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Specific method for determination of gefitinib in human plasma, mouse plasma and tissues using high performance liquid chromatography coupled to tandem mass spectrometry

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Abstract

A rapid, sensitive and specific method was developed and validated using liquid chromatography–tandem mass spectrometry (LC/MS/MS) for determination of gefitinib in human plasma and mouse plasma and tissue. Sample preparation involved a single protein precipitation step by the addition of 0.1 mL of plasma or a 200 mg/mL tissue homogenate diluted 1/10 in human plasma with 0.3 mL acetonitrile. Separation of the compounds of interest, including the internal standard (d8)-gefitinib, was achieved on a Waters X-Terra™ C_{18} (50 mm × 2.1 mm i.d., 3.5 μm) analytical column using a mobile phase consisting of acetonitrile–water (70:30, v/v) containing 0.1% formic acid and isocratic flow at 0.15 mL/min for 3 min. The analytes were monitored by tandem mass spectrometry with electrospray positive ionization. Linear calibration curves were generated over the range of 1–1000 ng/mL for the human plasma samples and 5–1000 ng/mL for mouse plasma and tissue samples with values for the coefficient of determination of >0.99. The values for both within- and between-day precision and accuracy were well within the generally accepted criteria for analytical methods (<15%). This method was subsequently used to measure concentrations of gefitinib in mice following administration of a single dose of 150 mg/kg intraperitoneally and in cancer patients receiving an oral daily dose of 250 mg.

Keywords: Gefitinib; ZD1839; LC/MS/MS; Pharmacokinetics

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 lead to the rational design and development of agents that selectively target this receptor [1]. Numerous 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.

Gefitinib, 4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy) quinazoline (ZD1839, Iressa®) (Fig. 1) is an orally active, selective EGFR-TK inhibitor (EGFR-TKI) that causes complete inhibition of EGF-stimulated EGFR autophosphorylation in cell lines. In preclinical studies, gefitinib has demonstrated antitumor activity against a variety of human cancer cell lines expressing EGFR, including lung, ovarian, breast, and colon [2–4]. In human xenograft models, ZD1839 in combination with standard cytotoxic agents resulted in both delayed tumor growth and tumor regression, leading to enhanced survival [5]. Gefitinib is indicated as monotherapy for the treatment of patients with locally advanced or metastatic non-small cell lung cancer

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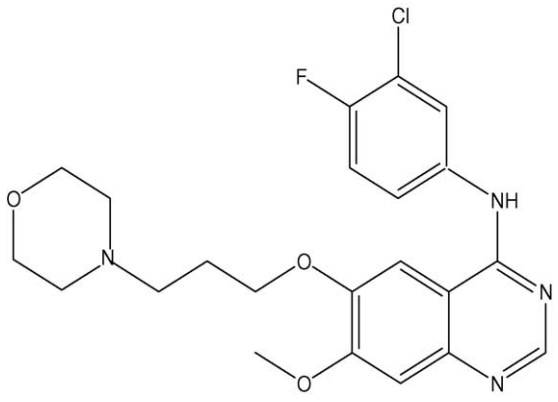

Fig. 1. Chemical structure of gefitinib.

after failure of both platinum based and docetaxel chemotherapies.

To comprehensively characterize the clinical pharmacokinetic (PK) profile of this drug, and to explore the relationship of PK with pharmacodynamics (PD) effects of gefitinib, a specific, reproducible and accurate method for the quantitation of gefitinib was necessary. Here, we describe a rapid, sensitive analytical method for the determination of gefitinib concentrations in human plasma and mouse plasma and tissue based on liquid chromatography–tandem mass spectrometry (LC/MS/MS) with electrospray positive ionization after a single protein precipitation with acetonitrile.

2. Experimental

2.1. Chemical and reagents

Gefitinib (Lot number 012X, 99.9% pure by HPLC) and the internal standard (d8)-gefitinib (Lot number 1S3, 99.6% pure by HPLC) were provided by AstraZeneca Co. (Macclesfield, Cheshire, UK). Formic acid (98%, v/v in water), methanol (HPLC grade) and acetonitrile (HPLC grade) were 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 from healthy donors originated from Pittsburgh Blood Plasma Inc. (Pittsburgh, PA, USA).

2.2. Stock solutions, calibration standards, and quality control samples

Stock solutions of gefitinib at a concentration of 1 mg/mL were prepared in duplicate by dissolving 10 mg in 10 mL of methanol and stored in glass vials at −20 °C. The stock solutions were diluted in blank human plasma on each day of analysis to prepare seven calibration standards containing gefitinib for human plasma samples at the following concentrations: 1 (duplicate), 5, 10, 50, 100, 500 and 1000 ng/mL (duplicate). Quality control (QC) samples were prepared independently in blank plasma at five different concentrations for gefitinib including: 1 ng/mL, the lower limit of quantitation (LLOQ); 3 ng/mL, the low QC (LQC); 70 ng/mL, the medium QC (MQC); 700 ng/mL, the high QC (HQC); and 7000 ng/mL diluted 1:10 with blank plasma, the above the upper limit of quantitation (AULQ) QC.

Gefitinib concentrations in mouse plasma and tissue were determined from a calibration curve prepared in human plasma consisting of six calibration standards containing gefitinib at the following concentrations: 5 (duplicate), 10, 50, 100, 500, and 1000 ng/mL (duplicate). Quality control (QC) samples were prepared independently in either blank mouse plasma or mouse tissue homogenates at four different concentrations for gefitinib including: 5 ng/mL, the LLOQ; 15 ng/mL, the LQC; 150 ng/mL, the MQC; and 800 ng/mL, the HQC.

2.3. Sample preparation

Prior to drug extraction, frozen samples were thawed in a water bath at ambient temperature. The tissues were homogenized with a Delta Shopmaster (Delta Machinery, Jackson, Tennessee, USA) in 4% (w/v) bovine serum albumin (Sigma Co. St. Louis, MO, USA) in phosphate buffer saline solution resulting in a final concentration of 200 mg/mL. The mouse plasma and tissue homogenates were diluted 1:10 with human plasma to achieve the same recovery as that observed from undiluted human plasma. A 0.1 mL aliquot of plasma or the diluted mouse plasma and tissue homogenates were added to a borosilicate glass test tube (13 mm × 100 mm) containing 0.3 mL of acetonitrile solution and (d8)-gefitinib (200 ng/mL), which was used as internal standard. The tube was mixed vigorously for 10 s on a vortex-mixer, followed by centrifugation at 2000 × *g* for 10 min at ambient temperature. A volume of 100 μL of the top organic layer was transferred to a disposable borosilicate glass culture tube (13 mm × 100 mm) and 100 μL deionized water was added to this tube. The tube was mixed vigorously for 10 s on a vortex-mixer, the sample was transferred to a 250 μL polypropylene autosampler vial, sealed with a Teflon crimp cap, and a volume of 20 μL was injected onto the HPLC instrument for quantitative analysis using a temperature-controlled autosampling device operating at 10 °C.

2.4. Chromatographic and mass-spectrometric conditions

Chromatographic analysis was performed using a Waters Model 2690 separations system (Milford, MA, USA). Separation of the analytes from potentially interfering material was achieved at ambient temperature using Waters X-Terra MS column (50 mm × 2.1 mm i.d.) packed with a 3.5-μM 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

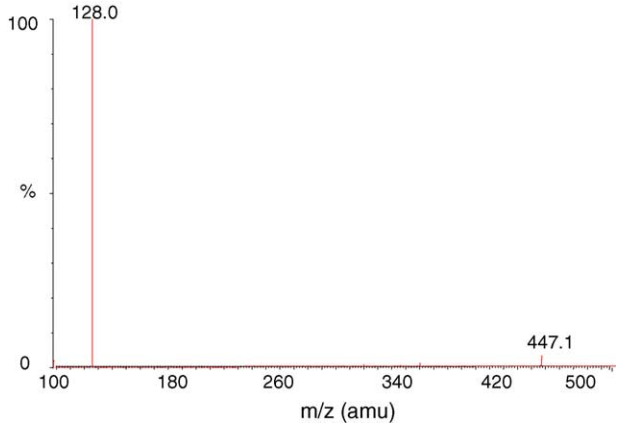

Fig. 2. Mass spectrum of gefitinib with monitoring at *m/z* 447.1 → 128.

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 both gefitinib and (d8)-gefitinib. Samples were introduced into the interface through a heated nebulized probe (350 °C). The spectrometer was programmed to allow the $[MH]^+$ ion of gefitinib at *m/z* 447.1 and that of the internal standard at *m/z* 455.1 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The collision energy was set at 25 eV for both gefitinib and (d8)-gefitinib. The product ions for gefitinib (*m/z* 128) (Fig. 2) and the internal standard (*m/z* 136) (data not shown) were monitored through the third quadrupole (Q3). 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.

2.5. Calibration curves

Calibration curves for gefitinib were computed using the ratio of the peak area of analyte and internal standard by using a least-squares linear regression analysis and uniform weighting. The parameters of each calibration curve were used to compute back-calculated concentrations and to obtain values for the QC samples and unknown samples by interpolation.

2.6. Method validation

Method validation runs for human plasma calibrator standards and QCs were performed on four consecutive days and included a calibration curve processed in duplicate and QC samples, at five different concentrations, in quadruplicate. Method validation runs for mouse plasma and tissue QCs, which were measured on a calibration curve prepared with human plasma, were performed on 3 consecutive days and included a calibration curve processed in duplicate and QC samples, at four different concentrations, in triplicate. The accuracy and precision of the assay was assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within- and between-run precision, respectively. The accuracy for each tested concentration was calculated as:

$$\mathrm{DEV}_{(\mathrm{gefitinib})} = 100 \times \left\{ \frac{([\mathrm{gefitinib}]_{\mathrm{mean}} - [\mathrm{gefitinib}]_{\mathrm{nominal}})}{[\mathrm{gefitinib}]_{\mathrm{nominal}}} \right\}$$

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:

$$\mathrm{BRP} = 100 \times \left\{ \frac{\sqrt{((\mathrm{Ms}_{\mathrm{bet}} - \mathrm{MS}_{\mathrm{wit}})/n)}}{\mathrm{GM}} \right\}$$

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:

$$\mathrm{WRP} = 100 \times \left\{ \frac{\sqrt{(\mathrm{MS}_{\mathrm{wit}})}}{\mathrm{GM}} \right\}$$

The specificity of the method was tested by visual inspection of chromatograms of extracted human plasma samples from six different donors and variety of tissue homogenates for the presence of endogenous or exogenous interfering peaks. The peak area needed to be less than 20% than the peak area for gefitinib 1 ng/mL in an aqueous solution. If not, plasma from six additional donors would be tested. The extraction efficiency of the assay was measured by comparison of the peak area ratio of gefitinib extracted from plasma and an aqueous solution in triplicate at concentrations of the low, middle, and high QCs. The stability of gefitinib in plasma was tested at concentrations of the low and high QCs in triplicate after three freeze–thaw cycles. The short-term stability of gefitinib in plasma was assessed in triplicate at room temperature (on the benchtop) for 4 h; long-term stability of gefitinib in plasma was assessed in duplicate at −20 °C. Stability of drug in neutral extracts on the autosampler at 10 °C was assessed in duplicate. Long-term stability of gefitinib in methanol at a concentration of 1 mg/mL at −20 °C was assessed in replicates of five.

2.7. Patient samples

The samples analyzed were from one cancer patient enrolled to a clinical trial where gefitinib monotherapy was administered at a dose of 250 mg once daily. Blood

samples were collected in heparinized tubes at baseline (pretreatment) and at 1–6 and 8 h following administration of the first dose of ZD1839. Additional samples were taken prior to gefitinib administration on days 2, 3, 8, 15, 22, and 28. Blood samples were immediately placed on ice or refrigerated then centrifuged at 4 °C, 1000 × *g* for 10 min. The resultant plasma was split into two aliquots, and stored at −20 °C until analysis. The clinical protocol was approved by the local institutional review board (The Sydney Kimmel Comprehensive Cancer Center at Johns Hopkins) and all patients provided written informed consent before entering the study.

Pharmacokinetic parameters after a single dose of gefitinib on day 1 were estimated using model-independent noncompartmental analysis as implemented in the computer software program WinNonlin (Version 3.1, Pharsight Corp.). The maximum plasma concentration (C_{max}) and the time of C_{max} after oral administration were obtained by visual inspection of the plasma concentration–time curve. The area under the plasma concentration–time curve (AUC) was calculated using the log–linear trapezoidal rule. Apparent oral clearance was calculated as dose/AUC.

2.8. Mouse specimens

Six-week-old female athymic nude mice (Harlan, IN, USA) housed in the animal facilities at Johns Hopkins University in compliance with current local and federal regulations, were used for this purpose. HuCCT1 human biliary cancer subcutaneous xenografts were created by injecting 5×10^6 cells suspended in a standard xenografting vehicle (Matrigel, BD Biosciences, MA, US). Tumors were allowed to grow to a size of 0.2 cm^3, when mice were stratified by tumor volume in two different groups: a control group treated with vehicle, and a group treated with a single dose of gefitinib 150 mg/kg intraperitoneally. Mice were euthanized with CO inhalation 24 h after treatment. Blood samples were collected in heparinized tubes, immediately placed on ice or refrigerated then centrifuged at 4 °C, 1000 × *g* for 10 min. The resultant plasma was stored at −20 °C until analysis. Tumor, liver and skin samples were obtained by surgical resection and immediately frozen at −20 °C.

3. Results and discussion

3.1. Detection and chromatography

The mass spectrum of gefitinib showed a protonated molecular ion ([MH^+]) at *m*/*z* 447.1. The major fragment observed was at *m*/*z* 128.0, which was selected for subsequent monitoring in the third quadrupole (Fig. 2). The mass spectrum of the internal standard (d8)-gefitinib, showed a [MH^+] at *m*/*z* 455.1, and the high collision energy gave one major product ion at *m*/*z* 136 (data not shown).

No peaks were observed in the chromatograms of blank plasma from six donors when monitored for gefitinib (data not shown). Representative chromatograms of blank human plasma and plasma spiked with internal standard and gefitinib are shown in Figs. 3 and 4. The mean (±standard deviation) retention times for gefitinib and (d8)-gefitinib under the optimal conditions were both 1.4 ± 0.1 min, with an overall chromatographic run time of 3 min. The selectivity for the analysis is shown by symmetrical resolution of the peaks, with no significant chromatographic interference around the retention times of the analytes and internal standard in drug-free specimens.

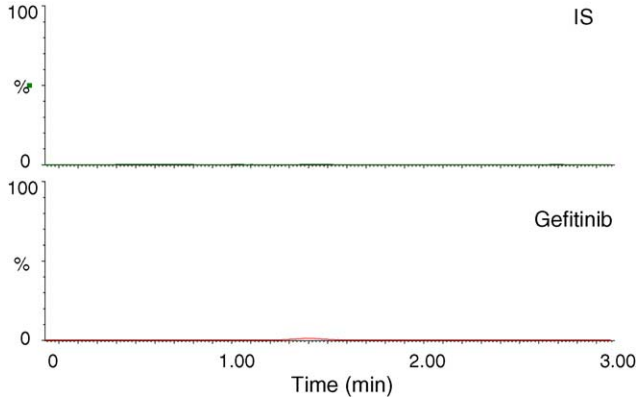

Fig. 3. Chromatograms of blank human plasma.

Representative chromatograms of blank mouse plasma and tissue homogenates, including live, skin, and tumor and murine specimens spiked with gefitinib and internal standard are shown in Figs. 5 and 6. No significant chromatographic interference was observed around the retention time of gefitinib.

3.2. Linearity of detector responses

The calculated peak area ratios of gefitinib to (d8)-gefitinib versus the nominal concentration of the analyte displayed a

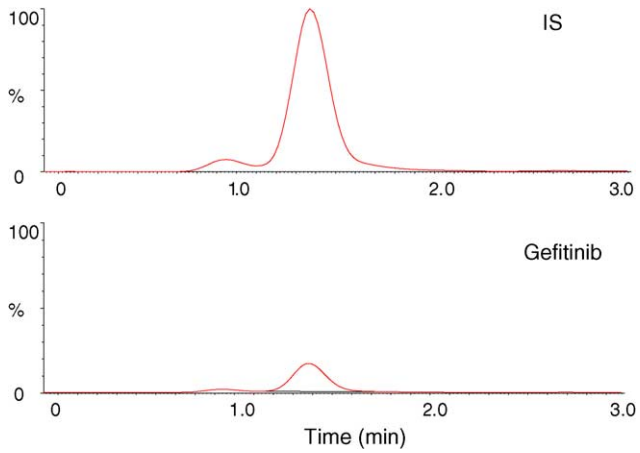

Fig. 4. Chromatograms of plasma spiked with gefitinib (1 ng/mL) and the internal standard (IS) (200 ng/mL) the retention times for gefitinib and internal standard were approximately 1.4 ± 0.1 min.

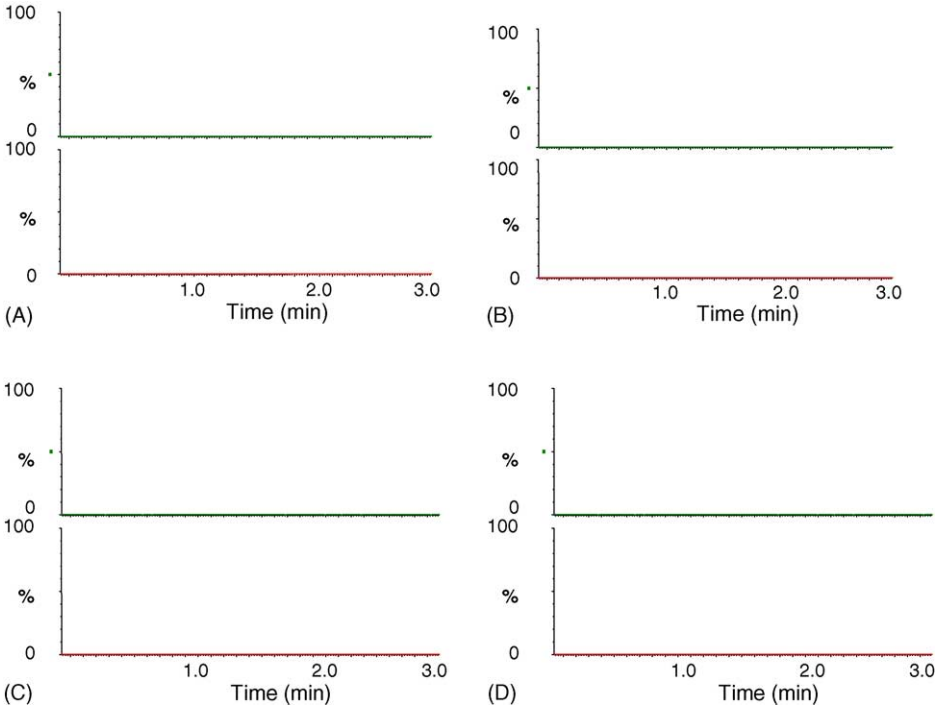

Fig. 5. Chromatograms of internal standard (top panel) and gefitinib (bottom panel) in blank (A) mouse plasma, (B) liver homogenate, (C) tumor homogenate, and (D) skin homogenate.

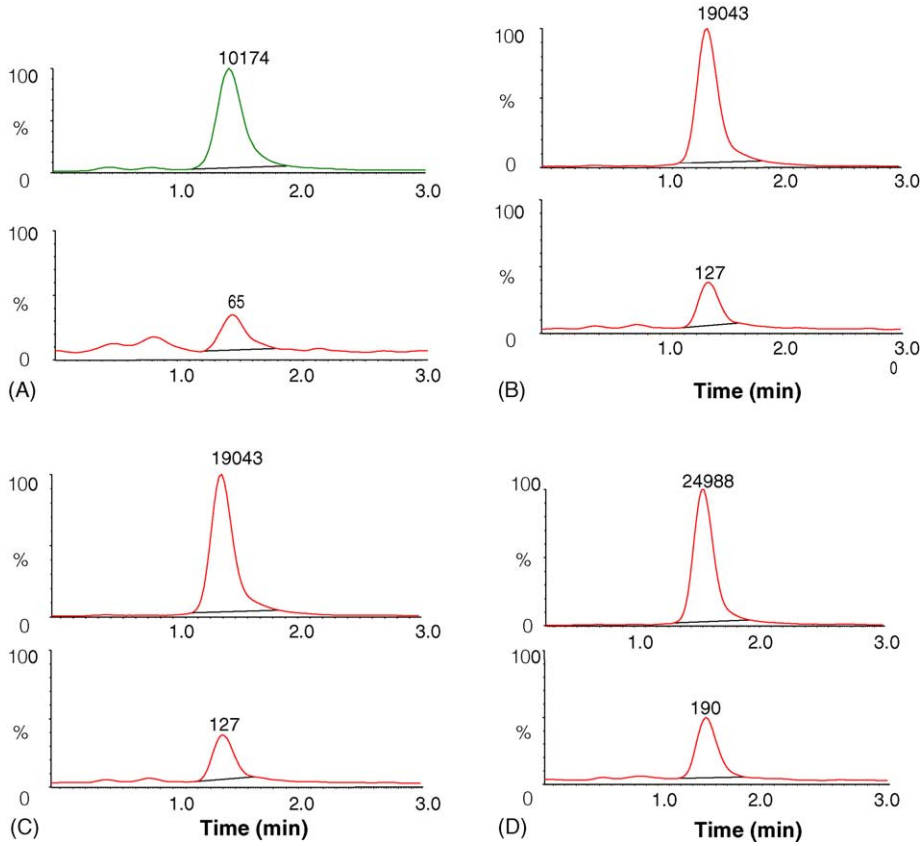

Fig. 6. Chromatograms of internal standard (top panel) and gefitinib 5 ng/mL (bottom panel) spiked in (A) mouse plasma, (B) liver homogenate, (C) tumor homogenate, and (D) skin homogenate.

Table 1
Back-calculated concentrations from calibration curves over the concentration range of 1–1000 ng/mL[a]

	Nominal concentration (ng/mL)						
	1	5	10	50	100	500	1000
Accuracy (%)	112.5	99.1	93.6	97.4	97.1	99.4	100.8
Concentration (ng/mL)[b]	1.13 ± 0.097	4.95 ± 0.25	9.36 ± 0.28	48.7 ± 2.69	97.1 ± 2.63	497 ± 16.7	1008 ± 25.9
Precision (%)							
Within-run	9.71	4.50	3.47	5.66	2.68	4.27	3.34
Between-run	[c]	2.37	[c]	[c]	0.38	[c]	[c]
Number of samples	8	8	8	8	8	8	8

[a] Performed in duplicate on 4 separate days.
[b] Values are mean ± standard deviation.
[c] No significant variation was observed as a result of performing the assay in different runs.

linear relationship in the tested range of 1–1000 ng/mL and 5–1000 ng/mL. A weighting factor, which is inversely proportional to the variance at the given concentration level (x) was used. This weighting factor was chosen compared to uniform weighting after evaluation of goodness-of-fit by assessment of the R^2 value, intercept closest to a zero value, % recovery of calibrators and QCs, and assessment of residuals. After applying the peak area ratio in combination with a weighting factor of $1/x$, a mean least-squares linear-regression correlation coefficient of great than 0.99 was obtained in all analytical runs. The statistical evaluation of the coefficients of the ordinary least-squares line indicated small bias in the slope and in the intercept, further indicating minor matrix effects and blank problems, respectively [7].

For each point on the calibration curves for gefitinib, the concentrations back-calculated from the equation of the regression analysis were always within 10% of the nominal value, except at 1 ng/mL, where the accuracy was within 12.5% of the nominal value (Tables 1 and 2). A linear regression of the back-calculated concentrations versus the nominal values provided a unit slope and an intercept not significantly different from zero (data not shown). The distribution of the residuals showed random variation, was normally distributed, and centered on zero (data not shown).

The LLOQ for gefitinib was established at 1 ng/mL for human plasma, which concentration was associated with a mean (±standard deviation) signal-to-noise ratio of 15.5 ± 3.9 from 20 observations.

3.3. Accuracy, precision, and recovery

For QC samples prepared by spiking human plasma, mouse plasma, or mouse tissue homogenates with gefitinib, the within- and between-run variability (precision), expressed as the percentage relative standard deviations, was less than 10%. Likewise, the mean predicted concentration (accuracy) was less than 10% of the nominal value (Table 3). The relative recovery of gefitinib from human plasma, mouse plasma, and mouse tissue homogenate was greater than 90% at low QC and high QC concentrations.

3.4. Analyte stability

QC samples prepared in human plasma undergoing three freeze–thaw cycles showed no significant degradation (<10%) for gefitinib. A concentration-dependent instability of gefitinib was noted at 4 h when stored at room temperature, with greater loss at the low QC concentration (Table 4). At −20 °C, gefitinib was stable in human plasma for up to 151 days; long-term stability experiments to assess stability for greater periods of time are ongoing. In neutral extracts, gefitinib was stable up to 18 h on the autosampler without any significant degradation, allowing for more than 250 samples to

Table 2
Back-calculated concentrations from calibration curves over the concentration range of 5–1000 ng/mL[a]

	Nominal concentration (ng/mL)					
	5	10	50	100	500	1000
Accuracy (%)	105.2	104.2	96.4	98.0	97.2	101.7
Concentration (ng/mL)[b]	5.16 ± 0.32	10.4 ± 0.61	48.2 ± 1.32	98.0 ± 2.34	486 ± 10.7	1017 ± 16.5
Precision (%)						
Within-run	6.69	4.90	2.15	3.05	1.13	1.53
Between-run	[c]	3.25	1.90	[c]	2.12	0.53
Number of samples	6	5	6	6	6	6

[a] Performed in duplicate on 3 separate days.
[b] Values are mean ± standard deviation.
[c] No significant variation was observed as a result of performing the assay in different runs.

Table 3
Assessment of accuracy, precision, and recovery

Nominal concentration (ng/mL)	Accuracy (%)	Precision (%)		Recovery (%)	Number of samples
		Within-run	Between-run		
Human plasma[a]					
1	106.2	10.6	2.31	[b]	20
3	94.7	6.27	5.70	93.3	20
70	101.3	3.17	3.39	94.4	20
700	104.07	3.18	3.29	91.7	20
7000[c]	106.8	2.42	2.56	[b]	20
Mouse plasma[d]					
5	96.0	9.59	[e]	[b]	9
15	98.1	6.25	[e]	95.3	9
150	97.5	2.58	2.16	89.8	9
800	94.0	3.20	2.73	105.5	9
Mouse liver[d]					
5	103.3	8.28	6.19	[b]	9
15	100.1	3.74	1.40	93.2	9
150	100.9	1.49	2.55	81.3	9
800	101.0	0.98	0.98	102.9	9
Mouse skin[d]					
5	94.5	7.45	4.47	[b]	9
15	102.3	1.88	[e]	97.6	9
150	101.8	2.91	[e]	87.6	9
800	103.7	1.66	2.47	100.9	9
Mouse tumor[d]					
5	99.3	8.97	3.04	[b]	9
15	99.1	4.442	[e]	98.4	9
150	99.5	1.95	3.01	92.3	9
800	97.5	1.25	8.72	107.2	9

[a] Performed in quintuplicate on 4 separate days.
[b] Not done.
[c] Sample diluted 1:10 with human plasma prior to extraction.
[d] Performed in triplicate on 3 separate days.
[e] No significant additional variation was observed as a result of performing the assay in different runs.

Table 4
Assessment of stability in human plasma[a]

Condition	Gefitinib		Number of samples
	3 ng/mL	700 ng/mL	
Freeze–thaw stability (−20 °C)			
Cycle 1	96.9	98.6	3
Cycle 2	99.9	92.1	3
Cycle 3	98.2	100.5	3
Short-term stability (room temperature)			
Time = 0.5 h	97.3	101.7	3
Time = 1 h	92.5	100.7	3
Time = 2 h	94.0	103.5	3
Time = 4 h	84.6	100.1	3
Long-term stability (−20 °C)			
Time = 51 days	104.5	99.7	2
Time = 151 days	92.5	105.9	2
Autosampler stability (10 °C)			
Time = 18 h	99.6	100.1	2

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

be analyzed simultaneously within a single chromatographic run. Stock solutions of gefitinib 1 mg/mL in methanol were stable for up to 142 days.

3.5. Plasma concentration–time profiles

The present LC/MS/MS method was successfully applied to study the pharmacokinetics of gefitinib in a cancer patient receiving oral gefitinib as a daily dose of 250 mg. Fig. 7 shows a gefitinib plasma concentration–time profile on day 1 (panel A) and pre-treatment trough concentrations on days 2, 3, 8, 22, and 28 (panel B). Following a single oral dose of gefitinib 250 mg, the maximum plasma concentration achieved was 212 ng/mL, which occurred at 5 h; the apparent oral clearance and terminal half-life were 36 L/h and 20 h, respectively.

3.6. Applicability of the assay for preclinical studies

The assay was suitable to measure gefitinib concentrations in mouse liver, tumor and skin following intraperitoneal administration of gefitinib 143 mg/kg; drug concentration; drug concentrations are listed in Table 5.

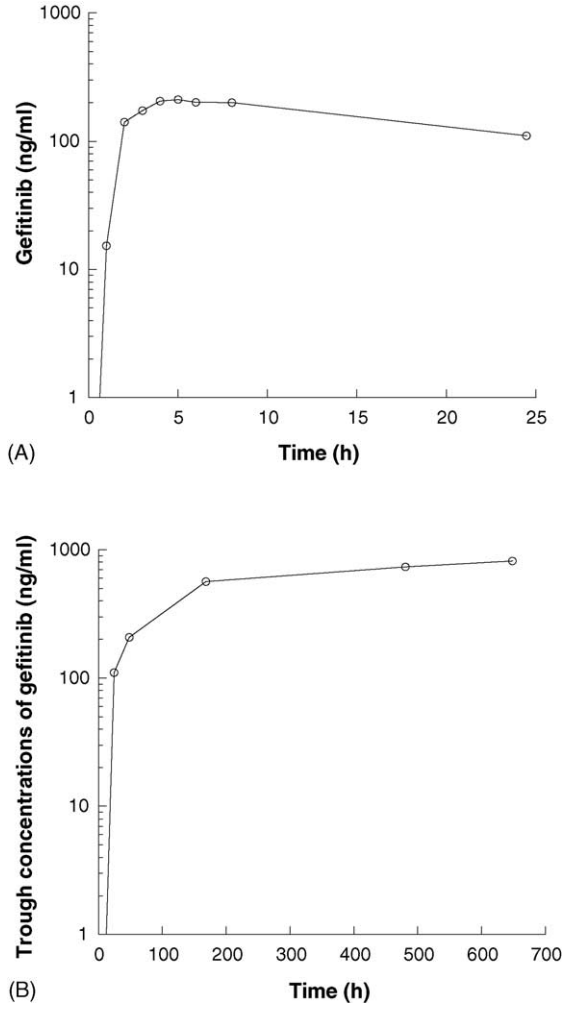

Fig. 7. (A) Gefitinib plasma concentration-time profile on day 1 and (B) pre-treatment trough concentrations on days 2, 3, 8, 22, and 28 (B) in a patient receiving an oral daily dose of 250 mg.

Table 5
Gefitinib concentrations in murine tissue after intraperitoneal administration of 150 mg/kg

Specimens	Gefitinib (ng/g)
Liver	227.2
Skin	17.3
Tumor	17.9

4. Conclusion

In conclusion, we have developed and validated an assay for measuring gefitinib in human plasma and mouse plasma and tissue. In comparison to a published method with an assay lower limit of quantitation of 0.5 ng/mL [6], the current assay uses less sample (100 μL versus 500 μL) and the sample preparation procedure is simpler and faster (protein precipitation versus liquid–liquid extraction and sample concentration). These characteristics combined with an overall chromatographic run time of 3 min, allows this assay to be easily applied to the quantitation of gefitinib in a large number of plasma and tissue samples. The described method for quantitation over the concentration range of 1–1000 ng/mL is sufficient to allow plasma pharmacokinetic monitoring of gefitnib during daily, continuous administration. This method is being used to characterize the plasma PK and PD of gefitinib as a single agent or in combination therapy in cancer patients and in preclinical studies to further optimize gefitinib treatment schedules for future clinical evaluation.

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