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A liquid chromatography with tandem mass spectrometry method for quantitating total and unbound ceritinib in patient plasma and brain tumor

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ABSTRACT

A rapid, sensitive, and robust reversed-phase liquid chromatography with tandem mass spectrometry method was developed and validated for the determination of total and unbound ceritinib, a second-generation ALK inhibitor, in patient plasma and brain tumor tissue samples. Sample preparation involved simple protein precipitation with acetonitrile. Chromatographic separation was achieved on a Waters ACQUITY UPLC BEH C_{18} column using a 4-min gradient elution consisting of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile), at a flow rate of 0.4 mL/min. Ceritinib and the internal standard ([$^{13}C_6$]ceritinib) were monitored using multiple reaction monitoring mode under positive electrospray ionization. The lower limit of quantitation (LLOQ) was 1 nM of ceritinib in plasma. The calibration curve was linear over ceritinib concentration range of 1–2000 nM in plasma. The intra- and inter-day precision and accuracy were within the generally accepted criteria for bioanalytical method (< 15%). The method was successfully applied to assess ceritinib brain tumor penetration, as assessed by the unbound drug brain concentration to unbound drug plasma concentration ratio, in patients with brain tumors.

1. Introduction

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that can be aberrantly activated by genetic mutation, gene amplification, or chromosomal rearrangement, leading to the expression of a potent oncogenic driver [1]. Genetic alterations in the *ALK* are implicated in the pathogenesis of at least 20 types of human cancers including non-small-cell lung cancer (NSCLC), renal cell carcinomas, anaplastic thyroid cancer, breast cancer, head and neck cancer, and glioblastoma [1–6]. Tumors with aberrant ALK activation often depend on ALK for growth and survival and can show marked sensitivity to ALK inhibitors.

Ceritinib (LDK378, Zykadia®) is an oral, highly potent and selective second-generation ALK inhibitor. It shows a greater preclinical antitumor potency than first-generation ALK inhibitor crizotinib, and has shown efficacy in patients with ALK-positive NSCLC who have acquired resistance to crizotinib [7]. Ceritinib inhibits autophosphorylation of ALK and its downstream signaling proteins in a dose-dependent manner. It has been approved by the United States Food and Drug Administration (FDA) for the treatment of patients with ALK-positive locally advanced or metastatic NSCLC who have progressed on or are intolerant to crizotinib [7].

Given the aberrant activation of ALK in many human cancers, ceritinib is under active preclinical and clinical investigations for the treatment of various ALK-positive cancers including primary brain cancer (in particular glioblastoma) and brain metastasis. Penetration of a chemotherapeutic drug across human blood-brain tumor barrier (BBTB) is the prerequisite for effective treatment of brain cancer. To prospectively assess the penetration of ceritinib across the BBTB, a phase 0 clinical trial is being conducted in patients with glioblastoma or brain metastasis (ClinicalTrials.gov identifier: NCT02605746). Patients are treated with daily oral ceritinib (750 mg) for 14 days prior to surgical resection of their tumors. The extent of drug penetration into brain tumor is assessed by the pharmacologically relevant, unbound drug brain tumor-to-plasma partition coefficient ($K_{p,uu}$), which is estimated as the ratio of unbound drug brain tumor concentration to unbound plasma concentration at the steady state [8].

A specific, sensitive, and reliable bioanalytical method is essentially required for the accurate determination of the $K_{p,uu}$ of ceritinib. Here, we report a fully validated liquid chromatography

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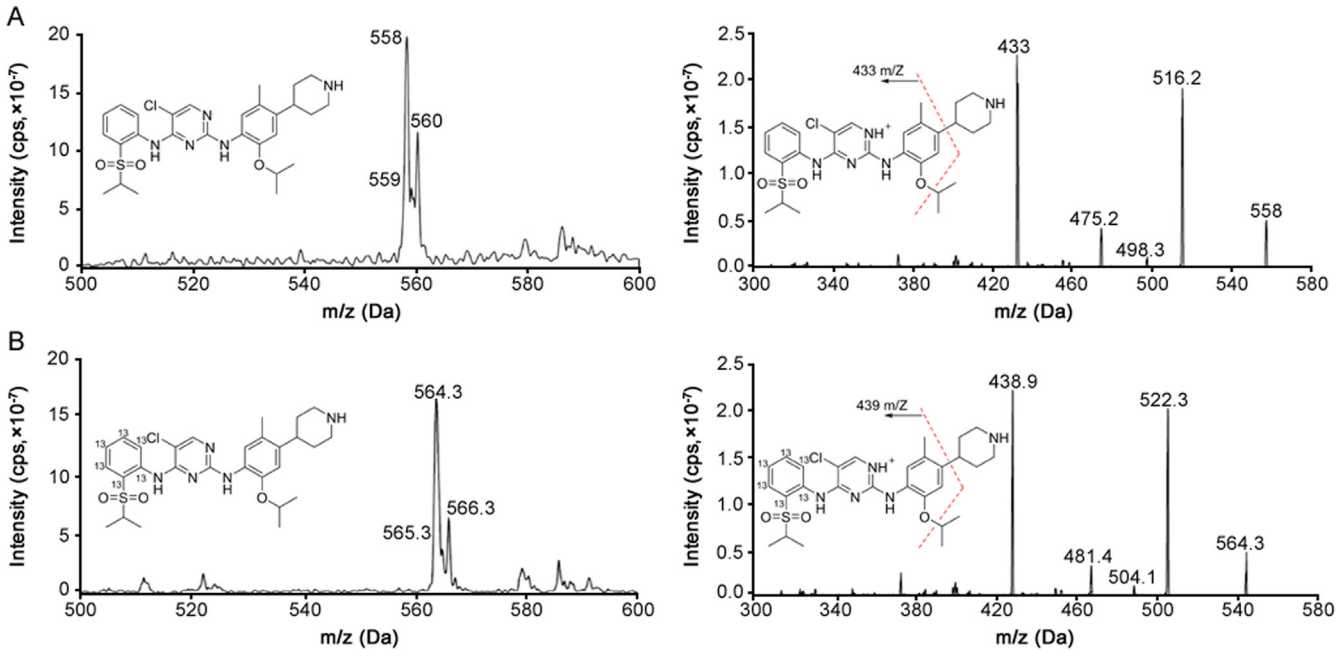

Fig. 1. The parent ion scan (left) and product ion scan mass spectra (right) of (A) ceritinib for which mass transition of *m/z* 558.0→433.0 was selected for monitoring, and (B) [$^{13}C_6$]ceritinib for which mass transition of *m/z* 564.3→438.9 was selected for monitoring.

coupled with tandem mass spectrometry (LC–MS/MS) method for the determination of total and unbound ceritinib in patient plasma and brain tumor tissue. Compared to the published assay [9,10], the present method demonstrated a better sensitivity (1 nM versus 1.79 nM), a larger dynamic range (1–2000 nM versus 1.79–900 nM), and negligible post-injection carryover, while the sample preparation involved a simple one-step protein precipitation.

2. Experimental

2.1. Chemicals and reagents

Ceritinib and the stable isotope-labeled internal standard, [$^{13}C_6$] ceritinib, were provided by Novartis Pharmaceuticals (Basel, Switzerland). All other chemicals and reagents were purchased from VWR International (Radnor, PA, USA) (LC-MS grade). Water was filtered and deionized with a US Filter PureLabPlus UV/UF system (Siemens, Detroit, MI, USA) and used throughout in all aqueous solutions. Drug-free (blank) human plasma from six different healthy donors and pooled plasma (with Na-EDTA anticoagulant) were purchased from Innovative Research Inc. (Novi, MI, USA).

2.2. Chromatographic and mass-spectrometric conditions

2.2.1. Instrumentation

All LC–MS/MS analyses were performed on an AB SCIEX (Foster City, CA) QTRAP 6500 LC–MS/MS system, which consists of a SHIMADZU (Kyoto, Japan) Nexera ultra-high performance liquid chromatography (UHPLC) coupled with a hybrid triple quadrupole/linear ion trap mass spectrometer. The UHPLC system is equipped with two X2 LC-30 CE pumps, an X2 SIL-30AC autosampler, a CBM-20A communication bus module, an X2 CTO-30A column oven, and two DGU-20A degassing units. Analyst® 1.6 software was used for system control and data acquisition, and MultiQuant 3.0 software was used for data processing and quantitation.

2.2.2. Liquid chromatography

Chromatographic separation was achieved on a Waters ACQUITY UPLC BEH C_{18} (2.1 mm × 50 mm, 1.7 μm) column using an optimized gradient elution consisting of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile), at a flow rate of 0.4 mL/min. The elution gradient program was as follows [shown as the time (min), (% mobile phase B)]: 0, 40%; 0.5, 40%; 2, 100%; 2.5, 100%; 2.7, 40%; 4, 40%. Column oven temperature was maintained at 35 °C. To minimize the carryover, external and internal washes were implemented prior to and after the injection for both the auto-sampler syringe and injection port, as follows: methanol (for R3) was used for external wash with one second rinse dip and 500 μL volume, and isopropanol (for R1) and 40% acetonitrile (R0 and R2) were used in the internal wash with sequence R1 to R0 to R2.

2.2.3. Mass spectrometry (MS)

The QTRAP 6500 mass spectrometer was operated in electrospray positive ionization using multiple reaction monitoring mode (MRM). The MS parameters were optimized to obtain the most sensitive and specific MS transitions for ceritinib and [$^{13}C_6$]ceritinib by direct infusion of 1 μM of the standard solutions into the ion source with a syringe pump. The Turbo ion-spray voltage was set at 5500 V and the source temperature was set at 500 °C. Collision gas was optimized at medium level with curtain gas, ion source gas 1 and ion source gas 2 delivered at 20, 30 and 30 psi, respectively. Decluttering potential, collision energy, collision cell exit potential, and entrance potential were optimized at 143, 43.2, 10, and 10 V, for ceritinib and 130, 43.8, 35, and 10 V for [$^{13}C_6$] ceritinib. The dwell time was set for 100 ms. The MS spectra of the parent ions and product ions for ceritinib and [$^{13}C_6$]ceritinib, under positive electrospray ionization mode, are presented in Fig. 1. The most sensitive MS transitions of *m/z* 558.0 → 433.0 and 564.3 → 438.9 were selected for monitoring ceritinib and [$^{13}C_6$]ceritinib, respectively.

2.3. Sample preparation

2.3.1. Stock solutions, calibration standards, and quality control (QC) samples

Ceritinib and [$^{13}C_6$]ceritinib stock solutions were prepared in methanol at a final concentration of 5 mM, and stored in brown glass vials at − 80 °C. Ceritinib working solutions were prepared freshly by serial dilutions of the stock solution with 50% acetonitrile in water on each day of analysis. [$^{13}C_6$]ceritinib working solution was prepared in acetonitrile at the concentration of 100 μM. For the determination of ceritinib in plasma and brain tissue samples, the calibration standards were prepared by spiking 5 μL of ceritinib working solution into 95 μL of blank human plasma to make the final concentrations at 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 nM. For the determination of unbound ceritinib in post-dialysis phosphate buffer solution (PBS, pH 7.4), the calibration standards were prepared in PBS. QC samples were prepared in blank human plasma at the concentrations of 1 (LLOQ), 3 (LOQ), 160 (MOQ), 1600 (HOQ), and 30,000 nM (50-fold dilution). All standards and QC samples were prepared fresh daily. For long-term and freeze-thaw stability, QC samples were prepared as a batch and stored at − 80 °C.

2.3.2. Plasma samples for the determination of total ceritinib plasma concentrations

Frozen plasma samples were thawed at room temperature. An aliquot of 100 μL plasma was transferred into a micro centrifuge tube, and 400 μL ice-cold acetonitrile containing 50 nM [$^{13}C_6$]ceritinib was added. The mixture was vortex-mixed for 10 s and centrifuged at 9000 *g* at 4 °C for 10 min. The supernatant was transferred to an autosampler vial and 5 μL was injected into the LC–MS/MS system.

2.3.3. Brain tissue samples for the determination of total ceritinib brain concentrations

Patient brain tumor tissue samples were thawed at ambient temperature. Tissue homogenate was prepared by adding 4 volumes of distilled water into the tissue sample, followed by homogenizing in a Precellys® homogenizer (at 2000 *g* for two 10 s with 5 s pause). An aliquot of 100 μL of tissue homogenate was protein precipitated using the same procedure as that for plasma samples, and 5 μL supernatant was injected into the LC–MS/MS system.

2.3.4. Equilibrium dialysis for the determination of ceritinib fraction unbound in plasma and brain tissue

The fraction unbound of ceritinib in either plasma or brain tissue homogenate was determined using equilibrium dialysis on a 96-Well Equilibrium DIALYZER™ with 5 kDa molecular weight cut-off regenerated cellulose membrane (Harvard Apparatus Holliston, MA), as described previously with modifications [11]. Briefly, equilibrium dialysis was performed with 200 μL of plasma or tissue homogenate against an equal volume of PBS (pH 7.4) containing 10% acetonitrile on a rotator (Harvard Apparutus Holliston, MA) at 37 °C. The optimal time to equilibrium was assessed with pooled human plasma at ceritinib concentrations of 1 and 10 μM for dialysis of 6, 16, and 24 h. At the end of dialysis, 200 μL acetonitrile (containing [$^{13}C_6$]ceritinib, 50 nM) was added into each PBS and plasma compartment, and the post-dialysis PBS and plasma samples were transferred into Eppendorf tubes. The mixture was vortex-mixed and centrifuged (9000 *g*, 4 °C, 10 min). 5 μL of the supernatant was injected into the LC–MS/MS system for determining ceritinib concentrations in the post-dialysis buffer solution (C_u) and post-dialysis plasma (C_p) or post-dialysis tissue homogenate (C_{hom}).

Fraction unbound in plasma ($f_{u,plasma}$) and in tissue homogenate ($f_{u,hom}$) was calculated using Eqs. (1) and (2), respectively. Fraction unbound in undiluted brain tissue was calculated based on the measured fraction unbound in diluted tissue homogenate ($f_{u,\ hom}$) and dilution factor (D_f) using Eq. (3) [12]. The total drug recovery was calculated by comparing the total drug concentration recovered in the buffer and plasma (or homogenate) compartments after dialysis with that initially added to the plasma (or homogenate) compartment.

$$f_{u,plasma} = \frac{C_u}{C_p} \tag{1}$$

$$f_{u,hom} = \frac{C_u}{C_{hom}} \tag{2}$$

$$f_{u,tissue} = \frac{1/D_f}{\left(\frac{1}{f_{u,hom}}-1\right)+1/D_f} = \frac{f_{u,hom}}{D_f-(D_f-1)f_{u,hom}} \tag{3}$$

2.4. Method validation using pooled human plasma

2.4.1. Specificity and selectivity

The presence of endogenous interfering peaks was inspected by comparing the chromatograms of the blank matrix (i.e., plasma from 6 donors or PBS) and those spiked with ceritinib at the LLOQ (1 nM in human plasma) and [$^{13}C_6$]ceritinib at 50 nM. The interfering peak area should be less than 10% of the peak area for the analyte at the LLOQ and less than 5% of the peak area for the internal standard.

2.4.2. Calibration curve, accuracy, and precision

Linearity was assessed at ceritinib concentration ranging from 1 to 2000 nM in human plasma. Calibration curves were built by fitting the analyte concentrations versus the peak area ratios of the analyte to internal standard using linear regression analysis with different weighting scheme (i.e., 1, 1/x, and $1/x^2$), where x represents the concentrations. The selection of weighting scheme was guided by evaluation of goodness-of-fit criteria including correlation coefficient (R^2), deviations of the back-calculated concentrations, and residual plots.

Intra- and inter-day precision and accuracy were assessed for the calibrator standards (each in duplicate) and QCs (including LLOQ, low, medium, high, and 50-fold dilution QCs, each in quintuplicate) on three days. The accuracy was assessed as the relative percentage of the determined concentration to nominal concentration. The intra- and inter-day precisions were estimated by one-way analysis of variance (ANOVA) using the JMP™ statistical discovery software version 5 (SAS Institute, Cary, NC, USA). The inter-day variance (VAR_{inter}), intra-day variance (VAR_{intra}), and grand mean (GM) of the observed concentrations across runs were calculated from ANOVA analysis. The intra-day precision (P_{intra}) was calculated as: $P_{intra} = 100 \times \left(\sqrt{(VAR_{intra})}/GM\right)$. The inter-day precision (P_{inter}) was defined as: $P_{inter} = 100 \times \left(\sqrt{\left((VAR_{inter} - VAR_{intra})/n\right)}/GM\right)$, where n represents the number of replicate observations within each day.

2.4.3. Matrix effect and recovery

Matrix effect and recovery were assessed in human plasma from 6 different donors, as described previously with modifications [13]. Three sets of QC samples (including low, medium and high QCs) were prepared. Set 1 QCs were prepared by spiking ceritinib (at 3, 160, and 1600 nM) into 100 μL of 50% acetonitrile. Set 2 QCs were prepared by spiking the same concentrations of ceritinib as Set 1 in 100 μL of blank matrix extract (i.e., post-

precipitation supernatant solution of blank plasma). Set 3 QCs were prepared by spiking the same concentrations of ceritinib as Set 1 in 100 μL of blank human plasma. All 3 set samples were processed by adding 400 μL acetonitrile containing [$^{13}C_6$]ceritinib (50 nM), followed by centrifugation (9000 *g* at 4 °C for 10 min), and 5 μL of the supernatants were injected into the LC–MS/MS system. The matrix effect was expressed as the ratio of the mean peak area of the analyte spiked post-precipitation (set 2) to that from neat solution (set 1). The recovery was calculated as the ratio of the mean peak area of the analyte spiked prior to precipitation (set 3) to that from post-precipitation solution (set 2).

2.4.4. Stability tests

The short-term (bench-top) stability of ceritinib in 50% acetonitrile (working solution) at the concentration of 1 and 100 μM as well as in plasma at the concentration of 3 and 1600 nM was tested at ambient temperature (25 °C) for 6 h. The autosampler stability of processed samples was tested at 4 °C for 9 h. The freeze-thaw stability of ceritinib in plasma (at 3 and 1600 nM) was evaluated after three cycles of freezing and thawing with at least one day of storage at − 80 °C between each thawing. The long-term stability of ceritinib in plasma (at 3 nM and 1.6 μM) was tested for 8 months.

2.5. Applications

The developed method was used to assess the tumor penetration of ceritinib in patients with glioblastoma or brain metastasis in a phase 0 clinical trial of ceritinib (ClinicalTrials.gov identifier: NCT02605746). The first cohort of 3 patients has been enrolled up to date. The patients were treated with oral ceritinib daily at the dose of 750 mg for 14 days prior to the surgical resection of their tumors at 2–4 h following the administration of the last dose. Blood and tumor samples were collected at the same time. Plasma was separated from whole blood by centrifugation (at 4 °C, 1500 *g*, for 10 min), and plasma samples were stored at − 80 °C until analysis. Brain tumor tissue was washed off blood with ice-cold PBS, blot-dried on tissue paper, snap-frozen in liquid nitrogen, and stored at − 80 °C until analysis. The protocol was approved by the Institutional Review Board of the Barrow Neurological Institute, St. Joseph's Hospital&Medical Center (Phoenix, AZ). All the patients provided a written informed consent.

Brain tumor tissue homogenates were prepared, as described in Section 2.3.3. Ceritinib total concentrations in plasma and tumor tissue homogenate samples were determined using the validated LC–MS/MS method. Unbound fractions of ceritinib in plasma and brain tumor tissue were determined using the equilibrium dialysis method as described in Section 2.3.4. Ceritinib unbound concentration in plasma or tumor tissue was calculated by multiplying the total drug concentration and fraction unbound. The $K_{p,uu}$ was calculated as the ratio of unbound drug brain tumor tissue concentration to unbound drug plasma concentration.

3. Results and discussion

3.1. Method development

Ceritinib is a weak base with pK_a values of 9.7 and 4.1. It has good solubility in DMSO, organic solvents (e.g., methanol and acetonitrile), and very acidic aqueous solution (e.g., 0.1 N hydrochloric acid). Its aqueous solubility decreases significantly with increased pH. The measured unionized species octanol/water partition coefficient (LogP) of ceritinib is 4.6, and the total species octanol/buffer (pH 6.8) distribution coefficient (LogD) is 1.69.

Based on its physicochemical properties, a reversed-phase liquid chromatography method using a Waters ACQUITY UPLC BEH C_{18} column was optimized for the separation of ceritinib. Under the optimized gradient elution with a total running time of 4 min, ceritinib was eluted at the retention time of ~ 1.6 min, with a sharp, symmetrical peak (Fig. 2). There was no apparent interference peak in the blank human plasma or PBS (Fig. 2).

Post-injection carryover was noted in the published study and during our assay development. To minimize the carryover, the external and internal washes with isopropanol (R1) and 40% acetonitrile (R0 and R2) were implemented for the syringe and injection port. Using the optimized washing condition, the mean peak area in the blank sample following the highest ceritinib calibrator injection was 1272 ± 196.5 (9 injections on 3 days), which is less than 5.0% of the mean peak area of the LLOQ (25,387 ± 7921, 9 injections on 3 days), suggesting the carryover was abolished.

Notably, ceritinib, when present in protein-free aqueous solution (e.g., PBS), exhibited significant adsorption to equilibrium dialysis plates, probably due to its high hydrophobicity (LogP, 4.6). To eliminate the adsorption, we modified equilibrium dialysis procedures by adding 10% acetonitrile into the PBS compartment during the dialysis, and adding 200 μL acetonitrile into both PBS and plasma compartments at the end of dialysis, followed by the collection of post-dialysis PBS and plasma samples. These procedures effectively reduced the adsorption, as indicated by an average of 91% (range, 86%–93%) recovery of ceritinib at the end of 24 h dialysis for the QC samples (at 1 and 10 μM).

3.2. Method validation

The developed method was fully validated using pooled human plasma for the selectivity, sensitivity, linearity, accuracy and precision, matrix effect and recovery, as well as stability according to the US Food and Drug Administration guidance to bio-analytical method validation.

The selectivity for the analysis was shown by symmetrical resolution of the chromatographic peaks, with no significant interference from 6 different donors of plasma (Fig. 2). Representative chromatograms of blank and spiked with ceritinib in human plasma samples at LLOQ as well as a patient plasma sample collected at 8 h after the oral administration of ceritinib 750 mg are shown in Fig. 2.

The plasma linear calibration curves were constructed using the peak area ratio of ceritinib to [$^{13}C_6$]ceritinib over ceritinib concentration range of 1–2000 nM in human plasma. The calibration curve was best fitted by a weighted ($1/x^2$) least-squares linear regression, expressed as $y = a \cdot x + b$, where y is peak area ratio, x is the analyte concentration, with a and b as fitted parameters. A linear correlation coefficient (R^2) of > 0.99 was obtained in all analytical runs.

For all calibrator standards (including LLOQ), the average accuracy in terms of the percentage of the back-calculated concentrations relative to the nominal concentrations ranged from 94.5% to 106.7%; the intra- and inter-day assay precisions were less than 8.8% (Table 1). For the QC samples (i.e., at the LLOQ, low, medium and high QC concentrations), the average accuracy ranged from 93.1% to 100.7%, and the intra- and inter-day precisions were within 10.3% (Table 2). In addition, accuracy and precision were evaluated for 50-fold dilution QC samples (at 30 μM in human plasma), which demonstrated the average accuracy of 96.1%, and intra- and inter-day precisions of 1.7% and 2.7%, respectively (Table 2).

The matrix effect was examined in human plasma from 6 different donors to assess the potential of ionization suppression or enhancement for ceritinib and [$^{13}C_6$]ceritinib. The average matrix factor of ceritinib determined from 6 different donors of plasma ranged from 0.72 to 0.82 at the low, medium and high QC

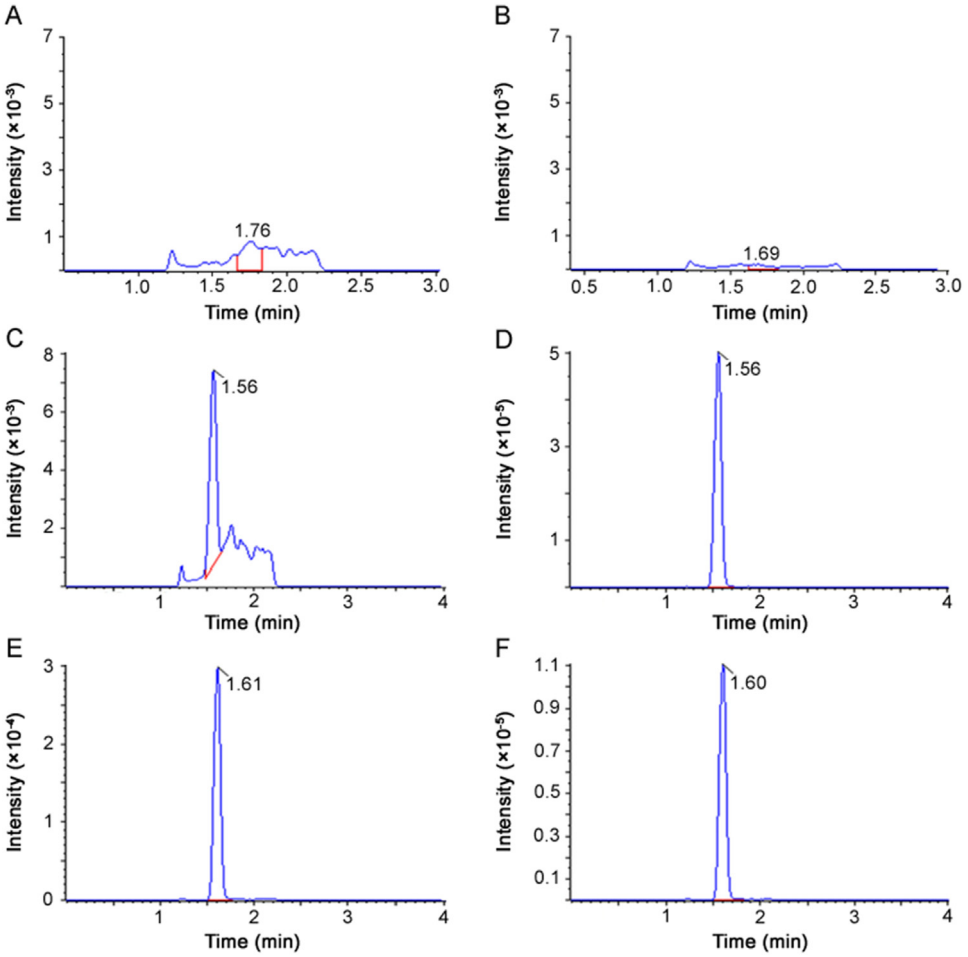

Fig. 2. Extracted ion chromatograms of (A and B), blank plasma (C and D) human plasma spiked with ceritinib at LLOQ (1 nM) and (E and F) a 20-fold diluted patient plasma sample collected at 8.0 h after oral administration of a single dose of ceritinib (750 mg). All samples were monitored at *m/z* 558.0→433.0 for ceritinib and at *m/z* 564.3→438.9 for [$^{13}C_6$]ceritinib. All plasma samples except for the blank were precipitated with 4 volumes of acetonitrile containing the internal standard [$^{13}C_6$]ceritinib (50 nM).

Table 1
Accuracy, intra- and inter-day precisions of ceritinib calibrator standards in plasma.[a]

Nominal concentration (μM)	Determined concentration (μM)	Average accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
0.001 (LLOQ)	0.0010 ± 0.0000	100.0	0.0	0.0
0.002	0.0020 ± 0.0001	98.3	8.8	–[b]
0.005	0.0052 ± 0.0001	103.0	3.3	–[b]
0.01	0.0099 ± 0.0006	99.0	4.8	3.5
0.02	0.0195 ± 0.0008	97.5	3.7	2.3
0.05	0.0533 ± 0.0029	106.7	6.5	–[b]
0.1	0.1033 ± 0.0018	103.3	1.7	0.5
0.2	0.1945 ± 0.0054	97.3	3.1	–[b]
0.5	0.5261 ± 0.0109	105.2	1.1	1.9
1	1.0202 ± 0.0172	102.0	1.0	1.6
2	1.8891 ± 0.0418	94.5	2.8	–[b]

[a] Each calibrator standard was evaluated in duplicate on three different days.
[b] No additional variation was observed as a result of performing assay on different days.

Table 2
Accuracy, intra- and inter-day precisions of ceritinib quality control samples in human plasma.[a]

Nominal concentration (μM)	Determined concentration (μM)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
0.001(LLOQ)	0.0010 ± 0.0001	100.7	10.3	–[b]
0.003	0.0028 ± 0.0002	93.1	5.3	3.7
0.16	0.1567 ± 0.0072	97.9	4.0	2.8
1.6	1.5428 ± 0.0742	96.4	3.0	4.5
30 (50-fold dilution QC)	28.8267 ± 0.8232	96.1	1.7	2.7

[a] Each QC was performed in quintuplicate on three different days.
[b] No additional variation was observed as a result of performing assay on different days.

concentrations, and the interindividual variability, assessed as the coefficient of variation (CV%) from 6 donors of plasma, was < 24.7% (Table 3). The matrix factor (expressed as average ± standard deviation) of [$^{13}C_6$]ceritinib (at 50 nM) from 6 different donors of plasma was 0.89 ± 0.23. The relative matrix factor of ceritinib, calculated as the ratio of the peak area ratio of ceritinib and [$^{13}C_6$]ceritinib in post-extracted solution (set 2) relative to that in neat solution (set 1) was > 0.84 at three QC concentrations, with the interindividual variability < 5.4% (Table 3), suggesting that the isotope-labeled internal standard adequately corrected for the variation of matrix effect from different sources of plasma.

The average recovery of ceritinib at three QC concentrations from 6 donors of plasma ranged from 96.1% to 105.1%, and the inter-individual variability was within 15.9% (Table 3). The recovery of [$^{13}C_6$]ceritinib at the concentration of 50 nM from 6 different donors of human plasma was 82.3% ± 9.2% (Table 3). The relative recovery of ceritinib, estimated as the ratio of the peak area ratio of ceritinib to [$^{13}C_6$]ceritinib in plasma (set 3) relative to

Table 3
Matrix effect and recovery of ceritinib and [$^{13}C_6$]ceritinib from 6 different donors of human plasma.

Analyte	Conc. (μM)[a]	Matrix factor[b]	Recovery (%)[c]	Relative matrix factor[d]	Relative recovery (%)[e]
Ceritinib	0.003	0.82 (21.7)	96.1 (15.9)	0.88 (5.4)	109.5 (5.5)
	0.16	0.78 (24.7)	99.3 (15.7)	0.84 (2.5)	113.1 (2.6)
	1.6	0.72 (10.1)	105.1 (5.6)	0.90 (4.5)	110.8 (3.8)
[$^{13}C_6$]ceritinib	0.05	0.89 (22.5)	82.3 (9.2)	–	–

[a] The nominal concentrations of the analyte spiked in plasma before precipitation (set 3). The same amounts of the analyte as in set 3 were spiked in the mobile phase and in plasma extract for set 1 and set 2.
[b] Matrix effect is expressed as the ratio of the mean peak area of ceritinib spiked post-precipitation (set 2) to the mean peak area of the same amount of analyte spiked in the mobile phase (set 1). Data are shown as the mean (% CV) from six donors of plasma extract.
[c] Recovery is calculated as the ratio of the mean peak area of ceritinib spiked in plasma before precipitation (set 3) to that spiked post-extraction (set 2). Data are shown as the mean (% CV) from six different donors of plasma.
[d] Relative matrix effect (or internal standard-normalized matrix effect) is expressed as the mean peak area ratio (the peak area of ceritinib to that of $^{13}C_6$ ceritinib) spiked in post-precipitation (set 2) to that in set 1. Data are shown as the mean (% CV) from six donors of plasma extract.
[e] Relative recovery (or internal standard-normalized recovery) is calculated as the mean peak area ratio in plasma before precipitation (set 3) to that in post-extraction (set 2). Data are shown as the mean (% CV) from six different donors of plasma.

that in the post-extracted solution (set 2), was consistently (~ 110%) at three QC concentrations, with the interindividual variability < 5.5% (Table 3), suggesting that the isotope-labeled internal standard well corrected for the variation of the extraction recovery from different matrices.

Table 4
Assessment of stability of ceritinib.

Condition	Ceritinib stability (%)	
Bench-top stability (in stock solution) (25 °C)[a]	1 μM[c]	100 μM[c]
1.0 h	103.5	102.0
2.0 h	106.9	108.1
4.0 h	106.2	101.2
6.0 h	108.8	101.1
Bench-top stability (in plasma) (25 °C)[a]	0.003 μM[c]	1.6 μM[c]
1.0 h	100.0	99.3
2.0 h	105.4	101.2
4.0 h	102.2	99.3
6.0 h	104.3	99.5
Auto-sampler stability (in the precipitation solution) (4 °C)[a]	0.003 μM[c]	1.6 μM[c]
1.0 h	103.5	100.0
3.0 h	104.7	100.2
6.0 h	101.2	100.3
9.0 h	100.0	100.5
Freeze-thaw stability (in plasma) (− 80 °C)[b]	0.003 μM[c]	1.6 μM[c]
Cycle 1	107.1	99.0
Cycle 2	105.9	96.8
Cycle 3	104.7	98.7
Long-term stability (in plasma) (− 80 °C)[b]	0.003 μM[c]	1.6 μM[c]
1 months	105.9	89.7
5 months	108.2	80.7
8 months	101.2	83.6

[a] Stability data is expressed as the mean percentage of the peak area determined at certain time relative to that at time zero.
[b] Stability data is expressed as the mean percentage of the analyte concentration determined at certain time point relative to the nominal concentration (%).
[c] Each concentration at each time point was assessed in triplicate.

The short- and long-term stability data of ceritinib are summarized in Table 4. Bench-top stability test suggested that ceritinib was stable in both 50% acetonitrile (at 1 and 100 μM) and human plasma (at 3 nM and 1.6 μM) at ambient temperature (~ 25 °C) for at least 6 h. Autosampler stability test suggested that ceritinib was stable in the protein precipitation solution at 4 °C for at least 9 h, allowing the assay to be performed continuously during the night for a large number of samples. Freeze-thaw stability test of plasma samples at low and high QC concentrations showed that no significant amount of ceritinib degraded after three freeze-thaw cycles. The long-term stability tests suggested that the ceritinib was stable in human plasma at low and high QC concentrations (3 and 1600 nM) at − 80 °C for 1 month, suggesting clinical samples should be analyzed within 1 month of storage at − 80 °C.

3.3. Applications

While the present method was fully validated in human plasma only, the method was successfully applied in determining ceritinib concentrations in patient plasma, brain tumor tissue homogenate, and post-dialysis PBS samples. The broad application of the method was attributable to the use of a stable isotope-labeled internal standard that can adequately correct for the variability in the matrix effects and extraction recovery of the analyte across different matrices.

To determine the unbound concentrations of ceritinib in patient plasma and brain tumor tissue, an equilibrium dialysis method using 96-well microdialysis plates was optimized. The optimum equilibrium time was determined at 24 h, at which the dialysis reached equilibrium (Fig. 3). Ceritinib was highly bound to human plasma proteins, with an average $f_{u,plasma}$ value of 2.5% in pooled human plasma at clinically relevant concentrations (1–10 μM) (Fig. 3). In the first cohort of 3 patients receiving daily ceritinib treatment at 750 mg for 14 days, the average $f_{u,plasma}$ of ceritinib at the steady-state plasma samples was 1.68%. Interestingly, compared to the plasma protein binding, ceritinib showed a higher binding to brain tumor tissue in the patient with neck/head cancer brain metastasis ($f_{u,brain}$, 0.19%) and with melanoma brain metastasis ($f_{u,brain}$, 0.23%), but not in the patient with breast cancer brain metastasis ($f_{u,brain}$, 7.6%). These data indicated a large interindividual variability in ceritinib binding to brain tumor tissue among different tumor types of brain metastasis. Consistently, ceritinib tumor penetration showed a large heterogeneity among various tumor types of brain metastasis, with the highest penetration in breast cancer brain metastasis ($K_{p,uu}$, 40.6), followed by melanoma brain metastasis ($K_{p,uu}$, 2.75) and head/neck brain metastasis ($K_{p,uu}$, 0.34). Further studies are needed to understand the mechanisms underlying the heterogeneous brain tumor penetration of ceritinib.

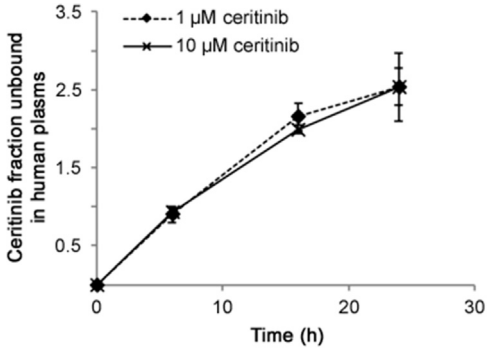

Fig. 3. Fraction unbound of ceritinib in pooled human plasma determined at the equilibrium time of 6, 16, and 24 h. Values are the mean ± standard deviation of triplicate measurements.

4. Conclusion

A rapid, sensitive, and robust LC–MS/MS method based on reversed-phase liquid chromatography was developed for the quantitation of total and unbound ceritinib in plasma and brain tissue samples. The method was fully validated with human plasma. Sample preparation involved a simple protein precipitation. The chromatography running time was 4 min. The LLOQ was 1 nM of ceritinib in human plasma. The linear calibration curve was established over ceritinib concentration range of 1–2000 nM in human plasma. This method was successfully applied to assess ceritinib brain tumor penetration, as assessed by the $K_{p,uu,}$ in patients with metastatic brain tumors.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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