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LC-MS/MS assay for the simultaneous quantitation of the ATM inhibitor AZ31 and the ATR inhibitor AZD6738 in mouse plasma

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Abstract

The ATM kinase inhibitor AZ31 and ATR kinase inhibitor AZD6738 are in various phases of preclinical and clinical evaluation for their ability to potentiate chemoradiation. To support the preclinical evaluation of their pharmacokinetics, we developed and validated an LC-MS/MS assay for the simultaneous quantification of AZ31 and AZD6738 in mouse plasma. A “dilute and shoot” method was used to precipitate proteins from a sample volume of 50 μ L. Chromatographic separation was achieved using a Phenomenex Polar-RP column and a gradient mobile phase consisting of methanol-water with 0.1% formic acid. Detection was accomplished using a Waters Quattro Micro mass spectrometer in positive ionization mode. The assay utilizing 50 μ L sample was linear from 10–5,000 ng/mL and determined to be both accurate (–8.2 to 8.6%) and precise (<5.4% CV) and achieved the criteria for U.S. FDA guidance for bioanalytical method validation. Quantification was achieved in mouse tissue homogenate using a separate 200 μ L sample preparation. This LC-MS/MS assay will be essential for determining the tissue distribution and pharmacokinetics in future mouse studies.

Keywords

AZ31; AZD6738; ATM; ATR; assay; validation

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1 Introduction

Ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are apical DNA damage signaling serine/threonine kinases that phosphorylate a broad and overlapping catalogue of several thousand substrates [1–3]. ATM kinase activity is increased at DNA double-strand breaks (DSBs) and has been widely studied since individuals with the disease ataxia telangiectasia, who express no ATM protein, are the most radiosensitive humans yet identified [4–6]. Lung cancer cells are radiosensitized by pharmacologic ATM kinase inhibitors in tissue culture [7–9], and it is widely believed that ATM kinase inhibitors will be tolerated in the clinic as ATM is not an essential protein [6, 10]. However, ATM kinase inhibition does not phenocopy ATM protein disruption, and while *atm*^{−/−} mice that express no ATM protein are viable, mouse embryos expressing kinase-inactive ATM protein die before embryonic day 9.5–10.5 [11–13].

ATR kinase activity is increased at damaged replication forks and resected DSBs [14]. ATR has been widely studied, but advances have been complicated by the finding that ATR is an essential protein in mice and mammalian cells [15–18]. Overexpression of kinase-inactive ATR increases sensitivity to cisplatin and ionizing radiation (IR) in tissue culture [19, 20]. Consistent with these findings lung cancer cells are sensitized to cisplatin and IR by ATR kinase inhibitors in tissue culture [21–25]. ATR kinase activity is also increased after hypoxia and ATR kinase inhibitors sensitize radiation-resistant hypoxic cells to IR in tissue culture [25–28]. Furthermore, in tissue culture and xenograft models, ATR kinase inhibitors synergize with DNA damaging chemotherapy to kill cancer cells that have mutations that inactivate ERCC1, ATM and XRCC1 [29–31]. Thus, while it is anticipated that ATR kinase inhibitors will generate toxicities in the clinic, acquired mutations and the microenvironment may render certain tumors sensitive to ATR kinase inhibitors in select tumors.

AZ31 and AZD6738 are highly selective, pharmacologic ATP competitors that inhibit ATM and ATR protein kinase activities, respectively. AZ31 is the first selective orally active and bioavailable ATM kinase inhibitor [32]. The ATR kinase inhibitor AZD6738 is also orally active and has been taken into clinical development (ClinicalTrials.gov Identifier: NCT02223923; NCT02630199; NCT01955668; NCT02264678). To support ongoing preclinical studies we have developed and validated an LC-MS/MS assay to quantitate the concentrations of AZ31 and AZD6738 in mouse plasma and performed a limited cross validated for mouse tissues.

2 Experimental

2.1 Chemicals and reagents

AZ31 (ATZ13535704) and AZD6738 were provided by AstraZeneca. Imatinib was purchased from LC Laboratories (Woburn, MA, USA). Methanol and water (all HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Control pooled mouse plasma was purchased from Lampire Biological Lab Inc. (Pipersville, PA, USA). Argon was purchased from Valley National Gases, Inc. (Pittsburgh, PA, USA). The following tyrosine kinase inhibitors were screened for use as internal standard: trametinib, lapatinib, nilotinib,

dabrafenib, imatinib, dasatinib, sorafenib, crizotinib, gefitinib, sunitib, erlotinib, and axitinib.

2.2 Chromatography

The LC system consisted of an Agilent (Palo Alto, CA, USA) 1100 SL autosampler and 1100 Binary Pump, a Phenomenex (Torrance, CA, USA) Polar-RP (4 μ m, 2 mm $\times$ 50 mm) column kept at ambient temperature. Mobile phase solvent A was 0.1% (v/v) formic acid in methanol and mobile phase solvent B was 0.1% (v/v) formic acid in water. Using a gradient method, the initial mobile phase composition was 35% solvent A pumped at a rate of 0.3 mL/min and increased linearly to 95% over 3.5 min where it was held until 7.0 min. Between 7.0 and 7.01 min, the percentage of solvent A was decreased to 35% and the flow rate was increased to 0.5 mL/min. These conditions were held constant until 10 min, followed by injection of the next sample. The total run time was 10 min.

2.3 Mass spectrometry

A solvent switch added to the method diverted LC flow to waste between 0–3 minutes and again between 6 to 10 min. Mass spectrometric detection was carried out using a Waters (Milford, MA, USA) Quattro Micro triple-stage, benchtop quadrupole mass spectrometer with electrospray ionization in positive-mode, multiple reaction monitoring (MRM) mode. The settings of the mass spectrometer were as follows: capillary voltage 3.0 kV; cone voltage 30.0 V; source temperature 120 °C; and desolvation temperature 400 °C. The cone and desolvation gas flows were 50 and 550 L/h, respectively. The collision voltage was 30 V. Quadrupoles 1 and 3 each had low mass and high mass resolution set at 12.0. The dwell time was 0.20 s, and the interscan delay was 0.02 s for AZ31 and AZD6738 and 0.10 s for imatinib. The MRM m/z transitions monitored were: m/z 413.0>332.0 for AZD6738; m/z 421.0>309.0 for AZ31; and m/z 494.4>394.2 for imatinib. The LC system and mass spectrometer were controlled by Waters MassLynx software (version 4.0), and data were collected with the same software.

2.4 Preparation of calibration standards and quality control samples

Stock solutions of analytes AZ31, AZD6738, and internal standard imatinib were prepared independently at 1 mg/mL in DMSO and stored at –80 °C. A combined 0.1 mg/mL stock solution of AZ31 and AZD6738 was prepared in methanol. On the day of analysis, the 0.1 mg/mL mix stock solution was serially diluted (in steps of 10-fold) in methanol to obtain the lower calibration working solutions. These calibration working solutions were diluted in mouse plasma to produce the following analyte concentrations: 10, 30, 100, 300, 1000, 3000, 5000 ng/mL. For each calibration series, zero and blank samples were also prepared.

Quality control (QC) stock solutions were prepared independently from separate weighings and stored at –80 °C. These solutions were diluted in mouse plasma to produce the following QC samples of either: QC Low (QCL) 25 ng/mL; QC Mid (QCM) 250 ng/mL, and QC High (QCH) 4,000 ng/mL.

2.5 Plasma sample preparation

50 µL of each standard, QC, or sample were pipetted into separate microcentrifuge tubes. 10 µL of 5 µg/mL internal standard (imatinib) working stock was added to each tube followed by addition of 500 µL of ice-cold methanol. Samples were vortexed for 1 min on a Vortex Genie-2 set at 10 (Model G-560 Scientific Industries, Bohemia, NY, USA) and then centrifuged at $13,000 \times g$ at 5 °C for 5 min. 100 µL of supernatant was then transferred to autosampler vials, followed by injection of 10 µL into the LC-MS/MS system.

2.6 Tissue sample preparation

200 µL of each standard, QC, or sample were pipetted into separate microcentrifuge tubes. 10 µL of 5 µg/mL internal standard (imatinib) working stock was added to each tube followed by addition of 500 µL of ice-cold methanol. Samples were vortexed for 1 min on a Vortex Genie-2 set at 10 (Model G-560 Scientific Industries, Bohemia, NY, USA) and then centrifuged at $13,000 \times g$ at 5 °C for 10 min. 200 µL of the resulting supernatants was transferred to new microcentrifuge tubes and placed in a refrigerator at 5 °C overnight. The following day, samples were centrifuged at $13,000 \times g$ at 5 °C for 20 min. 100 µL of supernatant was then transferred to autosampler vials, followed by injection of 1 µL into the LC-MS/MS system

2.7 Validation procedures

2.7.1 Calibration curve and lower limit of quantitation (LLOQ)—Decreasing concentrations of analytes were injected into the analytical system to determine the minimal concentration with a signal-to-noise ratio of at least 5:1. Calibration standards and blanks were prepared (see paragraph 2.4 and 2.5) and analyzed in triplicate to establish the calibration range with acceptable accuracy and precision. The analyte-to-internal standard ratio (response) was calculated for each sample by dividing the area of the analyte peak by the area of the internal standard peak. Standard curves were constructed by plotting the analyte-to-internal standard ratio *versus* the known concentrations in each sample. Standard curves were fitted by linear regression with weighting by $1/y^2$, followed by back-calculation of concentrations. The deviations of these back-calculated concentrations from the nominal concentrations were expressed as percentage of the nominal concentration.

2.7.2 Accuracy and precision—The accuracy and precision of the assay were determined by analyzing samples at the LLOQ, QCL, QCM, and QCH concentrations in 6 replicates each in 3 analytical runs, in addition to independently prepared, triplicate calibration curves. Accuracy was expressed as bias and was calculated at each test concentration as: $(\text{measured concentration} - \text{nominal concentration}) / \text{nominal concentration} \times 100\%$.

Assay precision was calculated by ANOVA as previously described [33], by using SPSS 23 for Windows (SPSS Inc., Chicago, IL, USA). Back-calculated concentrations of calibration and QC samples were entered with the run number as factor. From the resulting mean squares of the within runs and mean squares of the between runs, the intra-assay and inter-assay precisions were calculated.

2.7.3 Selectivity and specificity—To investigate whether endogenous matrix constituents interfered with the assay, six individual batches of control, drug-free heparinized mouse plasma were processed and analyzed according to the described procedure. Responses of analytes at the LLOQ concentrations were compared with the response of the blank samples. Cross-talk of each analyte was characterized by detection on other MRM channels.

2.7.4 Extraction recovery and matrix effect—We determined the extraction recoveries of AZ31 and AZD6738 from plasma by comparing the absolute response of an extract of control plasma, to which these analytes had been added after extraction, with the absolute response of an extract of plasma to which the same amounts had been added before extraction. The matrix effect was defined as the change in the absolute response of an extract of control plasma to which analyte had been added after the extraction relative to the absolute response of reconstitution solvent to which the same amount of each respective analytes had been added. Experiments were performed at the three QC concentrations, in quadruplicate.

2.7.5 Stability—Long-term stability experiments were performed in plasma and in stock solution after storage at -80°C . Stability in the stock solution was expressed as the percentage recovery of the stored solution (20 months) relative to a freshly prepared solution. The stabilities of AZ31 and AZD6738 in plasma at 80°C were determined by assaying samples before and after storage. In addition, the stabilities of AZ31 and AZD6738 in stock solution at room temperature for 4 h were determined in triplicate. All stability testing in plasma was performed in triplicate at the QCL, QCM and QCH concentrations. The effect of 3 freeze/thaw cycles on analyte concentrations in plasma was evaluated by assaying samples after they had been frozen (-80°C) and thawed on 3 separate days and comparing the results with those of freshly prepared samples. The stabilities of AZ31 and AZD6738 in plasma during sample preparation were evaluated by assaying samples before and after 4 h of storage at room temperature. To evaluate the stabilities of AZ31 and AZD6738 in prepared samples in the autosampler, QC samples and calibration curves were reinjected approximately 72 h after the first injection and compared the concentrations derived from the second injection with those derived from the first injection. The results of the second runs were expressed as a percentage of their respective values in the first runs.

2.7.6 Dilutional Integrity—To demonstrate dilutional integrity, the ability to dilute samples from above the upper limit of quantitation to within the validated concentration range, plasma samples containing AZ31 and AZD6738 above the upper limit of quantitation were diluted to within the assay range. Plasma samples with analyte concentrations of 10,000 ng/mL were diluted 100-fold (to 100 ng/mL) with control plasma and assayed in triplicate.

2.7.7 Anti-coagulantia cross validation—To demonstrate the ability of our heparin plasma based assay to quantitate EDTA plasma samples, QCL, QCM, and QCH samples prepared in heparin plasma were quantitated against a duplicate EDTA plasma calibration curve in quadruplicate.

2.7.8 Tissue homogenate limited cross validation—To investigate the validity of the method in different matrices, an array of tissues were collected from untreated male C57BL/6 mice. The following tissues were harvested: heart, lung, liver, spleen, small intestine (jejunum), colon, kidney, muscle, and brain. Tissues were homogenized using 1 part tissue (g) to 3 parts PBS (mL). Homogenates were then spiked at the QCM level and processed using 200 μ L of homogenate (see paragraph 2.6). Calibration curves and QCs were prepared using 200 μ L of control mouse plasma.

2.8 Application of the assay

To demonstrate the application of the assay, samples obtained from mice receiving a 1:1 mix of AZ31 and AZD6738 were analysed. The AZ31 was made up with 10% DMSO + 90% Captisol (made up at 30% w/v in de-ionized sterile water). The AZD6738 was made up in 10% DMSO + 40% propylene glycol + 50% de-ionized sterile water. Four female C57BL/6 mice were dosed orally with this 1:1 mixture at 10 μ L/g. The effective doses received for the AZ31 and AZD6738 were 50 mg/kg and 37.5 mg/kg respectively. After 45 min, mice were euthanized using CO₂ inhalation. Plasma was obtained by collecting blood using cardiac puncture and transferred to EDTA lined Greiner (Monroe, NC) MiniCollect® tubes and centrifuged at 2,000 $\times$ g for 10,000 min to separate plasma and red blood cells. The following tissues were collected: brain, heart, lung, liver, spleen, kidney, small intestine (flushed with PBS), colon (flushed with PBS), muscle, and fat. Tissues and plasma were immediately stored at –80 °C upon collection.

Prior to analysis, tissues were homogenized as detailed above. The samples were then processed and, when needed, diluted with control plasma to fall within the calibration range.

3 Results and Discussion

3.1 Validation of the assay

3.1.1 Chromatography—The approximate retention times of each compound were as follows: AZ31 at 3.7 min, AZD6738 at 4.7 min, and imatinib at 4.2 min with corresponding capacity factors of 2.7, 3.7 and 3.2 respectively, and a void time 1.0 min. Representative chromatograms of each compound (at the LLOQ), and internal standards in plasma are displayed in Fig. S. 1.

3.1.2 Calibration curve and LLOQ—The selected assay range of 10–5,000 ng/mL fulfilled the FDA criteria [34] for the LLOQ concentration and the calibration curve. Accuracies and precisions at the different concentrations from triplicate calibration curves on 3 separate days are reported in Table S 1. Representative calibration curves and corresponding correlation and regression coefficient are shown in Fig. S. 2.

3.1.3 Accuracy and precision—The range of QC based accuracies was –7.0 to 4.6% for AZ31 and –0.6 to 8.6% for AZD6738. The intra- and inter-assay precisions for the tested concentrations (LLOQ, QCL, QCM, QCH) were all within the defined acceptance criteria (Table S 2) [34].

3.1.4 Selectivity and specificity—Chromatograms of six individual control plasma samples contained no co-eluting peaks >20% of the analyte areas at the LLOQ concentration. Mean interference relative to the LLOQ for AZ31 and AZD6738 was 6.7 % and 6.9%, respectively.

Cross-talk calculations were performed and revealed that cross talk for all analytes into other channels are <0.1%, while they did not co-elute, indicating cross talk does not appear to be a major issue with any of the analytes.

3.1.5 Extraction recovery and matrix effect—The recoveries of AZ31 and AZD6738 were approximately 90 and 115%, respectively, with CVs between 1.8 and 9.4%. Matrix effects for AZ31 and AZD6738 were approximately 15 (i.e. ionization enhancement) and –9% (i.e. ionization suppression), respectively, with CVs between 1.4 and 9.6% (Table S 3).

3.1.6 Stability—The stabilities of AZ31 and AZD6738 stock solutions at room temperature for 4 h were 100.6% and 100.2% respectively (Table 1). Stabilities of stock solutions for 20 months at –80 °C were 104.2 and 96.4% for AZ31 and AZD6738 respectively. The stability in plasma after 3 freeze/thaw cycles (–80 °C to RT) at QC levels were between 99.6 to 105.9% for AZ31 and 97.9 to 103.7% for AZD6738. Long-term stabilities of the analytes in plasma at –80 °C were adequate with recoveries between 90.7 and 111.7. The absolute responses of plasma extracts of analytes at QC concentrations, when reconstituted and kept in the autosampler for 72 h, ranged from 90.4 to 96.5% (CV 4.3–12.9%). The relative response (analyte to internal standard ratio) ranged from 100.5 to 104.9% (CV 5.4–10.3%). Importantly, the reinjection run passed the requirements of any run set by the FDA guidance [34].

3.1.7 Dilutional Integrity—The samples diluted 100-fold from 10,000 ng/mL to 100 ng/mL displayed accuracies of 12.4% (CV 1.2%) for AZ31 and 14.4% (CV 0.3%) for AZD6738, indicating dilutional integrity for both analytes.

3.1.8 Anti-coagulantia cross validation—Accuracy and precision of back-calculated concentrations at QCL, QCM, and QCH were within 15%. Results are provided in Table S 4.

3.1.9 Tissue homogenate limited cross validation—Recovery of the analytes in various tissue homogenates analysed against a plasma calibration curve ranged from 90.1 to 109.1% (CV 2.8–7.7%) for AZ31 and recovery from 86.1 to 103.2% (CV 1.1–9.9%) for AZD6738 (Table 2).

3.2 Method Development

The method development of the assay included multiple variations in column type, extraction solvents, HPLC gradients, and strategies to select an optimum internal standard.

3.2.1 Extraction and sample preparation—To expedite sample preparation, a “dilute and shoot” direct injection method was used by diluting 200 µL of sample with 500 µL of ice-cold methanol to precipitate proteins. Methanol was chosen as the extraction solvent for

compatibility with the mobile phase and ionization optimization. Methanol also yielded similar extraction recoveries between analytes.

The resulting mixture was vortexed, centrifuged and 100 μ L of sample's supernatant transferred to an HPLC vial for injection. This method proved to lack the amount of extraction solvent necessary for complete protein precipitation. Therefore, after the protein precipitation, vortexing, and centrifugation, 200 μ L of the resulting supernatant was transferred to separate microcentrifuge tubes and placed in a refrigerator set at 5 $^{\circ}$ C overnight. The following day, samples were centrifuged again at $13,000 \times g$ at 5 $^{\circ}$ C for 20 min. This additional step resulted in more complete protein precipitation and the resulting supernatant was transferred to autosampler vials for injection. This method was fully validated and met FDA guidance criteria (data not shown). However, we separately attempted to increase the proportion of methanol by decreasing the sample volume to 50 μ L during extraction, at the risk of having too high a percentage of organic solvent and potentially jeopardizing compatibility with initial HPLC conditions. This sample preparation allowed direct injection of the supernatant without the need for overnight incubation and proved successful for plasma sample analysis but application to tissue homogenate failed acceptance criteria because of tissue specific systemic bias. For example, liver homogenate at all QC levels were approximately -25% compared to plasma for both AZ31 and AZD6738. AZD6738 appeared the most sensitive to tissue specific bias with liver, spleen, lung and small intestine homogenate all falling below approximately -20% . Therefore, we were required to apply the more laborious approach for tissue homogenate processing and analysis. This simplified plasma assay was also fully validated and met FDA guidance.

The matrix effect of various tissue homogenates likely explains why the 50 μ L sample preparation failed acceptance for tissue quantitation. There was a much larger plasma matrix effect in the 50 μ L sample preparation compared to the 200 μ L sample preparation. This is especially true for AZ31 where the 200 μ L sample preparation almost completely negated any matrix effect. Although having two sample preparations is less than ideal, complete plasma collection from mice rarely yields more than 500 μ L, and the 50 μ L sample preparation allows for a more practical method of quantifying these smaller available volumes. Smaller collection volumes are less of a concern for tissue homogenate which in our case represents a 3:1 dilution of tissue on PBS.

3.2.2 Internal Standard Selection—No isotopic internal standard was available for either AZ31 or AZD6738, so a series of experiments were used to select an ideal internal standard to be used for both compounds. We originally intended to have AZ31, AZD6738 and internal standard co-elute, but none of the three columns, and no chromatographic method, was able to achieve co-elution of AZ31 and AZD6738. To minimize potential ionization suppression and matrix effects, we aimed to identify an internal standard which eluted between AZ31 and AZD6738 with equidistant baseline separation. Twelve tyrosine kinases inhibitors (TKis) were each analysed for potential use as an internal standard by monitoring their mass transitions in an MRM method and subjecting them to 8 separate 15 min HPLC methods on three reverse phase column types. The following TKis were analysed: trametinib, lapatinib, nilotinib, dabrafenib, imatinib, dasatinib, sorafenib, crizotinib, gefitinib, sunitib, erlotinib, and axitinib. A neat sample was prepared containing a

mixture of AZ31, AZD6738, and all potential internal standards at identical concentrations, and the sample was repeatedly injected under varying flow types and columns. Dasatinib, imatinib and erlotinib all demonstrated the desired elution between AZ31 and AZD6738, and imatinib demonstrated the most equidistant relationship between AZ31 and AZD6738 using a gradient HPLC method. Based on these results imatinib was chosen as the optimal internal standard.

3.2.3 Chromatography—We initially evaluated chromatography using following three columns: Phenomenex Luna Phenyl-Hexyl 100A (100 × 2.0 mm, 3 μm), Synergi polar RP 80A (100 × 2.0 mm, 4 μm), Phenomenex Hydro RP 80A (100 × 2.0 mm, 4 μm). None of these columns were able to achieve our original goal of co-elution.

8 separate HPLC flow methods were analysed on each column type to prioritize the selection of an internal standard, as well as optimizing AZ31 and AZD6738 chromatography. Each method used methanol with 0.1% formic acid and water with 0.1% formic acid either as a gradient or isocratically, a flow rate of 0.3 mL/min, and a run time of 15 min. Methanol was chosen as the organic solvent based on its ionization efficiency determined during MS tuning. The optimal HPLC method was a gradient elution that consistently held at 0.3 mL/min and began with 40% organic that was increased to 95% over the course of 3.5 minutes. This composition was held constant for 5 minutes during which all analytes eluted. After this period the mobile phase composition returned to initial conditions and equilibrated for 6.5 minutes.

Each of the three columns resulted in separation of AZ31, AZD6738, and the internal standard so that the minimal cross-talk between MRM channels was not relevant to quantitation. The Hydro RP column allowed insufficient retention of AZ31 with elution occurring soon after the void time. The Luna Phenyl-Hexyl resulted extensive separation. The 100 mm Polar RP achieved baseline separation of AZ31 and AZD6738 as well as allowing the internal standard to elute with equidistant baseline separation between them. To shorten the total run time, a shorter Synergi polar RP 80A (50 × 2.0 mm, 4 μm) was used and found to allow the same characteristic chromatogram, but with a shorter run time of 10 min. Chromatography of the 200 μL sample preparation method gave identical retention times and peak shapes.

Carry-over was assessed by injecting 50,000 ng/mL of AZ31, AZD6738, and IS, followed by injection of 4 control plasma samples. Carry-over amounted to less than 0.5%.

3.2.4 Mass Spectrometry—AZ31 and AZD6738 were tuned on separately using 10 μg/mL, in either a solution of acetonitrile or methanol, to compare and optimize ionization efficiency as well as monitor fragmentation. Methanol achieved slightly higher Q1 signals, sufficient fragmentation, and higher Q3 signals compared to acetonitrile and was chosen as the organic solvent moving forward.

While analysing tissue homogenate samples using the 200 μL sample preparation, it was observed that injecting more than 1 μL injection led to detector saturation resulting in the upper limit of the calibration curve tipping over. Maintaining a 1 μL injection achieved a

linear analyte to internal standard response ratio up to 5000 ng/mL while also allowing the sensitivity to detect the lower limit of 10 ng/mL.

AZ31 and AZD6738 responses remained consistent during analysis but the internal standard imatinib signal would often display increasing sensitivity for the first 5 injections, at which point the signal would plateau and remain constant for the remainder of analysis. To allow for this, a set of 5 dummy injections, containing the working concentration of imatinib, were injected prior to the first calibration curve in order to condition the system. This allowed for consistent imatinib response for the duration of sample analysis.

3.3 Application of the assay

The tissues collected 45 min after simultaneous oral dosing of AZ31 (37.5 mg/kg) and AZD6738 (50 mg/kg) were brain, heart, lung, liver, spleen, kidney, small intestine, colon, muscle, fat, and plasma. Analyte concentrations quantitated in these tissues are depicted in Figure 1. Appropriate dilutions placed all samples within the calibration range and no samples fell below the LLOQ. Both AZ31 and AZD6738 distributed similarly between tissues. The oral administration resulted in high small intestine concentrations as well relatively high concentrations in highly perfused tissues such as liver and kidney. Both compounds had concentrations above the LLOQ in the brain and skeletal muscle, demonstrating distribution across the blood brain barrier and poorly perfused tissues, respectively.

4 Conclusion

The objective of this study was to develop and validate an analytical method for the simultaneous quantitation of AZ31 and AZD6738 in mouse plasma. This was accomplished using reversed phase chromatography for separation with tandem quadrupole mass spectrometric MRM detection. A separate sample preparation was used to successfully quantitate the compounds in tissue homogenates.

Employing LC-MS/MS allowed for quantitation of AZ31 and AZD6738 with a run time of 10 min across a concentration range of 10–5000 ng/mL. This allowed for the evaluation of AZ31 and AZD6738 distribution in mouse plasma and tissues. To our knowledge, this is the first assay published to date to simultaneously quantitate AZ31 and AZD6738 that is validated according to FDA guidance [34]. The analytical method presented herein will be a valuable tool in quantitating AZ31 and AZD6738 in mice as these drugs undergo further preclinical and clinical development either as monotherapy or in combination with other anticancer agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Chemical structures of AZ31, AZD6738 and imatinib.

Fig. 2. Concentrations (mean±SD, N=3) of AZ31 (○) and AZD6738 (◇) in murine plasma (ng/mL) and tissues (ng/g), 45 min after oral dosing of 50 mg/kg AZ31 and 37.5 mg/kg AZD6738.

Table 1

Stability of AZ31 and AZD6738 under varying conditions.

Storage condition	Concentration (ng/mL)	Stability (%)	CV (%)	Replicates
AZ31				
Stock Solution Stability				
Bench (RT) 4 h	100,000	100.6	2.2	3
Long Term (-80 °C) 20 months	100,000	104.2	3.1	4
Plasma Stability				
Bench (RT) 4 h	QCL 25	100.3	8.9	3
	QCM 250	105.6	3.6	3
	QCH 4000	99.3	3.6	3
F-T Cycle (3x)				
	QCL 25	105.9	6.9	3
	QCM 250	105.6	3.6	3
	QCH 4000	99.6	3.7	3
Long Term (-80 °C) 3 months				
	QCL 25	100.8	5.4	4
	QCM 250	90.7	2.5	4
	QCH 4000	106.1	1.9	4
Autosampler Stability 72 h				
Absolute Response				
	QCL 25	93.2	12.0	6
	QCM 250	90.4	6.8	6
	QCH 4000	92.9	4.7	6
Ratio				
	QCL 25	104.9	9.6	6
	QCM 250	100.5	6.7	6
	QCH 4000	102.1	5.9	6
AZD6738				
Stock Solution Stability				
Bench (RT) 4 h	100,000	100.2	0.7	3
Long Term (-80 °C) 20 months	100,000	96.4	16.4	4
Plasma Stability				
Bench (RT) 4 h	QCL 25	108.6	16.1	3

Storage condition	Concentration (ng/mL)	Stability (%)	CV (%)	Replicates
F-T Cycle (3x)	QCM 250	103.7	4.1	3
	QCH 4000	100.4	4.0	3
	QCL 25	97.9	4.8	3
	QCM 250	103.7	2.7	3
	QCH 4000	101.2	3.2	3
	QCL 25	90.7	8.3	4
Long Term (-80 °C) 3 months	QCM 250	93.0	6.2	4
	QCH 4000	111.7	5.7	4
	QCL 25	96.2	12.9	6
	QCM 250	96.3	6.6	6
Autosampler Stability 72 h	QCH 4000	96.5	4.3	6
	QCL 25	101.1	10.3	6
	QCM 250	101.5	6.5	6
	QCH 4000	100.7	5.4	6

Table 2

Recovery of AZ31 and AZD6738 from various mouse tissue homogenates relative to plasma using the using the 200 µL sample preparation.

Analyte	Tissue	Concentration (ng/mL)	Recovery (%)	CV (%)
AZ31	Heart	25 (QCL)	100.7	4.9
		250 (QCM)	98.2	2.8
		4000 (QCH)	91.5	5.8
	Lung	25 (QCL)	100.5	7.0
		250 (QCM)	94.7	4.5
		4000 (QCH)	90.1	5.0
	Liver	25 (QCL)	104.3	6.1
		250 (QCM)	96.9	5.7
		4000 (QCH)	106.4	4.8
	Spleen	25 (QCL)	99.6	5.5
		250 (QCM)	103.4	4.3
		4000 (QCH)	101.1	6.2
	Small Intestine	25 (QCL)	103.5	6.0
		250 (QCM)	96.6	5.2
		4000 (QCH)	105.9	5.0
	Colon	25 (QCL)	97.2	5.4
		250 (QCM)	99.4	4.4
		4000 (QCH)	96.9	7.1
	Muscle	25 (QCL)	103.0	4.8
		250 (QCM)	98.2	7.7
		4000 (QCH)	93.7	6.4
AZD6738	Brain	25 (QCL)	98.7	4.9
		250 (QCM)	103.4	3.8
		4000 (QCH)	100.3	7.3
	Kidney	25 (QCL)	91.4	6.7
		250 (QCM)	109.1	4.0
		4000 (QCH)	98.9	4.9
	Heart	25 (QCL)	103.0	4.6
		250 (QCM)	100.4	2.1
		4000 (QCH)	99.4	3.4
	Lung	25 (QCL)	90.0	4.9
		250 (QCM)	89.5	1.9
		4000 (QCH)	88.6	1.1
	Liver	25 (QCL)	100.4	6.8
		250 (QCM)	87.9	4.4
		4000 (QCH)	92.9	3.4
	Spleen	25 (QCL)	96.1	4.2

Analyte	Tissue	Concentration (ng/mL)	Recovery (%)	CV (%)
	Small Intestine	250 (QCM)	88.8	4.5
		4000 (QCH)	87.0	5.5
		25 (QCL)	96.5	2.0
	Colon	250 (QCM)	86.1	3.9
		4000 (QCH)	93.4	4.5
		25 (QCL)	103.2	3.6
	Muscle	250 (QCM)	100.8	4.6
		4000 (QCH)	98.7	4.6
		25 (QCL)	94.5	7.5
	Brain	250 (QCM)	91.6	9.9
		4000 (QCH)	88.1	2.1
		25 (QCL)	101.5	5.2
	Kidney	250 (QCM)	101.3	4.2
		4000 (QCH)	97.7	6.0
		25 (QCL)	103.2	4.5
		250 (QCM)	102.2	4.8
		4000 (QCH)	99.9	4.6

N=4.