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Simultaneous determination of OSI-774 and its major metabolite OSI-420 in human plasma by using HPLC with UV detection

Wenjiang Zhang[a], Lillian L. Siu[b], Malcolm J. Moore[a,b], Eric X. Chen[b,*]

[a] *Department of Experimental Therapeutics, Ontario Cancer Institute, Ontario, Canada*
[b] *Department of Medical Oncology and Hematology, Princess Margaret Hospital, Room 5-221A, 610 University Avenue, University Health Network, Toronto, Ontario, Canada M5G 2M9*

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Abstract

A new method was developed and validated for quantitating OSI-774 and its metabolite OSI-420 in human plasma. Sample preparation involved initial extraction with methyl *t*-butyl ether followed by back extraction with HCl and re-extraction with methyl *t*-butyl ether. This extraction process resulted in significant improvement in the specificity, reproducibility and sensitivity. The analytes were separated on a Water Symmetry C18 analytical column and the mobile phase consisted of acetonitrile–0.05 M potassium phosphate buffer (42:58, v/v) (pH 4.8), and monitored at a wavelength 345 nm. Values of between- and within-day precision and accuracy for both OSI-774 and OSI-420 were <20%. This method was successfully applied to study steady-state pharmacokinetics of OSI-774 and OSI-420 in a phase II clinical trial.

Keywords: OSI-774; OSI-420; HPLC

1. Introduction

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein composed of an extracellular ligand binding domain, a short transmembrane domain and an intracellular domain that has tyrosine kinase activity. Four members of the EGFR family exist, including EGFR/erbB1, HER2/erbB2, HER3/erbB3 and HER4/erbB4. Binding of a specific ligand, such as EGF or transforming growth factor-alpha (TGF-alpha), to the extracellular domain of EGFR results in receptor dimerization and autophosphorylation of tyrosine residues in the intracellular domain [1–3]. Tyrosine phosphorylation provides docking sites on the EGFR for recruitment of proteins that are either direct substrates for EGFR-mediated phosphorylation, or adapter proteins that link the receptor to a cascade of signalling pathways, resulting in cell proliferation, differentiation, migration, transformation, and protection from apoptosis [1–3]. EGFR is over-expressed in many tumors, including cancers of head and neck, esophageal, lung, colon, pancreas, ovary, kidney, and gliomas and EGFR over-expression is associated with a worse prognosis in many solid tumors [4]. Therefore, EGFR has become an attractive candidate for molecular targeted anticancer therapy. The inhibition of EGFR can be achieved either by preventing ligand binding with an anti-EGFR monoclonal antibody such as C225 (Cetuximab, Erbitux) [5] or by inhibiting tyrosine kinase activities with small molecules such as OSI-774 (Erlotinib, Tarceva) or ZD 1839 (Gefitinib, Iressa) [6–9].

OSI-774 [6,7-bis(2-methoxy-ethoxy)-quinazolin-4-yl]-[3-ethylphenyl] amino hydrochloride, an quinazolinylamine analogue, is an orally active, potent, selective inhibitor of the EGFR tyrosine kinase [3]. Based on results from xenograft models, the steady-state plasma OSI-774 concentration required for EGFR inhibition is estimated to be 500 ng/ml.

OSI-774 is currently undergoing evaluation in multiple phases II and III studies, either alone or in combination with

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[*] Corresponding author. Tel.: +1 416 946 2263; fax: +1 416 946 2082.
E-mail address: eric.chen@uhn.on.ca (E.X. Chen).

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other chemotherapeutic agents [8,9]. A recently completed randomized, placebo-controlled trial of OSI-774 versus best supportive care demonstrated a survival benefit using this agent in patients with advanced non-small-cell lung cancer after prior platinum-based and docetaxel therapies [10]. In support of the clinical development, several methods of measuring plasma OSI-774 have been developed and reported [6,11,12]. These methods either have interfering peaks [6], low sensitivity [11] or requiring liquid chromatography with tandem mass spectrometry (LC–MS) [12] that might limit their routine application. In this report, we describe a sensitive and specific method of simultaneously determining plasma concentrations of OSI-774 and its major metabolite OSI-420 using reverse phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection.

2. Experimental

2.1. Chemicals and reagents

OSI-774 (lot #45574-1-3F; purity 91.4% free base), its metabolite OSI-420 (lot #1-05-007-2598; purity 94.9%) and OSI-597 (lot #OMS-M0012-SD-18-28; purity 99.7%), the internal standard, were obtained from OSI Pharmaceuticals Inc. (Boulder, CO, USA). HPLC grade methyl *t*-butyl ether and acetonitrile were purchased from Fisher Scientific Inc. (Nepean, Ont., Canada) and EMD Chemical Inc. (Gibbstown, NJ, USA), respectively. Triethylamine was purchased from BDH Inc. (Toronto, Ont., Canada). Purified deionized water was prepared with the Milli-Q Academic Quantum Purification system (Millipore, Milford, MA, USA). Human plasma used for the preparation of controls and standards was obtained from the Canadian Blood Services (Toronto, Ont., Canada).

2.2. Standard stock solutions

OSI-774 and OSI-420 stock solutions were prepared separately in duplicate by dissolving 2.0 mg of each drug in 10 ml of pure methanol to a concentration of 200 μg/ml, respectively, and stored at −20 °C. The stock solution of OSI-597 was prepared by dissolving 2.0 mg of drug in 10 ml of pure acetonitrile to a concentration of 200 μg/ml and stored at −20 °C. Drug concentrations in each of the duplicate stock solutions were estimated from the mean peak area following repeat analysis of each stock solution, and the difference was determined to be within 5% for each of the duplicate stock solutions.

Calibration standards were prepared by diluting stock solutions further with blank human plasma each day to obtain OSI-774 concentrations of 12.5, 100, 500, 1000, 2000, 3000, 4000 ng/ml and OSI-420 concentrations of 5, 25, 100, 200,300, 400, 500 ng/ml. The internal standard was further diluted with acetonitrile to a final concentration of 10 μg/ml. Calibration curves were computed by linear regression using ratios of peak areas of OSI-774 or OSI-420 and the internal standard with a weighting factor of (1/[nominal concentration]). Pools of quality control (QC) samples of OSI-420 and OSI-774 were prepared by mixing appropriate amounts of stock solutions and blank human plasma to obtain OSI-420 concentrations of 25, 100 and 500 ng/ml and OSI-774 concentrations of 50, 500, 4000 ng/ml, and stored in batch at −20 °C for the duration of the validation procedure.

2.3. Sample preparation

Plasma samples were thawed at room temperature. A 250 μl volume of plasma was transferred to a polypropylene tube (100 mm × 14 mm), and 25 μl of the internal standard was added and mixed for 30 s on a vortex-mixer. After addition of 2.5 ml of methyl *t*-butyl ester, these tubes were capped, shaken on a shaker (New Brunswick Scientific Corp., Eddison, NJ, USA) for 10 min and then centrifuged at 3000 × *g* for 10 min at ambient temperature. The supernatants were transferred to a clean glass tube, and 0.6 ml of 5% HCl was added. The mixtures were shaken for 30 min followed by centrifugation at 3000 × *g* for 10 min at ambient temperature. The organic layer was discarded, and 0.6 ml of 1N NaOH and 2.5 ml of methyl *t*-butyl ester were added to the remaining aqueous layer. The samples were again shaken for 30 min followed by centrifugation at 3000 × *g* for 10 min under ambient temperature. The supernatants were transferred to 1.5 ml polypropylene tubes, and evaporated to dryness using the Savant Universal vacuum system (Thermo Electron Corp., Milford, MA, USA). Residues were reconstituted with 0.25 ml of the mobile phase, vortexed for 60 s on a vortex-mixer and centrifuged at 4000 × *g* for 30 min at ambient temperature. A volume of 100 μl was transferred to a 250 μl polypropylene auto-sampler vial, and 50 μl was injected onto the HPLC system for quantitative analysis.

2.4. Chromatographic conditions

The chromatographic system consisted of a LC-10ADvp liquid chromatography system equipped with a SPD-10Avp UV detector (Shimadzu Corp., Kyoto, Japan). Separation of the analytes was achieved at ambient temperature using a Waters Symmetry C18 reversed-phase analytical column (150 mm × 4.6 mm i.d., 5 μm particles) protected by a matching Waters Symmetry C18 guard column (Milford, MA, USA). The mobile phase consisted of acetonitrile–0.05 M potassium phosphate buffer (42:58, v/v) with the pH adjusted to 4.8 with 0.2% triethylamine, and the flow rate was set at 1.0 ml/min. The detector was set at a wavelength of 345 nm. Data were collected and analyzed using the Shimadzu Class VP software (version 7.1.1.SP).

2.5. Method validation

Method validation runs were performed on five consecutive days, and included a calibration curve processed in duplicate and QC samples at low, medium and high concentrations in quadruplicate. The accuracy or percentage deviation (DEV) of the assay was calculated as:

$$\mathrm{DEV}\,(\%) = \left(1 - \frac{\text{observed concentration}}{\text{nominal concentration}}\right) \times 100 \qquad (1)$$

Estimates for the between-run precision (BRP) were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square ($\mathrm{MS_{bet}}$), the within-group mean square ($\mathrm{MS_{wit}}$), and the grand mean (GM) of the observed concentrations across runs were calculated using S-Plus (Version 6.0, Insightful Corp., Seattle, WA, USA). The BRP was defined as:

$$\mathrm{BRP} = \sqrt{\frac{(\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n}{\mathrm{GM}}} \times 100 \qquad (2)$$

where n represents the number of replicate samples within each run. The within-run precision (WRP) was calculated as:

$$\mathrm{WRP} = \sqrt{\frac{\mathrm{MS_{wit}}}{\mathrm{GM}}} \times 100 \qquad (3)$$

To determine the extraction efficiency of OSI-774 and OSI-420 from plasma samples, five QC samples at each concentration were processed as described above. The recovery was determined by comparing areas obtained from QC samples with those from spiked solutions of same concentrations, and expressed as a percentage.

The specificity of the method was evaluated using blank human plasma samples from six different donors. Chromatograms were visually inspected for the presence of endogenous or exogenous interfering peaks. The stabilities of OSI-774 and OSI-420 in plasma were evaluated using QC samples following three full freeze–thaw cycles.

2.6. Patient samples

Blood samples were collected from eight patients participating in a phase II study of OSI-774 in combination with cisplatin as first line therapy for recurrent or metastatic squamous cell carcinoma of the head and neck. Cisplatin was administered intravenously over 60 min at 75 mg/m^2 every 21 days. OSI-774 was administered orally at a continuous daily dose of 150 mg, with a run-in period of 7 days prior to the first dose of cisplatin in cycle one (days −6 to 0) such that steady-state concentrations of OSI-774 can be achieved at the time of cisplatin administration (day 1). The protocol was approved by the Ethics Review Board of the Princess Margaret Hospital, University Health Network, Toronto, Canada, and patients gave written informed consent prior to treatment.

Blood samples (5 ml each) were collected in 7 ml heparinized tubes prior to OSI-774 dosing on days 1, 15, 22, and 43. The specimens were centrifuged at 3000 × *g* for 15 min at room temperature. Plasma was separated and stored at −70 °C until analysis.

3. Results and discussion

3.1. Chromatography

The retention times for OSI-774, OSI-420 and the internal standard were approximately 5.0, 3.0 and 11.0 min, respectively. The total run time for each sample analysis was 15 min. Chromatograms of HPLC analysis of a blank human plasma sample, a human plasma sample spiked with OSI-420 and OSI-774 at LLOQs, and a patient sample are shown in Fig. 1.

The first reported HPLC method of OSI-774 analysis by Hidalgo et al. [6] involved a one-step plasma sample extraction with methyl *t*-butyl ether. However, this simple extraction resulted in a major interfering peak around the retention time of the internal standard. Lepper et al. [11] reported an improved sample extraction method with a mixture of acetonitrile and *n*-butyl chloride. However, this method did not measure OSI-420, and the lower limit of quantitiation of OSI-774 was 100 ng/ml. Zhao et al. [12] developed a liquid chromatography with tandem mass spectrometry for measuring OSI-774 and OSI-420, and their method was showed to be superior in sensitivity with lower limits of quantitation (LLOQ) of 10 and 1 ng/ml, respectively, for OSI-774 and OSI-420. However, this technology is not available in every laboratory.

Considering that OSI-774 is an alkaloid quinazoline derivative, we used 0.5N HCl to back-extract OSI-774 and OSI-420 following initial plasma sample extraction with

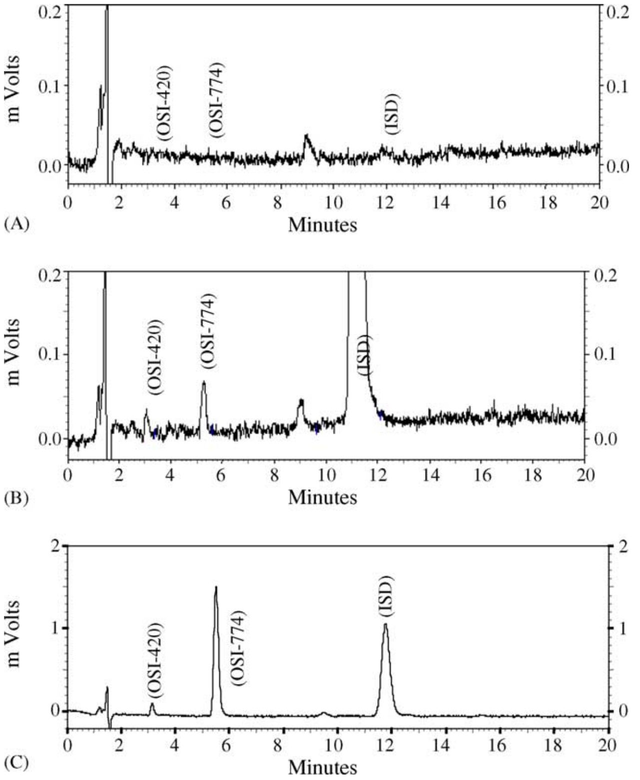

Fig. 1. Chromatograms of a blank human plasma sample (A); a human plasma sample spiked with OSI-420 (5 ng/ml), OSI-774 (12.5 ng/ml) and IS (2000 ng/ml) (B); and a plasma sample from a patient (C).

methyl *t*-butyl ether. The pH of the aqueous phase was then adjusted with NaOH and the sample was extracted once again using methyl *t*-butyl ether. As a result, the interfering peak around the retention time of the internal standard was eliminated, and the specificity of the analytical method was significantly improved. In addition, triethylamine was added to the mobile phase to improve the peak symmetry and effectively overcome the peak tailings of OSI-774 and OSI-420 observed previously [11]. Consequently, both specificity and sensitivity of this method were improved compared to those reported previously.

In human plasma, calibration curves were linear over the range of 5.0–500 ng/ml for OSI-420, and over the range of 12.5–4000 ng/ml for OSI-774 for this method. The correlation coefficients for the calibration curves were more than 0.99 for each validation run. The LLOQs of OSI-420 and OSI-774 were determined to be 5.0 and 12.5 ng/ml, respectively. At the LLOQ, the values for accuracy and precision were 8.35% and 19.0% for OSI-420, and −1.28% and 10.8% for OSI-774. The between- and within-run precision values of QC samples at three different concentrations were less than 7.0% for both OSI-774 and OSI-420. The accuracy of QC samples for the OSI-420 and OSI-774 were between 0.19% and 10.6% (Tables 1 and 2). These values of precision and accuracy confirmed that this assay is reproducible and valid.

By comparing ratios of peak areas of OSI-774 and OSI-420 from QC samples with those from spiked solutions of the same concentrations, the mean extraction recovery was found to be from 65.1% to 68.3% for OSI-774 from 50.0% to 64.8% for OSI-420 (Tables 1 and 2). In addition, there appears to be no significant change for OSI-774 and OSI-420 through three full cycles of freeze–thaws.

3.2. Pharmacokinetic analysis

The method was used to determine steady-state plasma OSI-774 and OSI-420 concentrations from eight patients administered OSI-774150 mg daily. The mean observed concentrations are shown in Fig. 2. The steady-state OSI-774 concentrations are well above the 500 ng/ml level which is thought to be the concentration for significant EGFR inhibition. The concentration of OSI-420 is approximately 10% of OSI-774, consistent with the previous report [6].

Table 1
Validation characteristics of OSI-420 in human plasma

	Nominal concentration (ng/ml)		
	25	100	500
Accuracy (DEV, %)[a]	1.67	2.60	0.19
Precision (%)[a]			
Between-run	5.04	3.25	1.90
Within-run	6.52	4.14	2.86
Extraction recovery (%)[b]	50.0	59.6	64.8
Stability (percent of initial)[c]			
Freeze–thaw cycles			
1	100.4	99.5	99.4
2	99.2	99.7	100.2
3	103.6	92.6	103.2

[a] Validation was performed over 5 days. A minimum of two samples were analyzed for each condition on each day.

[b] Mean of five samples at each concentration.

[c] Two samples were analyzed for each condition.

Table 2
Validation characteristics of OSI-774 in human plasma

	Nominal concentration (ng/ml)		
	50	500	4000
Accuracy (DEV, %)[a]	10.6	2.35	0.31
Precision (R.S.D., %)[a]			
Intra-day	2.73	2.34	1.98
Inter-day	3.77	4.03	3.14
Extraction recovery (%)[b]	66.3	65.1	68.3
Stability (percent of initial)[c]			
Freeze–thaw cycles			
1	110.0	102.7	98.3
2	103.0	99.0	96.2
3	103.0	98.1	97.2

[a] Validation was performed over 5 days. A minimum of two samples were analyzed at each concentration on each day.

[b] Mean of five samples at each concentration.

[c] Two samples were analyzed for each condition.

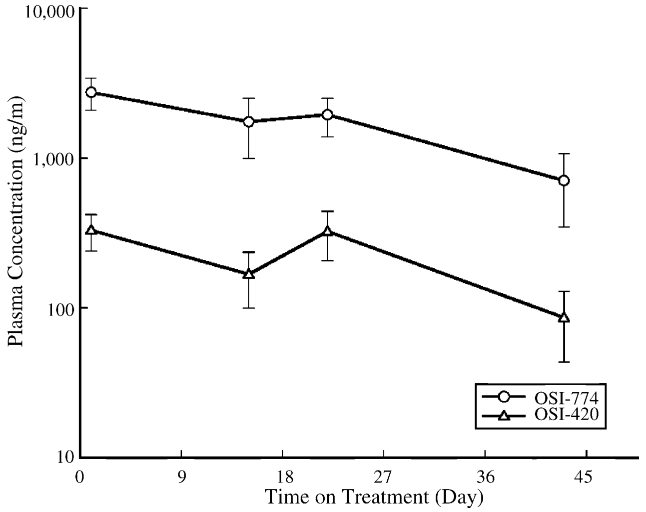

Fig. 2. Mean steady-state OSI-774 and OSI-420 concentrations following daily oral administration of 150 mg OSI-774.

4. Conclusion

In conclusion, the method presented for quantitating OSI-774 and OSI-420 in human plasma samples is specific, accurate and precise. Two extra extraction steps were added to the previously reported one-step sample preparation method [6,11]. Although the sample extraction process is slightly more time-consuming, as a result, the sensitivity of the method is significantly improved. The lower limit of quantitation of OSI-774 was improved from 100 to 12.5 ng/ml. This sensitivity is comparable to that obtained with a LC–MS method [12], and this method can be easily adopted in more clinical pharmacology laboratories for pharmacokinetic studies of OSI-774. Furthermore, OSI-420 concentrations can be simultaneously determined. This method has been successfully applied in measuring plasma samples from patients taking OSI-774 at a daily dose of 150 mg. As the therapeutic benefit of OSI-774 becomes established and utilization increases in the clinical setting, the development of an improved method to measure its plasma concentrations would facilitate future pharmacokinetic evaluations of this agent, given alone or in combination with conventional or targeted treatments.

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