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A Novel uHPLC-MS/MS Method for the Quantitation of AZD7451 (AZ12607092) in Human Plasma

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Abstract

Tropomyosin-related kinases (Trk) are tyrosine kinase receptors implicated in tumor proliferation, invasion, and survival signaling across a number of tumors, making them potentially attractive targets for the treatment of cancer. AZD7451 is a potent and selective inhibitor of Trk kinases currently undergoing a Phase I dose escalation in glioblastoma multiforme at the National Cancer Institute. A key part of early clinical testing for AZD7451 involves demonstrating that pharmacokinetic half-life and clinical exposures of AZD7451 are sufficient to inhibit Trk receptors in preclinical models. To address this need, an ultra sensitive analytical method was developed to measure the AZD7451 profile in human plasma. A liquid-liquid extraction recovered >80% of AZD7451 before quantitative analysis by ultra HPLC-MS/MS. A Varian Polaris® C18-A column and a mass transition of m/z 383.5→340.5 (m/z 389.6→342.0 for the internal standard [²H₆]-AZD7451) was used, and a dynamic calibration range of 0.5–1000 ng/mL was established, which provided a sensitive (<8.5% deviation), and precise (<6%) quantitative assay for AZD7451. AZD7451 demonstrated stability in human plasma at room temperature for 24 hrs (<7% change) and after extraction at 4 °C for 24 hrs (<8% change), and was stable through 4 freeze/thaw cycles (<8% change). This method was used to measure AZD7451 plasma levels in clinical samples to confirm the sensitivity at several time points following AZD7451 treatment in subjects with glioblastoma.

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Keywords

Tropomyosin-related kinase; ultra-high performance liquid chromatography; tandem mass spectrometry

1. Introduction

The tropomyosin-related kinases (Trk) are a family of membrane-associated tyrosine kinases frequently found in neuronal cells whose activation and expression have been implicated in neural growth, development, and function [1]. The Trk family is also involved in the survival and signaling in many tumors, including non-small cell lung cancer (NSCLC), neuroblastomas, invasive gliomas, papillary thyroid, pancreatic carcinomas, and other neuroendocrine tumors [2–4]. Several isoforms (A, B, and C) exist for the Trk family, each activated by specific neurotrophins (NT) where the Trk-NT pair correlates with a different cellular outcome. In particular, TrkA-NGF (nerve growth factor) and TrkC-NT3 are associated with better prognosis and less advanced tumors if normally expressed in neuroblastomas [5, 6].

Mutations in Trk receptors, such as splice variants in TrkA, contribute to the pathogenesis of several non-neural malignancies by promoting tumor and angiogenic effects via ligand-independent activity [6]. Similarly, increased levels of TrkB and its corresponding neurotrophin, BDNF, serve as indicators for a poorer prognosis in neuroblastoma and other cancers [1]. One study showed that TrkB overexpression might be directly involved in the ability of pancreatic adenocarcinoma to metastasize and suggested TrkB as a potential therapeutic target in pancreatic cancer [5].

Tropomyosin-related kinases have clearly been linked to various cancers, making them an attractive target for therapeutic modulation. AZD7451 is a novel, first-in-class, small molecule Trk inhibitor that may have efficacy for patients with cancers expressing Trk. Moreover, AZD7451 may be effective against recurrent or unresponsive malignant gliomas or glioblastomas in adults based on the fact that gliomas appear to utilize TrkA signaling for invasion and associated disease progression [7]. This ATP-competitive inhibitor works via binding Trk and subsequently blocking any neurotrophin-Trk interaction and secondary signal activation. Based on *in vitro* and cell based inhibition profiles against TrkA, TrkB, and TrkC, with IC₅₀ ranging 0.2 – 3 nM, binding of AZD7451 to TrkA, TrkB, and TrkC would be expected to inhibit responses associated with NGF, BDNF, and NT3, respectively [8].

Recent reports of closely related Trk inhibitors in mice implanted with adenoid cystic carcinoma tumors or neuroblastomas demonstrated single agent efficacy at doses and schedules that were well tolerated [9]. In preclinical studies, AZD7451 demonstrates good aqueous solubility (> 50 mg/mL in water), thus it was given orally once or twice daily to mice, rats and dogs to study pharmacokinetics and pharmacodynamics. It was shown to be rapidly absorbed in rats and dogs (maximum plasma concentration occurring between 0.25–1 hr), had an oral bioavailability ranging from 21–30% (with high permeability established in Caco-2 cells and no evidence of efflux by transporters), and plasma protein binding around 97% (data not shown). CYP3A4 accounted for 99% of all circulating metabolites (data not shown), none of which exhibited any biological activity. Furthermore, a key pharmacodynamic endpoint, the phosphorylation of TrkA, was inhibited 80% with a plasma concentration of 5 nM (~2 ng/mL), which is consistent with *in vitro* IC₅₀ values for TrkA phosphorylation of 0.2 nM (taking into account 97% plasma protein binding *in vivo*).

AZD7451 was moderately cleared in rats and dogs, with hepatic clearance being the main driver (~40% of hepatic blood flow) and minimal renal clearance (<1 mL/min/kg).

AZD7451 is currently being evaluated in a first-in-humans phase I clinical trial at the National Institutes of Health (NIH) Clinical Center in Bethesda, MD in patients with recurrent glioblastomas multiforme. Establishing the clinical pharmacokinetics of AZD7451 is a key endpoint in the Phase I clinical program, and given the relatively low ~5nM target plasma concentrations, an ultra-sensitive and bioanalytical method to quantify AZD7451 in plasma needed to be developed and validated in order to perform a robust pharmacokinetic analysis in humans.

The objective of the current study is to describe the validation of a novel, fast, sensitive, selective, accurate, and precise method for quantifying AZD7451 in human plasma over the calibration range of 0.5–1000 ng/mL. Pharmacokinetic data for the phase I study will be reported separately; the objective of this study is to describe the bioanalytical assay. Because this is a first-in-humans phase I study, the expected plasma concentrations following a dose escalation scheme could not be adequately predicted. For this reason, the method was calibrated as low as possible for the available instrumentation (0.5 ng/mL); the upper limit of calibration was 1000 ng/mL, with a validated 10-fold dilution up to 10,000 ng/mL.

2. Experimental

2.1. Materials

AZD7451 and [$^2\text{H}_6$]-AZD7451 were provided by AstraZeneca (Waltham, MA). The chemical structures of AZD7451 and [$^2\text{H}_6$]-AZD7451 are shown in Figure 1. Optimagrade acetonitrile and methanol were obtained from Fisher Scientific (Fairlawn, NJ, USA) and de-ionized water was generated with a Hydro-Reverse Osmosis system (Durham, NC, USA) connected to a Milli-Q UV Plus purifying system (Billerica, MA, USA). Drug-free human heparinized plasma was obtained from the NIH Clinical Center Blood Bank (Bethesda, MD).

2.2. Preparation of stock solutions

Master stock solutions were prepared individually by dissolving AZD7451 and [$^2\text{H}_6$]-AZD7451 in methanol at free base concentrations of 1 mg/mL. After vortex mixing and brief sonication, each stock solution was stored in glass vials at -80°C . Serial dilutions (working stocks) were prepared in methanol from the master stock and stored in glass vials at -80°C for the preparation of calibration and quality control (QC) samples.

For each analytical run, calibration standards in drug-free human heparinized plasma were freshly prepared in duplicate at concentrations of 0.5, 1.0, 2.0, 10, 50, 100, 500, 800, and 1000 ng/mL. Quality control (QC) samples were prepared in batch at concentrations of 0.5 (lower limit of quantification; LLOQ), 1.5 (low-range; LQC), 400 (mid-range; MQC), 800 (high-range; HQC), and 10,000 ng/mL (10-fold dilution; DQC) by adding drug-free heparinized plasma to the required amount of working stock in a volumetric flask. QC samples were vortex-mixed, then subdivided into aliquots and stored at -80°C .

2.3. Sample preparation

Frozen standards and samples were thawed on wet ice before homogenization by vortex-mixing. One hundred microliters of 1% formic acid (aq) was added to 100 μL of plasma calibrator aliquots, QC samples, and unknown samples. A liquid-liquid extraction was performed by adding 1 mL of methyl t-butyl ether (MTBE) containing 25 ng/mL [$^2\text{H}_6$]-AZD7451 (internal standard). This mixture was briefly vortexed, then centrifuged for 10

min at 13,200 rpm ($11,700 \times g$) in a mini-centrifuge before the supernatant was dried down and reconstituted with (50/50, v/v) H₂O/ACN.

2.4. Instrument conditions

Ten microliters of each sample was injected onto a Waters ACQUITY UPLC system (Waters Corporation, Milford, MA, USA), which included a binary pump, refrigerated autosampler (4 °C) and a temperature-controlled column compartment (30 °C). Chromatographic separation was achieved on a Varian Polaris® C18-A, 2.0×50 mm, 3.0 µm preceded by a Varian Polaris® C18-A guard column packed with 3.0-µm packing material. The mobile phase consisted of A: 0.1% formic acid in water, and B: 0.1% formic acid in acetonitrile with a flow rate of 0.8 mL/min and a total run time of 5 min. A gradient of mobile phase B was utilized by maintaining the initial 20% for the first 0.3 min, then increasing linearly to 70% over 3.0 min, up to 100% by 3.2 min and held there until 4.0 min, before returning to 20% by 4.1 min. The column eluent was directed into a Waters Quattro Premier XE triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The mass spectrometer was set to monitor AZD7451 (m/z 383.5→340.5) and the internal standard [²H₆]-AZD7451 (m/z 389.6→342.0) using multiple reaction monitoring (MRM) in the positive ion mode. Universal mass spectrometric settings included capillary voltage of 500 V, cone voltage of 45 V, extractor voltage 7 V, RF Lens 1.0, source temperature of 120 °C, desolvation temperature 450 °C, cone gas flow 100 L/hr, desolvation gas flow 800 L/hr, collision energy of 13, and dwell times of 150 msec. MRM peak integrations and data analyses were performed using the QuanLynx program in MassLynx 4.1 (Waters Corp, Milford, MA).

2.5. Validation

2.5.1. Linearity—Calibration curves for AZD7451 were constructed by least-squares linear regression analysis of a nine-point calibration curve (0.5–1000 ng/mL) by plotting the ratio of the analyte peak area versus the internal standard peak area, using $1/x^2$ as a weighting factor, where x is the ratio of the nominal analyte:IS concentration. Calibrator response functions and choice of regression analysis were investigated by calculating correlation coefficients (r) and the percent deviation (% DEV) for all calibrators.

2.5.2. Accuracy and precision—Accuracy and precision were evaluated by determining AZD7451 at five different concentrations of QC samples (lower limit of quantitation (LLOQ), low-range, mid-range, high-range, 10-fold dilution), in five replicates analyzed over four different days. Each run consisted of blank plasma samples, internal standard only, and calibration standards in duplicate; QC and LLOQ samples in replicates of five, with the LLOQ prepared independently in five different lots of plasma each of the four days ($n=20$). Accuracy (% DEV) was defined as the percent difference between the mean observed concentration and the nominal concentration. The repeatability of the assay was determined by the within-run precision (WRP) and between-run (BRP), as calculated below.

$$WRP = 100 \times [(MS_{WIT})^{0.5}/GM]$$

$$BRP = 100 \times [(\{MS_{BET}-MS_{WIT}\}/n)^{0.5}/GM]$$

GM represents the grand mean, MS_{wit} represents within-group mean squared, MS_{bet} represents between-group mean squared, and n represents the number of repetitions. FDA guidelines for bioanalytical validation were followed, with $\pm 15\%$ variability in accuracy and precision allowed except for the LLOQ, where $\pm 20\%$ variability is acceptable [10].

2.5.3. Stability—The stability of AZD7451 in plasma at room temperature was assessed over a 24-hr period. Samples at three concentrations (1.5, 400, 800 ng/mL) were either

extracted immediately (fresh) or kept at room temperature in plasma for 24 hours before extraction, each in triplicate. The analyte concentration after 24 hours was compared to the concentration of freshly prepared samples in the same analytical run.

Stability tests were performed to examine the potential for degradation of AZD7451 in plasma during freeze/thaw cycles. Samples were assayed at three concentrations (1.5, 400, 800 ng/mL) in triplicate. The samples were subjected to four freeze/thaw cycles at -80°C , with each freeze cycle lasting at least 12 hr. The analyte concentration after each storage period was compared to the concentration of freshly prepared samples in the same analytical run.

The post-preparative stability of AZD7451 and $[\text{}^2\text{H}_6]$ -AZD7451 in the injection vials pending analysis in the autosampler was assessed. Samples were re-injected and re-analyzed 24 hr after the initial analysis and compared to the original values obtained from those same samples prepared 24 hr prior.

Long-term stability of AZD7451 in methanol at $^{\text{TM}}80^{\circ}\text{C}$ was assessed at 2 months and 16 months, compared to freshly prepared solutions.

2.5.4. Extraction Recovery and Matrix Effects—The extraction efficiency, or recovery, of the liquid-liquid extraction was assessed by comparing analyte peak areas from both a low (1 ng/mL; $n=5$) and high (800 ng/mL; $n=5$) concentration, both before and after the addition of the liquid extraction solvent. For each set, both concentrations (low and high) were assayed in quintuplet by spiking analyte into five different lots of plasma to further demonstrate selective and matrix effects.

Matrix effects from the plasma on the mass spectrometric signals for AZD7451 and the internal standard $[\text{}^2\text{H}_6]$ -AZD7451 were assessed through direct comparison of samples spiked in plasma to samples spiked in neat solution (reconstitution solution). AZD7451 and $[\text{}^2\text{H}_6]$ -AZD7451 peak areas were first analyzed using a low (1 ng/mL; $n=3$) and a high (800 ng/mL; $n=3$) calibrator sample spiked into the reconstitution solution (50/50, v/v; ACN/ H_2O). These values were used for comparison to the same concentrations by spiking drug into each of 5 plasma lots after extraction (to avoid discrepancies from extraction efficiency).

2.6. Clinical Application

To demonstrate the applicability of this method for measuring AZD7451 clinically, escalating oral doses were administered either once daily (QD) or twice daily (BID). For this demonstration, the plasma concentration vs time data was collected on three patients on their first oral dose (12 mg) of AZD7451. This was the first clinical administration of this compound, thus the pharmacokinetics were based on unpublished preclinical studies. The National Cancer Institute Institutional Review Board approved this clinical protocol.

3. Results and Discussion

3.1. Selectivity

Figure 2 depicts LC-MS/MS chromatograms of a drug-free plasma extract (Fig 2A), the internal standard only (Fig 2B), the LLOQ at 0.5 ng/mL (Fig 2C), and a clinical pharmacokinetic sample taken 15 min following a single 20-mg oral dose (Fig 2D). The retention times of AZD7451 and its deuterated analog, $[\text{}^2\text{H}_6]$ -AZD7451, were 1.45 and 1.43 min, respectively. The use of ultra-high performance liquid chromatography (uHPLC) provided sharp, symmetrical peaks that were well-resolved and separated from interfering matrix peaks that were eluted following an organic solvent gradient to cleanse the column.

3.2. Validation

The calibration standards, ranging from 0.5–1000 ng/mL, were run in duplicate on each of four days (n=8). The nine calibration standards proved accurate and precise (Table 1), with a mean ($\pm$ SD) calibration curve linear correlation (r^2) of 0.9929 ± 0.0019 (n=4). Four quality control (QC) samples, at low (1.5 ng/mL), mid (400 ng/mL), high (800 ng/mL), and 10-fold diluted (10,000 ng/mL) concentrations, plus a LLOQ QC were run in quintuplet daily over four days (n=20). All QC levels proved accurate and precise, within the required 15% (20% for LLOQ samples) (Table 2).

3.3. Stability

AZD7451 demonstrated sufficient bench-top stability in plasma at room temperature through 24 hrs, with <10% difference in measured concentrations from freshly extracted samples (Table 3). Freeze/thaw stability was demonstrated through four freeze/thaw cycles in human plasma, where changes in measured values at each of the three concentrations after each freeze/thaw cycle were well below 10%, indicating no significant degradation (Table 4). The 24-hour post-preparative stability measurements were consistent with the initial run (<10% change), allowing samples extracted from human plasma to be reanalyzed on the following day when necessary without significant degradation (Table 5). Long-term freezer stability in methanol demonstrated no significant (<15%) decrease at either 2 months or 16 months.

3.4. Extraction Recovery and Matrix Effects

The mean overall recoveries for AZD7451, estimated by comparing the mass spectrometric signal response of the analyte spiked into 5 different lots of human plasma before extraction versus analyte spiked after extraction at both a low (1 ng/mL; n=5) and a high (800 ng/mL; n=5) concentration, were approximately 85.0% and 80.1%, respectively. Comparison of analyte peak areas between post-extraction plasma and mobile phase resulted in 50.2% and 57.8% signal reduction from plasma matrix effects (compared to clean solution) for the low and high concentrations, respectively. Negligible matrix effects were observed via post-column infusion of analyte and injection of blank plasma extracts.

3.5. Clinical application

As shown in Figure 3, the average AZD7451 plasma concentration vs. time curves for patients receiving once daily oral doses of AZD7451 was determined in human plasma. These initial results suggest the analytical methods and assays developed here are sufficient to detect AZD7451 plasma concentrations in clinical trials with a sensitivity of 0.5 ng/mL and should allow further investigation of AZD7451 plasma concentrations as part of the Phase I clinical pharmacokinetic study. The clinical results presented here demonstrate the applicability of this assay; a pharmacokinetic analysis of the drug will be published separately after the completion of the phase I trial.

Conclusions

This paper describes the first analytical methods associated with quantitative detection of AZD7451, a novel, potent inhibitor of the Trk family of kinase receptors, currently undergoing early clinical testing in glioblastoma at the NCI. This method proved sensitive (LLOQ 0.5 ng/mL), selective, accurate, precise, and efficient at recovering drug (~80%) from human plasma. AZD7451 was found to be stable in plasma through 4 freeze/thaw cycles, at room temperature for 24 hours, and demonstrated 24 hr post-preparative stability at 4 °C. This novel assay is currently being applied to the first in human clinical study

associated with oral administration of AZD7451 following once or twice daily dose escalation.

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Abbreviations

uHPLC	Ultra-high performance liquid chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
QC	Quality control
LLOQ	Lower limit of quantification

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Highlights

We developed and validated a novel uHPLC-MS/MS assay for the quantification of AZD7451. >This method is accurate, precise, and uses a simple liquid extraction procedure that recovers over 80% of drug from human plasma. >AZD7451 demonstrated excellent freeze/thaw, plasma, and post-preparative stability. >Successfully applied to a first-in-humans clinical pharmacokinetic study.

AZD7451

[²H₆]-AZD7451

Figure 1.
Structures of AZD7451 and the internal standard ²[H₆]-AZD7451.

Figure 2.

Chromatograms of A) a blank plasma extract, B) internal standard only extract, C) the lower limit of quantification (LLOQ), and D) a clinical pharmacokinetic sample measured 15 min following a 20-mg oral dose.

Figure 3 Mean (n=3) Plasma Concentration vs Time Curve

Figure 3.
AZD7451 plasma concentration vs time curve.

Table 1

Calibration Linearity

Nominal (ng/ml)	GM (ng/ml)	SD (ng/ml)	DEV (%)	CV (%)	n
0.5	0.46	0.07	-7.50	16.1	8
1.0	1.03	0.10	2.50	10.1	8
2.0	2.11	0.15	5.62	6.90	8
10	10.5	0.61	4.75	5.81	8
50	50.9	1.52	1.75	2.99	8
100	102	4.51	2.06	4.42	8
500	481	12.0	-3.64	2.49	8
800	745	30.0	-6.82	4.03	8
1000	966	30.0	-3.78	3.12	8

Abbreviations: GM, grand mean; SD, standard deviation; DEV (%), relative deviation from nominal value; CV (%), coefficient of variation; *n*, number of replicate observations within each validation run, i.e. two samples at each concentration were run on four separate occasions, for a total (*n*) of eight samples at each concentration.

Table 2

Accuracy and Precision

Nominal (ng/ml)	GM (ng/ml)	SD (ng/ml)	DEV (%)	WRP (%)	BRP (%)	n
0.5 (LLOQ)	0.48	0.09	-3.16	15.8	10.7	20
1.5 (LQC)	1.63	0.12	8.33	5.33	5.84	20
400 (MQC)	389	13.8	-2.76	3.13	1.90	20
800 (HQC)	780	26.9	-2.45	3.45	0.23	20
10,000 (DQC)	9794	435	-2.05	4.44	0.23	20

Abbreviations: GM, grand mean; SD, standard deviation; DEV (%) relative deviation from nominal value; WRP, within-run precision; BRP, number of replicate observations within each validation run.

Table 3

Plasma Stability at Room Temperature

	Immediately after preparation	After 24 hours	Mean Change after 24h (%)
Nominal (ng/ml)	GM (ng/ml)	GM (ng/ml)	
1.5 (LQC)	1.56	1.45	−6.81
400 (MQC)	381	368	−3.38
800 (HQC)	769	733	−4.78

Abbreviations: GM, grand mean

Table 4

Freeze/Thaw Stability in Plasma

Freeze/Thaw Cycles	1.5 ng/mL (LQC)		400 ng/mL (MQC)		800 ng/mL (HQC)	
	GM (ng/ml)	DEV from fresh (%)	GM (ng/ml)	DEV from fresh (%)	GM (ng/ml)	DEV from fresh (%)
0 (Fresh)	1.58	-	373	-	748	-
1	1.50	-5.19	395	6.00	759	1.52
2	1.58	0.28	386	3.52	764	2.17
3	1.69	7.03	388	4.10	736	-1.63
4	1.51	-4.31	385	3.44	760	1.67

Abbreviations: GM, grand mean; DEV (%) relative deviation from nominal value

Table 5

Post-Preparative Stability

	Immediately after preparation	After 24 hours	Mean Change after 24h (%)
Nominal (ng/ml)	GM (ng/ml)	GM (ng/ml)	
0.5 (LLOQ)	0.40	0.43	6.84
1.5 (LQC)	1.56	1.67	7.03
400 (MQC)	381	386	1.36
800 (HQC)	770	763	−0.91
10,000 (DQC)	9765	9834	0.70

Abbreviations: GM, grand mean