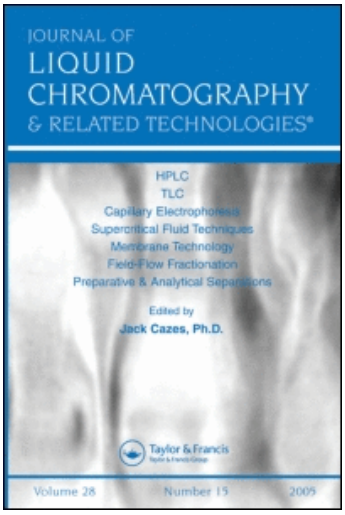

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Determination of Gefitinib in Plasma by Liquid Chromatography with a C_{12} Column and Electrospray Tandem Mass Spectrometry Detection

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ABSTRACT

A highly sensitive liquid chromatography electrospray tandem mass spectrometry (LC-ESI-MS/MS) method has been developed for the measurement of gefitinib (ZD1839) in human plasma. The method was validated over a linear range of 0.5–1000 ng/mL, using deuterated gefitinib (D_8-ZD1839) as the internal standard (IS). Compounds were extracted from 500 μL of sodium heparin plasma by 6.0 mL butyl methyl ether liquid–liquid extraction. The dried residue was reconstituted with 250 μL of 20% acetonitrile with 1.0% formic acid and 30 μL

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injected onto the LC-ESI-MS/MS system. Chromatographic separation was achieved on a Phenomenex® Synergi 4μ MAX-RP 80 Å C_{12} column (75 × 2.0 mm^2) with an isocratic mobile phase of acetonitrile–1.0% formic acid (30 : 70, v/v). The analytes were detected with a PE Sciex API 365 triple quadrupole mass spectrometer using turbo ion spray® source with positive ionization. Ions monitored in the multiple reaction monitoring (MRM) mode were m/z 447.2 (precursor ion) to m/z 127.8 (product ion) for gefitinib and m/z 455.2 (precursor ion) to m/z 136.0 (product ion) for D_8-ZD1839. The lower limit of quantitation (LLOQ) of gefitinib was 0.30 ng/mL ($S/N \geq 10$), and results from a 5-day validation study demonstrated acceptable within-day and between-day precision (CV% values ≤6.0% and ≤5.2%, respectively) and accuracy (range 91.0–97.7%). This method is now used to analyze plasma samples from pediatric pharmacokinetic studies of ZD1839, and the wide linear range (~4 log) of this method provides a distinct advantage, as shown by the results of a representative patient.

Key Words: Gefitinib; Plasma; Liquid chromatography; Electrospray tandem mass spectrometry.

INTRODUCTION

Gefitinib, Iressa® (ZD1839) is a novel, low molecular weight, synthetic 4-anilinoquinazoline derivative (Fig. 1) that is orally active as a selective epidermal growth factor receptor (EGFR, erbB1) tyrosine kinase inhibitor. Gefitinib selectively binds to the ATP-binding site of EGFR, preventing autophosphorylation and other downstream events, such as cell growth, proliferation, and cellular motility. In vitro and in vivo, gefitinib potently inhibits the tyrosine kinase activity of EGFR.[1,2] Clinical antitumor activity has been seen in a variety of cancer cell lines and in mouse xenografts after treatment with gefitinib, and currently clinical trials are underway in adults and children. Results of preclinical studies have also shown synergistic antitumor activity when gefitinib was combined with a variety of other anticancer agents, such as paclitaxel, topotecan, and doxorubicin. We have reported synergistic activity when gefitinib was administered concomitantly with other anticancer agents to mice bearing neuroblastoma, osteosarcoma, and rhabdomyosarcoma xenografts.[3]

In adults with solid malignant tumors, who received gefitinib daily for 14 days, gefitinib was absorbed slowly with a median t_{max} of 3 hr (range 1–7 hr).[4] In this dose escalation study, the authors noted that drug exposure (C_{max} and AUC_{0-24}) increased with dose, and interpatient variability in exposure within a dose group was 2.5–8-fold. Over the entire dose range

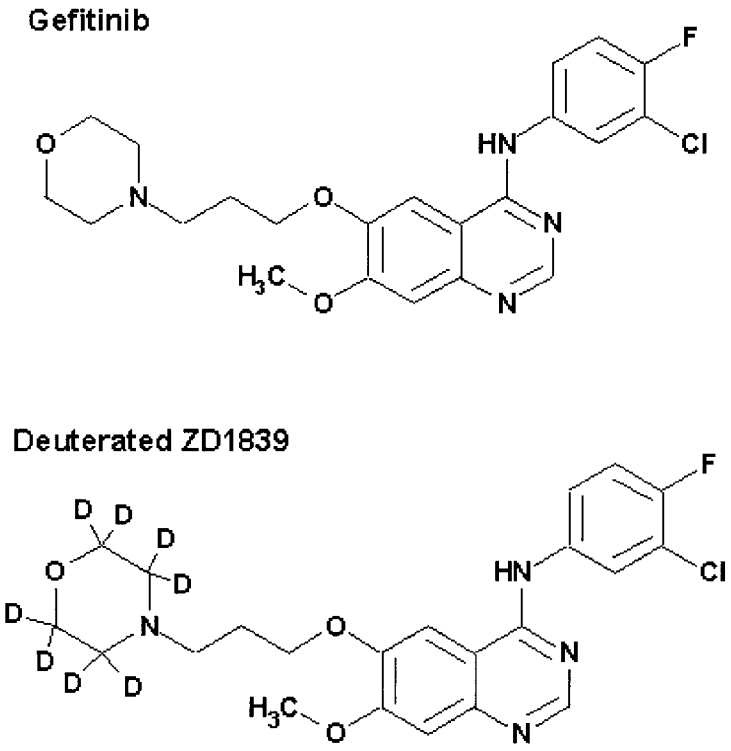

Figure 1. Chemical structure of gefitinib and D_8-ZD1839.

(50–700 mg/day) the terminal half-life ranged from 24–85 hr, which is comparable to that seen in healthy volunteers.[5] No change in half-life was observed as the dose increased or on multiple dosing. Steady state was reached in all patients between 7 and 10 days with daily dosing at all dosage levels. Biologically, relevant plasma concentrations were achieved after oral dosing (doses greater than 100 mg), and were maintained across the 24-hour dosing interval.[6] In humans, gefitinib is approximately 90% protein bound, primarily to serum albumin.[4]

Since no data are available regarding the disposition of gefitinib in children, we are conducting extensive clinical pharmacokinetic studies of gefitinib to accompany the dose-ranging Phase I clinical trials of this compound. We needed a sensitive, robust, and specific method to analyze gefitinib in plasma samples from this patient population. Therefore, we developed and validated a highly sensitive liquid chromatography electrospray tandem

mass spectrometry (LC-ESI-MS/MS) method for measurement of gefitinib in human plasma over a wide concentration range.

EXPERIMENTAL

Chemicals

Gefitinib and D_8-ZD1839, used for preparation of standards and quality control samples, were supplied by AstraZeneca Pharmaceuticals (Cheshire, England; ZD1839: Batch C253/1P1, 99.6% purity, date 9/1998; D_8-ZD1839: Batch 1S2, 100% purity, Date 7/2001). The following chemicals were used: acetonitrile, HPLC grade (Fisher Scientific, Fair Lawn, NJ 07410), formic acid, minimum 95% (Sigma, St. Louis, MO). *tert*-Butyl methyl ether, 99.8% HPLC grade (Aldrich, Milwaukee, WI). Blank human plasma was obtained from Lifeblood (Memphis, TN). All water was distilled, deionized, and further purified via a Millipore Milli-QUV plus and Ultra-Pure Water System (Tokyo, Japan) (resistance: 18.2 MΩ).

Chromatographic Conditions

The HPLC system consisted of a Shimadzu (Kyoto, Japan) system controller (SCL-10AVP), pump (LC-10ADVP), autosampler (SIL-10ADVP), and on-line degasser (DGU-14A). A 30 μL of reconstituted plasma extract was injected onto a Phenomenex® (San Francisco, CA) Synergi 4μ MAX-RP 80 Å analytical column (75 × 2.0 mm^2), preceded by a pre-column of the same material (4.0 × 2.0 mm^2). The flow-rate was 0.15 mL/min with a mobile phase that consisted of acetonitrile–1% formic acid (30 : 70, v/v). The column was maintained at room temperature (20–25°C). Under these conditions, typical retention time was 1.93 min for both gefitinib and D_8-ZD1839, and backpressure values of approximately 35–40 bar were observed between the columns. The total run time for each sample was 4.0 min.

Mass Spectrometric Conditions

Detection was performed with a PE SCIEX API 365 triple quadrupole mass spectrometer (Toronto, Canada) equipped with a turbo ion spray® source (thermally and pneumatically assisted electrospray), which was run at the unit-resolution of Q1 and the low-resolution of Q3 in positive mode with multiple reaction monitoring (MRM). Full-scan positive-ion mass

spectra showed the parent molecular ion for gefitinib at m/z 447.2 to the predominant ion m/z 128.0, and for D_8-ZD1839 at m/z 455.2 to m/z 135.9, respectively (Fig. 2). The optimized conditions of MS/MS with the turbo ion spray source were as follows: 400°C ion spray temperature, 11 psi nebulizer gas (NEB) pressure, 13 psi curtain gas (CUR) pressure, 5 L/min turbo gas flow, 5200 V ionspray voltage (IS), and 9.0 U collision gas (CAD); 44 V declustering potential (DP), 213 V focusing potential (FP), −5 V

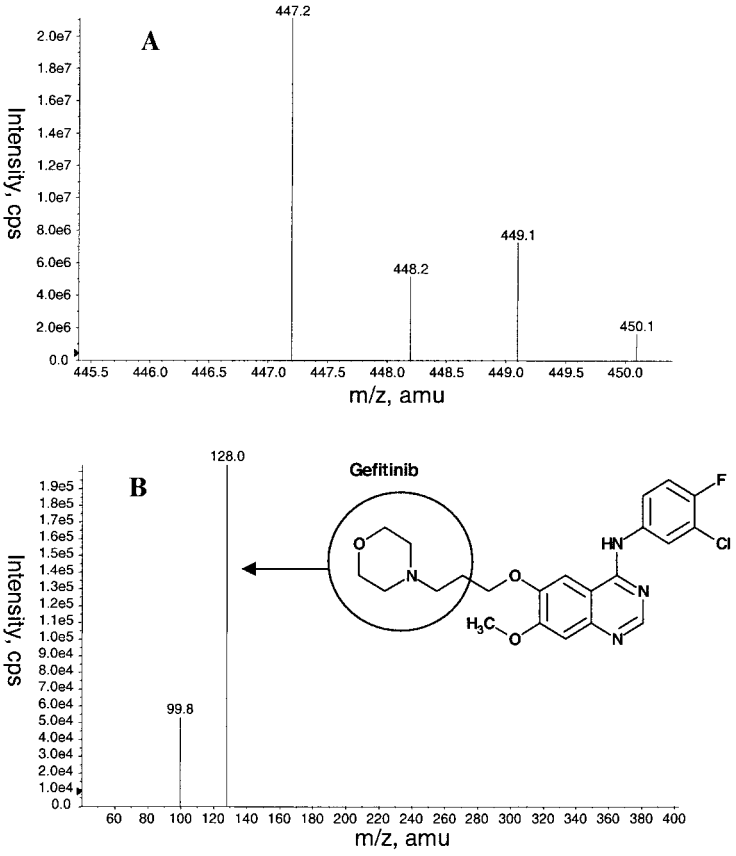

Figure 2. (A) Scan positive-ion mass spoectra for parent ion for gefitinib at m/z 447.2; (B) collision induced dissociation spectra for gefitinib to the predominant ion m/z 128.0; (C) scan positive-ion mass spectra for parent molecular ion for D_8-ZD1839 at m/z 455.2; and (D) collision induced dissociation spectra for D_8-ZD1839 to the predoiminant ion m/z 128.0.

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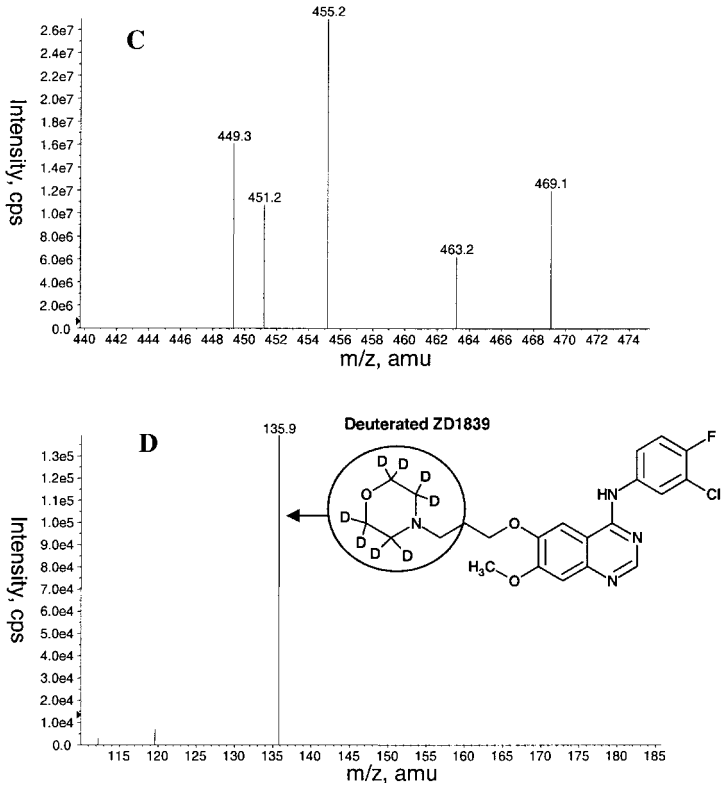

Figure 2. Continued.

entrance potential (EP), 24 V collision cell entrance potential (CEP), 34 V collision energy (CE), and 4.0 V collision cell exit potential (CXP). The mass spectrometer was interfaced to a computer workstation running Analyst software (Version 1.0; Applied Biosystems, Foster City, CA) for data acquisition and processing.

Sample Preparation

Standard Solutions

Stock solutions were prepared by separately dissolving either gefitinib or D_8-ZD1839 in methanol to yield a concentration of 1.0 mg/mL. Then, the solutions were sonicated for a minimum of 5 min to completely dissolve

these compounds. The stock solutions were stored at −80°C, and less than 5% of their nominal value was lost over 3 months. The working solutions (0.01, 0.1, 1.0, and 10.0 μg/mL) were prepared at the time of assay from the 1.0 mg/mL stock solutions by making dilutions with cold methanol.

Calibration Standards and Quality Controls

Calibration standards were made by adding gefitinib working solutions to plasma to give final concentrations of 0.5, 5.0, 50, 100, 300, 500, and 1000 ng/mL. Quality controls were prepared to give final concentrations of 3.0, 30, and 600 ng/mL.

Plasma Sample Preparation

A 500 μL of spiked gefitinib plasma sample or patient plasma was added into a 15 mL amber glass tube at ambient temperature, then 10 μL of 1.0 μg/mL D_8-ZD1839 and 6.0 mL *t*-butyl methyl ether were added into each tube. The samples were vortexed at high speed for at least 5 min, and then centrifuged at 4000 *g* for 10 min at 4°C. The organic layer was transferred to a clean 15 mL amber glass tube and evaporated to dryness under a stream of nitrogen. The residues were immediately dissolved in 250 μL of 20% acetonitrile in 1.0% formic acid, and a 30 μL aliquot was injected onto the LC-ESI-MS/MS system by the autosampler.

Method Validation

The method developed for gefitinib measurement in human plasma was validated over 5 days by analysis of quality control samples to determine within-day and between-day precision and accuracy. Two calibration curves were analyzed during this validation. The linear regression of the ratio of gefitinib/D_8-ZD1839 peak area was weighted by $1/x^2$. The coefficient of determination (R^2) was used to evaluate the linearity of the calibration curve.

The limit of detection (LOD) and lower limit of quantitation (LLOQ) were defined as the minimum value at which the ratio of signal/noise was $\geq$3 and 10, respectively. These were determined by triplicate analysis of an extensive calibration curve in the low concentration range (0.01–0.5 ng/mL).

The stability of gefitinib in plasma at −80°C was evaluated at three concentrations (3.0, 30, and 600 ng/mL) over three months. For gefitinib in reconstituted extracted plasma, we evaluated stability at three concentrations (3.0, 30, and 600 ng/mL), three different storage conditions (25°C, 4°C, and −80°C) for up to 1 month.

Resolution of Extra Plasma Peaks

Given our final chromatographic conditions, we still observed a peak in front of, but separated from, the primary gefitinib peak (see Fig. 3). Although with a fresh mobile phase and new analytical column, this peak did not interfere with our chromatography, we still chose to evaluate the cause of this peak to avoid the potential for interfering peaks later when the analytical column or mobile phase may have deteriorated slightly. Sodium ions in the form of sodium formate were added to the gefitinib reconstituted solution (20% acetonitrile in 1.0% formic acid) to make 0.02, 0.1, and 0.5 mM sodium solutions. A gefitinib stock solution was diluted with the gefitinib reconstituted solution and each of the three sodium solutions described above, to make the 100 ng/mL gefitinib samples, respectively. Injections were made, and the gefitinib peak area was quantitated with and without sodium to assess the effect of sodium on the gefitinib peak area.

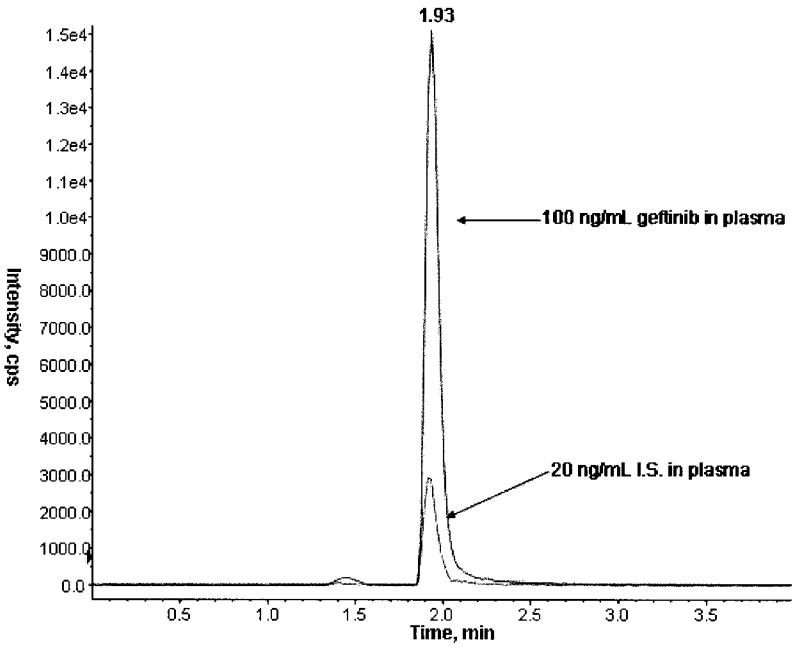

Figure 3. MRM chromatogram of plasma spiked with gefitinib and D_8-ZD1839 (IS), showing that both gefitinib and internal standard are eluted at the same time.

Patient Sample Collection

Patient blood samples (2 mL) were collected in green-top (Heparin sodium) Vacutainer tubes (Franklin Lakes, NJ) and centrifuged at 7000*g* for 2 min to separate the plasma. The plasma samples were then processed as described for calibrators and controls.

RESULTS

Chromatography and Mass Detection

We used reversed-phase chromatography (Phenomenex® Synergi 4μ C12 column) with a mobile phase consisting of 30% acetonitrile in 1.0% formic acid water for best results. Mobile phases consisting of 10–80% acetonitrile were evaluated. Acetonitrile 30% in mobile phase at an ambient temperature (22–25°C) produced the best resolution and a favorable retention time. The MRM chromatograms of plasma spiked with gefitinib and D_8-ZD1839 are shown in Fig. 3. As shown, gefitinib and IS were eluted at the same time. The specificity of the method was established by the absence of chromatographic components at the retention time of both gefitinib and IS in blank plasma samples from six different sources.

Method Validation

To assess within-day and between-day precision and accuracy, we evaluated validation parameters for gefitinib (Table 1). Ten injections of low,

Table 1. Validation parameters of gefitinib in human plasma.

Quality control gefitinib (ng/mL)	Within-day ($n = 10$)		Between-day ($n = 12$)	
	RSD (%)	Error (%)	RSD (%)	Error (%)
3.0	5.8	6.7	5.0	9.0
30.0	4.9	2.3	5.2	2.3
600	2.8	4.0	3.6	2.3

Note: Ten injections of low, medium, and high quality control samples were run within 1 day to assess within-day variability and 12 injections were run within 3 days to evaluate between-day variability. Variability reported as relative standard deviation (%RSD) and percentage error (%error).

medium, and high quality control samples, as described previously, were made on day-one and day-two to assess within-day variability and again on day-three, four, and five to evaluate between-day variability. The LOD and LLOQ in plasma for this method were 0.10 ng/mL ($S/N = 3.6$, $n = 3$) and 0.30 ng/mL ($S/N = 10.8$, $n = 3$, CV $\leq 9.4\%$), respectively. The extraction recovery was assessed in triplicate at concentrations of 3.0 and 600 ng/mL and was 99.2% $\pm$ 3.0%. The calibration curves for human plasma were linear from 0.50 to 1000 ng/mL, with correlation coefficients (R^2) greater than 0.995.

Stability of Gefitinib in Plasma and Extract

To test the stability of gefitinib in plasma at -80°C, we evaluated three concentrations (3.0, 30, and 600 ng/mL) over 3 months. As depicted in Table 2, for gefitinib in plasma at -80°C, the decrease of peak area ratio (Δgefitinib : D_8-ZD1839) remained less than 8.8% within 3 months. Thus, gefitinib is stable in plasma for 3 months at -80°C. To assess the stability of gefitinib in reconstituted extracted plasma, we evaluated three concentrations (3.0, 30, and 600 ng/mL) and three different storage conditions (25°C, 4°C, and -80°C) for up to 1 month. As depicted in Table 3, for gefitinib in extracted plasma samples, we observed less than a 7.7% decrease in peak area within 6 hr at 25°C and a 7.9% decrease within 24 hr at 4°C. When stored at -80°C, we observed a 9.1% decrease in peak area within 19 days as presented in Table 4. Therefore, gefitinib is stable in extracted plasma samples for 6 hr at 25°C, for 24 hr at 4°C, and for 19 days at -80°C.

Table 2. Stability of gefitinib in plasma at -80°C.

Plasma gefitinib (ng/mL) ($n = 2$)	1 hr	30 days	60 days	90 days
3.0	99.4	93.3	95.6	91.2
30	96.8	98.6	92.7	92.1
600	106.3	107.8	106.1	96.2

Note: Spiked plasma samples with high, medium, and low gefitinib concentrations were aliquotted in duplicate and stored in -80°C. The samples were then assayed over 3 months. Stability was assessed by the ratio of peak area (Δgefitinib : D_8-ZD1839). Data presented is mean percent of initial peak ratio of two samples tested.

Table 3. Stability of gefitinib in extracted plasma.

Extracted gefitinib (ng/mL)	25°C ($n = 2$)			4°C ($n = 2$)		
	1 hr	6 hr	24 hr	1 hr	6 hr	24 hr
3	102.2	92.3	88.5	102.2	98.1	92.1
30	95.6	95.7	87.4	95.6	99.1	94.1
600	107.7	103.2	92.5	107.7	107.8	104.2

Note: The reconstituted gefitinib samples were separately stored (in duplicate) at 25°C and 4°C and analyzed over 24 hr. Stability was assessed by the ratio of peak area (Δgefitinib : D_8-ZD1839). Data is expressed as mean percent of initial peak ratio of two samples tested.

Resolution of Plasma Extract Peaks

The peak area of gefitinib at 1.95 min decreased with an increase of sodium ion concentration in the 100 ng/mL gefitinib samples. The peak area of gefitinib at 1.45 min increased proportionally to the decrease observed in peak area of gefitinib at 1.93 min (Table 5).

Application of Method to Patient Samples

To show the applicability of the method, we analyzed plasma samples from a pediatric patient enrolled on a Phase I clinical trial of gefitinib. After oral gefitinib administration, serial plasma samples were collected, over

Table 4. Stability of gefitinib in extracted plasma at −80°C.

Extracted gefitinib (ng/mL) ($n = 2$)	1 day	5 days	19 days	27 days
3.0	99.4	96.7	90.9	88.1
30	96.8	96.6	93.2	89.9
600	106.3	106.6	109.3	106.3

Note: Reconstituted gefitinib samples were stored at −80°C in duplicate and analyzed by the over 27 days. Stability was assessed by the ratio of peak area (Δgefitinib : D_8 -ZD1839). Data is expressed as mean percent of initial peak ratio of two samples tested.

24 hr, in green-top (Heparin sodium) Vacutainer tubes, and centrifuged at 7000*g* for 2 min to separate the plasma. After liquid–liquid extraction, samples were reconstituted and then analyzed by the method described in this report. A representative plasma concentration–time profile for gefitinib after oral administration is depicted in Fig. 4.

DISCUSSION

The current method has several advantages over the previously published method.[7] First, the wide linear range of our method (e.g., 0.5–1000 ng/mL) represents an improvement over the previously published method (0.5–100 ng/mL).[7] In addition, children enrolled on Phase I gefitinib clinical trials were expected to receive a wide range of dosages, so we expected that gefitinib plasma concentrations in our clinical pharmacokinetic studies would extend over a broad range. Thus, for these reasons we needed an extremely robust analytical method. With this LC-ESI-MS/MS method, plasma

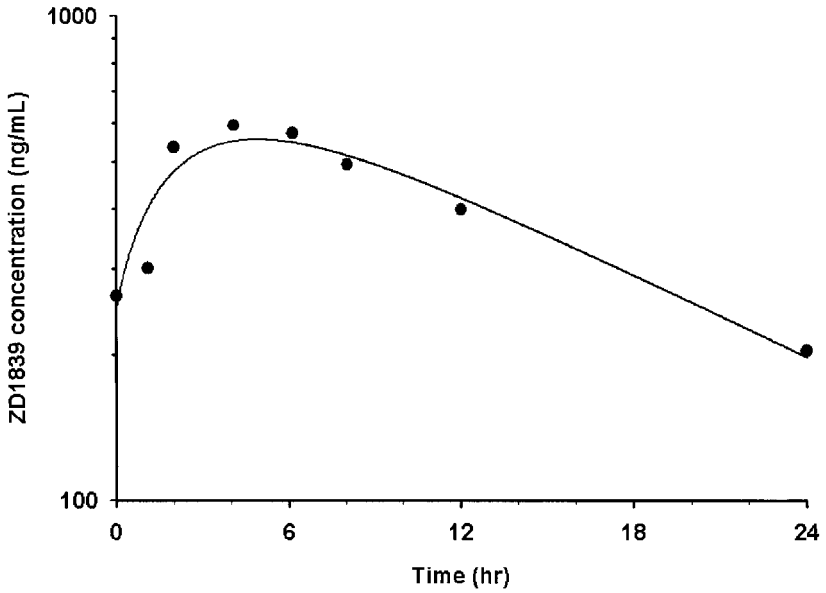

Figure 4. Concentration–time plot from a patient who received oral gefitinib, 100 mg/m^2. Plasma concentration–time points (●) are plotted and the solid line represents the best-fit line from the pharmacokinetic analysis.

gefitinib concentrations can be directly determined in support of clinical pharmacokinetic studies of gefitinib in children. Moreover, in the present method, we chose to use thermally and pneumatically assisted ESI-MS/MS instead of APCI MS/MS[7] for several reasons. The thermally and pneumatically assisted electrospray ionization source is more efficient and precise than APCI for analysis of polar compounds, such as gefitinib.[8–10] The electrospray LC-MS/MS approach is widely used recently for drug analysis in biological fluids, especially in therapeutic drug monitoring and the pharmaceutical industry,[8,11–17] enhancing the likelihood that this method could be implemented at other institutions. Finally, the triple quadrupole mass analyzer with thermally ESI ionization source and collisionally activated dissociation is highly sensitive and specific.

In the case of gefitinib, the m/z measurement is parent and daughter-compound specific, which makes this method especially useful. The thermally and pneumatically assisted electrospray ionization source utilizes an ion spray inlet with an additional probe blowing hot dry nitrogen (400°C) at right angles to the plume of charged droplets from the sprayer. The heated gas increases the efficiency of ion evaporation, resulting in increased sensitivity and the ability to handle higher liquid sample flow rates (up to 500 μL/min). Additionally, using the MS/MS, the resolving power of HPLC becomes less demanding and is mainly used to separate the analyte and IS from the bulk of the matrices. This provides the advantage of a short run time as with the method described herein.

Initially, after the mass scan of both gefitinib and D_8-ZD1839, we employed five different reverse phase HPLC columns to completely resolve gefitinib and the IS. We then used formic acid (0.1%, 0.5%, and 1%) with acetonitrile (20%, 30%, 40%, and 50%), instead of ammonium acetate/acetonitrile as a mobile phase, to yield a preferable peak shape, peak height, and retention time. Later, using 30% acetonitrile in 1% formic acid water, we were able to shorten the retention time of the target compounds to less than 2 min at a flow-rate of 150 μL/min with an overlap between gefitinib and the IS, without affecting the specificity of detection or quality of the results. Out of the five columns tested, the Synergi C12 column showed the most stable performance using the conditions of our method. We also varied the percentage of acetonitrile (from 10% to 50%) in reconstitution solution (1% formic acid water) for a dried extraction plasma sample, and found that using 20% acetonitrile in the reconstitution solution produced the best separation and peak shape with 30% acetonitrile mobile phase.[18]

Using the sample preparation procedures described above and the optimal HPLC conditions, we observed a peak in front of and completely separate from gefitinib. Our preliminary studies showed that this peak was not a

degradation product or a by-product from the manufacturing process (data not shown). We also determined that this peak was not an isomer of gefitinib. We performed a series of experiments that showed the size of this peak was in proportion to the sodium ion concentration and inversely proportionate to the size of the primary gefitinib peak. Therefore, we speculate that sodium ions form an adduct with gefitinib during plasma sample preparation or even during column separation. This adduct is more polar and elutes earlier than gefitinib, and will be fragmented upon heating in the ESI source. Based upon this speculation, we have minimized the extra addition of cations (e.g., sodium, potassium) during sample and mobile phase preparation to minimize formation of this adduct. However, the presence of trace amounts of cations still account for a peak at 1.45 min, although the peak area is less than 5% of the gefitinib peak at 1.95 min.

In summary, we have developed and validated an LC-ESI-MS/MS method for the rapid and precise quantitation of gefitinib in plasma samples from pediatric pharmacokinetic studies. The method is also sensitive and specific, which further enhances its utility as an analytical method for use in clinical pharmacokinetic studies of gefitinib. We used this HPLC ESI-MS/MS method to measure gefitinib in plasma samples obtained from a representative patient receiving oral gefitinib, and showed the importance of a wide linear range ($\sim$4 log) in this application. Moreover, given the relatively small sample requirement and sensitivity of this method ($S/N \geq 5$ at 0.15 ng/mL) it may also be useful for in vitro studies of gefitinib (e.g., tissue culture studies), where low concentrations or small sample volumes may be expected.

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