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Rapid determination of gefitinib and its main metabolite, O-desmethyl gefitinib in human plasma using liquid chromatography–tandem mass spectrometry

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ABSTRACT

A novel, rapid and specific liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed and validated for the simultaneous quantification of gefitinib and its predominant metabolite, O-desmethyl gefitinib in human plasma. Chromatographic separation of analytes was achieved on an Alltima C18 analytical HPLC column (150 mm × 2.1 mm, 5 μm) using an isocratic elution mode with a mobile phase comprised acetonitrile and 0.1% formic acid in water (30:70, v/v). The flow rate was 300 μL/min. The chromatographic run time was 3 min. The column effluents were detected by API 4000 triple quadrupole mass spectrometer using electrospray ionization (ESI) in positive mode. Linearity was demonstrated in the range of 5–1000 ng/mL for gefitinib and 5–500 ng/mL for O-desmethyl gefitinib. The intra- and inter-day precisions for gefitinib and O-desmethyl gefitinib were ≤10.8% and the accuracies ranged from 89.7 to 104.7% for gefitinib and 100.4 to 106.0% for O-desmethyl gefitinib. This method was used as a bioanalytical tool in a phase I clinical trial to investigate the possible effect of hydroxychloroquine on the pharmacokinetics of gefitinib. The results of this study enabled clinicians to ascertain the safety of the combination therapy of hydroxychloroquine and gefitinib in patients with advanced (Stage IIIB–IV) non-small cell lung cancer (NSCLC).

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1. Introduction

Gefitinib (Iressa®) is an orally active and selective inhibitor of the epidermal growth factor receptor (EGFR; HER1) tyrosine kinase [1]. EGFRs initiate signals that are important in the proliferation and survival of cancer cells. EGFRs are frequently over-expressed in non-small cell lung cancer (NSCLC). Lung cancer is a major cause of morbidity as the overall 5-year survival is at a dismal 15% [2]. Consequently, EGFR inhibitors such as gefitinib are attractive therapeutic agents as they provide a targeted treatment approach by interfering with the signal transduction pathway implicated in cancer cell proliferation [3]. Gefitinib is used as an oral monotherapy in patients with advanced NSCLC (Stage IIIB–IV) after failure of both platinum-based and docetaxel chemotherapies. However, gefitinib has modest activity, attaining median survival duration of 6.5–7.6 months and 1-year survival rate of 29–35% [4,5]. Efficacy of gefitinib is limited by primary (de novo) resistance and acquired resistance after gefitinib therapy [6].

Preliminary experimental results from cell lines suggested the addition of hydroxychloroquine may result in re-sensitization to gefitinib and higher efficacy [7,8]. The findings from an ongoing phase I clinical trial also suggested that a combination of hydroxychloroquine and gefitinib may potentially delay or reverse the acquired resistance to gefitinib [9]. Co-administration of hydroxychloroquine and gefitinib has been proposed to improve the clinical efficacy of gefitinib. Therefore, in order to ascertain the safety of this combination of drugs, it is imperative to study the possible effect of hydroxychloroquine on the pharmacokinetics of gefitinib.

In this study, a rapid and specific (LC–MS/MS) method was developed and validated for this purpose. Up to date, only two assays of the respective HPLC-UV and LC–MS/MS approach are available for the quantification of gefitinib, but there is no assay available for the simultaneous quantification of gefitinib and O-desmethyl gefitinib [10,11]. The quantification of O-desmethyl gefitinib would help to discern the possible effect of hydroxychloroquine on the metabolism of gefitinib. Hence, the development and validation of this novel LC–MS/MS method will serve as a novel bioanalytical platform for the simultaneous quantification of gefitinib and O-desmethyl gefitinib in human plasma.

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2. Materials and methods

2.1. Chemicals and reagents

Gefitinib (Lot number 6-YM-127-1, 98% pure by HPLC) and O-desmethyl gefitinib (Lot number 5-DHL-3-4, 98% pure by HPLC) were purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). The internal standard (IS), O-methyl gefitinib-d3 (Lot number 143, 99% isotopically pure) was purchased from Medical Isotopes, Inc. (Pelham, NH03076, USA). Formic acid (98–100%), methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q Plus system (Millipore, Milford, MA, USA). Drug-free (blank) human plasma from healthy donors was provided by National University Hospital (NUH), Singapore.

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

Stock solutions of gefitinib, O-desmethyl gefitinib and IS were prepared in methanol at 1 mg/mL and stored at 4 °C. The working solutions for the six calibration standards containing gefitinib and O-desmethyl gefitinib were prepared by dilution of the stock solution with 50% methanol at the following concentrations: 0.025, 0.05, 0.25, 1.0, 2.5, 5.0 μg/mL and 0.025, 0.05, 0.25, 1.0, 2.0, 2.5 μg/mL, respectively. The working solutions for the three quality control (QC) samples containing gefitinib and O-desmethyl gefitinib were prepared at the following concentrations: 0.075, 0.5, 3.5 μg/mL and 0.075, 0.5, 1.5 μg/mL, respectively.

2.3. Calibrators and QC samples preparation

For the preparation of a calibrator, 10 μL of gefitinib, O-desmethyl gefitinib and IS were each added to a 1.5 mL microcentrifuge tube. Next, 50 μL of blank human plasma was added and the mixture was vortexed for 30 s. Subsequently, 100 μL of 100% acetonitrile was added for direct precipitation of the proteins in the human plasma samples. The mixture was vortexed vigorously for one minute, followed by centrifugation at 35,000 × *g* for 15 min at 4 °C. Thereafter, 50 μL of the supernatant was transferred to a 1.5 mL microcentrifuge tube containing 70 μL of 0.1% formic acid in water. The tube was vortexed for 3 s before the sample was transferred to a 250 μL glass insert placed in an autosampler vial. A volume of 10 μL was injected for quantitative analysis by LC–MS/MS.

2.4. Chromatographic and mass-spectrometric conditions

Chromatographic analysis was performed on a high-performance liquid chromatographic system, which comprised Agilent 1100 series binary pump and auto-sampler (Agilent Technologies, Germany). Chromatographic separation of analytes was achieved using an Alltech Alltima C18 analytical HPLC column (150 mm × 2.1 mm, 5 μm; Alltech Associates Inc, Lexington, Kentucky USA). The mobile phase which comprised acetonitrile – water containing 0.1% formic acid (30:70, v/v) was delivered isocratically at a flow rate of 300 μL/min at 20 °C. The column effluent was detected by API (Atmospheric Pressure Ionization) 4000 triple quadrupole mass spectrometer (Applied Biosystems, MDS SCIEX, Ontario, Canada). The analytes were first nebulized by nitrogen gas and then introduced into the detector at 500 °C. The analytes were declustered at a potential of 86 V and analyzed by an electropositive ion spray (ESI +ve) of 5500 V. The optimized collision energies of gefitinib, O-desmethyl gefitinib and IS used were 37, 35 and 81 V, respectively. Multiple reaction monitoring (MRM) was used to monitor the precursor ion (Q1) and product ion (Q3). The mass spectrometer was tuned to allow the $[M+H]^+$ ions of gefitinib (m/z 447), O-desmethyl gefitinib (m/z 433) and IS, O-methyl gefitinib-d3 (m/z 450) to pass through the first quadrupole (Q1) and into the collision cell (Q2) for fragmentation. The product ions of gefitinib (m/z 128), O-desmethyl gefitinib (m/z 128) and IS (m/z 100) were monitored through the third quadrupole (Q3). The dwell time per channel was 200 milliseconds for data collection.

2.5. Construction of standard curves

The standard curve was constructed with six concentrations for both gefitinib and O-desmethyl gefitinib. The calibrators for gefitinib and O-desmethyl gefitinib were prepared at the following concentrations: 5, 10, 50, 200, 500, 1000 ng/mL and 5, 10, 50, 200, 400, 500 ng/mL, respectively. The standard curve was determined by using the least-squares linear regression which was drawn on the ratio of the peak area of either gefitinib or O-desmethyl gefitinib against the IS. The calibrators were weighted according to $1/x$ where x is the concentration. Quantification of patient samples was calculated using interpolation.

2.6. Method validation

Method validation was carried out according to guidelines for bioanalytical method validation by the Food and Drug Administration (FDA) [12]. Intra-day and inter-day accuracy and precision were established by analyzing QC samples of gefitinib and O-desmethyl gefitinib at the nominal concentrations: 15, 100, and 700 ng/mL and 15, 100, and 300 ng/mL, respectively. Intra-day variability was determined by analyzing the QCs in quadruplicate using one calibration curve. Inter-day variability was assessed by analyzing the QCs on five different days using calibration curves obtained daily. Accuracy was expressed as a percentage of the mean value measured over the nominal value at each concentration whereas precision was expressed in terms of coefficient of variation (CV), defined as a percentage of standard deviation divided by the mean.

2.7. Matrix effect and recovery

To identify the possible effect of matrix on the quantification of analytes, QC samples with and without plasma prepared in quadruplicate at each concentration were analyzed. The concentration levels evaluated were at 15, 100, and 700 ng/mL for gefitinib, 15, 100, and 300 ng/mL for O-desmethyl gefitinib and 200 ng/mL for IS. In the reference tubes, gefitinib, O-desmethyl gefitinib and IS, O-methyl gefitinib-d3 in 10 μL were each added into a microcentrifuge tube and dried under vacuum at 60 °C in a concentrator plus (eppendorf, Hamburg, Germany) for an hour. The reference tube was later reconstituted with 70 μL of acetonitrile–water containing 0.1% formic acid (30:70, v/v). In the matrix (50 μL plasma) containing tubes, 30 μL of 50% methanol in water was added followed by 100 μL of acetonitrile for protein precipitation. The tubes were then centrifuged at 35,000 × *g* for 15 min at 4 °C. Thereafter, 50 μL of the supernatant was transferred into a microcentrifuge tube, followed by the addition of 10 μL of gefitinib, O-desmethyl gefitinib and IS, respectively. The sample was dried under vacuum at 60 °C for an hour. The dried supernatant was reconstituted with 70 μL of acetonitrile–water containing 0.1% formic acid (30:70, v/v). The samples were analyzed for possible matrix effect based on the ratio of the peak area of the analyte in the matrix-based tube against that in the reference tube.

Recovery experiments were performed in quadruplicate at each QC sample concentration. A control mixture was prepared by the procedure mentioned in Section 2.3 with the exception that 50 μL of deionized water was used in place of the blank human plasma.

Percentage recovery of gefitinib and O-desmethyl gefitinib were calculated using the formula:

$$\text{percentage recovery} = \frac{\text{PeakArea_}\textit{sample}}{\text{PeakArea_}\textit{control}} \times 100\%$$

2.8. Dilution procedure

In the process of quantifying the concentration of gefitinib in the human plasma samples, there were samples that exceeded the highest concentration of 1000 ng/mL for gefitinib. Hence, assays of these samples were repeated using 25 μL of sample diluted with 25 μL with blank plasma instead of 50 μL of sample as mentioned earlier. This procedure was validated with spiked samples at three concentrations, specifically at 1200, 1500 and 2000 ng/mL. The working solutions prepared for these three concentrations were 60, 75 and 100 μg/mL. The procedure for sample preparation was identical to the one described in Section 2.3 with the exception that 10 μL of O-desmethyl gefitinib was replaced with 50% methanol and the spiked sample was diluted with 25 μL of blank human plasma. The dilution factor for this procedure is two. The procedure was repeated in quadruplicate at each concentration.

2.9. Protein precipitation efficiency

Protein precipitation is a simple, fast and cost-effective plasma sample preparation technique in bio-analysis. However, protein residues in purified samples may result in matrix ion suppression and damage to the mass spectrometer. In general, the volume of acetonitrile used in protein precipitation is in the range of two to four times the volume of the plasma. Thus, in order to attest the effect of acetonitrile volume on the protein precipitation efficiency, volume ratio of acetonitrile: plasma (1:1, 2:1, 3:1 and 4:1) was tested in triplicates. The mixture of acetonitrile and plasma was vortexed for a minute and then centrifuged at 35,000 × *g* for 15 min. All the supernatant was transferred and dried in vacuum for 2 h. Subsequently, 50 μL of deionized Millipore water was added and the tube was vortexed for 30 s. The plasma which served as a control, was diluted 2X, 5X, 10X, 50X and 100X. The calibration standard samples for the Bradford assay [13] were prepared by using serial dilutions of bovine serum albumin (BSA) in the range of 0–1000 μg/mL. The diluted plasma, BSA and acetonitrile processed plasma samples were transferred onto the 96-well plate. The absorbance at 595 of the respective samples was measured and their protein contents were then estimated by referring to the protein standard curve.

2.10. Drug measurement in patient plasma

The Phase I clinical trial received approval by the Institutional Ethics Review Board, National University Hospital (NUH) and all patients were provided written informed consent. Human plasma samples were obtained from eight patients with advanced NSCLC enrolled to a phase I clinical trial at the NUH, Singapore [9]. An oral dose of gefitinib (250 mg) was administered daily. On day 7, a series of plasma samples were collected from the respective patients to characterize their pharmacokinetic profiles. On day 35, a similar pharmacokinetic study was carried out in the same batch of patients, except an oral dose of hydroxychloroquine (600 mg) was given 2 h after gefitinib administration. On day 7 and 35, blood samples were collected in BD Vacutainer® containing lithium heparin 68 USP units (Franklin Lakes, USA) at time points 2, 3, 4, 5, 7 and 24 h after gefitinib administration. The blood samples were centrifuged at 1300 × *g* at 4 °C for 10 min and the plasma (supernatant) was then transferred into a cryovial to be stored at −80 °C. Procedure for sample preparation was identical to the method in Section 2.3 with an exception in the initial step in which 10 μL of gefitinib and 10 μL of O-desmethyl gefitinib were replaced by 20 μL of 50% methanol.

3. Results and discussion

3.1. Protocol optimization

It was found that the protein precipitation efficiency of acetonitrile: plasma in the volume ratio of 2:1 was comparable to that of 3:1 and 4:1. The results showed that an equal volume of acetonitrile and plasma has the lowest protein precipitation efficiency of 94.4%. Hence, an efficient protein precipitation (>99.9%) could be achieved with the acetonitrile: plasma ratio greater than 2 fold.

3.2. Chromatographic optimization

The mass spectra of gefitinib and O-desmethyl gefitinib showed a protonated precursor ion $[M+H]^+$ at *m*/*z* of 447 and 433, respectively and have similar major fragment at *m*/*z* 128 as observed in the third quadrupole (Fig. 1). O-methyl gefitinib-d3, the internal standard was identified by MRM at a different transition (*m*/*z* 450 $[M+H]^+ \rightarrow 100$ (product ion)) to avoid potential interference from gefitinib with a small mass difference of 3 amu. Injection of blank human plasma and patient samples showed no interfering peaks at the retention times when gefitinib, O-desmethyl gefitinib and IS were eluted. Under optimized conditions, the chromatographic run was completed within 3 min with the three analytes being eluted at 1.9 min (Fig. 2). The solvent front peak appeared in about 1 min did not overlap with the other analytes under the current chromatographic conditions. The column selection and choice of mobile phase played important roles in method development. Two commonly used octyl (C8) and octadecyl (C18) silyl columns were tested for the chromatographic peak symmetry and retention. C18 column with suitable hydrophobic nature was selected to provide acceptable retention of the analytes. For the mobile phase, 0.1% formic acid was used to improve chromatographic peak shape and more importantly, to increase the signal intensity as well as to provide a source of protons [14]. However, a very low concentration (0.1%) of formic acid was used in order to preserve the lifespan of the column and also to avoid adverse effects on the peak as the acid may donate protons that potentially alter the charge of the ions.

The flow rate is also an important factor in chromatographic optimization. After testing a series of flow rates (250, 300, 350, 400, 450 μL/min), 300 μL/min was selected to achieve a rapid overall chromatographic run time and also to ensure that the elution of the peaks were not within the first minute of the run. This is to prevent possible interference with endogenous substances when this method was applied to patient samples. The mobile phase was optimized at 30:70 of acetonitrile–water containing 0.1% formic acid for several reasons. The 30:70 ratio allowed a rapid chromatographic run time as a lower percentage of organic phase (e.g. 20:80) would result in a longer run time (approximately 6–7 min) and a higher percentage of organic phase (e.g. 40:60) would result in insufficient time for partition and separation in the column, which would cause the analytes to be co-eluted with solvent front into the mass spectrometer too rapidly. The analytes were eluted with good peak shape at appropriate retention times separated from the solvent front.

3.3. Selectivity and linearity

The selectivity for the optimized method was observed from the chromatographic analysis of human plasma of all 13 patients. At the LLOQ of 5 ng/mL, symmetrical peaks of gefitinib, O-desmethyl gefitinib and IS were clearly identified without any significant matrix

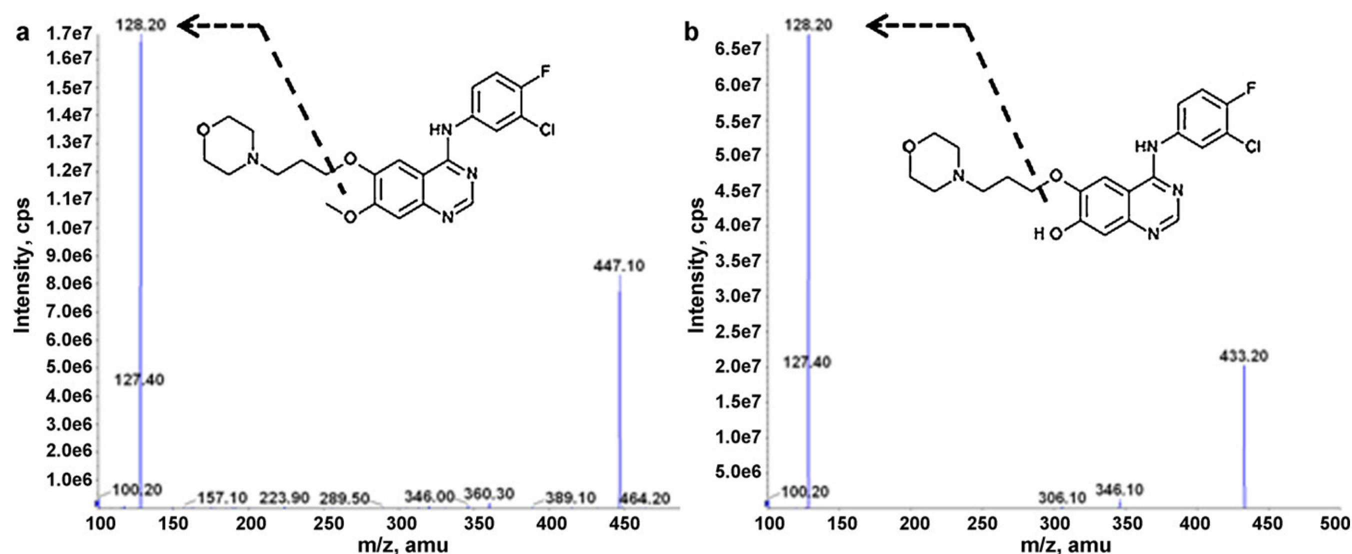

Fig. 1. Mass spectra of (a) gefitinib at m/z 447 → 128; (b) O-desmethyl gefitinib at m/z 433 → 128.

interference near the retention times of these analytes. This selectivity can also be observed in patient samples, with identical peak shapes and retention times compared to those in the blank plasma at LLOQ.

The good linearity was demonstrated within the range of 5–1000 ng/mL for gefitinib and 5–500 ng/mL for O-desmethyl gefitinib. However, the narrower range for O-desmethyl gefitinib applied because O-desmethyl gefitinib presents as much lower concentrations compared to gefitinib in patient plasma samples. Moreover, the regression line tends to non-linearity at higher concentrations exceeding 500 ng/mL, probably due to the structural difference between O-desmethyl gefitinib and the internal standard. Therefore, a narrower range was used for the standard curve of O-desmethyl gefitinib.

In the construction of the standard curve, as the quantitative data being heteroscedastic, the absolute errors of the data would vary with the sample concentrations. Without appropriate weighting scheme, the large standard deviations of data at higher concentrations would dominate in the calculation of the regression of the curve. In our study, a simple weighting factor of $1/x$ where x is the concentration was found to give the best fitting of the standard curve. The standard curves of gefitinib and O-desmethyl gefitinib provided reliable response within the range of 5–1000 ng/mL and 5–500 ng/mL, respectively. The typical equations of the standard curves are as follows: gefitinib: $y = 0.00559x + 0.0164$, $r^2 = 0.9998$; O-desmethyl gefitinib: $y = 0.00213x + 0.00306$, $r^2 = 0.9995$, where y represents the ratios of gefitinib and O-desmethyl gefitinib area that of IS and x represents plasma concentrations of gefitinib and O-desmethyl gefitinib.

3.4. Accuracy and precision

Accuracy was expressed as a percentage of the amount of analyte quantified over the amount that was spiked into the plasma. Intra-day and inter-day method validation established using QC samples spiked with both gefitinib and O-desmethyl gefitinib achieved an accuracy of within 15% of the nominal concentrations for all QC samples. The intra-day and inter-day precisions in RSD for gefitinib and O-desmethyl gefitinib were $\leq$10.8 and the accuracies ranged from 89.7 to 104.7% for gefitinib and 100.4 to 106.0% for O-desmethyl gefitinib (Table 1). The assay robustness was evaluated through monitoring the patient plasma samples before drug ingestion. No significant interfering peaks were co-eluted with two analytes with or without hydroxychloroquine. Validation of this dilution procedure was done with spiked samples at three concentrations, specifically at 1200, 1500 and 2000 ng/mL in quadruplicates. Excellent accuracy was achieved with less than 4% difference from the spiked (nominal) concentrations. The procedure also proved to be very precise with a CV <4% at all three concentrations (Table 2). Hence, steps were taken to ensure accurate quantification of analytes in the patient samples and no assumptions were made on the linearity of the standard curve at concentrations that exceeded the range (5–1000 ng/mL). Therefore, this ensured that the quantitative assay was robust.

3.5. Stability

Validation of the stability of analytes in the plasma was done with QC samples in quadruplicates at each nominal concentration for both gefitinib and O-desmethyl gefitinib. The QC samples were prepared in plasma the day before the analysis and were stored at −80 °C. Short-term stability was assessed on bench top at intervals of 0, 3 and 6 h; room temperature and 4 °C; and after 3 and 6 freeze–thaw cycles. QC samples of gefitinib and O-desmethyl gefitinib showed no significant degradation in all experiments on assessing their short-term stability (Table 3). The processed samples were stable up to 56 h on the autosampler, except at the lowest QC sample for gefitinib. In our scenario, the patients' gefitinib concentrations were much higher than 15 ng/mL. This suggested that both gefitinib and its main metabolite are stable in abovementioned conditions.

3.6. Matrix effect and recovery

Ion suppression, a form of matrix effect is an inherent problem in LC–MS analysis. This could be a result of interference from endogenous substances from matrices and exogenous substances such as polymers extracted from pipette tips introduced during sample preparation [12,15]. Matrix ion suppression is more common with direct precipitation method compared to liquid–liquid extraction (LLE) and solid-phase extraction (SPE) [16]. Matrix effect was evaluated with quadruplicate QC samples. Our finding indicated minimal matrix effect on gefitinib and IS, as the percentage of analyte present in the matrix to the analyte reconstituted in mobile phase was greater than 90% (Table 4). However, the matrix seemed to have a significant ion suppression effect on O-desmethyl gefi-

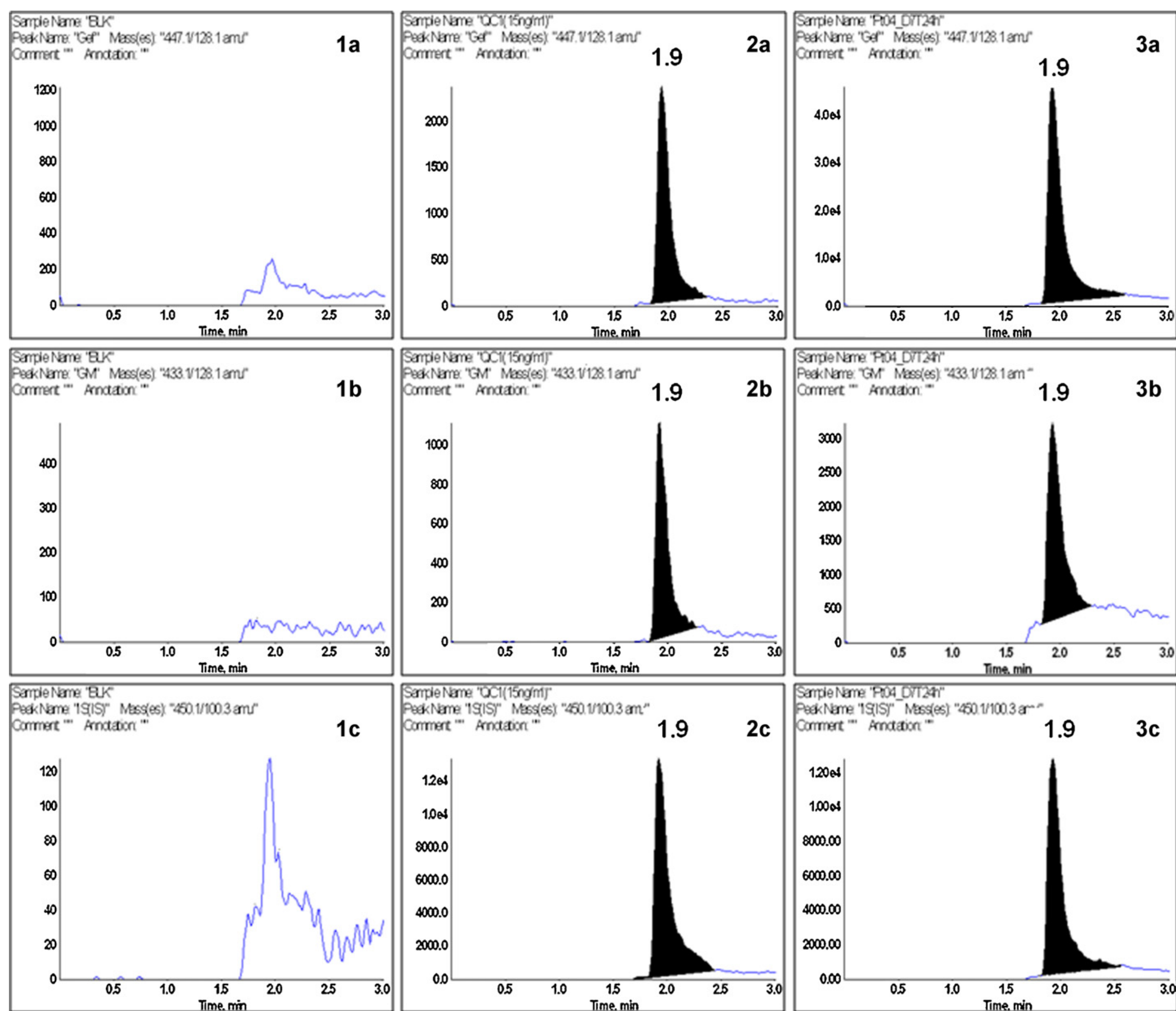

Fig. 2. Representive chromatograms of gefitinib (a), O-desmethyl gefitinib (b) and O-methyl gefitinib-d3 (c). Sample 1: blank plasma; Sample 2: QC1 spiked in blank plasma; Sample 3: patient plasma taken at 24 h after ingestion of gefitinib.

Table 1
Intra-day and inter-day accuracy and precision for (a) gefitinib and (b) O-desmethyl gefitinib ($n = 5$).

Nominal conc. (ng/mL)	Gefitinib QC samples					
	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
	Quantified conc. (mean ± S.D., ng/mL)	Accuracy (%)	CV (%)	Quantified conc. (mean ± S.D., ng/mL)	Accuracy (%)	CV (%)
15	15.7 ± 1.2	104.7	7.7	14.6 ± 0.8	97.7	9.4
100	98.5 ± 4.4	98.5	4.4	96.6 ± 1.5	96.6	10.8
700	628.0 ± 21.3	89.7	3.4	687.8 ± 37.2	98.3	5.4

Nominal conc. (ng/mL)	O-Desmethyl gefitinib QC samples					
	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
	Quantified conc. (mean ± S.D., ng/mL)	Accuracy (%)	CV (%)	Quantified conc. (mean ± S.D., ng/mL)	Accuracy (%)	CV (%)
15	15.3 ± 1.1	101.9	7.1	15.1 ± 1.4	100.6	9.3
100	100.4 ± 3.7	100.4	3.7	102.1 ± 11.0	102.1	10.8
300	317.6 ± 6.0	106.0	1.9	310.6 ± 23.5	103.5	7.6

Table 2
Dilution effect on the quantification of high concentration plasma samples.

Spiked concentration (ng/mL)	Dilution factor	Quantified concentration (mean ± S.D., ng/mL); CV (%)	Accuracy (%)[a]
1200	2	587.25 ± 10.08; 1.72	97.9
1500	2	740.00 ± 27.14; 3.67	98.7
2000	2	985.50 ± 5.92; 0.60	98.6

[a] [(quantified concentration) × 2/spiked concentration] × 100.

Table 3
Stability assessment of a gefitinib and O-desmethyl gefitinib.

Condition	Gefitinib QC samples		
	15 ng/mL	100 ng/mL	700 ng/mL
Short-term stability (25 °C)			
0 h	94.00	95.25	98.57
3 h	85.33	96.10	96.00
6 h	85.33	95.15	95.07
Freeze–thaw stability (−80 °C)			
3 cycles	90.00	95.80	98.33
6 cycles	89.33	96.87	94.43
Autosampler (4 °C)			
Time = 56 h	81.33	95.70	95.79
Temperature			
25 °C	96.67	98.40	97.07
4 °C	99.67	100.05	94.79
Condition	**O-Desmethyl geftinib QC samples**		
	15 ng/mL	100 ng/mL	300 ng/mL
Short-term stability (25 °C)			
0 h	100.73	88.90	87.67
3 h	88.80	96.00	97.67
6 h	105.67	105.50	102.67
Freeze–thaw stability (−80 °C)			
3 cycles	89.51	99.73	99.33
6 cycles	103.47	110.87	98.44
Autosampler (4 °C)			
Time = 56 h	101.27	113.20	115.67
Temperature			
25 °C	106.93	109.60	101.00
4 °C	100.33	106.40	105.67

[a]Expressed as a mean percentage change from nominal concentration.

tinib, as the peak area of the analyte present in the matrix was only 40–50% of the nominal concentration. The substantial matrix effect on O-desmethyl gefitinib could be due to the presence of a hydroxyl group in the compound. A high percentage of endogenous substances in plasma are hydrophilic compounds which could have significant interaction with O-desmethyl gefitinib through hydrogen bonding. However, due to its simplicity and cost-effectiveness, direct protein precipitation method was still adopted for sample preparation in this study [17]. The use of an isotopic IS of gefitinib with the same retention time as O-desmethyl gefitinib may help to circumvent the influence of matrix effect to a certain extent, as both the internal standard and O-desmethyl gefitnib were exposed to the co-eluted environment [17,18]. Nevertheless, the inter-day and intra-day accuracy and precision for O-desmethyl gefitinib were consistent below 15% in spite of the proposed matrix effect. Good average recoveries were achieved for gefitinib (106%) and

Table 4
Matrix effect evaluated by QC samples.

	QC samples (%)[a]		
	QC1	QC2	QC3
Gefitinib	97.5	103.3	93.0
O-Desmethyl gefitinib	50.2	47. 6	41.6
Internal standard (d3) – gefitinib	92.0	96.8	92.4

[a] Expressed as a percentage of peak area ratio of analyte present in the matrix to analyte reconstituted in mobile phase.

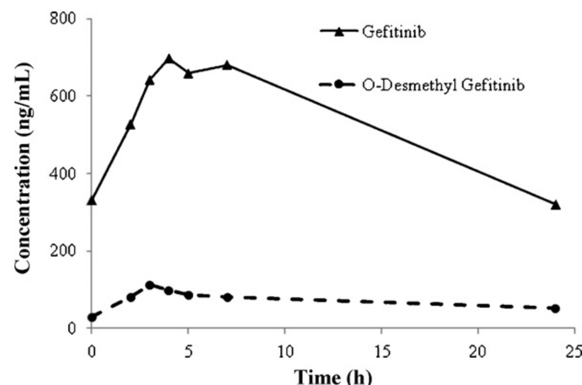

Fig. 3. Plasma concentration–time profiles of gefitinib (solid lines) and O-desmethyl gefitinib (dotted line) of a patient on day 7 after ingestion of 250 mg of gefitinib daily.

O-desmethyl gefitinib (102%) were achieved. This also suggested that 2 fold volume of acetonitrile can result in satisfactory protein precipitation efficiency.

3.7. *Application of validated LC–MS/MS method*

The method was applied for simultaneous quantification of gefitinib and O-desmethyl gefitinib in plasma samples from a phase I clinical trial. The analytical method was demonstrated sensitive enough to determine the concentrations for all plasma samples taken within 24 h. The plasma concentration–time profiles of gefitinib and desmethyl gefitinib in one patient on the drug for 7 days are shown in Fig. 3. The plasma concentrations of gefitinib were found much higher than that of O-desmethyl gefitinib. The results indicated that the elimination rate of gefitinib was slow with a moderate extent of metabolism.

4. Conclusions

A rapid, specific and robust LC–MS/MS assay has been developed and fully validated based on FDA guidelines. The method described is suitable for the determination of gefitinib and its main metabolite in patient samples. This method has been successfully applied to an ongoing phase I clinical trial to discern the possible effect of hydroxychloroquine on the pharmacokinetics of gefitinib in patients.

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