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Short communication

A simple HPLC-UV method for the simultaneous quantification of gefitinib and erlotinib in human plasma

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ABSTRACT

Gefitinib and erlotinib are two oral tyrosine kinase inhibitors (TKI) approved for the treatment of advanced non-small cell lung cancer (NSCLC). Published methods for simultaneous analysis of erlotinib and gefitinib in plasma are exclusively based on mass spectrometry. The purpose of this study was to develop a simple and sensitive HPLC-UV method to simultaneously quantify these two TKI in plasma. Following liquid–liquid extraction, gefitinib, erlotinib and sorafenib (internal standard), were separated with gradient elution (on a C8+ Satisfaction® using a mobile phase of acetonitrile/20 mM ammonium acetate pH 4.5). Samples were eluted at a flow rate of 0.4 ml/min throughout the 15-min run. Dual UV wavelength mode was used, with gefitinib and erlotinib monitored at 331 nm, and sorafenib at 249 nm. The calibration was linear in the range 20–1000 ng/ml and 80–4000 ng/ml for gefitinib and erlotinib, respectively. Inter- and intra-day imprecision were less than 7.2% and 7.6% for gefitinib and erlotinib, respectively. This analytical method was successfully applied to assess the steady state plasma exposure to these TKI in NSCLC patients. This simple, sensitive, accurate and cost-effective method can be used in routine clinical practice to monitor gefitinib or erlotinib concentrations in plasma from NSCLC patients.

1. Introduction

Gefitinib and erlotinib are two orally bioavailable synthetic anilinoquinazolines that selectively and reversibly bind to the intracellular ATP-binding site of the epidermal growth factor receptor (EGFR) tyrosine kinase [1]. These two tyrosine kinase inhibitors (TKI) are currently approved for the treatment of non-small-cell lung cancer (NSCLC) in patients harbouring EGFR activating mutations.

Both drugs are associated with large interindividual pharmacokinetic variability in cancer patients (~60%) [1]. This substantial pharmacokinetic variability is likely to impact treatment outcomes, further that different clinical studies reported a relationship between clinical outcomes and plasma exposure to erlotinib [2–4] and gefitinib [5,6]. Besides, these two TKIs primarily metabolized through CYP3A4 pathway are candidates to pharmacokinetic drug–drug interactions with inducers and inhibitors of CYP3A4 [1,7]. Taken together, these elements support the need to perform a therapeutic drug monitoring (TDM) for these two TKI. Otherwise, the over-expression of cellular drug efflux pumps, such as P-glycoprotein (P-gp) in tumour cells is thought to play a significant role in the resistance phenotype of tumour [8]. Given erlotinib and gefitinib are substrates of P-gP [1], measuring intracellular concentration of these drugs in tumour cells may be also suitable.

Several liquid chromatography–tandem mass spectrometry (LC/MS/MS) methods have been described to determine erlotinib and/or gefitinib in human plasma [9–13]. Because of their high sensitivity, these methods could be adapted to quantify intracellular concentrations of these drugs in tumour cells. As LC/MS/MS is not available in most hospital laboratories, high performance liquid

Abbreviations: AUC_{0-24}, area under the plasma drug concentration–time curve from 0 to 24 h; C_{minss}, trough concentration at steady state; CYP3A4, cytochrome P4503A4; DMSO, dimethylsulfoxide; EGFR, endothelial growth factor receptor; HPLC, high performance liquid chromatography; IQC, in-house quality control; IS, internal standard; LC/MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower limit of quantification; NSCLC, non-small cell lung cancer; TDM, therapeutic drug monitoring; TKI, tyrosine kinase inhibitor.

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chromatography with ultraviolet detection (HPLC-UV) may be used instead to measure drug concentration in plasma. However, in contrast with erlotinib [14–16], no HPLC-UV method has been reported for the determination of gefitinib concentrations in human plasma.

The aim of this work was to develop a simple, sensitive and cost-effective HPLC-UV method to simultaneously quantify gefitinib and erlotinib in human plasma from NSCLC patients.

2. Materials and methods

2.1. Reagents

Erlotinib, gefitinib and sorafenib were purchased from LC Laboratories (Woburn, USA), ethyl acetate, HPLC grade acetonitrile, sodium hydroxide (NaOH) 1 N and dimethylsulfoxide (DMSO) from VWR (Fontenay-sous-Bois, France). Ammonium acetate was purchased from Sigma (St. Louis, MO, USA). Deionised purified water was prepared in the laboratory using an ELGA system (Veolia, Le Plessis Robinson, France).

2.2. Stock and working standard solutions

Stock solutions containing 1 mg/ml of gefitinib, erlotinib and sorafenib (internal standard, IS) were prepared in DMSO. These stocks solutions were aliquoted, then stored at −20 °C in the dark. Each day, working solutions of gefitinib (10 and 1 μg/ml) and erlotinib (40 and 4 μg/ml) were freshly prepared with drug-free plasma for a set of calibrating standards at 20, 50, 100, 250, 500 and 1000 ng/ml for gefitinib and 80, 200, 400, 1000, 2000 and 4000 ng/ml for erlotinib. In-house quality controls (IQC) were prepared using different stock solutions of gefitinib and erlotinib. Concentrations of IQC were 20, 200 and 1000 ng/ml for gefitinib, and 80, 800 and 4000 ng/ml for erlotinib. Finally, a working solution of IS (25 μg/ml) was also freshly prepared with deionised purified water.

2.3. Chromatographic apparatus and conditions

The chromatography system consisted of Dionex Ultimate 300 equipped with a gradient pump with degas option and gradient mixer, a UV-visible detector, an autosampler, and a Chromeleon® chromatography workstation (Dionex Corporation, Sunnyvale, CA, USA). Chromatographic separation was achieved on a C8+ Satisfaction® (250 mm × 3 mm, 5 μm; Cluzeau Info Labo, Courbevoie, France) associated with a guard column packed with the same bonded phase. The composition of the mobile phase at time zero (the time of injection) was 45% ammonium acetate (20 mM, pH 4.5) and 55% acetonitrile. After 5-min run, the percentage of acetonitrile was gradually increased to 75% over 6 min. Then, the composition was changed back to ammonium acetate–acetonitrile (45:55; v:v) within 6 s. Finally, the chromatographic system was equilibrated during 5 min before the next injection. A flow rate of 0.4 ml/min was used throughout the 15-min run. Chromatography was performed at 40 °C. Dual UV wavelength mode was used, with erlotinib and gefitinib monitored at 331 nm, and sorafenib at 249 nm.

2.4. Sample preparation

First, 100 μl of IS at 25 μg/ml was added to 400 μl of plasma (calibration standard, IQC or plasma sample). After mixing, 500 μl of NaOH 1 N was added, then 3 ml of ethyl acetate. After an agitation of 10 min with a mechanical shaker, the tubes were centrifuged 10 min at 4000 rpm at room temperature. The supernatant was transferred into a glass tube and evaporated to dryness at 40 °C under nitrogen stream. The residue was reconstituted in 70 μl of mobile phase and 50 μl of each sample was injected into the chromatographic system.

2.5. Specificity and selectivity

Only blood samples from subjects not receiving any of the drugs of the interest (erlotinib, gefitinib and sorafenib) were used to test the specificity and the selectivity of the method. Interferences with endogenous compounds have been evaluated from plasma samples from patients experiencing severe hepatic ($n = 5$) or renal ($n = 5$) impairment. Drug interferences were investigated in plasma from 10 cancer patients and 20 hospitalized patients. The patient samples were run without being spiked with drug. Overall, this analysis allowed testing potential interferences of 59 drugs (assessed at steady state) with gefitinib, erlotinib and sorafenib (Table 1).

2.6. Method validation

The method was validated according to the FDA guidelines for bioanalytical method validation [17].

Linearity of the method was determined by replicate analysis of 6 complete standard curves on 6 separate days. The three levels of IQC for each compound were assayed thrice with each standard curve. A linear regression was used to plot the peak area ratio (y) of analyte to IS vs. analyte concentration. Homoscedasticity of the model was assessed by the Levene test. The best weighting factor for linear regression was determined according to the result of the Levene test and to the variation of variance with respect to concentration. Intra- and inter-day imprecision (coefficient of variation (CV%)) and accuracy expressed as bias were evaluated using the three levels of IQC. Six replicates of each level were assayed in one run for the intra-day experiment. Three replicates of each level were assayed within six different days for the inter-day experiment. According to FDA guidelines, the accuracy and imprecision for all tested concentrations should be within ±15% except for the lower limit of quantification (LLOQ), in which case these parameters should not exceed 20%.

Table 1
Drugs listed for the specificity.

Acepromazine	Losartan
Acetaminophen	Metoclopramide
Ajmaline	Naproxen
Alfuzosin	Nefopam
Allopurinol	Oycodone
Alprazolam	Paroxetine
Amiodarone	Pefloxacin
Amlodipine	Pegfilgrastim
Budesonide	Phenobarbital
Cholecalciferol	Piperacillin
Ciprofloxacin	Potassium Chloride
Clonazepam	Prazepam
Clorazepate dipotassium	Propranolol
Cloxacillin	Rifampin
Cyclosporine	Rosuvastatin
Digoxin	Sodium chloride
Enoxaparin	Sulfamethoxazole
Escitalopram	Sunitinib
Esomeprazole	Tacrolimus
Everolimus	Tazobactam
Fluconazole	Teicoplanin
Formoterol	Theophylline
Fosfomycin	Tramadol
Fusidic Acid	Trimethoprim
Gentamicin	Valaciclovir
Heparin	Vancomycin
Hydrochlorothiazide	Verapamil
Hydrocortisone	Zopiclone
Isradipine	
Levetiracetam	

Recovery of gefitinib and erlotinib was evaluated at concentrations corresponding to the three IQC values analysed in six replicates. The peak areas of extracted IQC were compared with peak areas of reference standards prepared in mobile phase and injected directly into the column. The recovery was expressed as percentage area of the extracted IQC relative to the directly injected reference standard.

The stability of erlotinib and gefitinib was assessed using the three levels of IQC (in triplicate) for freeze–thaw, short-term stability and stability after sample preparation. The freeze–thaw samples underwent 3 cycles of freeze and thaw before preparing the samples for analysis, the short term stability samples were kept for 24 h at room temperature (20 °C) or at 4 °C before extraction, and post-preparation samples were extracted and kept in mobile phase for 24 h in the autosampler at 4 °C. For all these experiments, stability was acceptable when ≥85% of the analyte was recovered.

2.7. Application

The method was applied to determine gefitinib and erlotinib concentrations over 24 h-period in two patients with relapsed NSCLC harbouring EGFR activating mutations. Patients received either 150 mg of erlotinib or 250 mg of gefitinib on a once daily schedule. Pharmacokinetic sessions were performed after drug exposure had reached steady state (i.e., 5 half-lives). Samples were collected into 5-ml lithium heparinized vacutainer tubes at different times (i.e., trough level, 1, 2, 4, 8, 12 and 24 h after dosing). The area under the plasma drug concentration–time curve from 0 to 24 h (AUC_{0-24}) was calculated using the linear trapezoidal rule. Steady state trough concentration (C_{minss}) was also measured in 29 adult NSCLC patients treated in a routine outpatient setting with gefitinib ($n = 8$) or erlotinib ($n = 21$). After centrifugation at 3000 rpm for 5 min at 4 °C, plasma was transferred to propylene tubes and stored at −20 °C until analysis. This study was approved by the local Review Board for Oncology, and all patients provided informed consent and approved the protocol and the sampling in compliance with the ethical principles originating from the revised Declaration of Helsinki (Edimburg, 2000) and according to French regulations.

3. Results

3.1. Chromatography

Representative chromatograms of blank plasma, LLOQ and patient treated with gefitinib or erlotinib are shown in Fig. 1. No significant interference from endogenous or exogenous compounds was observed in the chromatograms of plasma from 30 patients (10 cancer patients and 20 hospitalized patients) and patients experiencing severe renal or hepatic impairment.

3.2. Linearity

The Levene statistic test showed a significant difference ($p < 0.05$) between variances of each standard concentration. As the variance grew proportionally to the concentration, the best weighting factor was 1/(peak area ratio). In human plasma, calibration curves were linear over the range of 20–1000 ng/ml for gefitinib, and over the range of 80–4000 ng/ml for erlotinib. The assay showed good correlation coefficients ($r^2 > 0.99$ for two analytes), with regression intercepts not statistically different from zero.

3.3. Accuracy, imprecision and LLOQ

Intra- and inter-assay bias and imprecision are summarized in Table 2. The LLOQs for gefitinib and erlotinib were 20 ng/ml and 80 ng/ml, respectively.

3.4. Recovery

The mean absolute recoveries (±SD) for gefitinib were 82.9% (±15.0%), 83.5% (±6.9%) and 81.6% (±4.1%) at 20, 200 and 1000 ng/ml, respectively. Concerning erlotinib, the mean absolute recoveries were 81.3% (±3.3%), 80.7% (±5.6%) and 83.7% (±3.3%) at 80, 800 and 4000 ng/ml. Finally, the mean recovery of the IS was 84.1% (±5.3%).

3.5. Stability

The results of the stabilities under varying storage conditions are summarized in Table 3. All results well met the criterion for the stability measurements. The long-term stability of gefitinib and erlotinib in plasma was not studied because erlotinib and gefitinib are stable at −20 °C in human plasma for up to 12 months and 5 months, respectively [11,12].

3.6. Application in clinical settings

Fig. 2 presents the pharmacokinetic profile of gefitinib (250 mg/day) and erlotinib (150 mg/day) in two Caucasian patients. The AUC_{0-24} values were 8746 ng/ml h and 96.9 μg/ml h for gefitinib and erlotinib, respectively. In daily clinical practice, mean C_{minss} was 211 ± 63.9 ng/ml in 8 patients administered 250 mg gefitinib per day, and 1609 ± 895 ng/ml in 21 patients administered erlotinib (dose range: 150–300 mg/day).

4. Discussion

To date, published methods for gefitinib quantification in plasma are exclusively based on mass spectrometry [9–12]. Here, we describe for the first time a simple, sensitive and cost-effective HPLC method using UV detection and liquid–liquid extraction for quantification of gefitinib in plasma from NSCLC patients. Additionally, this method presents the great advantage to allow a simultaneous quantification of erlotinib.

Sorafenib was selected as internal standard because it is a structurally similar TKI. Additionally, it is not currently prescribed in combination with erlotinib or gefitinib to NSCLC patients. The chromatographic and extraction conditions from a previously published HPLC-UV method for sorafenib quantification [18] were initially tested but an interfering peak in human blank plasma co-eluted at or around the retention time of gefitinib. The substitution of C18 ultrasphere ODS column (250 mm × 4.6 mm, 5 μm; Beckman Coulter, Fullerton, USA) for C8+ Satisfaction® significantly improved the peak shape and resolution, but not enough to separate gefitinib with the interfering peak in human blank plasma. Decreasing the pH of ammonium acetate from 6.5 to 4.5 allowed achieving this complete separation. Under these new chromatographic conditions, the closeness of gefitinib peak to the solvent front required to decrease the flow rate of mobile phase (from 1 ml/min to 0.4 ml/min). Finally, initial extraction conditions [18] were slightly modified to achieve the analytical sensitivity required for the quantification of gefitinib. During the pre-treatment of samples, the substitution of water for NaOH 1 N significantly improved the gefitinib recovery yield (by a 1.4-fold factor) and therefore allowed achieving the concentrations range required. Overall, the present liquid–liquid extraction is efficient, reproducible and rapid.

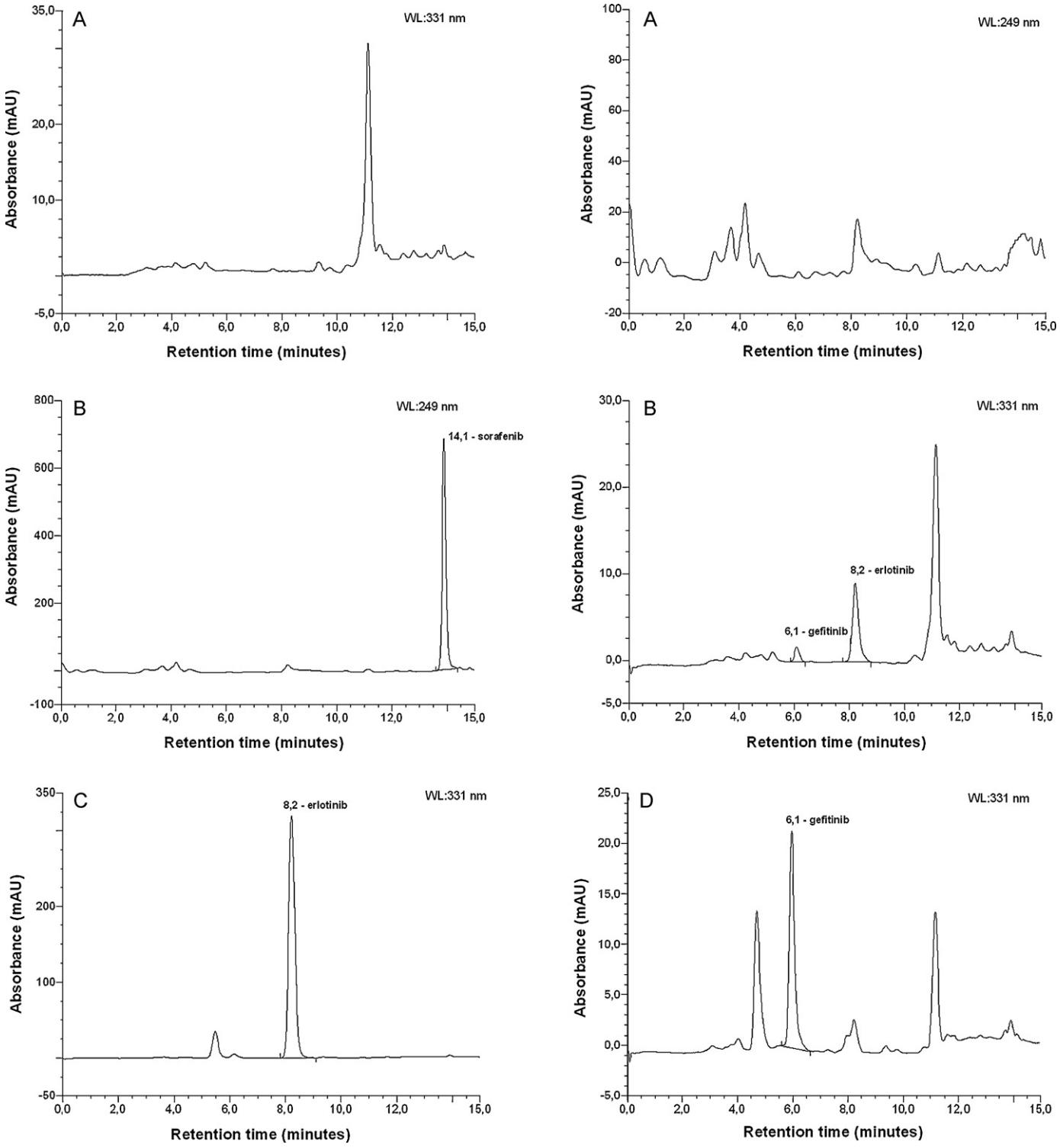

Fig. 1. Representative chromatograms. (A) Blank human plasma at 331 nm (figure) and 249 nm (insert), (B) lower limit of quantification of gefitinib (20 ng/ml) and erlotinib (80 ng/ml) at 331 nm (figure) and sorafenib (internal standard) at 249 nm (insert), (C) plasma from NSCLC patient treated with 150 mg erlotinib once daily (concentration of erlotinib: 3531 ng/ml), (D) plasma from NSCLC patient treated with 250 mg gefitinib once daily (concentration of gefitinib: 316 ng/ml).

The present method has a satisfactory selectivity and specificity. However, as we did not ensure whether interfering drugs were at a therapeutic level in plasma, our methodology to test the specificity presents a limit. The values of imprecision and bias for two drugs prove both the reliability and the reproducibility of the method. The LLOQs found with our method (20 ng/ml and 80 ng/ml for gefitinib and erlotinib, respectively) are substantially higher than those previously reported with LC/MS/MS methods (5 ng/ml for both TKIs) [9,10]. However, the high sensitivity of LC/MS/MS methods is further useful for the quantification of these TKIs in tumour cells than in plasma. Our LLOQs are clinically relevant for the quantification of gefitinib and erlotinib in plasma. Indeed, two investigations in patients received 250 mg gefitinib once daily reported an average C_{minss} of 266 ng/ml

Table 2
Imprecision and accuracy of gefitinib and erlotinib determination in human plasma.

Gefitinib			Erlotinib		
Theoretical (ng/ml)	Bias[a] (%)	Imprecision (%)	Theoretical (ng/ml)	Bias[a] (%)	Imprecision (%)
Within-day (n = 6)					
20	−2.3	7.2	80	5.7	7.6
200	−9.5	4.1	800	−2.4	3.5
1000	−6.5	3.0	4000	2.8	3.6
Between-day (n = 18)					
20	−4.4	6.0	80	−3.3	7.1
200	1.1	4.9	800	−2.2	4.9
1000	−0.1	5.3	4000	−1.1	5.0

[a] Accuracy is expressed as bias.

Table 3
Stability of gefitinib and erlotinib under various storage conditions (n = 3 for each value).

Gefitinib			Erlotinib		
Concentration (ng/ml)	Stability[a] (%)	CV (%)	Concentration (ng/ml)	Stability[a] (%)	CV (%)
Freeze–thaw cycle 1					
20	110.7	8.3	80	95.1	6.0
200	103.2	7.9	800	91.8	3.2
1000	100.4	1.1	4000	99.7	0.2
Freeze–thaw cycle 2					
20	107.0	6.7	80	97.4	8.2
200	102.4	4.0	800	91.1	2.3
1000	99.1	3.4	4000	101.1	1.8
Freeze–thaw cycle 3					
20	102.2	5.0	80	111.0	9.0
200	106.5	2.3	800	99.2	3.6
1000	114.5	0.5	4000	99.6	1.1
Before extraction at RT (24 h)					
20	103.5	7.4	80	105.9	1.4
200	111.1	1.3	800	95.1	4.5
1000	112.4	2.9	4000	101.9	4.3
Before extraction at 4 °C (24 h)					
20	96.4	13.4	80	107.2	1.6
200	118.1	2.1	800	106.4	4.0
1000	102.9	5.4	4000	104.6	1.1
Stability in autosampler (24 h)					
20	103.0	3.5	80	102.7	0.7
200	95.7	3.8	800	100.8	6.0
1000	96.6	2.7	4000	97.1	3.5

RT, room temperature.
[a] Expressed as the mean percentage change from time zero (nominal concentration).

(range 94–538 ng/ml) and 406 ng/ml (range 104–1846 ng/ml) [5,6]. Regarding erlotinib, phase I studies reported a C_{minss} of 1200 ± 620 ng/ml and 1642 ± 1085 ng/ml in Western and Japanese NSCLC patients treated with the recommended 150 mg daily dose, respectively [2,19]. Overall, these data support that our LLOQs are sufficient to quantify plasma erlotinib or gefitinib plasma C_{minss} in NSCLC patients.

Finally, the present method was successfully applied in clinical settings. At a daily dose of 250 mg gefitinib, AUC_{0-24} and C_{minss} measured in 8 patients are in the range to those previously documented in several investigations [5,6,20]. Concerning erlotinib, AUC_{0-24} and C_{minss} measured are also in the range of those previously reported in a PK/PD investigation carried out in 1047 NSCLC patients under erlotinib [3]. Therefore, these results prove that our method can be used in clinical practice to monitor plasma gefitinib and erlotinib concentrations in NSCLC patients.

In conclusion, we have developed the first HPLC-UV method for the simultaneous quantification of gefitinib and erlotinib in plasma from NSCLC patients. This simple and cost-effective method may contribute to the spreading of gefitinib and erlotinib monitoring in hospital laboratories not having LC/MS/MS.

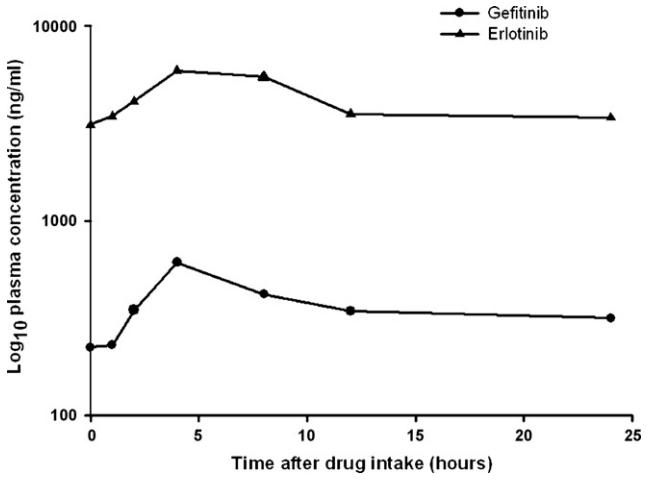

Fig. 2. Plasma concentration vs. time plot of erlotinib (▲) from NSCLC patient treated with 150 mg erlotinib once daily and gefitinib (●) from NSCLC patient treated with 250 mg gefitinib once daily. Pharmacokinetic sessions were performed at steady-state.

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Not applicable.

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