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# Determination of lapatinib (GW572016) in human plasma by liquid chromatography electrospray tandem mass spectrometry (LC–ESI-MS/MS)

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**Abstract**

A sensitive method for the determination of lapatinib (GW572016) in human plasma was developed using high-performance liquid chromatographic separation with tandem mass spectrometric detection. Plasma samples (100 μL) were prepared using solid phase extraction (SPE) columns, and 6.0 μL of the reconstituted eluate was injected onto a Phenomenex® CuroSil-PFP 3 μ analytical column (50 mm × 2.0 mm) with an isocratic mobile phase. Analytes were detected with a PE SCIEX API-365 LC–MS/MS system at unit (Q1) and low (Q3) resolution in positive multiple reaction monitoring mode (*m*/*z* 581 (precursor ion) to *m*/*z* 364 (product ion) for lapatinib). The mean recovery for lapatinib was 75% with a lower limit of quantification of 15 ng/mL (S/N = 11.3, CV ≤ 14%). This method was validated over a linear range of 100–10,000 ng/mL, and results from a 5-day validation study demonstrated good within-day and between-day precision and accuracy. This method has been used to measure plasma lapatinib concentrations in a Phase I study in children with cancer.


*Keywords:* Plasma; Laptinib (GW572016); Liquid chromatography; Electrospray tandem mass spectrometry (LC–ESI-MS/MS)

## 1. Introduction

The ErbB or epidermal growth factor (EGF) family of receptor tyrosine kinases plays an important role in both normal and aberrant cellular proliferation and survival. Two prominent members of the ErbB family include the epidermal growth factor receptor (EGFR or ErbB1) and HER-2/*neu* (ErbB2) receptors, which are expressed in a variety of tissues. Overexpression or constitutive activation of EGFR and ErbB2 have been noted in several malignancies, and are associated with poor clinical outcomes. Ligand binding to an EGFR monomer results in homodimerization or heterodimerization between ligand-bound EGFR and another EGFR or ErbB family monomer, respectively [1,2]. ErbB2, which seems to have no direct ligand, preferentially heterodimerizes with EGFR and other ErbB monomers. In both instances, dimerization leads to autophosphorylation of adjacent ErbB monomer tyrosine residues [3,4], and recruitment of signaling proteins that stimulate cell division, growth, and survival [1,5]. Since both ErbB1 and ErbB2 are catalytically capable of activating such proliferative pathways, a compound that would simultaneously target both receptors would have theoretical advantages.

Lapatinib (GW572016; GlaxoSmithKline, Research Triangle Park, NC) is an orally active 4-anilinoquinazoline inhibitor of both ErbB1 and ErbB2 tyrosine kinases. Lapatinib has exhibited cytostatic or cytotoxic activity in various cell lines and xenografts [4,6–8], and has demonstrated its tolerability in early phase clinical trials in adults with cancer [9]. Two Phase I and pharmacokinetic clinical trials of lapatinib are being planned in both adults and children with brain tumors. The character-

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ization of lapatinib disposition in these two study populations will require a highly sensitive and specific assay; one that also exhibits sufficient accuracy and precision. To date only one analytical method (i.e., LC–MS/MS) for lapatinib has been reported in the literature [10]. However, this complex method mainly focuses on an on-line extraction technique and utilizes custom equipment not available to most biomedical laboratories. Here we describe a sensitive and specific LC–MS/MS method with a deuterated lapatinib (GW572016R) as internal standard for quantification of lapatinib (GW572016) in human plasma.

## 2. Experimental

### 2.1. Chemicals

Lapatinib (Fig. 1; GW572016, 98.6% purity) and deuterated lapatinib (Fig. 1; GW572016R, 98.0% purity), as the internal standard (ISTD), were supplied by GlaxoSmithKline (Collegeville, PA, USA) and used for preparation of calibrators and control samples. HPLC grade acetonitrile and methanol were obtained from Burdick & Jackson (Muskegon, MI, USA), whereas formic acid (minimum 95%) and *N-N*-dimethylformamide were purchased from Sigma (St. Louis, MO, USA). Ammonium hydroxide and ammonium formate were obtained from Fisher (Fairlawn, NJ, USA). Blank human plasma was obtained from Lifeblood (Memphis, TN, USA). 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). Other chemicals were purchased from standard sources and were of the highest quality available.

### 2.2. Apparatus and chromatographic conditions

#### 2.2.1. Chromatographic conditions

The HPLC system consisted of a Shimadzu (Kyoto, Japan) system controller (SCL-10AVP), pump (LC-10ADVP), autosampler (SIL-10ADVP), and online degasser (DGU-14A). Reconstituted plasma extract (6 μL) was injected onto a Phenomenex (San Francisco, CA) CuroSil-PFP 3 μm analytical column (50 mm × 2.0 mm) preceded by a guard column of the same material (30 mm × 2.0 mm). Flow rate was 0.20 mL/min with a mobile phase consisting of acetonitrile/5.0 mM ammonium formate (pH 3.0)/formic acid (v/v/v = 1000:50:1). The column was maintained at 60 °C by HotPocket (Keystone Scientific, Inc., Bellefonte, PA). Under these conditions, the typical retention time was 1.63 min for both lapatinib and its internal standard, with a total run time for each sample of 3.0 min. We observed backpressure values of approximately 32 bars.

#### 2.2.2. Mass spectrometric conditions

Detection was performed with a PE SCIEX API 365 triple quadrupole mass spectrometer (Toronto, Canada) equipped

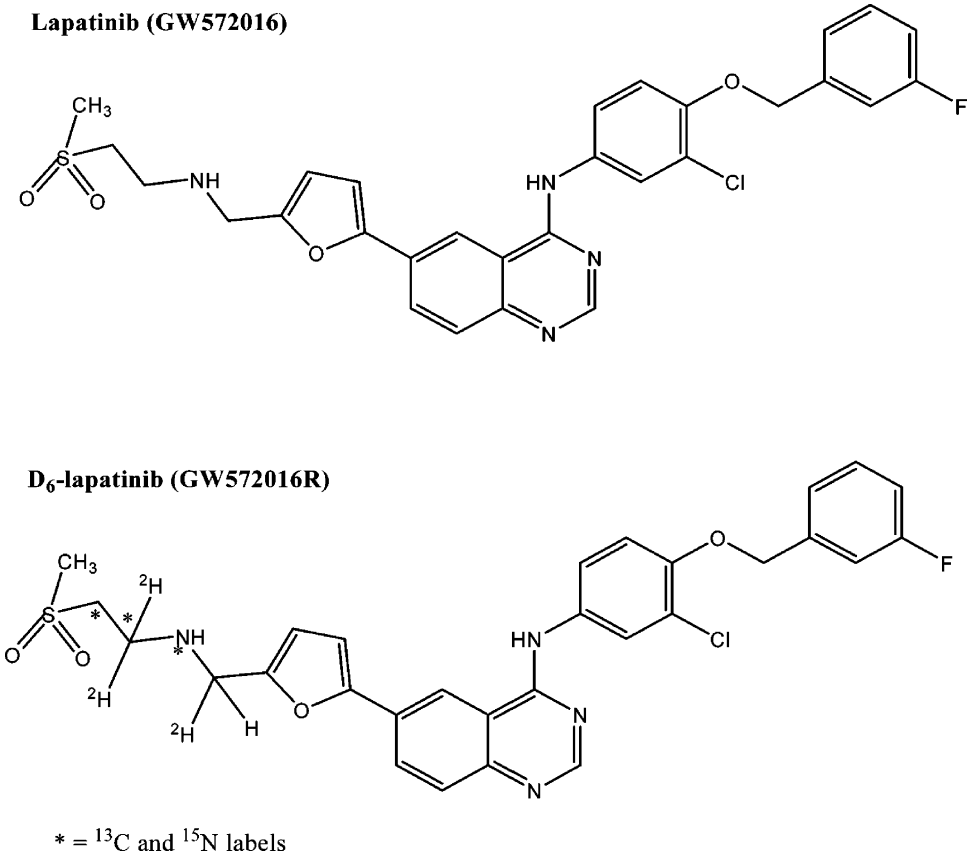


Fig. 1. Chemical structure of lapatinib and deuterated-lapatinib.

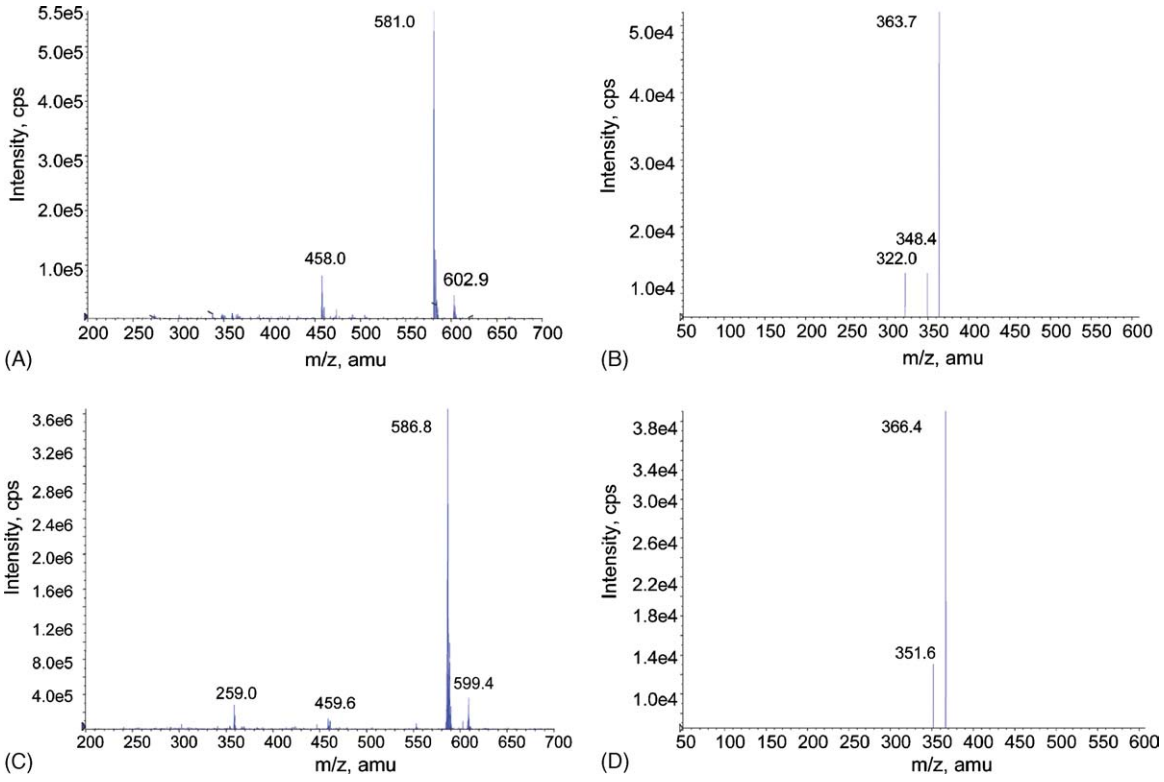


Fig. 2. (A) Scan positive-ion mass spectra for parent molecular ion for lapatinib at *m/z* 581.0; (B) collision induced dissociation spectra for lapatinib to the predominant ion *m/z* 363.7; (C) scan positive-ion mass spectra for parent molecular ion for $D_6$-lapatinib at *m/z* 586.8, and (D) collision induced dissociation spectra for $D_6$-lapatinib to the predominant ion *m/z* 366.4.

with a Turbo IonSpray® 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 (Fig. 2) showed the parent molecular ion for lapatinib at *m/z* 581 to the predominant ion at *m/z* 364, and the parent molecular ion for GW572016R (ISTD) at *m/z* 587 to the predominant ion at *m/z* 366, respectively. The optimized conditions of MS/MS with electrospray conditions were as follows: 500 °C ion spray source temperature, 55 kPa nebulizer (NEB) pressure, 41 kPa psi curtain (CUR) gas pressure, 5 L/min turbo gas flow, 4900 V ionspray voltage (IS), and 3.0 units collision gas (CAD); 45 V declustering potential (DP), 232 V focusing potential (FP), 10 V entrance potential (EP), 20 V collision cell entrance potential (CEP), 60 V collision energy (CE), and 38 V collision exit potential (CXP). The mass spectrometer was interfaced to a computer workstation running Analyst software (Version 1.4 Applied Biosystems, Foster City, CA) for data acquisition and processing.

### 2.3. Sample preparation

#### 2.3.1. Stock solutions

Stock solutions were prepared by separately dissolving either lapatinib or ISTD in *N-N*-dimethylformamide to yield a concentration of 1.0 mg/mL. The stock solutions were stored at −80 °C, and less than 5% of their nominal values were lost over 6 months. The lapatinib working solutions (2.0, 10.0, and 100.0 μg/mL) were prepared at the time of assay from the 1.0 mg/mL stock solutions by making dilutions with 80% methanol water. An ISTD working solution (50.0 μg/mL) was prepared from its 1.0 μg/mL stock solution in the same manner.

#### 2.3.2. Calibration curve and quality controls

Calibrators were made by adding lapatinib working solutions to blank plasma in correct proportions to give final concentrations of 100, 250, 500, 1000, 2500, 5000, 7500, and 10,000 ng/mL with 2000 ng/mL ISTD. Plasma controls were prepared in triplicate using the same methodology at concentrations at 200, 1500, and 9000 ng/mL.

#### 2.3.3. Plasma sample preparation

A total of 100 μL of plasma or patient plasma was spiked with ISTD, and added to a 2.0 mL amber microcentrifuge tube. The spiked plasma was then treated with 20 μL 95% formic acid followed by 500 μL deionized water. The solution was vortexed and then 500 μL of the mixture was transferred to a vacuum-assisted Phenomenex (San Francisco, CA) Strata-X tube (60 mg/3 mL; PN: 8B-S100-UBJ) preconditioned with 3 × 1 mL methanol washes followed by 3 × 1 mL washes with water. The loaded sample was then washed with 4 × 0.5 mL of 5% acetonitrile in water. The sample was eluted with a mixture of acetonitrile/methanol (v/v = 60/40) and dried under nitrogen for 30 min. The sample was reconditioned with 100 μL mobile

phase and a volume of 6.0 μL injected by the autosampler onto the LC–ESI-MS/MS system.

#### 2.3.4. Patient sample collection

Patient blood samples (2 mL) were collected in green-top (heparin sodium) Vacutainer tubes (Franklin Lakes, NJ), aliquoted into microcentrifuge tubes, and centrifuged at 7000 × *g* for 2 min to separate the plasma that was stored in −80 °C. The plasma samples were then processed as described for calibrators and controls for analysis.

### 2.4. Ion suppression effects

Ion suppression was evaluated with a comprehensive approach that uses a post-column infusion of the analyte [11]. Briefly, a neat lapatinib sample (100 ng/mL) was infused, post-column, through a Valco zero dead volume tee using a Harvard Apparatus syringe pump 11 (Harvard Apparatus, Holliston, MA, USA) at a constant flow rate of 5.0 μL/min into the LC effluent (150 μL/min) prior to entering the mass spectrometer. Different blank plasma samples ($n = 3$) and mobile phase solution were then injected onto CuroSil-PFP column. Effluent from the HPLC combined with the infused neat lapatinib, entered the electrospray interface and was analyzed under the operating conditions for lapatinib to measure the "matrix effect," not only from one run, but also from late-eluting compounds that may not be detected until after several sequential analyses had been performed.

### 2.5. Assay validation

The method developed for lapatinib quantitation in human plasma was validated over 5 days by analysis of lapatinib plasma quality control samples, and the within-day and between-day precision and accuracy for the method were determined. Two calibration curves were analyzed during this validation. The linear regression of the ratio of lapatinib/ISTD 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 limit of quantitation (LOQ) were defined as the minimum value at which the ratio of signal/noise was ≥3 and ≥10, respectively. These were determined by triplicate analysis of an extensive calibration curve in the low concentration range (5–100 ng/mL).

The stability of lapatinib at 4 and −20 °C in the reconstituted extracted plasma sample for up to 72 h, and at −80 °C for three months were evaluated at two concentrations (200 and 9000 ng/mL).

### 2.6. Application of method to patient samples

Serial blood samples (2 ml) were collected from a pediatric patient following a 300 mg oral lapatinib dose. Each blood sample collected into a green-top (heparin sodium) vacutainer tube (Franklin Lakes, NJ) and centrifuged at 7000 × *g* for 2 min to separate the plasma. All plasma samples were stored at −80 °C until processing for analysis. After solid phase extraction (SPE), samples were reconstituted and then analyzed using the described method.

## 3. Results and discussion

### 3.1. Chromatography

Recently, the PFP column (pentafluorophenyl bonded to silica) has been preferentially used in place of traditional $C_8$ or $C_{18}$ columns for reversed-phase HPLC separation of molecules containing aromatic groups primarily due to its higher selectivity for, and improved retention of, such molecules [12,13]. Lapatinib is a small molecule (MW = 581) that contains five aromatic rings (Fig. 1), and thus a small PFP column presents itself as a logical choice for the HPLC separation linked to a mass spectrometer. Therefore, we chose a 50 mm × 2.0 mm CuroSIL PFP 3 μm column to achieve the most efficient separation of lapatinib prior to detection with mass spectroscopy. Beyond column selection, other conditions including the composition of mobile phase and column temperature were optimized to increase mass signal strength for lapatinib in terms of counts per second. Increasing the percentage of acetonitrile in the mobile phase from 80 to 95% improved the signal in a proportional manner, whereas adjusting the ammonium formate buffer (pH 3.0) concentration to 5.0 mM resulted in an optimal signal response. Additionally, a trace amount of formic acid (MP/HCOOH = v/v = 1000/1) was added to improve protonation of lapatinib in the positive ionization mode. Column temperature was tested at 25, 40, 50, 60, and 70 °C, respectively. According to ion intensity, retention time, and peak shapes of both lapatinib and ISTD, the PFP column performaned best at 60 °C. Therefore, final HPLC conditions selected were a mobile phase consisting of acetonitrile/ammonium formate buffer (5.0 mM, pH 3.0)/formic acid = v/v/v = 95/5/0.1 and a column temperature of 60 °C.

### 3.2. Solid phase extraction

Lapatinib is a strong non-polar hydrophobic compound with a log $P'$ greater than 4.6; thus, it should be retained on a non-polar $C_{18}$ SPE stationary phase. During our studies to develop an extraction method for lapatinib in plasma, we observed that lapatinib is indeed strongly retained in a Strata-X $C_{18}$ SPE tube even when using 100% methanol or acetonitrile as eluting solution. We pretreated plasma lapatinib with base and acid before solid phase extraction process. Our results showed that base pre-treatment (15–30% $NH_4OH$) yielded an average recovery of 45% lapatinib from plasma in comparison to acid pretreatment (20–95% HCOOH), which yielded an average recovery of 62%. We hypothesized that under the basic conditions, the lapatinib molecule was essentially neutral and less polar. Thus, when the solid phase was pretreated with base the lapatinib became more hydrophobic, and increased its binding to the $C_{18}$ media. However, when the sample is pretreated with acid, lapatinib becomes charged, which reduces the overall hydrophobicity of the molecule, and makes the compound more polar.

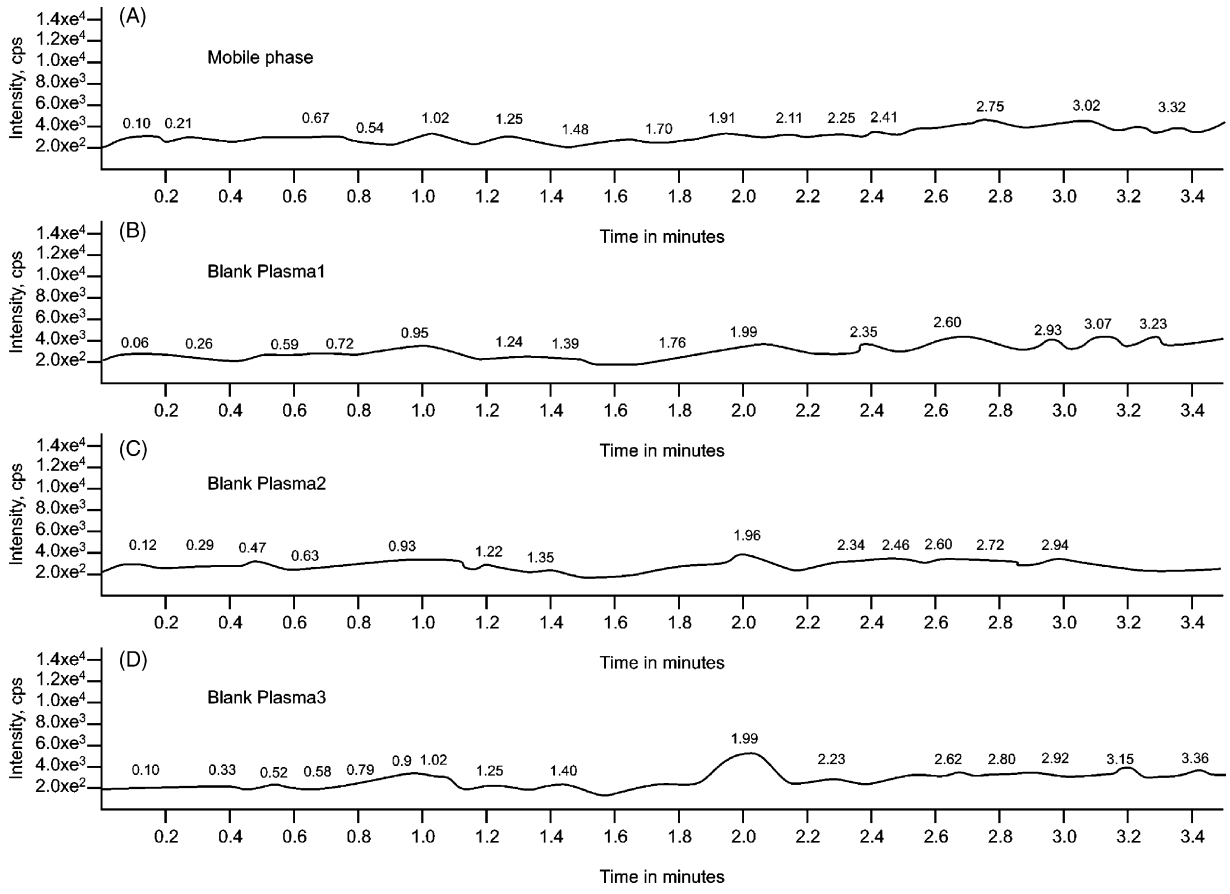


Fig. 3. Infusion chromatogram for lapatinib—a neat lapatinib (100 ng/mL) was constantly infused, post-column, at a flow rate of 5.0 μL/min into the LC effluent that included the tested samples: (A) mobile phase solution, (B) blank human plasma 1, (C) blank human plasma 2, and (D) blank human plasma 3.

This results in easy elution of lapatinib from the $C_{18}$ stationary phase by an organic solvent. It was finally determined that pretreating the plasma lapatinib sample with formic acid (20 μL 95% formic acid with 100 μL plasma lapatinib sample), and subsequently eluting with acetonitrile/methanol (v/v = 60/40) mixture produced an acceptable average lapatinib solid phase extraction recovery of 75% over the concentration range of 100–10,000 ng/mL. Moreover, using an acidified mobile phase (ammonium formate buffer pH 3.0) with formic acid 0.1%) resulted in a favorable elution of lapatinib from the CuroSIL PFP column (i.e., improved retention and peak shape).

### 3.3. Ion suppression

Ion suppression is an important factor affecting the quantitative performance of a mass detector, especially with electrospray interface [14]. We studied this ion suppression effect from both SPE extracted human blank plasma ($n = 3$) and mobile phase solution. Depicted in Fig. 3A–D are the results from these studies that illustrate the response obtained from the applied post-column infusion. Since lapatinib and its isotope (ISTD) coeluted at the same time in this method, only lapatinib at 100 ng/mL was infused in this ion suppression study. The results demonstrated the absence of either relevant suppression or enhancement of lapatinib ion intensity at retention time of 1.62 min (Fig. 4), and in the relative time range in this developed LC–MS/MS method.

### 3.4. Assay validation

To assess within-day and between-day precision and accuracy, we evaluated validation parameters for lapatinib (Table 1). Ten injections of low, medium, and high lapatinib concentration control samples as described previously were made on days 1 and 2 to assess within-day variability and again on days 3–5 to evaluate between-day variability. The LOD and LLOQ in plasma for this method were 4.2 ng/mL (S/N = 3.2,

Table 1
Validation parameters of lapatinib in human plasma

| Quality control lapatinib (ng/mL) | Within-day ($n = 10$) | | Between-day ($n = 5$) | |
|---|---|---|---|---|
| | %R.S.D. | %Error | %R.S.D. | %Error |
| 200 | 2.5 | 4.9 | 7.9 | −1.63 |
| 1500 | 2.8 | −1.1 | 6.9 | −4.7 |
| 9000 | 2.0 | 3.7 | 3.4 | 2.4 |

Ten injections of low, medium, and high quality control of lapatinib plasma samples were run within 1 day to assess within-day variability and fifteen injections were run within 5 days to evaluate between-day variability. Variability reported as relative standard deviation (%RSD) and percentage error (%Error).

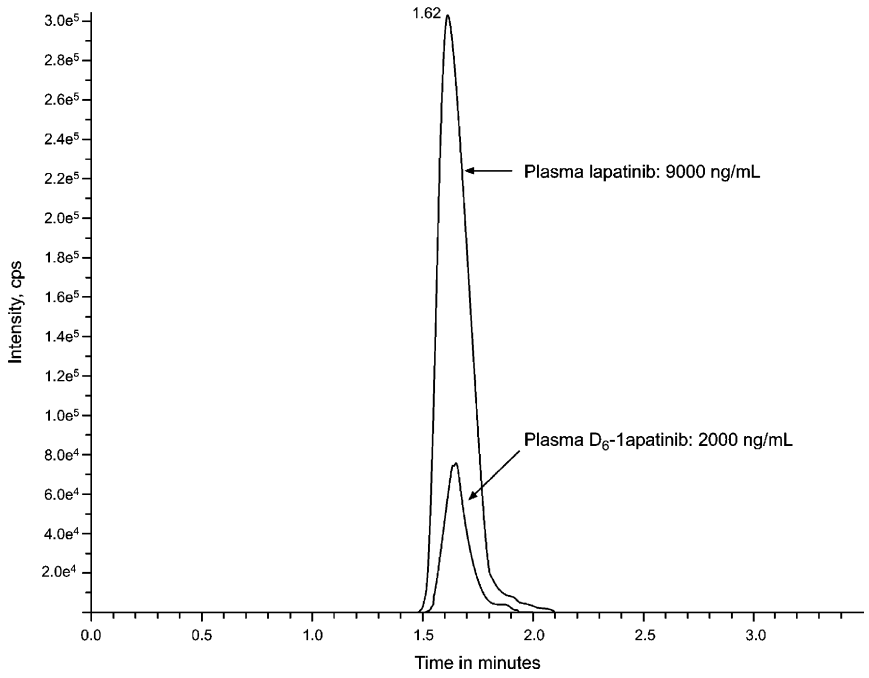


Fig. 4. MRM chromatogram of human plasma spiked with lapatinib and $D_6$-lapatinib (internal standard). Note that both components are eluted simultaneously at 1.62 min.

$n = 3$) and 15 ng/mL (S/N = 11.3, $n = 3$, CV ≤ 11%), respectively. The extraction recovery of plasma lapatinib in comparison to the neat lapatinib at the same concentration was ∼75% ($n = 3$, S.D. = 3.06, CV% = 4.09) at the spiked lapatinib concentrations of 200, 1500, and 9000 ng/mL in triplicate. The calibration curves for human plasma were linear from 100 to 10,000 ng/mL, with correlation coefficients ($R^2$) greater than 0.995.

### 3.5. Stability

To assess the stability of lapatinib in reconstituted extracted plasma, we evaluated three lapatinib concentrations (200, 1500, and 9000 ng/mL) at 4 °C for 24 h and at −20 °C for up to 72 h after solid phase extraction and then reconstituted in mobile phase. As depicted in Table 2a, we observed less than a 6% decrease in lapatinib peak area at both testing conditions. Thus, lapatinib is considered stable in extracted plasma samples at 4 °C for 24 h and at −20 °C for 72 h.

To test the long-term stability of lapatinib in plasma at −80 °C, we evaluated two concentrations (200 and 9000 ng/mL) over three months. Internal standard was added to stability samples prior to SPE extraction and analysis. As depicted in Table 2b, for lapatinib in plasma at −80 °C, the decrease of peak area ratio (Δlaptinib:ISTD) was less than 10% within 3 months. Thus, lapatinib is stable in plasma at −80 °C for at least 3 months.

### 3.6. Application of assay in patient blood sample

To show the applicability of the method, we analyzed plasma samples from a child enrolled on a Phase I pharmacokinetic trial of lapatinib. Serial plasma samples were collected, processed, and analyzed by the methods described in this report. All plasma samples were stored at −80 °C until analysis. A representative plasma concentration–time profile for lapatinib after oral administration is depicted in Fig. 5. This method developed to quantitate lapatinib in human plasma samples possesses sufficient precision (CV% ≤ 7.9%) and accuracy (−5 ≤ error% ≤ 5.0), for large-scale application in clinical studies of lapatinib pharmacokinetics in both pediatric and adult populations.

Table 2a
Stability of lapatinib in extracted human plasma

| Extracted lapatinib (ng/mL) | <1 h | 24 h at 4 °C ($n = 2$) | 72 h at −20 °C ($n = 2$) |
|---|---|---|---|
| 200 | 102.2 | 98.8 | 97.9 |
| 1500 | 95.6 | 98.3 | 95.6 |
| 9000 | 107.7 | 94.8 | 104.7 |

The reconstituted lapatinib samples were separately stored (in duplicate) at 4 and −20 °C and analyzed up to 72 h. Stability was assessed by the ratio of peak area (Δlapatinib:ISTD). Data is expressed as mean percent of initial peak ratio of two samples tested.

Table 2b
Stability of lapatinib in human plasma at −80 °C

| Plasma lapatinib | <1 day | 15 days | 45 days | 90 days |
|---|---|---|---|---|
| 200 ng/mL ($n = 2$) | 100 | 96 | 92 | 92 |
| 9000 ng/mL ($n = 2$) | 100 | 91 | 91 | 92 |

Spiked plasma samples with high and low lapatinib concentrations were aliquotted in duplicate and stored at −80 °C. The samples were then assayed over 3 months. Stability was assessed by the ratio of peak area (lapatinib/ISTD). Data presented is mean percent of initial peak ratio of two samples tested.

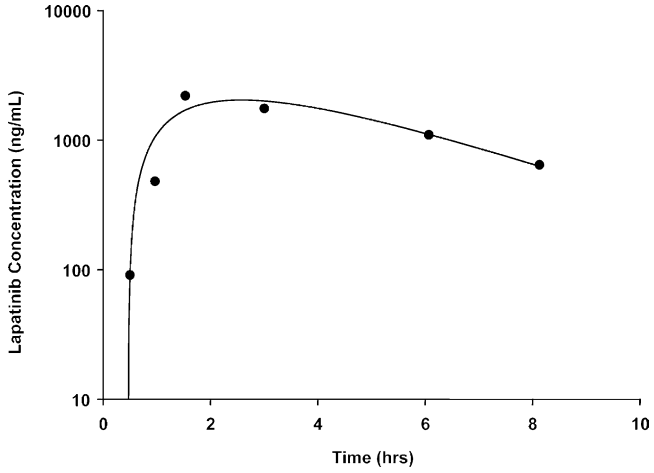


Fig. 5. Lapatinib plasma concentration–time profile in a pediatric patient after one oral dose of lapatinib (400 mg). Plasma concentration–time points (●) are plotted and the solid line represents the best-fit curve resulting from the pharmacokinetic analysis.

## 4. Conclusion

We have developed and validated an LC–ESI-MS/MS method for the rapid and precise quantitation of lapatinib in human 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 lapatinib. Moreover, given the relatively small sample requirement and sensitivity of this method ($S/N \geq 11.3$ at 15 ng/mL) it may also be useful for in vitro studies of lapatinib (e.g., tissue culture studies) where low concentrations or small sample volumes may be expected. In addition, by using a narrow-bore column instead of 4.6 i.d. column with lower flow rate, this assay reduces solvent costs and minimizes environmental impact of the toxic solvent. Finally, we have successfully applied this LC–ESI-MS/MS method by measuring lapatinib in human plasma from a clinical pharmacokinetic study in a child treated with oral lapatinib.

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