylene tube and evaporated to dryness under a nitrogen gas stream at 40°C. The residue was reconstituted in 50 μL of mobile phase, and 10-μL aliquots were injected into the UHPLC-UV system.

2.5. Method validation

The method was validated according to internationally accepted recommendations for validating bioanalytical methods [20]. The selectivity of the method was assessed by comparing the chromatograms of blank and spiked human serum from six individuals. Calibration curves
consisted of seven points for erlotinib and six points for OSI-420, in concentrations from 6–6,000 ng/mL for erlotinib and 6–2,000 ng/mL for OSI-420. QC samples at four concentrations (LLOQ, LQC, MQC, and HQC) in five replicates were analyzed on three different days to evaluate reproducibility. The intra-assay (n = 5) and inter-assay (3 days) accuracy and precision were assessed by relative error (RE) and coefficient of variation (CV), respectively. RE was calculated by the following formula: [(measured concentration – nominal concentration)/nominal concentration] × 100 (%).

The stability of analytes was assessed by comparing the quantitative value of LQC and HQC samples analyzed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples). The residual ratio of erlotinib and OSI-420 was defined as the measured value of reference samples, as a percentage of the stability samples. The residual ratio of IS was calculated by comparing the peak area ratio of reference samples, as a percentage of the stability samples. Short-term stability was assessed at room temperature for 4 or 24 h. Freeze–thaw stability was assessed after three cycles of freezing and thawing (−80°C to room temperature). Long-term stability was assessed after storage at −80°C for 1 or 4 month(s). Post-preparative stability was assessed at 4°C for 24 h in an auto-sampler.

Recoveries from pooled blank serum were assessed three times at four concentrations (LLOQ, LQC, MQC, and HQC) for analytes and IS. Recovery was calculated by comparing the peak area ratios of extracted (pre-spiked) samples with those of unextracted (post-spiked) samples.

2.6. Clinical application

Serum samples were collected from an NSCLC patient who gave written informed consent according to the protocol adopted by the institutional review board of the Graduate School of Medicine at Tohoku University (2014-1-131). The human blood samples (2 mL) were obtained in glass tubes at 3, 6, 12, and 24 h and 24 days after dosing. These tubes were allowed to stand at room temperature for 30 min, and were then centrifuged at 1,660 × g for 10 min to separate the serum supernatant. The serum was collected in a 1.5 mL polypropylene tube and stored at −80°C until analysis. The area under the concentration–time curve for 0–24 h (AUC0–24) was estimated by the linear trapezoidal rule.

3. Results and discussion

3.1. Method development

Erlotinib and OSI-420 are quinazolinamines, and thus possess a lone electron pair that might be affected by the pH of the mobile phase. Therefore, we first examined the UV spectra of erlotinib, OSI-420, and IS under various pH conditions. In neutral pH, the compounds yielded two absorbance bands in the range from 190 to 400 nm, with maxima at 246 and 333 nm, whereas in acidic pH the compounds yielded maxima at 246 and 345 nm (data not shown). In order to avoid interference peaks from endogenous compounds as much as possible, which could be expected at lower wavelengths, we selected a UV wavelength of 345 nm. Accordingly, all subsequent UHPLC-UV assays were performed using a UV wavelength of 345 nm and a mobile phase with a pH of 2.5. The retention times for erlotinib, OSI-420, and IS were approximately 2.2, 1.1, and 3.5 min, respectively, using 20 mM potassium phosphate buffer (pH 2.5)/acetonitrile (74:26, v/v) as a mobile phase and Inertsil ODS-3 (100 mm × 2.1 mm I.D., 2 μm) as an analytical column. The total run time for each sample analysis was less than 5 min.

Next, we investigated sample preparation of erlotinib and OSI-420 from serum, which required 1) removal of interference peaks derived from endogenous compounds, and 2) efficient extraction of the compounds from serum.
We first performed protein precipitation with acetonitrile to remove endogenous compounds. However, interference peaks from endogenous compounds in human blank serum consistently appeared at the retention times of the analytes. To address this issue, we performed a one-step solvent (1 mL) extraction using ethyl acetate, diethyl ether, n-hexane, or t-butyl methyl ether. The use of ethyl acetate, diethyl ether, or n-hexane was insufficient to remove those interference peaks (data not shown). On the other hand, after extraction using t-butyl methyl ether, no endogenous compounds were found at the retention times (Fig. 1 (A)), and the analytes were recovered efficiently (≥ 93%) with good reproducibility (CV < 2.9%) in human serum without interference from contaminants.

### 3.2. Method validation

No significant interfering peaks derived from endogenous compounds were observed on any chromatograms, suggesting that this method has sufficient specificity for quantitation of erlotinib and OSI-420 concentrations in human serum (Fig. 1 (B)). Three calibration curves for erlotinib and OSI-420 were obtained during method validation. This method covered a linear concentration range of 6–6000 ng/mL for erlotinib and 6–2000 ng/mL for OSI-420 (correlation coefficients > 0.999). Intra- and inter-day accuracies (RE, %) for erlotinib and OSI-420 were within ± 7.4 and ± 5.7%, respectively, and intra- and inter-day precisions (CV, %) were ≤ 6.8 and ≤ 5.1%, respectively (Table 1). Taken together, these results indicate that our method has suitable performance for quantitation of erlotinib and OSI-420 concentrations in human serum.

Erlotinib and OSI-420 were stable in human serum (criteria, 100 ± 15%) over three freeze–thaw cycles, for up to 24 h at room temperature, for up to 4 months at −80°C, and for 24 h at 4°C in processed samples in the auto-sampler (Table 2).

Extraction recovery rates of erlotinib, OSI-420, and IS were 85.9 ± 8.3, 89.8 ± 5.1, and 89.5 ± 1.8%, respectively, which were within the range of quantitation (Table 3).
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All results satisfied the criteria described in the guidelines for bioanalytical method validation. Hidalgo et al. [3] reported the first method for using HPLC-UV to quantitate erlotinib and OSI-420 levels using a one-step plasma sample extraction with 5 mL of t-butyl methyl ether. However, this method did not achieve effective separation from plasma-derived contamination. Lepper et al. [18] reported an improved plasma sample extraction using a mixture of acetonitrile and n-butyl chloride (1:4, v/v). Although this method provided better separation with a shorter run time (< 3 min), it did not measure OSI-420, and the LLOQ of erlotinib was 100 ng/mL. Zhang et al. [19] reported a multi-step sample preparation, resulting in high specificity and sensitivity. In their method, the LLOQ values of erlotinib and OSI-420 were 12.5 and 5.0 ng/mL, respectively. However, the sample extraction processing and run times were much longer. Advantages of our method in comparison with these previous HPLC-UV methods [3,18,19] include the use of a small sample volume (100 µL), one-step liquid–liquid extraction with high recovery, and short chromatographic run times.

### 3.3. Clinical pharmacokinetics

We sequentially analyzed the serum concentration of erlotinib and OSI-420 in an NSCLC patient (female, 58 years) administered the recommended daily dose (150 mg) of erlotinib. As shown in the UHPLC-UV chromatogram (Fig. 1 (C)), OSI-420 and erlotinib with retention times of 1.1 and 2.2 min, respectively, could be separated from other peaks. Previous studies demonstrated that OSI-413 (desmethyl erlotinib), an isomeric metabolite of OSI-420, was observed between the retention times of OSI-420 and erlotinib in reversed-phase HPLC [16,17]. In those studies, the peak area of OSI-413 was larger than that of OSI-420. Therefore, the taller peak with the retention time of 1.3 min might represent OSI-413.

The observed concentration–time profile of erlotinib and OSI-420 is shown in Fig. 2. The steady-state trough concentrations of erlotinib and OSI-420 were 0.26 and 0.010 µg/mL, respectively, and the AUC₀–₂₄ value of erlotinib was 4.2 µg h/mL. A previous study indicated that the steady-state trough concentrations of erlotinib and OSI-420 are approximately 1.2 and 0.15 µg/mL, respectively, and that the mean AUC₀–₂₄ value of erlotinib is approximately 20 µg h/mL [21]. This patient was taking erlotinib in combination with esomeprazole and carbamazepine, which respectively influence the absorption and metabolism of erlotinib. Therefore, the reductions in the serum concentrations of erlotinib and OSI-420 might be due to drug-drug interactions.

### 4. Conclusions

We developed and validated a rapid and simple UHPLC-UV method for quantitative determination of erlotinib and its metabolite, OSI-420. This method allowed quantitation of erlotinib and OSI-420 in the low ng/mL range, and could therefore be used to measure these concentrations in serum samples from patients following administration of erlotinib at a reduced daily dose, in order to optimize treatment with this agent. Additionally, the advantages of this method include the use of a small serum sample volume, liquid–liquid extraction with high recovery, and short chromatographic run times. This method has been successfully used in analyses of erlotinib and OSI-420 in human serum samples to support real-time drug monitoring for patients administered erlotinib.

#### Table 3. Extraction recovery of erlotinib, OSI-420, and IS from human serum.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC sample</th>
<th>Nominal concentration (ng/mL)</th>
<th>Recovery (%; n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erlotinib</td>
<td>LLOQ</td>
<td>6</td>
<td>94.6 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>10</td>
<td>91.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>500</td>
<td>90.8 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>5000</td>
<td>85.9 ± 8.3</td>
</tr>
<tr>
<td>OSI-420</td>
<td>LLOQ</td>
<td>6</td>
<td>97.9 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>10</td>
<td>94.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>500</td>
<td>91.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>1500</td>
<td>89.8 ± 5.1</td>
</tr>
<tr>
<td>OSI-597  (IS)</td>
<td>–</td>
<td>1000</td>
<td>89.5 ± 1.8</td>
</tr>
</tbody>
</table>

IS, internal standard; QC, quality control; LLOQ, lower limit of quantitation; LQC, low QC; MQC, medium QC; HQC, high QC. Each data point represents mean ± standard deviation.
Conflict of interest
The authors declare no conflicts of interest, financial or otherwise.

References