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Liquid-chromatographic determination of erlotinib (OSI-774), an epidermal growth factor receptor tyrosine kinase inhibitor

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Abstract

A high-performance liquid-chromatographic (HPLC) assay with UV detection has been developed for the quantitative determination of erlotinib (OSI-774) in human plasma. Quantitative extraction was achieved by a single-solvent extraction involving a mixture of acetonitrile and *n*-butyl chloride (1:4, v/v). Erlotinib and the internal standard hydrochloride salt (OSI-597) were separated on a column packed with Nova-Pak C18 material and a mobile phase composed of acetonitrile and water, pH 2.0 (60:40, v/v). The column effluent was monitored with dual UV detection at wavelengths of 348 nm (erlotinib) and 383 nm (OSI-597). The calibration graph was linear in the range of 100–4500 ng/ml, with values for accuracy and precision ranging from 87.9 to 96.2% and 2.13 to 5.10%, respectively, for three different sets of quality control samples. The developed method was successfully applied to study the pharmacokinetics of erlotinib in a cancer patient at the recommended daily dose of 150 mg.

Keywords: Pharmacokinetics; Erlotinib; OSI-774

1. Introduction

Phosphorylation of tyrosine residues on the epidermal growth factor receptor (EGFR) is an important early event in signal transduction, leading to cell replication for major human carcinomas [1]. This receptor is widely expressed in advanced cancers, including glioma, breast, ovarian, renal, head and neck, and colon carcinoma, and higher levels of EGFR are inversely related to survival in cancer patients [2]. There is preclinical evidence that EGFR-mediated signaling plays a critical role in processes affecting tumor growth, including cell adhesion, cell motility, angiogenesis, and apoptosis [3]. Therefore, interruption of this growth signal represents a potential target for anticancer treatment. Over the last several years, at least two general strategies have been used to block the EGFR signaling: (i) by preventing ligand binding with anti-EGFR monoclonal antibodies (e.g. cetuximab) and (ii) by inhibiting its intrinsic tyrosine kinase with small molecules [3]. Among numerous EGFR tyrosine kinase inhibitors synthesized, two agents, viz. gefitinib (ZD1839, Iressa®) [4] and erlotinib hydrochloride (OSI-774, Tarceva®, formerly

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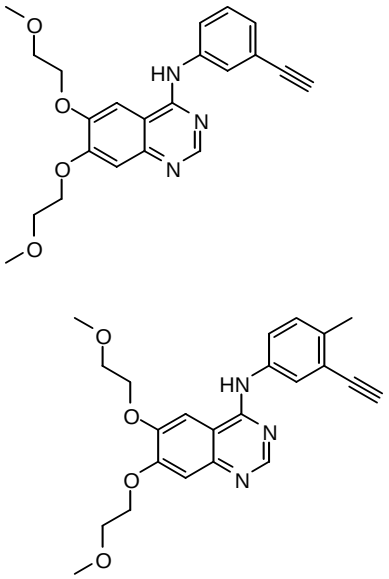

Fig. 1. Chemical structures of erlotinib (OSI-774) (top) and the internal standard (OSI-597) (bottom).

CP-358,774) [5], have advanced to Phase II/Phase III clinical development. The latter agent is an orally bioavailable quinazolinylamine analogue (Fig. 1), and has shown a broad spectrum of preclinical antitumor activity with an IC_{50} of 2 nM (i.e. 0.786 ng/ml) for inhibition of the human EGFR tyrosine kinase [6,7]. Phase I clinical trials with single-agent erlotinib hydrochloride have been completed using both intermittent and continuous dosing regimens [8], and established a recommended daily dose of 150 mg for further clinical evaluation. Phase II clinical trials of erlotinib suggest that the drug might be an effective treatment option either alone or in combination with standard therapies as first- or second-line therapy in a number of malignant diseases [9].

Although erlotinib is generally well absorbed after oral administration, there is large inter- and intrasubject variation in peak concentration and in area under the curve (AUC) after the same oral dose [8]. It has been proposed, therefore, that determination of erlotinib concentrations in patients is expedient before attempting to draw any correlation between drug dose and biologic effects. Hence, a specific and accurate analytical method for the determination of erlotinib in human plasma was considered obligatory. In this report, we describe a novel analytical method based on high-performance liquid chromatography (HPLC) with UV detection for the determination of erlotinib in human plasma samples, which can be implemented easily for routine analysis.

2. Experimental

2.1. Chemicals

Erlotinib hydrochloride, [6,7-bis(2-methoxy-ethoxy)-quinazolin-4-yl]-(3-ethynyl-phenyl)amine hydrochloride (lot # 45574-1-3F; purity, 91.4% free base), the internal standard OSI-597 (lot # OMS-M0012-SD-1-8-28; purity, 99.7%; Fig. 1) and OSI-420 (lot # 1050072598; purity 94.9%), a metabolite of erlotinib, were obtained from OSI Pharmaceuticals Inc. (Boulder, CO, USA). HPLC grade acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ, USA) and *n*-butyl chloride originated from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade or better. Water was purified and deionized by the Milli-Q-UF system (Millipore, Milford, MA, USA) and used throughout. Human plasma was obtained from healthy volunteers.

2.2. Preparation of stock solutions and standards

Stock solutions of OSI-774 and OSI-597 were prepared independently in triplicate by dissolving the appropriate amount of drug, corrected for purity, in neat ethanol at a concentration of approximately 0.64 mg/ml, and were then stored in glass at −20 °C for up to 4 weeks. The difference in drug concentration in each of the triplicate stock solutions, estimated from the mean peak area following repeat analysis of a dilution of the stock, was determined to be within 5%. Out of one of the erlotinib stock solutions, a working solution containing 100 μg/ml was prepared in ethanol, which was further used for the construction of calibration samples and quality control (QC) samples. An internal standard working solution of 20 μg/ml was prepared by dilution with ethanol, and was stored for later use at −20 °C.

With each analytical run, calibration standards in drug-free human heparinized plasma were prepared freshly in duplicate at erlotinib concentrations of 100,

500, 1000, 1500, 3000, and 4500 ng/ml, such that the total amount of ethanol added was identical in each sample (approximately 6%). Pools of QC samples of erlotinib in plasma were prepared similarly in polypropylene tubes at concentrations of 300, 2000, and 4000 ng/ml, and stored in batch at −20 °C for the duration of the validation procedure.

2.3. Sample pretreatment

Plasma samples containing known or unknown concentrations of erlotinib were allowed to thaw at room temperature, and then mixed for 5 s on a vortex-mixer. A 500 μl volume of plasma was transferred to a polypropylene tube (100 mm × 14 mm), followed by addition of the internal standard solution (30 μl to yield a final concentration of OSI-597 of 1200 ng/ml) and 5 ml of a mixture of acetonitrile and *n*-butyl chloride (1:4, v/v). The tube was capped, mixed for 20 s and centrifuged for 5 min at 2850 × *g*. After centrifugation, the organic layer was transferred to a clean glass tube with a Pasteur pipette, and evaporated to dryness under a constant stream of nitrogen at 45 °C. Next, a volume of 200 μl of a 1:4 (v/v) mixture of acetonitrile and water (pH 2.0, trifluoroacetic acid) was added to the residue, which was reconstituted by vortex-mixing for 10 s. The content of the tube was transferred to a low-volume glass insert and a 150 μl aliquot was subjected to chromatography.

2.4. Chromatography

The chromatographic system consisted of an 1100 system (Agilent, Palo Alto, CA, USA) equipped with a photodiode array detector. The stationary phase was composed of Nova-Pak C18 material (4 μm particles) packed in a 150 mm × 3.9 mm (internal diameter) stainless steel column (Waters, Milford, MA, USA), without a guard column. The mobile phase consisted of a mixture of acetonitrile and water (60:40, v/v), with the pH adjusted to 2.0 (trifluoroacetic acid), and a flow rate set at 1.00 ml/min. Preliminary experiments indicated that within a range of 190–400 nm, the peak wavelengths for erlotinib and OSI-597 occurred at 348 and 383 nm, respectively (not shown). Hence, the column effluent was monitored at a dual UV absorption wavelength of 348 and 383 nm to allow for optimal detector responses of each analyte. The column temperature was maintained 20 °C. Acquisition and integration of data was performed with a ChemStation chromatographic work station (Agilent) running on an IBM-compatible computer under Microsoft Windows XP version 5.1. Calibration graphs were calculated by least-squares linear regression analysis of the peak area ratio of erlotinib and the internal standard versus the drug concentration of the nominal standard using Microsoft Excel 2002. The zero concentration sample (blank) was used to visually verify the purity of the reagents and the lack of other potentially interfering (endogenous) substances, but was not considered for the regression analysis of standards. The goodness-of-fit of various calibration models was evaluated by visual inspection, the correlation coefficient and an ANOVA lack-of-fit test.

2.5. Validation procedures

Method validation was performed according to procedures described in detail elsewhere [10], with modifications. With each chromatographic validation run, duplicate calibration standards were prepared in blank human plasma by serial dilution at erlotinib concentrations of 100, 500, 1000, 1500, 3000, and 4500 ng/ml. Sets of QC samples were prepared separately in batch in the same manner at final concentrations of 300, 2000, and 4000 ng/ml, and were analyzed with each chromatographic run in quadruplicate. The complete validation procedure was performed on four separate occasions. The precision of the assay was assessed by the between-run and within-run precision (WRP). Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as 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 run days were calculated using the NCSS 2001 package (J. Hintze, Number Cruncher Statistical Systems, Kaysville, UT, USA). The between-run precision (BRP) was defined as:

$$\mathrm{BRP}\,(\%) = \left[\frac{(\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n}{\mathrm{GM}}\right]^{0.5} \times 100 \qquad (1)$$

where n represents the number of replicates within each validation run. The WRP was calculated as:

$$\text{WRP}\ (\%) = \frac{(\text{MS}_{\text{wit}})^{0.5}}{\text{GM}} \times 100 \quad (2)$$

The accuracy or percentage deviation (DEV) was calculated by the formula:

$$\text{DEV}\ (\%) = \left(1 - \frac{\text{observed concentration}}{\text{nominal concentration}}\right) \times 100 \quad (3)$$

The extraction recovery of erlotinib was established at concentrations of 500 and 3000 ng/ml, by comparing peak areas of samples prepared in human plasma with the spiking solution The recovery was determined in four independent analytical runs, and expressed as a percentage. This abbreviated procedure was performed to confirm an earlier observation that the applied solvent extraction for isolation of erlotinib results in >95% extraction efficiency [11].

Blank human plasma samples from five different individuals were used to investigate the potential interference of endogenous components. In addition, certain drugs that are commonly used in the pre- or postchemotherapy period were selected for potential chromatographic interference in the final assay for erlotinib and the internal standard.

2.6. Pharmacokinetic analysis

The patient studied was a female with a histologically confirmed diagnosis of advanced breast cancer, who received chemotherapeutic treatment with single-agent erlotinib hydrochloride tablets at a daily dose of 150 mg (OSI Pharmaceuticals). The tablets were taken once daily in the morning with up to 200 ml of water 1 h before or 2 h after food. Treatment was administered on an outpatient basis, with tablets dispensed to the patient on the first day of each 28-day treatment period. The current experiment was approved by the local Institutional Review Board, and the patient signed informed consent before study entry for the blood sampling procedure.

A total of eight blood samples (7 ml each) were obtained and collected in 10 ml glass tubes containing heparin as an anticoagulant. These samples were obtained before the first dose of erlotinib was administered, and then again on day 28 at 0, 2.67, 4.67, 9.67, 13.67, and 27.92 h after drug administration. Specimens were immediately centrifuged at 3000g for 5 min to separate the plasma supernatant, which was stored at $-70\,^{\circ}\text{C}$ until the time of analysis. Plasma concentration–time data of erlotinib were analyzed by noncompartmental methods using the software package WinNonlin v4.0 (Pharsight Corporation, Mountain View, CA), assuming steady-state conditions.

3. Results and discussion

3.1. Chromatography

Sample pretreatment of erlotinib in plasma was initially performed by a one-step solvent extraction with methyl *t*-butyl ether or diethyl ether, based on an earlier procedure [8]. This sample handling, however, consistently showed a major peak in human blank plasma co-eluting at or around the retention time of the internal standard. Furthermore, it resulted in an unusual artifactual chromatographic behavior of the analytes, with separation into two peaks of comparable peak area proportion, when analyzed according to the method by Hidalgo et al. Eventually, erlotinib and the internal standard were isolated with a single step extraction involving a mixture of acetonitrile and *n*-butyl chloride, which was initially developed for the isolation of taxane antineoplastic agents from human matrices [12]. This sample handling was also chosen for its optimal elimination of endogenous interferences, while maintaining high extraction efficiency for all compounds. In order to ensure sufficient selectivity and analyte separation in this assay, we have also slightly modified the mobile phase composition as compared to an earlier method [8] by increasing the organic modifier content from 30 to 60%. In fact, low contents of acetonitrile or the use of methanol in the mobile phase resulted in poor accuracy and precision due to severe front-tailing bands (asymmetry factor $\geq$ 2.0), arising from secondary retention effects on the reversed-phase column. In addition, a reduced diameter column with smaller silica particle size was used, providing better resolution with a shorter run time. Unlike the method developed by Hidalgo et al. [11], erlotinib and the internal standard were both monitored by DAD at their respective absorbance maxima, to provide the most reproducible absorbance.

3.2. Validation characteristics

Fig. 2 displays chromatograms of an extract of a blank human plasma sample with internal standard only (A), an extract of a plasma sample spiked with erlotinib at a concentration of 1000 ng/ml (B), and an extract of a trough plasma sample taken immediately before erlotinib intake on day 28 (dose, 150 mg) (C), all obtained under the optimized conditions. The selectivity for the analytes is shown by the sharp and symmetrical resolution of the peaks, with no significant interfering peaks for all compounds in drug-free specimens, obtained from different individuals. Erlotinib (t_R = 1.58 min) and the internal standard OSI-597 (t_R = 1.81 min) were well separated, and the overall chromatographic run time was established at 3 min. Several different drugs were tested for potential interference with erlotinib and the internal standard (Table 1), and none of the samples taken from patients on these drugs was found to give an interfering peak during the analysis around the retention time of erlotinib or the internal standard (not shown). The selectivity and resolution of the HPLC system was also confirmed by co-injection of erlotinib with one of its known metabolites, OSI-420 [8] (t_R = 1.40 min).

The measurement variance over the range of 100–4500 ng/ml increased proportionally with the erlotinib concentration, as detected by a one-sided *F*-test at an α-value of 5% [13]. Because of this heteroscedasticity, a weighting factor was used, which is inversely proportional to the variance at the given concentration level. After applying the peak area ratio of the internal standard and erlotinib in combination with a weighting factor of $1/x$, a mean (±standard deviation) linear regression correlation coefficient of 0.9949 ± 0.0029 (range, 0.9914–0.9985) was obtained. The statistical evaluation of the coefficients of the ordinary least-squares line indicated small bias in the slope (relative standard deviation, R.S.D., 0.0595) and in the intercept (R.S.D., 0.128), further indicating minor matrix effects and blank problems, respectively [13]. In blank human plasma spiked with erlotinib at a concentration of 100 ng/ml, only one out of eight samples was outside the acceptable ±20% deviation limits for accuracy [10], while the remaining samples were within 16%, with a mean percentage deviation from the nominal concentration and a between-run variability of −12.5 and 7.54%, respectively. Based on these results, the lower limit of quantitation was established at 100 ng/ml.

Table 1
Interference analysis of samples from subjects on various commonly administered drugs

Drug	Dosage[a]	Drug	Dosage[a]
Amlodipine besylate	10 mg (oral)	Loperamide	2 mg (oral)
Atenolol	50 mg (oral)	Metronidazole	0.75% (topical)
Ciprofloxacin	750 mg (oral)	Morphine sulfate	10 mg (oral)
Clotrimazole	15 mg (oral)	Omeprazole	20 mg (oral)
Cyanocobalamine	250 μg (oral)	Ondansetron	8 mg (i.v.)
Dexamethasone	8 mg (oral)	Oxycodone	5 mg (oral)
Diazepam	2 mg (oral)	Pamidronate disodium	90 mg (i.v.)
Diphenhydramine	50 mg (i.v.)	Phenytoin	400 mg (oral)
Docusate sodium	100 mg (oral)	Pseudoephedrine	30 mg (oral)
Epoietin alpha	40000 units (s.c.)	Pyridoxine hydrochloride	50 mg (oral)
Fluticasone proprionate	0.05% (nasal)	Raloxifene	–[b] (oral)
Folic acid	400 μg (oral)	Ranitidine	150 mg (oral)
Glucosamine sulfate	500 mg (oral)	Rofecoxib	25 mg (oral)
Hydromorphone	2 mg (i.v.)	Sertraline hydrochloride	100 mg (oral)
Hydroxyzine	25 mg (oral)	Verapamil	240 mg (oral)
Ketoconazole	2% (topical)	Warfarin	1 mg (oral)
Levofloxacin	500 mg (oral)	Zolpidem tartrate	5 mg (oral)
Levothyroxine	75 μg (oral)		

Abbreviations: i.v., intravenous; s.c., subcutaneous.
[a] Route of administration in parentheses.
[b] Drug dose unknown.

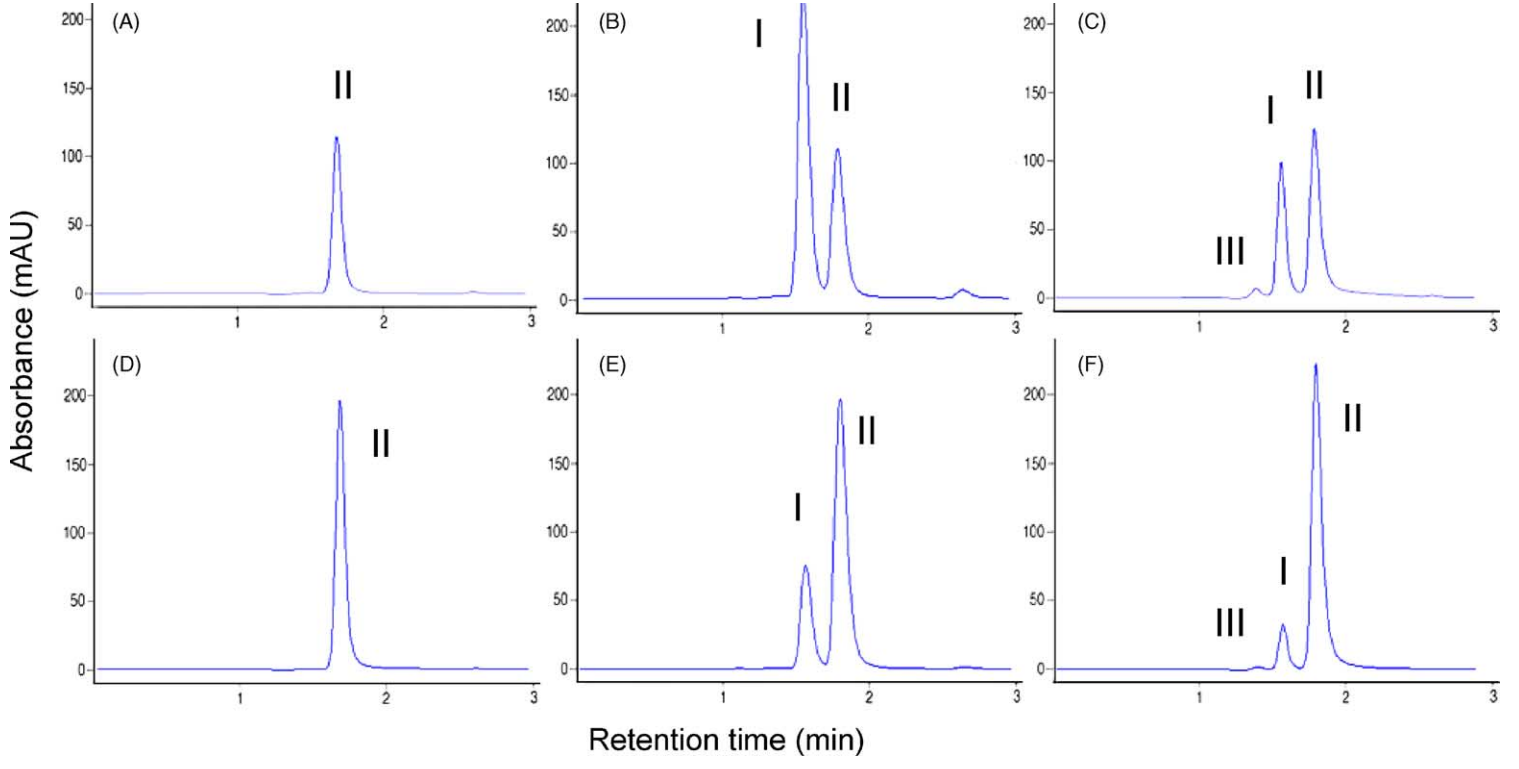

Fig. 2. Chromatograms from reversed-phase HPLC analysis of a blank human plasma sample (A and D), a human plasma sample spiked with erlotinib at a concentration of 1000 ng/ml (B and E), and a plasma sample obtained from a female patient with breast cancer immediately before oral administration of erlotinib on day 28 (dose, 150 mg) (C and F). The top and bottom chromatograms were obtained using the optimal UV absorption wavelengths for erlotinib (348 nm) and the internal standard OSI-597 (383 nm), respectively. The labeled chromatographic peaks indicate erlotinib (I), the internal standard OSI-597 (II), and the erlotinib metabolite OSI-420 (III), respectively. The calculated *R*-value for erlotinib and OSI-597 is 1.637.

Table 2
Back calculated concentrations from calibration curves[a]

Nominal (ng/ml)	GM (ng/ml)	S.D. (ng/ml)	DEV (%)	R.S.D.	*n*
100	87.5	6.60	−12.5	0.0754	2
500	547	38.5	+9.46	0.0702	2
1000	1021	76.2	+2.12	0.0747	2
1500	1493	117	−0.468	0.0783	2
3000	3086	280	+2.86	0.0909	2
4500	4364	385	−3.03	0.0883	2

Abbreviations: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; R.S.D., relative standard deviation; *n*, number of replicate observations within each validation run.

[a] Two samples at each concentration were run on four separate occasions, for a total of eight samples at each concentration.

Validation data of the analytical method in terms of accuracy (i.e. the percentage deviation from nominal) and precision are shown in Tables 2 and 3. At the upper limit of quantitation (i.e. 4500 ng/ml), the mean percentage deviation and the within-run variability were less than 9%. Based upon analysis of QC samples on four different occasions, the final method was shown to be accurate, with an average accuracy at the three tested concentrations within 13.1%, and precise, with a within-run and between-run precision always within 8.83%. The peak area response of the internal standard showed an R.S.D. of 0.0626 and a within-run precision of 6.04%, indicating an acceptable degree of bias and low random error. The mean overall extraction recoveries for erlotinib, determined in four separate analytical runs, was not significantly different from 100% and independent of the spiked concentration, similar to previous findings [11].

Table 3
Assessment of accuracy and precision from quality-control samples[a]

Nominal (ng/ml)	GM (ng/ml)	S.D. (ng/ml)	DEV (%)	WRP (%)	BRP (%)	*n*
300	288.5	25.96	−3.83	4.93	8.83	4
2000	1839	85.24	−8.04	5.10	–[b]	4
4000	3477	100.7	−13.1	2.13	2.90	4

Abbreviations: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; WRP, within-run precision; BRP, between-run precision; *n*, number of replicate observations within each validation run.

[a] Four samples at each concentration were run on four different days, for a total of 16 samples per concentration level.

[b] No additional variation was observed as a result of performing the assay on different days.

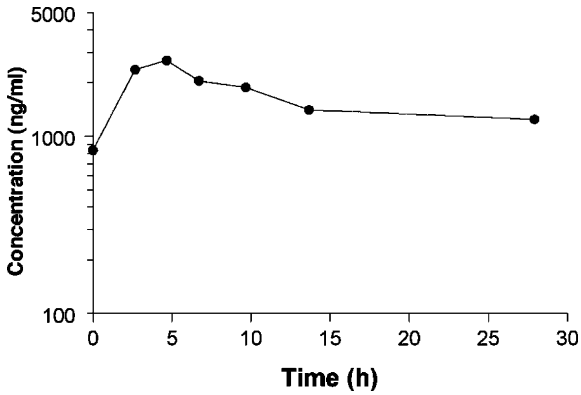

Fig. 3. Plasma concentration–time profile of erlotinib on day 28, following oral administration of a dose of 150 mg to a female patient with breast cancer, after 27 days of continuous once-daily dosing.

3.3. Clinical pharmacokinetics

The described analytical method was applied to a pharmacokinetic pilot study of erlotinib given orally to a patient with breast cancer on a once every day (continuous) schedule. The observed concentration–time profile of erlotinib, obtained on day 28, is shown in Fig. 3. The peak concentration of erlotinib was 2690 ng/ml, and the $AUC_{0–28}$ was 45.7 μg h/ml, which is similar to a mean $AUC_{0–24}$ (±standard deviation) value of 38.4 ± 29.6 μg h/ml previously obtained in three adults with various malignant diseases who were also given a dose of 150 mg for 23 days [8].

4. Conclusion

In conclusion, the method presented for the determination of erlotinib in human plasma is specific, accurate and precise, and is selective enough to be used in clinical trials without any extrapolation. As sample pretreatment consists of a simple one-step protein-precipitation and extraction, it avoids the use of a specific solvent extraction techniques involving methyl *t*-butyl ether that were used in a previous report. Although tandem triple-quadrupole

mass-spectrometric detection of erlotinib in extracted plasma samples was shown to provide superior assay sensitivity [11] as compared to the UV detection described here, this technique is still at the disposal of few laboratories only. Moreover, the current method provides sufficient sensitivity and permits the analysis of samples obtained from patients treated at the recommended daily dose of 150 mg, suggesting that a limit of quantitation below 100 ng/ml is not required. The assay is currently being used to further investigate the clinical pharmacologic behavior of erlotinib in cancer patients.

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