Specific method for determination of OSI-774 and its metabolite OSI-420 in human plasma by using liquid chromatography–tandem mass spectrometry

Ming Zhao[a], Ping He[a], Michelle A. Rudek[b], Manuel Hidalgo[b], Sharyn D. Baker[a],*

[a]Division of Experimental Therapeutics, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins,
Johns Hopkins University School of Medicine, Baltimore, MD, USA
[b]Division of Medical Oncology, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins,
Johns Hopkins University School of Medicine, Baltimore, MD, USA

Abstract

A new simple and specific method was developed and validated for the quantitative determination of OSI-774 (Tarceva™, Erlotinib) and its metabolite, OSI-420, in human plasma. Sample pretreatment involved a single protein precipitation step with acetonitrile. The analytes were separated on Waters X-Terra C_{18} (50×2.1 mm I.D., 3.5 μm) analytical column and eluted with acetonitrile–water mobile (70:30, v/v) containing 0.1% formic acid. The analytes of interest were monitored by tandem mass spectrometry with electrospray positive ionization. The overall extraction efficiency was greater than 88% for OSI-774 and 62% for OSI-420, with values for within-day and between-day precision and accuracy of <15%. Compared to previous assays, this method is simple, specific, and reproducible and will be used to characterize the plasma pharmacokinetics of OSI-774 at doses of 50 to 150 mg to optimize treatment with this agent.
© 2003 Elsevier B.V. All rights reserved.

Keywords: OSI-774; OSI-420

1. Introduction

The epidermal growth factor receptor (EGFR), a type I receptor tyrosine kinase (TK), is involved in the regulation of cellular differentiation and proliferation and is highly expressed by many types of human cancer [1]. Evidence that dysregulation of the EGFR-mediated signal transduction pathways play a role in pathogenesis of various cancers has led to the rational design and development of agents that selectively target this receptor [1]. An emerging number of drugs targeted to the EGFR are in clinical development including monoclonal antibodies against the extracellular domain of the receptor and small molecules that inhibit the receptor TK.

OSI-774 ([6,7-bis(2-methoxy-ethoxy)-quinazolin-4-yl]-3-ethynyl-phenyl)amine–HCL; Erlotinib, Tarceva™) (Fig. 1A) is an orally bioavailable quinazoline derivative that binds to the ATP pocket of the EGFR TK and inhibits the autophosphoryla-

*Corresponding author. The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Bunting–Blaustein Cancer Research Building, Room 1M86, 1650 Orleans Street, Baltimore, MD 21231-1000, USA. Tel.: +1-410-502-7149; fax: +1-410-614-9006.
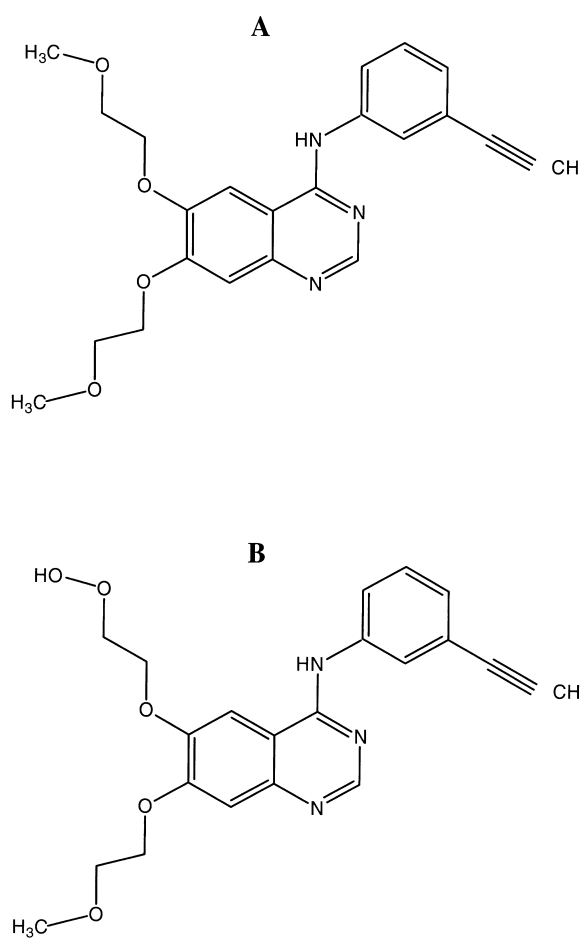
E-mail address: sdbaker@jhmi.edu (S.D. Baker).

Fig. 1. Chemical structures of OSI-774 (A) and OSI-420 (B).

tion of the receptor. This compound is 1000-fold more potent against EGFR TK than most other human kinases, including *c-src* and insulin receptor TK [2]. OSI-774 monotherapy has demonstrated clinical activity in non-small cell lung cancer, head and neck cancer, and ovarian cancer in Phase II studies [3–5]. At this juncture, the agent has completed phase III evaluation as first line treatment in combination with chemotherapy in patients with non-small cell lung cancer and pancreatic cancer. In addition, a larger number of clinical studies testing OSI-774 either alone or in combination with chemotherapy, radiation therapy and hormone therapy in a large variety of human cancers are underway [6–8].

In a phase I study of OSI-774 [9], the plasma pharmacokinetics of OSI-774 and its principal me-

tabolite OSI-420 (Fig. 1B) were characterized over the dose range of 50 to 200 mg. Plasma samples were extracted with methyl *t*-butyl ether, the extracts were reconstituted with acetonitrile and water (vol/vol, 30/70; pH 2.40), and quantitation was performed using HPLC and UV detection at 345 nm. The limit of quantitation for both OSI-774 and OSI-420 was 10 ng/ml. Validation of the method during assay implementation (in-study) included the incorporation of quality control samples in duplicate at three different concentrations. No comprehensive pre-study method validation was reported. Over the dose range of 50 to 200 mg, mean C_{max} and $C_{ss,min}$ values ranged from 427 to 1737 ng/ml and 283 to 1360 ng/ml, respectively. Exposure to OSI-420 relative to that of the parent compound was low and averaged 12%.

To comprehensively characterize the clinical pharmacokinetic (PK) profile of this drug, a prerequisite for optimizing its clinical use and to explore relationships with pharmacodynamics (PD) effects of OSI-774, a specific, reproducible and accurate method for the quantification of OSI-774 and its principal metabolite OSI-420 (Fig. 1B) was necessary. The assay reported in this paper utilizes a simple protein precipitation procedure with acetonitrile. A liquid chromatography–tandem mass spectrometry (LC–MS-MS) method with electrospray positive ionization achieves a specific, reproducible and accurate method, which can be easily applied to the quantitation of OSI-774 and OSI-420 in a large number of human plasma samples.

2. Experimental

2.1. Chemical and reagents

OSI-774 (lot number 45574-1-3F, 91.4% pure) and OSI-420 (lot number 1 05 007 2598, 94.9% pure) were gifts from OSI Pharmaceuticals (Uniondale, NY, USA). The internal standard, midazolam, was supplied by Hoffmann–La Roche (Nutley, NJ, USA). Formic acid (88%, v/v in water) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile (HPLC grade) was from obtained from EM Science (Gibbstown, NJ, USA). Deionized water was obtained from a Milli-Q-UF system

(Millipore, Milford, MA, USA) and used throughout in all aqueous solutions. Drug-free (blank) human plasma originated from Pittsburgh Blood Plasma (Pittsburgh, PA, USA).

2.2. Sample preparation

Prior to extraction, frozen plasma samples were thawed in a water bath at ambient temperature. A 0.1 ml aliquot of plasma was added to a borosilicate glass test tube (13×100 mm) containing 0.4 ml of acetonitrile solution of midazolam (100 ng/ml), used as internal standard. The tube was mixed vigorously for 10 s on a vortex-mixer, followed by centrifugation at 2000 *g* for 5 min at ambient temperature. A volume of 100 µl of the top organic layer was transferred to a disposable borosilicate glass culture tube (13×100 mm) and 100 µl deionized water was added to this tube. The tube was mixed vigorously for 10 s on a vortex-mixer and then the sample was transferred to a 250 µl polypropylene autosampler vial, and a volume of 10 µl was injected onto the HPLC instrument for quantitative analysis using a temperature-controlled autosampler device operating at 10 °C.

2.3. Chromatographic and mass-spectroscopic conditions

Chromatographic analysis was performed using a Waters Model 2690 separations system (Milford, MA, USA) equipped with a Waters Model 996 photodiode-array detector. Separation of the analytes from potentially interfering material was achieved at ambient temperature using Waters X-Terra MS column (50×2.1 mm I.D.) packed with a 3.5 µ*M* ODS stationary phase, protected by a guard column packed with 3.5 µm RP18 material (Milford, MA, USA). The mobile phase used for the chromatographic separation was composed of acetonitrile–water (70:30, v/v) containing 0.1% formic acid, and was delivered isocratically at a flow-rate of 0.15 ml/min. The column effluent was monitored using a Micromass Quattro LC triple-quadrupole mass-spectrometric detector (Beverly, MA, USA). The instrument was equipped with an electrospray interface, and controlled by the Masslynx version 3.4 software (Micromass), running under Microsoft Windows NT

on a Compaq AP200 Pentium III computer. The samples were analyzed using an electrospray probe in the positive ionization mode operating at a cone voltage of 40 V for OSI-774, and OSI-420 and 45 V for midazolam, the internal standard. Samples were introduced into the interface through a heated nebulized probe (350 °C). The spectrometer was programmed to allow the $[MH]^+$ ion of OSI-774 at m/z 394, OSI-420 at m/z 380 and that of the internal standard at m/z 326 to pass through the first quadrupole (Q1) and into the collision cell (Q2) (Fig. 2A). The collision energy was set at 32 eV for OSI-774, at 30 eV for OSI-420 and at 25 eV for the internal standard. The daughter ions for OSI-774 (m/z 278), OSI-420 (m/z 278) and the internal standard (m/z 286.1) were monitored through the third quadrupole (Q3) (Fig. 2B). Argon was used as collision gas at a pressure of 0.0027 mBar, and the dwell time per channel was 0.5 s for data collection.

2.4. Calibration curves

Stock solutions of OSI-774 were prepared by dissolving 4.0 mg, accurately weighed, in 4.38 ml of ACN (concentration=1.0 mg/ml) in duplicate, area counts for each of the duplicate aliquots were checked in quintuplicate, and if the mean value for area counts were within 5%, the stock solutions were then stored in a glass vial at −20 °C. The stock solutions were diluted further in blank human plasma on each day of analysis to prepare calibration samples containing OSI-774 at concentrations of 10, 50, 100, 500, 1000, 5000, and 10000 ng/ml. Calibration curves were computed using the ratio of the peak area of OSI-774 and internal standard by using a weighted (1/[nominal OSI-774 concentration]) linear regression analysis.

Stock solutions of OSI-420 was prepared by dissolving 4.0 mg, accurately weighed, in 8.43 ml of 50% ACN (concentration=0.5 mg/ml) in duplicate, area counts for each of the duplicate aliquots were checked in quintuplicate, and if the mean value for area counts were within 5%, the stock solutions were then stored in a glass vial at −20 °C. The stock solutions were diluted further in blank human plasma on each day of analysis to prepare calibration samples containing OSI-420 at concentrations of 1, 5, 10, 50, 100, 500, and 1000 ng/ml. Calibration
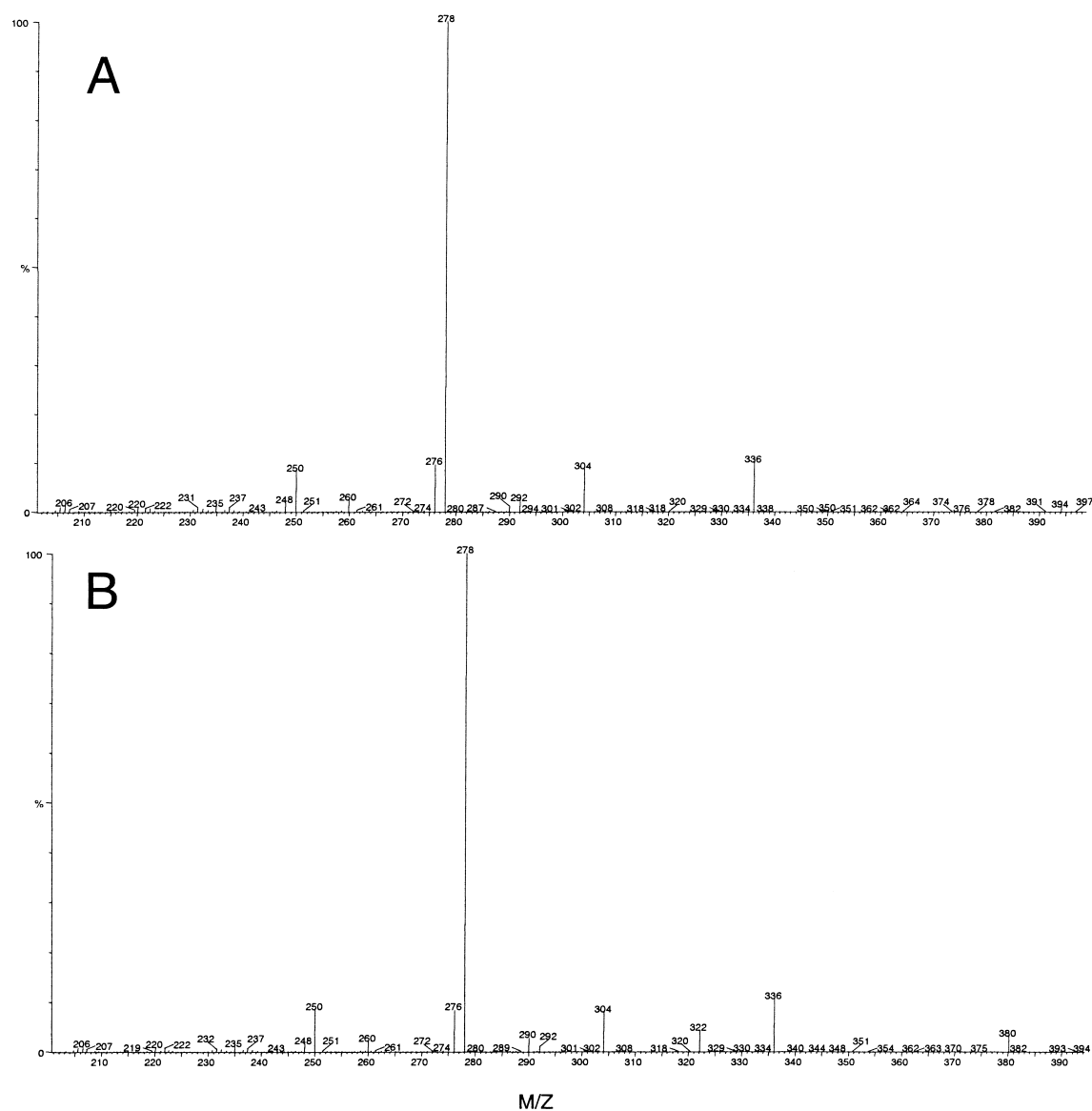
Fig. 2. Mass spectra of OSI-774 (A) and OSI-420 (B). A mass-to-charge (m/z) of 394→278 was monitored for OSI-774 and 380→278 for OSI-420, respectively.

curves were computed using the ratio of the peak area of OSI-420 and internal standard by using a weighted (1/[nominal OSI-420 concentration]) linear regression analysis.

The assay lower limit of quantitation (LLOQ) was determined to be 10 ng/ml for OSI-774 and 1 ng/ml for OSI-420, respectively. The LLOQ was determined by meeting the following two criterion: (i) the

signal-to-noise ratio larger than 5 and (ii) the values for precision and accuracy were less than 20%.

2.5. Method validation

Method validation runs were performed on four consecutive days, and included a calibration curve

processed in duplicate, and quality control (QC) samples, at four different concentrations, in quadruplicate. QC samples were prepared independently in blank plasma at OSI-774 concentrations of 10, 30, 800, and 8000 ng/ml and OSI-420 concentrations of 1, 3, 80, and 800 ng/ml. The accuracy and precision of the assay was assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-run precision, respectively. The accuracy for each tested concentration was calculated as:

$$DEV_{(OSI-774)} = 100$$
$$\times \{([OSI-774]_{mean}$$
$$- [OSI-774]_{nominal})/[OSI-774]_{nominal}\}$$

$$DEV_{(OSI-420)} = 100$$
$$\times \{([OSI-420]_{mean}$$
$$- [OSI-420]_{nominal})/[OSI-420]_{nominal}\}$$

Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}), and the grand mean (GM) of the observed concentrations across runs were calculated using the JMP™ statistical discovery software version 4 (SAS Institute, Cary, NC, USA). The between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as:

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

where n represents the number of replicate observations within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as:

$$WRP = 100 \times \{\sqrt{(MS_{wit})/GM}\}$$

The specificity of the method was tested by visual inspection of chromatograms of extracted human plasma samples from six different donors for the presence of endogenous or exogenous interfering peaks. The extraction efficiency of the assay was measured by comparison of extracted plasma samples and aqueous samples of OSI-774 at concentrations of 10, 30, 800, and 8000 ng/ml and OSI-420 at concentrations of 1, 3, 80, and 800 ng/ml, respectively. The stabilities of OSI-774 and OSI-420 in plasma were tested at the concentrations of QC samples by three freeze–thaw cycles. The short-term stabilities of OSI-774 and OSI-420 in plasma were also assessed on the benchtop for 2 h at room temperature.

3. Results and discussion

3.1. Analytical procedure

The mass spectrum of OSI-774 showed a protonated molecular ion ($[MH^+]$) at m/z 394 and that of OSI-420 at m/z 380 (Figs. 2A and B). The high collision energy fragmented OSI-774 and OSI-420 into several fragments. The major fragment observed was at m/z 278 for both analytes, which was selected for subsequent monitoring in the third quadrupole.

A single protein precipitation step with acetonitrile combined with direct injection into the HPLC system was our first choice of sample preparation; this gave clean extracts free from endogenous interference. Deionized water was added to make the organic solvent concentration in reconstitute solution less than that in mobile phase, which generated the most symmetric chromatographic peaks. For the chromatography, the large organic component of the eluent was employed to allow retention times of approximately 1.3 min for OSI-774, OSI-420, and internal standard to be achieved giving a high sample throughput. In the final procedure, only a small volume (10 µl) of the sample after extraction was injected on the column to maintain high efficiency and resolution therefore compromising assay sensitivity. However, the LLOQ of 10 and 1 ng/ml was sufficient to quantitate OSI-774 and OSI-420 concentrations, respectively, in plasma, following administration of the lowest dose level of 50 mg in a phase I trial [9]. Although increased injection volumes could achieve higher response factors, overloading the column resulted in various kinds of

distorted separation artifacts, including asymmetric sample bands.

3.2. Method validation

Chromatograms of blank and spiked human plasma samples are shown in Fig. 3. The selectivity for the analysis is shown by the sharp and symmetrical resolution of the peaks, with no significant interfering peak for both OSI-774, OSI-420 and internal standard in drug-free specimens, which were obtained from six different individuals. The retention times for OSI-774, OSI-420 and internal standard under the optimal concentrations were 1.3 ± 0.1 min, with an overall chromatographic run time of 2.5 min.

Calibration curves of OSI-774 in human plasma over the range of 10 to 10000 ng/ml and OSI-420 over the range of 1 to 1000 ng/ml were best fitted using a liner regression analysis, applying the peak area in combination with a weight factor (1/[nominal concentration]). The correlation coefficients for the calibration curves of ≥ 0.99 were observed throughout the validation. The lower limit of quantitation was established at 10 ng/ml for OSI-774 and
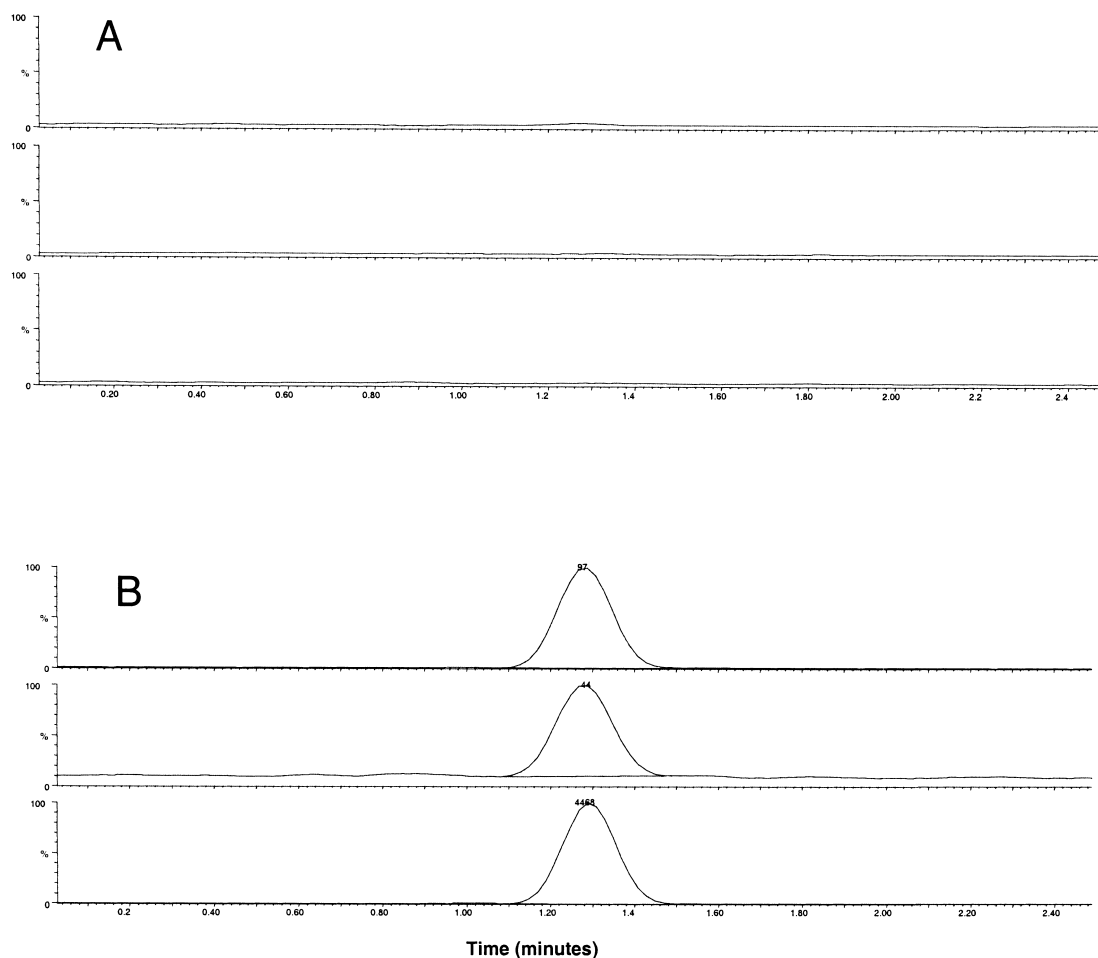

Fig. 3. Chromatograms of blank plasma (A), plasma spiked with 10 ng/ml OSI-774 and 1 ng/ml OSI-420, and plasma spiked with 100 ng/ml of midazolam, the internal standard (B). The retention times for OSI-774, OSI-420, and internal standard were approximately 1.3 ± 0.1 min.

1 ng/ml for OSI-420 with an average signal-to-noise ratio of 7, which was calculated from eight determinations. At the lower limit of quantitation, the values for accuracy, within-run, and between-run precision were 94%, 6.5%, and 3.1% for OSI-774 and 96%, 10% and 9.2% for OSI-420, respectively. The within-run and between-run precision of three different QC samples calculated in quadruplicate on 4 separate days for both OSI-774 and OSI-420 in human plasma were <15% over a wide range of OSI-774 and OSI-420 concentrations (Tables 1 and 2). The accuracy of QC samples for both OSI-774 and OSI-420 were between 95–107%. Precision and accuracy studies indicated that this assay is reproducible and accurate.

By comparing the peak area ratios of OSI-774 and OSI-420 containing plasma samples with those for non-processed samples prepared in the mobile phase, the mean extraction efficiency was found to be greater than 88% for OSI-774 and 62% for OSI-420 (Tables 1 and 2).

There appeared to be no evidence of analyte degradation in human plasma at various time periods and temperatures of storage (Table 1). Freeze–thaw stability tests were performed in duplicate and OSI-774 and OSI-420 were stable through three full cycles of freeze–thaws. Autosampler stability testing showed that the analyte could last 26 h without any degradation allowing for up to 500 samples to be analyzed within a run.

4. Conclusion

In conclusion, we have developed and validated a novel assay for measuring OSI-774 and its metabolite OSI-420 levels in human plasma. The method was shown to meet the current requirements as to validation of bioanalytical methodologies, providing good accuracy and precision [10]. To compare with the published HPLC–UV method [9], our method has the following advantages: (1) the sample preparation procedure is less complex; (2) requires half the sample size of plasma (100 versus 200 μl) without the loss of assay sensitivity; and (3) is specific, reproducible and accurate. This method will be used to characterize the plasma pharmacokinetics of OSI-774 at doses of 50 to 150 mg as a single agent or in combination therapy to optimize treatment with this agent.

Table 1
Validation characteristics of OSI-774 in human plasma

Nominal concentration (ng/ml)	10	30	800	8000
Accuracy (%)	94.50	95.2	102.9	109.0
Precision (%)				
Within-run	6.49	7.22	5.19	4.60
Between-run	3.04	*	*	2.82
Extraction recovery (%)		94.9	88.2	96.4
Stability (% of initial)				
Freeze–thaw cycles				
1		97.6	101.0	100.2
2		99.4	101.3	99.6
3		93.6	104.4	104.9
Two hours at room temperature		n.d.	105.9	n.d.
Autosampler stability (26 h, %)		93.7	87.7	91.8

*No significant additional variation was observed as a result of performing the assay in different runs. n.d., not done.

Table 2
Validation characteristics of OSI-420 in human plasma

Nominal concentration (ng/ml)	1	3	80	800
Accuracy (%)	95.6	99.2	105.1	101.8
Precision (%)				
Within-run	10.2	8.03	7.46	5.19
Between-run	9.18	5.90	6.31	*
Extraction recovery (%)		70.4	66.7	62.5
Stability (% of initial)				
Freeze–thaw cycles				
1		98.3	99.2	98.8
2		98.3	98.1	102.6
3		98.3	102.9	102.1
Two hours at room temperature		n.d.	102.7	n.d.
Autosampler stability (26 h, %)		87.7	95.3	94.0

*No significant additional variation was observed as a result of performing the assay in different runs. n.d., not done.

Acknowledgements

References

[1] V. Grunwald, M. Hidalgo, Curr. Probl. Cancer 26 (2002) 109.
[2] J.D. Moyer, E.G. Barbacci, K.K. Iwata, L. Arnold, B. Boman, A. Cunningham, C. DiOrio, J. Doty, M.J. Morin, M.P. Moyer, M. Neveu, V.A. Pollack, L.R. Pustilnik, M.M. Reynolds, D. Sloan, A. Theleman, P. Miller, Cancer Res. 57 (1997) 4838.
[3] N. Finkler, A. Gordon, M. Crozier, R. Edwards, J. Figueroa, A. Garcia, J. Hainsworth, D. Irwin, S. Silberman, L. Allen, K. Ferrante, D. Fisher, P. Nadler, Proc. Am. Soc. Clin. Oncol. 20 (2001) 208a.
[4] R. Perez-Soler, A. Chachoua, M. Huberman, D. Karp, J. Rigas, L. Hammond, E. Rowinsky, G. Preston, K.J. Ferrante, L.F. Allen, P.I. Nadler, P. Bonomi, Proc. Am. Soc. Clin. Oncol. 20 (2001) 310a.
[5] N.N. Senzer, D. Soulieres, L. Siu, S. Agarwala, E. Vokes, M. Hidalgo, S. Silberman, L. Allen, K. Ferrante, D. Fisher, C. Marsolais, P. Nadler, Proc. Am. Soc. Clin. Oncol. 20 (2001) 2a.
[6] L. Forero, A. Patnaik, L.A. Hammond, A. Tolcher, S. Schwartz, M. Hidalgo, S. Malik, T. Murphy, A. Goetz, T. Mays, A. Kiene, M. Hill, J.S. DeBono, M. Beeram, B. Forouzesh, D. Hao, A. Zitelli, D. Woods, P. Nadler, E.K. Rowinsky, Proc. Am. Soc. Clin. Oncol. 21 (2002) 25b.
[7] B. Forouzesh, M. Hidalgo, C. Takimoto, J.S. de Bono, L. Forero, M. Beeram, S. Malik, A. Patnaik, J. Rizzo, L.A. Hammond, G. Schwartz, A. Goetz, T. Mays, A. Kiene, J. Norris, A. Tolcher, E.K. Rowinsky, P. Nadler, D. Wood, A. Zitelli, Proc. Am. Soc. Clin. Oncol. 21 (2002) 21a.
[8] M.J. Ratain, C.M. George, L. Janisch, H.L. Kindler, C. Ryan, D.L. Wood, P.I. Nadler, E.E. Vokes, Proc. Am. Soc. Clin. Oncol. 21 (2002) 76b.
[9] M. Hidalgo, L.L. Siu, J. Nemunaitis, J. Rizzo, L.A. Hammond, C. Takimoto, S.G. Eckhardt, A. Tolcher, C.D. Britten, L. Denis, K. Ferrante, D.D. Von Hoff, S. Silberman, E.K. Rowinsky, J. Clin. Oncol. 19 (2001) 3267.
[10] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249.