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An aqueous normal-phase chromatography coupled with tandem mass spectrometry method for determining unbound brain-to-plasma concentration ratio of AZD1775, a Wee1 kinase inhibitor, in patients with glioblastoma

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Abstract

A rapid, sensitive, and robust aqueous normal-phase chromatography method coupled with tandem mass spectrometry was developed and validated for the quantitation of AZD1775, a Wee-1 inhibitor, in human plasma and brain tumor tissue. Sample preparation involved simple protein precipitation with acetonitrile. Chromatographic separation was achieved on ethylene bridged hybrid stationary phases (*i.e.*, Waters XBridge Amide column) under an isocratic elution with the mobile phase consisting of acetonitrile/ammonium formate in water (10 mM, pH 3.0) (85:15, v/v) at a flow rate of 0.8 mL/min for 5 min. The lower limit of quantitation (LLOQ) was 0.2 ng/mL of AZD1775 in plasma and tissue homogenate. The calibration curve was linear over AZD1775 concentration range of 0.2–1000 ng/mL in plasma and tissue homogenate. 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 the penetration of AZD1775 across the blood-brain tumor barrier, as assessed by the unbound brain-to-plasma ratio, in patients with glioblastoma.

Keywords

AZD1775; Aqueous normal-phase chromatography; Tandem mass spectrometry; LC-MS/MS; Unbound brain-to-plasma ratio; Fraction unbound

1. Introduction

Glioblastoma is the most frequently reported malignant brain tumor histology, with average survival after diagnosis ranging from 12 to 16 months. Although the conventional treatment with surgery, radiation, and temozolomide postpones tumor progression and extends patients survival to some extent, these tumors universally develop resistance to radiation and

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chemotherapy and unrelentingly result in patient death. The G2/M checkpoint plays a pivotal role in preventing the programmed cell death in glioblastoma [1]. A critical downstream mediator of G2/M checkpoint is the Wee1 kinase, a cellular tyrosine kinase that ensures maintenance of CDC2-cyclin B complex in an inactive state by phosphorylating the tyrosine-15 (Tyr15) residue of CDC2 [4]. Inhibition of Wee1 kinase activates CDC2 through removal of its inhibitory Tyr15 phosphorylation, thereby allowing cells to enter the mitotic phase of the cell cycle [4–6]. Thus, Wee1 inhibition offers a promising chemo- and radio-sensitizing strategy by forcing cancer cells with damaged DNA to enter unscheduled mitosis and to undergo cell death [7]. AZD1775 is a first-in-class, highly selective, potent, small molecule inhibitor of Wee1 kinase [2]. It inhibits Wee1 activity and induces DNA damage and G2 checkpoint escape in cell-based assays at an EC₅₀ of ~80 nM [2]. Based on promising preclinical results [3,4], several clinical trials are currently undergoing to evaluate AZD1775 in combination with radiation and/or chemotherapy in patients with brain tumors ([ClinicalTrials.gov](https://clinicaltrials.gov)).

Penetration of anticancer drugs across the blood-brain barrier is a prerequisite for effective treatment of brain tumors. AZD1775 has physicochemical properties that are relatively favorable for brain penetration, *i.e.*, molecular weight of 501.2 Da, octanol-water partition coefficient (logP) of 2.8, and fraction unbound of ~0.2 in human plasma. Surprisingly, a preclinical study with glioblastoma orthotopic xenograft models suggested that AZD1775 exhibited limited, heterogeneous penetration into orthotopic glioblastoma in mice, with the brain-to-blood concentration ratio of 0.05 [4]. To address the question whether AZD1775 can penetrate the human blood-brain tumor barrier, we conducted a phase 0 trial in patients with first-recurrence, *de novo* glioblastoma ([ClinicalTrials.gov](https://clinicaltrials.gov) identifier:). The patients were treated with a single dose of AZD1775 before surgical resection of their tumors, and the drug levels in plasma and brain tumor samples were determined at predefined time points. The total brain-to-plasma concentration ratio (K_p) was commonly used as a measure of brain penetration of a drug. However, it has been increasingly recognized that the K_p has limited relevance to pharmacodynamics because the K_p is mostly governed by nonspecific binding of a drug to proteins and lipids in plasma and brain. Given the notion that unbound drug concentration drives the *in vivo* pharmacological effect, the use of unbound brain-to-plasma concentration ratio ($K_{p,uu}$) as a measure of brain penetration is more pharmacologically relevant.

The purpose of the present study was to develop a specific, sensitive, and reliable bioanalytical method for the determination of not only total but also unbound concentrations of AZD1775 in patient plasma and brain tumor samples. Given the hydrophobic nature of AZD1775 (logP, 2.8), reversed-phase liquid chromatography methods were initially tested. However, under all tested reversed-phase chromatographic conditions, AZD1775 was poorly retained. Interestingly, AZD1775 was found to be well retained on a Waters Atlantis HILIC silica column under hydrophilic interaction liquid chromatography (HILIC) condition [5], which is usually applied for the analysis of polar, hydrophilic compounds. In this study, the retention of AZD1775 on ethylene bridged hybrid stationary phases (*e.g.*, Waters XBridge Amide column) instead of ordinary silica stationary phases (*e.g.*, Waters Atlantis HILIC silica column) was investigated. It was found that AZD1775 exhibited a unique dual hydrophilic-hydrophobic retention behavior on the ethylene bridged hybrid stationary phase.

The term “aqueous normal-phase chromatography” was thus used to distinguish the dual retention mechanism from HILIC retention mode [6]. Here, an aqueous normal-phase chromatography method coupled with tandem mass spectrometry was developed for the quantitation of total and unbound AZD1775 in plasma and brain tissue. The method was fully validated in human plasma. The present method, with the lower limit of quantitation (LLOQ) of 0.2 ng/mL, was 10-fold more sensitive than the published HILIC method [5]. The method was successfully applied to assess the penetration of AZD1775 across the blood-brain tumor barrier, as assessed by $K_{p,uu}$.

2. Experimental

2.1. Chemicals and reagents

AZD1775 and the stable isotope-labeled internal standard (AZD1775-D8) were provided by the AstraZeneca (Wilmington, DE, USA). All other chemicals and reagents were LC–MS grade. Water was filtered and deionized with a US Filter PureLab Plus 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 system, which consists of an enhanced high performance hybrid triple quadrupole/linear ion trap mass spectrometer, interfaced with a SHIMADZU (Kyoto, Japan) Nexera high performance liquid chromatography (HPLC) system that is equipped with two X2 LC-30AD pumps, a X2 SIL-30AC autosampler, a CBM-20A communication bus module, a X2 CTO-30A column oven, and 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 tested based on both reversed-phase and aqueous normal-phase chromatography. Reversed-phase liquid chromatography was performed on a Waters XTerra MS C18 (50 × 2.1 mm, 3.5 μm) or XBridge C18 (50 × 2.1 mm, 3.5 μm) column with the optimized isocratic elution with the mobile phase consisting of methanol-ammonium acetate (10 mM, pH 5.0) (40:60, v/v), at the flow rate of 0.4 mL/min. Aqueous normal-phase chromatography was performed on a Waters XBridge™ Amide column (100 × 4.6 mm, 3.5 μm) using the optimized isocratic elution with the mobile phase consisting of acetonitrile- ammonium formate (10 mM, pH 3.0) (85:15, v/v), at the flow rate of 0.8 mL/min. The running time was 5 min for both methods. Column oven temperature was set at 40 °C for both methods.

2.2.3. Mass spectrometry—The column effluent was monitored using the QTRAP 6500 mass spectrometer. Mass spectrometry conditions for AZD1775 and AZD1775-D8 were optimized by direct infusion of their standard solutions into the ion source under both positive and negative ionization mode. Both compounds underwent more efficient ionization in the positive electrospray ionization mode than in the negative ionization mode. Therefore,

AZD1775 and AZD1775-D8 were monitored at the positive ionization mode using multiple reaction monitoring at the mass transitions (m/z) 501.2 > 483.3 and 509.3 > 491.2, respectively (Fig. 1). The Turbo ion-spray voltage was set at 5500 V and the source temperature was set at 400 °C. Collision gas was optimized at Medium level, and curtain gas, ion source gas 1 and ion source gas 2 were delivered at 172, 207 and 276 kPa, respectively. Declustering potential, collision energy, collision cell exit potential, and entrance potential were optimized at 100, 31, 12, and 10 V, respectively, for both AZD1775 and AZD1775-D8. The dwell time was set for 50 ms.

2.3. Sample preparation

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

samples—Stock solutions of AZD1775 and AZD1775-D8 were prepared in methanol at a concentration of 1 mg/mL, and stored in brown glass vials at –20 °C. Working solutions were prepared freshly on each day of analysis as serial dilutions in methanol. For the determination of AZD1775 in plasma and brain tissue samples, the calibration standards were prepared by spiking 10 µL of AZD1775 working solution into blank human plasma at the final concentrations of 0.2, 0.5, 5, 20, 100, 200, 500 and 1000 ng/mL. For the determination of unbound AZD1775 in post-dialysis phosphate buffer solution (PBS, pH 7.4), the calibration curve was prepared in PBS. QC samples were prepared in blank plasma at the concentrations of 0.2 (LLOQ), 0.6, 80, and 800 ng/mL. 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 AZD1775 plasma concentrations

samples—Frozen plasma samples were thawed at ambient temperature. Into 100 µL of plasma, 500 µL acetonitrile containing AZD1775-D8 (5 ng/mL) was added. The mixture was vortex-mixed for ~1 min, and centrifuged at 9000g 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 AZD1775 brain concentrations

samples—Patient brain tumor samples were thawed at room temperature. The tumor homogenate was prepared by adding 4 vol of distilled water to a weighted tumor sample followed by homogenizing using a Precellys® homogenizer (at 2000 g for two 10 s with 5 s pause). A 100 µL aliquot of brain tumor homogenate was precipitated with 500 µL of acetonitrile containing AZD-D8 (5 ng/mL). The mixture was vortexed for 1 min and centrifuged at 9000g, at 4 °C, for 10 min. The supernatant was transferred to an autosample vial, and 5 µL was injected into the LC–MS/MS system.

2.3.4. Equilibrium dialysis for the determination of AZD1775 fraction unbound in plasma and brain tissue

samples—The fraction unbound of AZD1775 in either plasma or brain tissue homogenate was determined using equilibrium dialysis on a 96-Well Equilibrium DIALYZER™ with a 5- KDa cut-off regenerated cellulose membrane (Harvard Apparatus Holliston, MA), as described previously [7]. Briefly, equilibrium dialysis was performed with a 200 µL of plasma or tissue homogenate against an equal volume of PBS

(pH 7.4) on a rotator (Harvard Apparatus Holliston, MA) at 37 °C. The optimal time to equilibrium was assessed with pooled human plasma at AZD1775 concentrations of 50, 500, and 5000 ng/mL for dialysis of 2, 4, 6, 16, and 24 h. At equilibrium, 100 µL aliquots of both compartments were transferred into Eppendorf tubes, and 500 µL acetonitrile containing AZD1775-D8 (5 ng/mL) was added. The mixture was vortex-mixed and centrifuged (9000g, 4 °C, 10 min), and 5 µL of the supernatant was injected into the LC–MS/MS system for determining AZD1775 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 tissue homogenate ($f_{u,hom}$) was calculated using Eqs. (1) and (2), respectively. Fraction unbound in undiluted brain tissue can be calculated based on the measured fraction unbound in diluted tissue homogenate ($f_{u,hom}$) and dilution factor (D_f) using Eq. (3) [8].

$$f_{u, plasma} = \frac{C_u}{C_p} \quad (1)$$

$$f_{u, hom} = \frac{C_u}{C_{hom}} \quad (2)$$

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

2.4. LC–MS/MS 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 AZD1775 at the LLOQ (0.2 ng/mL in human plasma) and AZD1775-D8 (5 ng/mL). 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 AZD1775 concentration ranging from 0.2 to 1000 ng/mL 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/x²) 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, residual plots.

The intra-day and inter-day accuracy and precision were assessed for the calibrator standards (each in duplicate) and QCs (including LLOQ, low, medium, and high 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}), the intra-day variance (VAR_{intra}), and the grand mean (GM) of the observed concentrations across runs were calculated from ANOVA analysis. The intra-day precision (P_{intra}) was calculated as: $P_{intra} = 100 \times (\sqrt{VAR_{intra}}/GM)$. The intra-day precision (P_{inter}) was defined as: $P_{inter} = 100 \times (\sqrt{(VAR_{inter} - VAR_{intra})/n})/GM$, 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 [9]. Three sets of QC samples (including low, medium and high QCs) were prepared. Set 3 QCs were prepared by spiking AZD1775 (at the concentrations of 0.6, 80, and 800 ng/mL) and AZD1775-D8 (at the concentration of 5 ng/mL) into 100 μ L human plasma. Set 3 samples were processed by adding 500 μ L acetonitrile, followed by centrifugation (at 9000g, at 4 °C, for 10 min), and the supernatants were injected into the LC–MS/MS system. Set 1 and set 2 QC samples were prepared by spiking the same amounts of the analyte and internal standard as those for set 3 into 600 μ L mobile phase and blank matrix extract (*i.e.* post-precipitation supernatant solution of blank plasma), respectively. Set 1 and set 2 samples were directly injected into the LC–MS/MS system. The matrix effect is 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 is 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—The short-term (bench-top) stability of the AZD1775 in methanol (working solution) at the concentration of 100 and 1 μ g/mL as well as in plasma at the concentration of 0.6 (LQC) and 800 ng/mL (HQC) were tested at ambient temperature (25 °C) for 6 h. The stability of the processed samples in the autosampler was examined at 4 °C for 12 h at the concentration of 0.6 (LQC) and 800 ng/mL (HQC) in human plasma. The freeze-thaw stability of the AZD1775 in plasma was evaluated at 0.6 ng/mL and 800 ng/mL after three cycles of freezing and thawing with at least 1 day of storage at –80 °C between each thawing. The long-term stability of AZD1775 in methanol (1 mg/mL) and in plasma (0.6 and 800 ng/mL) was tested for 9 months.

2.5. Applications

Four patients with *de novo* glioblastoma participating in the phase 0 trial ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study?term=AZD1775&rank=1) identifier:) were given a single dose of AZD1775 (400 mg) orally before surgical resection of their tumors. Blood and tumor tissue samples were collected at 2–4 h following the administration of AZD1775. Plasma was separated from whole blood by centrifugation (at 4 °C, 1000g, 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. AZD1775 total concentrations in plasma and tumor tissue homogenate samples were determined using the validated LC–MS/MS method. Unbound fractions of AZD1775 in plasma and brain tumor tissue were determined using the equilibrium dialysis method as described in Section 2.3.4. AZD1775 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 tumor concentration to unbound plasma concentration.

3. Results and discussion

3.1. Retention mechanism

AZD1775 was poorly retained under the traditional reversed-phase liquid chromatographic condition, as suggested by wide, tailing peaks on the Waters XTerra MS C18 and XBridge C18 column (Fig. 2A and B). On the contrary, AZD1775 was well retained on ordinary silica stationary phases (*e.g.*, Atlantis HILIC silica column) under HILIC condition [5], and also on ethylene bridged hybrid stationary phases (*e.g.*, Waters XBridge Amide column) under aqueous normal-phase liquid chromatographic condition, as illustrated by a sharp, symmetrical peak (Fig. 2C).

Ethylene bridged hybrid material represents a new type of stationary phase, enabling retention of both polar and non-polar compounds. This dual hydrophilic-hydrophobic retention property is attributable to the interplay between the dominant and secondary separation mechanisms that occur with ethylene bridged hybrid stationary phases. By varying the percentage of water in the mobile phase, reversed-phase retention mode, normal-phase mode, or a combination of both can take place [6]. As shown in Fig. 3, when decreasing acetonitrile concentration from 90% to 10% in the mobile phase, AZD1775 exhibited a U-shape retention curve on the Waters XBridge Amide column, indicating the retention mechanism was changing from aqueous normal phase to reversed phase. This elution pattern is often observed for compound class that has both hydrophobic and hydrophilic properties [6]. AZD1775 fits to this class because its structure possesses both polar function groups (*i.e.*, –OH and –NH) and a significant hydrophobic component.

Retention mechanisms of aqueous normal-phase chromatography are not completely understood. Multimodal retention mechanisms can be involved, including liquid-liquid partitioning of the analyte between mobile phase and water-enriched solvent layer that is partially immobilized onto the surface of the stationary phase [10], hydrogen-bonding, hydrophobic and hydrophilic interaction, and ion-exchange interactions [11]. The predominant retention mechanism depends not only on the chemical structure of the analyte but also on the composition of the mobile phase and functional group on the stationary phase. In general, when the analyte has polar protic functional groups (*e.g.*, –OH, NH and –SH), hydrogen-bonding retention mechanism may be predominant the aqueous normal-phase retention [12], and this strong hydrogen bond capability could be weakened by switching acetonitrile to methanol in the mobile phase because of hydrogen-bonding competition [13]. Specifically, because polar protic solvents such as methanol (CH₃OH) have stronger ability to hydrogen bonds than polar aprotic solvents such as acetonitrile (CH₃CN), polar protic solvents (*e.g.*, methanol) can more effectively compete for polar

active sites on the surface of the stationary phase, thus perturbing the formation of water layers on the stationary phase and diminishing the hydrogen-bonding between the analyte and the stationary phase. Because AZD1775 structure has both hydrogen-bond donor and acceptor function groups (Fig. 1), hydrogen-bonding mechanism may play a role in the retention of AZD1775 under aqueous normal-phase condition. As shown in Fig. 4, replacing acetonitrile with methanol in the mobile phase reduced (but not completely abolished) retention of AZD1775, suggesting that hydrogen-bonding plays a role, yet other mechanisms may be also involved in the retention of AZD1775 under aqueous normal-phase condition.

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 bioanalytical method validation.

The selectivity for the analysis was shown by symmetrical resolution of the peaks, with no significant chromatographic interference from 6 different donors of plasma (Fig. 5). Representative chromatograms of blank and spiked with AZD1775 in human plasma samples at LLOQ as well as a patient plasma sample collected at 8 h after the oral administration of AZD1775 20 mg are shown in Fig. 5.

The linear calibration curves were established over the AZD1775 concentration range of 0.2–1000 ng/mL in human plasma. The relationship between peak area ratios of AZD1775 to AZD1775-D8 *versus* AZD1775 concentrations was best fitted by a linear equation, expressed as $y = a \times x + b$, where y is peak area ratio, x is the analyte concentration, a and b are fitted parameters. A weighting function of $1/x^2$ produced the best goodness-of-fit. A linear correlation coefficient (R) of >0.99 was obtained in all analytical runs.

The LLOQ was established at 0.2 ng/mL AZD1775 in plasma, at which the mean signal-to-noise ratios were 195 ± 88 ($n = 15$). 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 89.2% to 110.6%; the intra- and inter-day assay precisions were less than 8.9% (Table 1). For all QC samples (*i.e.*, at the LLOQ, low, medium and high QC concentrations), the average accuracy ranged from 94.3% to 104.8%, and the intra- and inter-day precisions were within 9.1% (Table 2).

The matrix effect was examined in 6 different donors of human plasma to assess the potential of ionization suppression or enhancement for AZD1775 and AZD1775-D8. The average matrix factor of AZD1775 determined from 6 different donors of plasma ranged from 0.60 to 0.72 at the low, medium and high QC concentrations, and the interindividual variability, assessed as the coefficient of variation (CV%) from 6 donors of plasma, was $<12\%$ (Table 3). The matrix factor (expressed as average $\pm$ standard deviation) of AZD1775-D8 (at the concentration of 5 ng/mL) from 6 different donors of plasma was 0.80 ± 0.003 . The relative matrix factor of AZD1775, calculated as the ratio of the peak area ratio of AZD1775 to AZD1775-D8 in post-extracted solution (set 2) relative to that in neat solution (set 1) ranged from 0.87 to 1.03 at three QC concentrations, with the interindividual

variability <5.0% (Table 3), suggesting that the isotope-labeled internal standard adequately corrected for the matrix effect from different sources of plasma.

The average recovery of AZD1775 at the concentrations of 0.6, 80, and 800 ng/mL from 6 donors of plasma ranged from 82% to 86%, and the interindividual variability was within 10% (Table 3). The recovery of AZD1775-D8 at the concentration of 5 ng/mL from 6 donors of human plasma was $87\% \pm 5.7$. These data suggested that the extraction recovery from plasma was consistent and reproducible for the analyte and internal standard. The relative recovery of AZD1775, estimated as the ratio of the peak area ratio of AZD1775 to AZD1775-D8 in plasma (set 3) relative to that in the post-extracted solution (set 2) ranged from 90 to 96% at three QC concentrations, with the interindividual variability <7% (Table 3), suggesting that the isotope-labeled internal standard well corrected for the variation (if any) in the extraction recovery of the analyte from different matrices.

The short- and long-term stability data of AZD1775 were summarized in Table 4. Bench-top stability test suggested that AZD1775 was stable in both methanol (at 1 and 100 $\mu\text{g/mL}$) and human plasma (at 0.6 and 800 ng/mL) at ambient temperature ($\sim 25^\circ\text{C}$) for at least 6 h. Autosampler stability test suggested that AZD1775 was stable in the protein precipitation solution at 4°C for at least 12 h, allowing the assay to be performed continuously overnight for a large number of samples. Freeze-thaw stability test of plasma samples at the low and high QC concentrations showed that <4% of AZD1775 degraded after three freeze-thaw cycles. The long-term stability tests suggested that AZD1775 was stable in methanol at 1 mg/mL (stock solution) at -20°C for at least 9 months, and it was stable in human plasma (at 0.6 and 800 ng/mL) at -80°C for at least 7 months.

3.3. Applications

While the present method was fully validated in human plasma only, the method was successfully applied to determine AZD1775 concentrations not only in patient plasma samples but also in patient brain tumor tissue 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/or extraction recovery of the analyte across different matrices.

To determine the unbound concentrations of AZD1775 in patient plasma and brain tumor samples, an equilibrium dialysis method using 96-well microdialysis plates was optimized to determine the fraction unbound of AZD1775 in patient plasma and brain tumor tissue samples. The optimum equilibrium time was determined at 16 h at which the dialysis reached equilibrium (Fig. 6). AZD1775 was modestly bound to human plasma proteins, with an average fraction unbound value of 0.20 in pooled human plasma (Fig. 6). In 4 patients with glioblastoma who received a single dose of AZD1775 (400 mg), the fraction unbound was 0.18 ± 0.02 (range, 0.15–0.20) and 0.06 ± 0.004 (range, 0.06–0.07) in plasma and brain tumor tissue homogenate samples, respectively, suggesting AZD1775 exhibits a higher binding affinity to brain tissue components than to plasma proteins. The total brain-to-plasma ratio (K_p) was determined as 7.56 ± 2.98 (range, 4.20–11.50) and the unbound brain-to-plasma ratio ($K_{p,uu}$) was 2.61 ± 0.79 (range, 1.58–3.34) in 4 patients. These data indicated

a good penetration of AZD1775 across the human blood-brain tumor barrier and supported further development of this drug for the treatment of brain tumors.

4. Conclusion

A rapid, sensitive, and robust LC–MS/MS method based on aqueous normal-phase liquid chromatography was developed for the quantitation of AZD1775 in plasma and brain tissue samples. The method is fully validated with human plasma. Sample preparation involved a simple protein precipitation. The chromatography running time was 5 min. The LLOQ was 0.2 ng/mL of AZD1775 in plasma and tissue homogenate. The linear calibration curve was established over the AZD1775 concentration range of 0.2–1000 ng/mL in plasma and tissue homogenate. This method was successfully applied to assess the penetration of AZD1775 across the blood-brain tumor barrier, as assessed by $K_{p,uu}$.

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Fig. 1. Product mass spectrum of AZD1775 at m/z 501.3 $\rightarrow$ 483.5 (A), and the internal standard AZD1775-D8 at m/z 509.4 $\rightarrow$ 491.4 (B).

Fig. 2.

Comparison of extracted ion chromatograms obtained from the reversed-phase and aqueous normal-phase liquid chromatography methods. (A) Chromatographic separation was performed on a Waters XTerra MS C18 column (2.1 × 50 mm, 3.5 μm) column. (B) Chromatographic separation was performed on a Waters XBridge C18 column (2.1 × 50 mm, 3.5 μm). (C) Chromatographic separation was performed on a Waters XBridge Amide column (4.6 × 100 mm, 3.5 μm). 5 μL of AZD1775 neat solution (20 ng/mL) was injected, and monitored at m/z 501.3 → 483.5.

Fig. 3. Dual hydrophilic-hydrophobic retention behavior of AZD1775 on the Waters XBridge Amide column. Mobile phase consisted of 10 mM ammonium formate in water (pH 3.0) and acetonitrile. 5 μ L of AZD1775 neat solution (20 ng/mL) was injected, and monitored at m/z 501.3 $\rightarrow$ 483.5.

Fig. 4.

Replacing acetonitrile with methanol in the mobile phase reduces the retention of AZD1775 on the Waters XBridge Amide column under aqueous normal-phase liquid chromatographic condition. (A) Separation was performed under an isocratic elution with the mobile phase consisting of 10 mM ammonium formate in water (pH 3.0) and acetonitrile (15:85, v/v). (B) Separation was performed under an isocratic elution with the mobile phase consisting of 10 mM ammonium formate in water (pH 3.0) and methanol (15:85, v/v). 5 μ L of AZD1775 neat solution (20 ng/mL) was injected, and monitored at m/z 501.3 $\rightarrow$ 483.5.

Fig. 5.

Extracted ion chromatograms of blank plasma (A and B), spiked plasma with AZD1775 at LLOQ (0.2 ng/mL) (C and D), and a patient plasma sample collected at 8.0 h after oral administration of a single dose of AZD1775 (20 mg) (E and F), monitored at m/z 501.3 $\rightarrow$ 483.5 for AZD1775 and at m/z 509.4 $\rightarrow$ 491.4 for AZD1775-D8. The blank plasma sample was precipitated with 5 vol of acetonitrile (without the internal standard), and the spiked plasma and patient plasma samples were precipitated with 5 vol of acetonitrile containing the internal standard AZD1775-D8 (5 ng/mL).

Fig. 6. Fraction unbound of AZD1775 in plasma determined at 2, 6, 16 and 24 h. Values are the mean of triplicate measurements (with coefficient variation <10%).

Table 1

Accuracy, intra- and inter-day precisions of AZD1775 calibrator standards in plasma^a.

Nominal concentration (ng/mL)	Determined concentration (ng/mL)	Average accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
0.2 (LLOQ)	0.2 ± 0.02	101	8.9	^b
0.5	0.5 ± 0.03	109	5.0	^b
5	5.5 ± 0.2	111	3.8	^b
20	21.8 ± 0.6	109	2.4	1.9
100	102.6 ± 2.4	103	2.2	0.8
200	193.7 ± 7.0	97	3.1	2.1
500	477.2 ± 5.4	95	0.9	0.7
1000	891.8 ± 16.8	89	1.6	0.9

^aEach calibrator standard was evaluated in duplicate on three different days.

^bNo additional variation was observed as a result of performing assay in different days.

Table 2

Accuracy, intra- and inter-day precisions of AZD1775 quality control samples in human plasma^a.

Nominal concentration (ng/mL)	Determined concentration (ng/mL)	Average accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
0.2 (LLOQ)	0.2 ± 0.02	98	9.1	^b –
0.6	0.6 ± 0.04	100	7.2	^b –
80	83.9 ± 2.5	105	2.7	1.5
800	754.3 ± 26.3	94	2.2	3.2

^aEach QC was performed in quintuplicate on three different days.

^bNo additional variation was observed as a result of performing assay in different days.

Table 3

Matrix effect and recovery of AZD1775 and AZD1775-D8 from 6 different donors of human plasma.

Analyte	Concentration (ng/mL) ^a	Matrix factor ^b	Recovery (%) ^c	Relative matrix factor ^d	Relative recovery (%) ^e
AZD1775	0.6	0.60 (12)	86 (6.5)	0.87 (4.6)	96 (6.9)
	80	0.70 (9.4)	82 (10.0)	1.00 (1.3)	91 (2.1)
	800	0.72 (9.3)	84 (7.8)	1.03 (5.0)	90 (5.0)

^aThe 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.

^bMatrix effect is expressed as the ratio of the mean peak area of AZD1775 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.

^cRecovery is calculated as the ratio of the mean peak area of AZD1775 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.

^dRelative matrix effect (or internal standard-normalized matrix effect) is expressed as the ratio of the mean peak area ratio in set 2 to that in set 1. Data are shown as the mean (%CV) from six donors of plasma extract.

^eRelative recovery (or internal standard-normalized recovery) is calculated as the ratio of the mean peak area ratio in set 3 to that in set 2. Data are shown as the mean (%CV) from six different donors of plasma.

Table 4

Assessment of stability of AZD1775.

AZD1775			
Bench-top stability (in stock solution) (25 °C) (%) ^d	1 µg/mL ^c	100 µg/mL ^c	
1.0h	102	101	
2.0h	103	102	
3.0h	105	104	
4.0h	107	107	
6.0h	108	108	
Bench-top stability (in plasma) (25°C) (%) ^a	0.6 ng/mL ^c	800ng/mL ^c	
1.0h	102	102	
2.0h	100	103	
3.0h	104	103	
4.0h	103	102	
6.0h	105	103	
Auto-sampler stability (in the precipitation solution) (4 °C) (%) ^a	0.6 ng/mL ^c	800 ng/mL ^c	
12.0	99	100	
Freeze-thaw stability (in plasma) (-80°C) (%) ^b	0.6 ng/mL	800 ng/mL	
Cycle 1	100	87	
Cycle 2	96	86	
Cycle 3	97	92	
Long-term stability (in plasma) (-80°C) (%) ^b	0.6 ng/mL ^c	800 ng/mL ^c	
2 months	95	92	
7 months	97	87	

^aStability data is expressed as the mean percentage of the peak area determined at certain time relative to that at time zero.^bStability data is expressed as the mean percentage of the analyte concentration determined at certain time point relative to the nominal concentration (%).^cEach concentration at each time point was assessed in triplicate.