ORIGINAL ARTICLE

Determination and pharmacokinetic study of AZD-3759 in rat plasma by ultra performance liquid chromatography with triple quadrupole mass spectrometer

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Abstract

Background: AZD-3759 is a new, potent, oral, active central nervous system-penetrant *EGFR* inhibitor. Despite promising clinical activity among patients pre-treated and never treated with EGFR-tyrosine kinase inhibitors, no time saving pharmacokinetic study method has been reported in an animal model.

Methods: Protein was precipitated with acetonitrile and then used for sample pre-processing. A CORTECS BEH C18 column was used to separate the analytes at 40°C. Acetonitrile and water (containing 0.1% formic acid) were chosen as the mobile phase at a flow rate of 0.4 mL/min. The analytes were quantified by multiple reaction monitoring mode with positive electrospray ionization.

Results: The target fragment ions were m/z 460.38→141 for AZD-3759 and m/z 285.1→193.1 for internal standard diazepam. The calibration curve exhibited good linearity for AZD-3759 at a range of 1–500 ng/mL. The intra-run and inter-run precision variations were both < 8.22%. The recovery rate of AZD-3759 from plasma was > 76.4%.

Conclusion: An accurate, simple ultra performance liquid chromatography with triple quadrupole mass spectrometer method was developed and validated to determine AZD-3759 in rat plasma. Our validated method can be applied to the pharmacokinetic study of AZD-3759 at an oral dosage of 10 mg/kg.

Introduction

AZD3759 is a new, potent, oral, active central nervous system (CNS)-penetrant EGFR inhibitor, which has good blood–brain barrier permeability for non-small cell lung cancer (NSCLC) patients with *EGFR* mutations.[1] It is mainly designed to effectively bypass the blood–brain barrier to address CNS metastasis in NSCLC patients carrying *EGFR* mutations, such as brain metastasis (BM) and dura mater (or pia mater, leptomeningeal metastases). AZD3759 is currently at a phase I/II research and development stage.[3,4] At Km ATP concentrations, the half maximal inhibitory concentrations (IC_{50}) were 0.3, 0.2, and 0.2 nM for wild-type *EGFR* and mutated variants L858R and exon19-Del, respectively.[2] AZD3759 exhibited inhibitory effects on the proliferation of the phosphorylated (p) EGFR pathway and *EGFR* mutant derived PC-9 and H3255 cells, with IC_{50} of 7.7 nm and 7 nm, respectively. However, it had no inhibitory effect on the proliferation rate of H838 cells.[3]

AZD3759 was tolerable up to 300 mg twice a day, with promising clinical activity in *EGFR*-mutant NSCLC with CNS metastases.[4] The recommended phase 2 dose was defined as 200 mg twice a day, at which level both AZD3759 and the metabolite AZ'1168 could reach efficacious concentrations for target inhibition. The promising clinical activity among patients pretreated and never treated with an EGFR-tyrosine kinase inhibitor warrants the future development of AZD3759.[5] However, no time saving pharmacokinetic study method has been reported in an animal model to date, which limits basic research regarding this new *EGFR* inhibitor drug. This study aimed to develop an ultra performance liquid chromatography with triple quadrupole mass spectrometer (UPLC-MS/MS) method for the quantification of AZD3759 in pharmacokinetic studies in rats. Our method has a short retention time, high sensitivity, and is portable for use in the clinic.

Methods

Chemicals and reagents

AZD-3759 (purity > 98%) was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Analytical reagent grade formic acid and diazepam (as the internal standard [IS]) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fisher Scientific Co. (Fair Lawn, New Jersey, USA) provided chromatography grade methanol and acetonitrile. Drug-free blank plasma was obtained from Wenzhou Medical University Laboratory Animal Center (Wenzhou, China).

Liquid chromatographic conditions

Chromatographic separation of AZD-3759 and diazepam was carried out using an ACQUITY UPLC I-Class and XEVO TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). Binary Solvent Manager (BSM) and a Sample Manager with Flow-Through Needle (SM-FTN) were used in the UPLC system. Two microliter aliquots of samples were separated onto a CORTECS BEH C18 column (Waters Corp.) at 40°C. The mobile phase consisted of eluent A (acetonitrile) and eluent B (water with 0.1% formic acid), with a flow rate of 0.4 mL/minutes in gradient eluent A as follows: from start to 1 minute, 10–30%; from 1 to 2 minutes, 30–95%; from 2 to 2.5 minutes, maintained at 95%; and from 2.5 to 2.6 minutes, 95–10%. The total running time for the analytes was three minutes. A strong wash (methanol–water, 50/50, V/V) followed by a weak wash (methanol–water, 10/90, V/V) was performed after each injection.

Mass spectrometric conditions

The triple-quadrupole mass spectrometer was operated in the positive electrospray ionization mode with an ESI interface. The capillary voltage was set at 1 kV and the cone voltage 35 V for AZD-3759 and IS diazepam; Collision Energy was set at 35 V for AZD-3759 and 30 V for IS. The source and desolvation temperature was 150°C. The desolvation gas flow was 1000 L/h and the cone gas (nitrogen) flow was 50 L/h. A multiple reaction monitoring (MRM) mode of m/z 460.38→141 for AZD-3759 and m/z 285.1→193.1 for diazepam (IS) was used for quantitative analysis, with a scan time of 0.108 seconds per transition. Masslynx 4.1 software (Waters Corp.) was used for data processing.

Preparation of standard solutions and quality control samples

The stock solutions of AZD-3759 (0.5 mg/mL) and diazepam (0.5 mg/mL, IS) were prepared using a methanol–water solution. The working solutions of IS (0.5 μg/mL, IS) and AZD-3759 were prepared by diluting corresponding stock solutions with methanol.

The concentration of calibration curves was prepared by spiking appropriate working solutions into rat blank plasma at a range of 1–500 ng/mL for AZD-3759 (1, 2.5, 5, 10, 25, 50, 100, 200 and 500 ng/mL). Quality control (QC) samples of AZD-3759 were prepared independently at three different levels (1.2, 60, and 300 ng/mL). All calibration and QC samples were frozen at −20°C before use.

Plasma sample preparation

Before analysis, the plasma sample was thawed to room temperature. An aliquot of 20 μL of the IS working solution (0.5 μg/mL) was added into 100 μL of collected plasma sample in a 1.5 mL centrifuge tube, followed by the addition of 200 μL of acetonitrile. The tubes were vortex mixed for one minute. After centrifugation at 13 000 rpm for 10 minutes, the supernatant (2 μL) was injected into the UPLC–MS/MS system for detection.

Method validation

Rigorous tests for selectivity, linearity, accuracy, precision, recovery, and stability were conducted according to the guidelines set by the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA), in order to thoroughly validate the proposed bioanalytical method.[6–19] All QC plasma samples were prepared on three consecutive days with six replicates.

Linearity and the lower limit of quantification (LLOQ)

The calibration curves were established based on the peak area ratio of AZD-3759 to IS. The calibration curves of

AZD-3759 were drawn using the weighted (1/x) least square linear regression method. The lowest standard on the calibration curve was accepted as the linearity and the lower limit of quantification (LLOQ), if the following conditions were convincing: the analyte response was at least five times the blank response; the analyte peak (response) was identifiable, discrete, and reproducible; and the back-calculated concentration had precision that did not exceed 20% of the CV and accuracy within 20% of the nominal concentration.

Precision, accuracy, recovery, and matrix effect

The precision and accuracy of the developed method were evaluated in rat plasma with QC samples at different concentrations (1.2, 60, and 300 ng/mL) before detection. The inter-day and intra-day precisions over consecutive days were determined by analyzing each QC sample with different concentrations of AZD-3759. Precision was expressed as relative standard deviation (RSD), which was required to be < 15%. The acceptance criteria for accuracy was within 85–115%, except for LLOQ (≤ 20%).

The recovery rate was calculated using the peak area ratios of analytes from whole blood samples spiked with known concentrations of dissolved samples, and precipitated blank whole blood samples at the same concentration at three QC levels as the percent extraction recoveries.

Parallelism of diluted study samples was evaluated with diluted standards to detect matrix effects. Nonspecific binding was also determined. The matrix effect was evaluated by comparing the ratio (A/B × 100%) of peak response of AZD-3759 containing an equivalent amount, both extracted from blank plasmas (A) and dissolved in pure standard solution (B).

Stability

Quality control samples with three different concentrations (*n* = 6 for each) were stored at different temperatures for different times to evaluate the short and long-term stability of AZD-3759. The QC samples were found stable after storage at room temperature for 24 hours and −20°C for three weeks, respectively. Samples were stable after three freeze and thaw cycles after storage at −20°C for 24 hours and thawing at room temperature.

Pharmacokinetic study

Twelve Sprague–Dawley rats (200–250 g) were used in the experiment. Before drug administration, all rats were fasted for 12 hours, only receiving water. A single oral dose of AZD-3759 (dissolved in CMC-Na solution) was administered at 10 mg/kg. Blood samples (about 0.3 mL) were collected at 0.0833, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, 24, and 36 hours. Samples were centrifuged at 3500 rpm for 10 minutes and plasma were kept at −20°C for storage. The pharmacokinetic parameters of the study were: the maximum plasma concentration (C_{max}), the maximum plasma time (T_{max}), the area under the plasma concentration–time curve (AUC), the plasma clearance (CL), and the half-life ($t_{1/2}$). These were calculated using DAS software version 3.2.8 (The People's Hospital of Lishui, Lishui, China).

Results

Method development

Good peak symmetry, a short retention time, and appropriate ionization were obtained using the mobile phase of acetonitrile and water (0.1% formic acid) with gradient elution. AZD-3759 and IS diazepam gave protonated precursor molecular ions $[M + H]^+$ in the positive ionization mode. As shown in Figure 1, the major ions detected were m/z 460.38→141 for AZD-3759 and m/z 285.1→193.1 for diazepam (IS), with retention times of 1.19 and 2.21 minutes, respectively. Compared to the liquid-liquid extraction method, protein precipitation with acetonitrile showed a higher recovery rate. The total running time for one sample was only three minutes using our method and exhibited high detection sensitivity.

To obtain the best chromatographic condition, the mobile phase modifier acetonitrile/methanol-water (0, 0.1, 0.2, 0.3 and 0.5% [v/v] formic acid), gradient elution type (isocratic elution and gradient elution), and flow rate (0.2, 0.3, 0.4 and 0.5 mL/min) were systematically investigated. The results indicated that the best condition was acetonitrile-0.1% formic acid with a flow rate of 0.4 mL/minute on a CORTECS BEH C18 column (2.1 × 50 mm, 1.6 μm). Under this condition, the spread of the peaks was reduced, whereas the sensitivity was improved compared to those on a UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm). Acetonitrile instead of methanol was used to quantify AZD-3759 because methanol could produce a strong peak response for AZD-3759 and diazepam.

Sample preparation was also important for the determination of AZD-3759. In this study, acetonitrile was chosen as the protein precipitation solvent because it exhibited a better precipitation effect than methanol, with acceptable recoveries. Our UPLC–MS/MS method for one sample can be completed within three minutes with an LLOQ of 0.5 ng/mL for AZD-3759.

Matrix effect and selectivity

The MRM chromatograms of AZD-3759 and diazepam, blank plasma sample (A), blank plasma sample spiked with
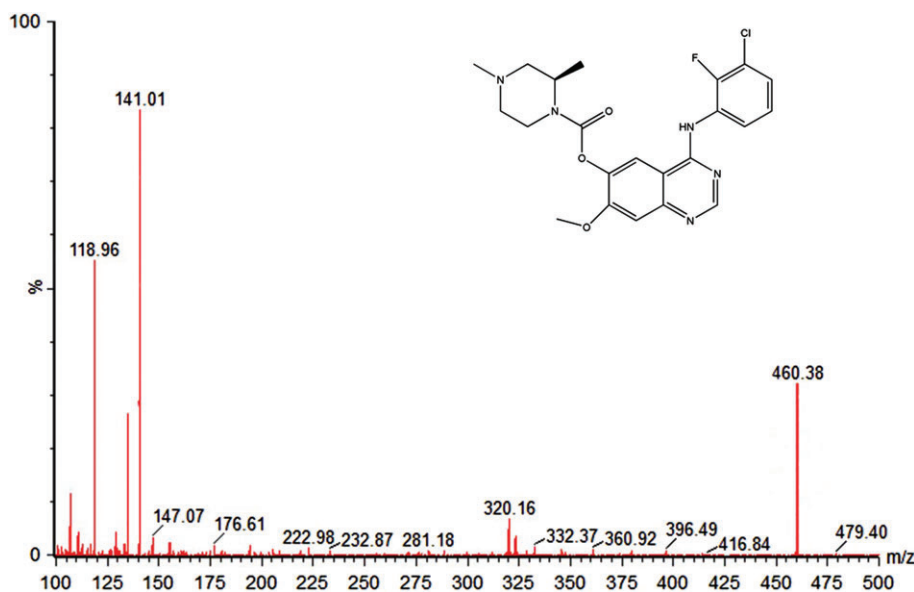
Figure 1 Mass spectrometer spectrum of the peaks corresponding to AZD-3759.

AZD-3759 and IS (B), and plasma sample at 0.5 hours after oral administration of 10 mg/kg AZD-3759 (C) are shown in Figure 2. No interfering peaks were found at the retention time of AZD-3759 and IS. To describe the matrix effect, low, medium, and high concentrations (1.2, 60, and 300 ng/mL) of AZD-3759 were prepared before detection. As a result, the measured concentrations were $94.17 \pm 8.2\%$, $118.46 \pm 10.6\%$, and $106.2 \pm 9.7\%$ of theoretical values of added AZD-3759, respectively ($n = 6$).

Linearity and the LLOQ

Calibration curves over three consecutive days showed excellent linearity ranging from 1 to 500 ng/mL, which could meet the needs very well for a determination of AZD-3759. The regression equation fitted was: $Y = 0.010471X + 0.0119161$, $r = 0.9995$, where Y represents the peak area ratios of analyte to IS, and X represents the AZD-3759 plasma concentration. The LLOQ of AZD-3759 in rat plasma was 0.5 ng/mL, which yielded a precision of 6.27%.

Precision, accuracy, and recovery

The RSDs of QC samples over three validation days were evaluated as the precision of the method. The inter-day and intra-day precision was < 10% at each QC level. The accuracy of the method ranged from 85.3% to 111.7% at each QC level. The mean recovery value of AZD-3759 was > 76.4%. The recovery of the IS was 92.5%. Assay performance data is presented in Table 1, which meets the criteria for biological analysis.

Carry-over

No background interfacing peaks (≥ 20% of the LLOQ, 5% of the IS) were left in the blank plasma samples on the chromatogram. In addition, strong wash (methanol–water, 50/50, *V/V*) and weak wash (methanol–water, 10/90, *V/V*) were automatically performed after each injection in the program to ensure that no carry over material could be detected in the assay.

Stability

No significant loss of stability of AZD-3759 in rat plasma was observed at low, medium, or high concentrations (1.2, 60, and 300 ng/mL). Long-term, room temperature storage, and freeze-thaw cycle conditions were evaluated and are shown in Table 2. All stability tests of AZD-3759 in rat plasma indicated that the RSDs of the responses were < 15%.

Pharmacokinetic study

The pharmacokinetic study on rats orally administered AZD-3759 (dissolved in CMC-Na solution) with 10 mg/kg was successfully performed using the validated UPLC-MS/MS method. Detailed pharmacokinetic parameters are shown in Table 3. The mean plasma concentration–time curve of AZD-3759 is shown in Figure 3.

Discussion

Since the application of AZD-3759 in clinics, most studies have focused on the effectiveness of this drug in NSCLC

Figure 2 The multiple reaction monitoring chromatograms of AZD-3759 and diazepam. (**a**) Blank plasma sample, (**b**) blank plasma sample with AZD-3759 and internal standard (IS), and (**c**) plasma sample at 0.5 hours after oral administration of 10 mg/kg AZD-3759. 1: ADZ-3759; 2: diazepam; 3: Total ion chromatogram.
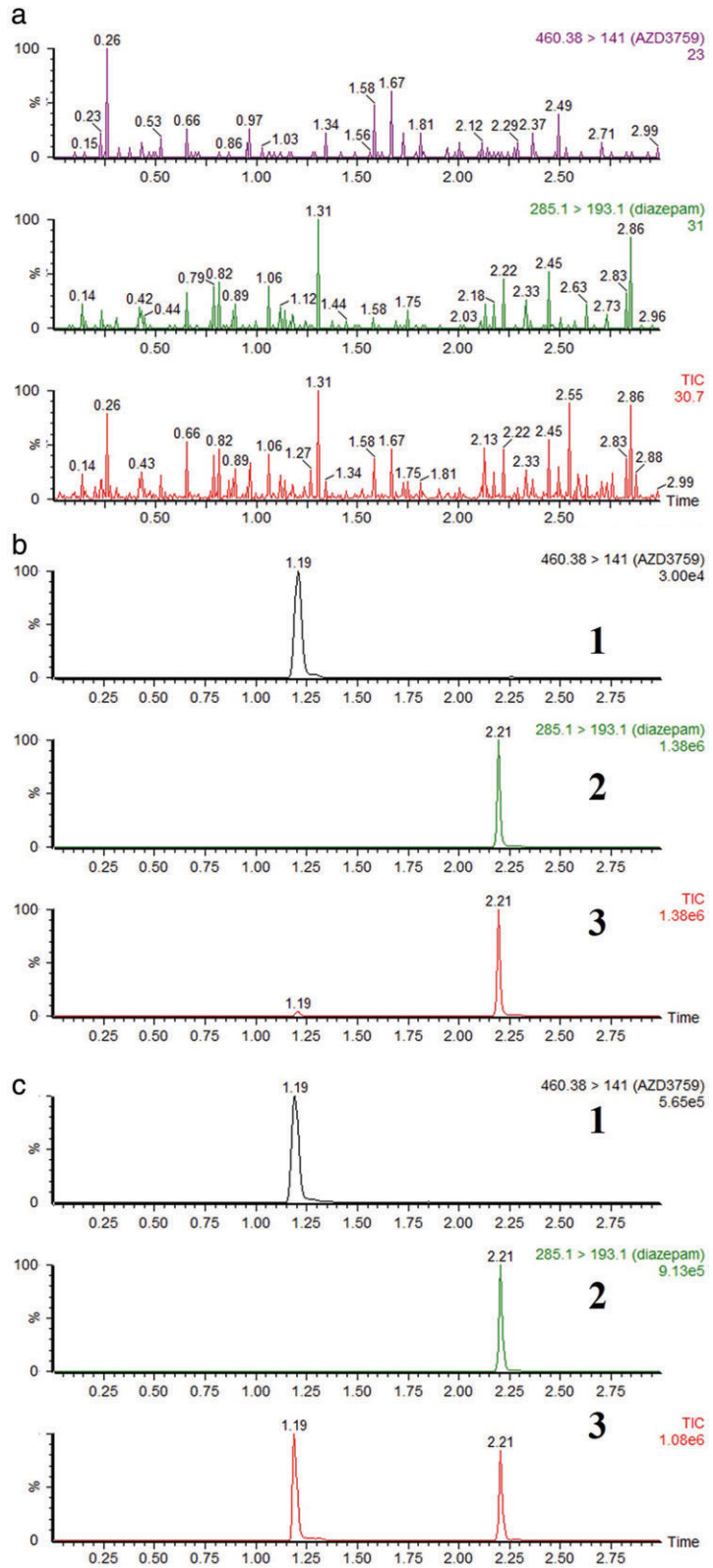
Table 1 The inter-day and intra-day precision, accuracy, and recovery of AZD-3759 in rat plasma (*n* = 6)

	Concentration (ng/ml)	RSD (%)		Accuracy (%)		Recovery (%)
		Intra-day	Inter-day	Intra-day	Inter-day	
Compound	1.2	5.23	7.19	106.33 ± 5.56	102.22 ± 7.35	84.92 ± 4.5
	60	8.22	4.48	97.86 ± 8.04	98.79 ± 4.42	80.51 ± 3.97
	300	3.01	3.01	103.90 ± 3.13	102.73 ± 3.1	83.68 ± 3.35

RSD, relative standard deviation.

Table 2 Stability of AZD-3759 under various conditions (*n* = 6)

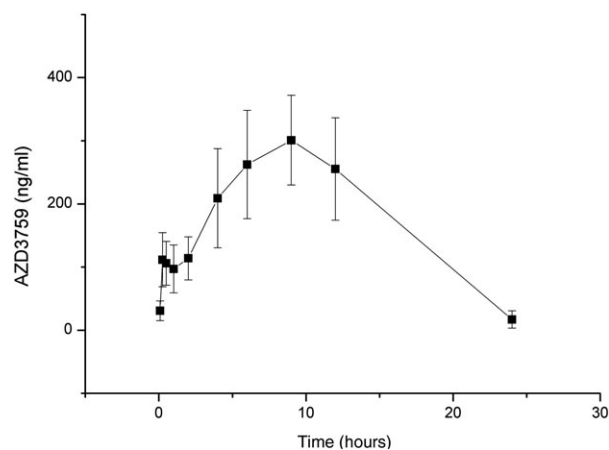| Condition	Concentration (ng/ml)		RSD (%)	Accuracy (%)
	Added	Measured		
Room temperature,	1.2	1.236	9.04	103.0
24h	60	60.079	3.52	100.1
	300	297.481	2.73	99.2
−20°C,	1.2	1.283	4.77	106.9
3 weeks	60	60.841	6.23	101.4
	300	298.063	5.93	99.4
3 freeze-thaw	1.2	1.286	12.28	107.2
cycles	60	61.841	4.27	103.1
	300	318.076	3.99	106.0

RSD, relative standard deviation.

Table 3 Primary pharmacokinetic parameters after oral administration of AZD-3759 in rats (*n* = 6, Mean ± SD)

Pharmacokinetics parameters	Oral administration (n = 6)
$AUC_{(0-t)}$ (ng/mL·h)	4301.756 ± 890.667
$AUC_{(0-\infty)}$ (ng/mL·h)	4403.952 ± 904.901
$MRT_{(0-t)}$ (h)	9.178 ± 0.932
$MRT_{(0-\infty)}$ (h)	9.649 ± 1.358
$t_{1/2}$ (h)	3.481 ± 0.91
Tmax (h)	9 ± 1.897
CL (L/h/kg)	2.339 ± 0.403
Vz (L/kg)	11.781 ± 3.872
C_{max} (ng/mL)	325.983 ± 63.936

AUC, area under the plasma concentration–time curve; CL, plasma clearance; C_{max}, maximum plasma concentration; MRT, mean residence time; SD, standard deviation; $t_{1/2}$, half-life; T_{max}, maximum plasma time.

Figure 3 Mean plasma concentration time profile after oral administration of AZD-3759 (10 mg/kg) in rats (mean ± standard deviation). (—■—) AZD3759

Acknowledgments

Disclosure

No authors report any conflict of interest.

patients.[1,5] To date, no time-saving pharmacokinetic study method has been reported in an animal model to facilitate a pharmacokinetic study in the clinic. In the present study, a simple, precise, and accurate UPLC–MS/MS method for the quantitation of AZD-3759 in rat plasma was established, using 100 µL of plasma with an LLOQ of 0.5 ng/mL. The UPLC–MS/MS method can successfully be applied to a pharmacokinetic study of AZD-3759 after oral administration in rats and this method provides a foundation for the pharmacokinetic detection of AZD-3759 in a clinical setting.

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