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Pharmacokinetics and bioavailability of ipatasertib in dog plasma by LC/MS/MS

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Abstract

A rapid, sensitive and reliable liquid chromatography-tandem mass spectrometric method was established to quantify ipatasertib in dog plasma. The dog plasma sample was deproteinated by using acetonitrile with ulixertinib as internal standard followed by separation on a Spursil C_{18}-EP column with a gradient mobile phase comprised of 2 mM ammonium acetate containing 0.1% formic acid and acetonitrile. Positive ion electrospray was used and multiple reactions monitoring transitions were *m/z* 458.2 > 387.2 for ipatasertib and *m/z* 433.1 > 262.1 for the internal standard, respectively. The developed method was validated with a linear range of 0.3-1500 ng/mL, with correlation coefficient > 0.9989. The lower limit of quantification was 0.3 ng/mL. The intra- and inter-day precision ranged from 3.58 to 14.32%, whereas the intra- and inter-day accuracy was in the range of -2.50-13.25%. No carry-over and matrix effects were observed under the current conditions. The extraction recovery was demonstrated to be >85.43%. Ipatasertib was proved to be stable during the storage, processing and determination. The validated assay was further successfully employed to a pharmacokinetic study of ipatasertib after oral and intravenous treatments to dogs. The bioavailability of ipatasertib was determined to be 19.3%.

Keywords: ipatasertib, pharmacokinetics, bioavailability, liquid chromatography tandem mass spectrometry

1. Introduction

AKT (Protein kinase B) is one of the most frequently activated kinases in cancers, which play a key role in a variety of cellular processes such as glucose metabolism, apoptosis, tumor proliferation and cell migration (Tokunaga et al., 2008; Bellacosa, et al., 1995; Manning and Cantley, 2007; Jiang and Liu, 2008). AKT has emerged as a promising target for cancer therapy in recent years and several AKT inhibitors have been developed. Ipatasertib (also known as GDC-0068) is a highly selective, potent, ATP-competitive AKT inhibitor (Blake et al., 2012), which showed strong antitumor effects in a variety of carcinoma including colon cancer (Sun et al., 2018). It has been reported that ipatasertib induced colon cancer cell apoptosis by activating p53 upregulated modulator of apoptosis (PUMA) (Sun et al., 2018). Currently, Ipatasertib is under clinical development for the treatment of solid tumors (Isakoff et al., 2020; Bang et al., 2019).

Though the pharmacological effects of ipatasertib have been extensively studied, investigation on the pharmacokinetic property of this drug is sparsely conducted. Pharmacokinetics refers to the time course of drug concentrations in the body and knowledge of pharmacokinetics aids understanding of drug efficacy, safety and toxicity (He and Wan, 2019; Fan and de Lannoy, 2014). Pharmacokinetic study provides quantitative analysis of absorption, distribution, metabolism and excretion of a drug after administration, which plays a key role not only in drug discovery but also in drug developmental stages. To better elucidate the pharmacokinetic properties of ipatasertib and to support its clinical study, it was necessary to develop and validate a selective and sensitive assay for the determination of ipatasertib. Liquid chromatography combined with electrospray ionization tandem mass

spectrometry (LC/MS/MS) has been viewed as one of powerful techniques for drug monitoring due to its high selectivity and sensitivity (Liu et al.,2011; Zhao et al., 2014). To the best of our knowledge no prior LC/MS/MS base methods were reported for the determination of ipatasertib in biological matrices. In this paper, we described the development and validation of a rapid, sensitive and reliable LC/MS/MS method for the quantification of ipatasertib in dog plasma. Furthermore, the validated LC/MS/MS method was applied to the pharmacokinetic investigation of ipatasertib in dogs after oral and intravenous administration.

2. Materials and methods

2.1. Chemicals and reagents

Ipatasertib and ulixertinib (Internal standard, IS) with purity > 98% were purchased from MedChemExpress (Shanghai, China). Acetonitrile was of HPLC grade and supplied by Fisher Scientific (Fairlawn, NJ, USA). Formic acid was purchased from Fluka BioChemika (Buchs, Switzerland). Double distilled water used for LC/MS/MS analysis was prepared by a Milli-Q system (Millipore, MA, USA). Other chemicals were of analytical grade and commercially available.

2.2. LC/MS/MS conditions

The liquid chromatography performed on a Dionex U3000 LC system (Thermo Fisher Scientific, CA, USA). Chromatographic separation was obtained on a Spursil C_{18}-EP column (2.1 × 50 mm, 3 μm; Dikma Technologies Inc., CA, USA) that was maintained at 40 ^{o}C. Mobile phase consisting of 2 mM ammonium acetate fortified with 0.1% formic acid (A) and acetonitrile (B) was delivered at a flow rate of 0.4 mL/min. The gradient elution procedure

was optimized as follows: 0-0.3 min 20% B, 0.3-1.5 min 20-90% B, 1.5-2 min 90% B and 2-2.5 min 20% B. The injection volume was set at 2 μL.

A Vantage TSQ triple quadrupole tandem mass spectrometer (Thermo Fisher Scientific, CA, USA) connecting to an LC system through an electrospray ionization operated in the positive ion mode was employed for mass spectrometric analysis. The optimized source parameters were as follows: spray voltage 3.0 kV; vaporizer temperature 200 ^{o}C; sheath gas flow rate 40 arb, auxiliary gas flow rate 10 arb; capillary temperature 300 ^{o}C. Quantification was conducted in multiple reactions monitoring (MRM) mode with precursor-to-product transitions at *m/z* 458.2 > 387.2 for ipatasertib and 435.2 > 262.1 for IS. The collision energy was optimized at 32 eV. All operations were controlled by Xcalibur software (version 2.2).

2.3. Preparation of stock solutions, calibration standards and quality control samples

Stock solution of ipatasertib (1.5 mg/mL) was prepared in acetonitrile. Stepwise dilution with acetonitrile was made to obtain the working solutions in the concentration range of 3-15000 ng/mL for calibrators. A solution of internal standard ulixertinib at 1000 ng/mL was prepared by diluting the ulixertinib stock solution (1 mg/mL) with acetonitrile. An aliquot of 5 μL of working solution was spiked into a 1.5-mL tube and then evaporated to dryness by nitrogen blowing. The residue was reconstituted with 50 μL of blank dog plasma to prepare the plasma calibrators in the concentration range of 0.3-1500 ng/mL. The quality control (QC) samples were prepared from a separated stock solution in the same manner to obtain the concentrations of 0.6, 80 and 1200 ng/mL.

2.4. Sample preparation

An aliquot of 5 μL of IS working solution at 1000 ng/mL was spiked into 50 μL of dog

plasma, in 1.5-mL Eppendorf tubes. Each mixture was then vortexed for 1 min. Afterwards, the samples were deproteinated by adding 200 μL of acetonitrile, followed by vortexing of the mixture for 2 min. The denatured protein was removed by centrifugation at 15000 rpm for 10 min. 200 μL of the supernatant was separated and evaporated to dryness by nitrogen gas blowing. The residue was re-dissolved in 100 μL of acetonitrile-water (1:4, v/v) solution. After centrifuging at 15000 rpm for 5 min, 2 μL of the supernatant was injected into LC/MS/MS system for quantification.

2.5. Validation procedures

The method validation was carried out based on the guideline set by the US Food and Drug Administration (US Food and Drug Administration, 2018), including selectivity, carry-over, linearity, lower limit of quantification (LLOQ), precision, accuracy, extraction recovery, matrix effect, incurred sample reanalysis and storage stability. Glucuronidation is one of the metabolic pathways of ipatasertib. The glucuronide conjugates may be degraded into the aglycone in plasma during the storage and analysis, which makes the quantification questionable. Therefore, the incurred plasma samples from pre-experiment were used to confirm the selectivity, short-term and long-term stability during the method validation.

2.5.1. Selectivity

To investigate the selectivity of the method, blank dog plasma, blank dog plasma spiked with analyte at LLOQ and IS and the actual plasma samples that were taken at 2 h after ipatasertib administration were analyzed by the developed LC-MS/MS method. There should be no interference at the retention times of ipatasertib and IS.

2.5.2. Carry-over effect

Carry-over was investigated during the validation by injecting a blank sample after the upper limit of quantification (ULOQ) sample to ensure that carry-over does not affect the determination. The carry-over did not exceed 20% of the LLOQ for ipatasertib and 5% of the IS.

2.5.3. Calibration curve, linearity and LLOQ

Eight non-zero concentrations were employed to determine the linearity (0.3, 1, 10, 50, 100, 500, 1000 and 1500 ng/mL). A weighted ($1/x^2$) least square regression algorithm was used to plot the peak area ratio of the analyte to the IS versus the nominal concentrations. The back-calculated concentrations of calibration standards should be within 85-115% of the nominal concentration at each level. The LLOQ was defined as the lowest concentration of the calibration curve, at which the precision and accuracy should meet the required limits.

2.5.4. Accuracy and precision

The inter-day precision and accuracy were evaluated by sextuple analysis of each QC on three separate days. The intra-day precision and accuracy were evaluated by sextuple analysis of each QC on the same day. The precision expressed as relative standard deviation (RSD) should be within 15%; accuracy expressed as relative error (RE) should be in the range of ±15%.

2.5.5. Extraction recovery and matrix effect

The extraction recovery of ipatasertib at three concentration levels was determined by comparing the peak areas of the ipatasertib from regularly prepared QC samples (A) with those of post-extraction blank plasma spiked at the same corresponding concentrations (B). The matrix effect was evaluated by comparing the peak areas of post-extraction blank plasma

spiked QC samples from six different dogs (B) with those of QC samples prepared in the neat solution (acetonitrile) solutions at the same concentrations (C). If the ratio <85% or >115%, a matrix effect was suggested. The extraction recovery and matrix effect of IS were also determined in the same manner.

2.5.6. Stability

The stability of ipatasertib in dog plasma was determined under different storage conditions in six replicates after storage at -80 ^{o}C for 30 days, after storage at 25 ^{o}C for 12 h and following three freeze-thaw cycles (-80 ^{o}C to 37 ^{o}C). The stability of the processed QCs was evaluated following storage at 4 ^{o}C for 12 h in auto-sampler vials. If the determined values were within an acceptable deviation (±15%) from the nominal concentration, ipatasertib was considered stable.

Incurred samples were obtained at 0.5, 2 and 12 h after oral administration. Each sample was divided into 12 replicates. Four samples were immediately processed and analyzed after collection (T_0 sample). The other samples were analyzed after storage at 25 ^{o}C for 12 h or at -80 ^{o}C for 30 days. The concentration of the stability samples should be within 85-115% of the T_0 samples.

2.6. Pharmacokinetic study

Male beagle dogs (body weight 8-10 kg) were provided by Animal Experimental Center of Zhengzhou University (Zhengzhou, China). Dogs were placed in standard conditions of temperature (25 ± 2 ^{o}C), humidity (55-65%) and specific pathogen-free. They were allowed to acclimatize in the laboratory for five days before experiment with free access to food and water. Before administration, the animals were fasted overnight but water was available. All

the animal experiments were approved by the Ethics Committee of Henan Zhengzhou University (Zhengzhou, China). The dogs were randomly divided into two groups. One group was given 10 mg/kg ipatasertib by intragastric administration and the other group was intravenously administered with 1 mg/kg ipatasertib through tail vein. The blood samples were collected into EDTA-Na_2-containing tubes at 0, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h post-dose. After centrifuging the blood samples at 5000 rpm for 5 min, the resulting plasma samples were immediately separated and then stored at -80 ^{o}C until analysis.

The pharmacokinetic parameters were calculated by using WinNonlin software based on non-compartmental analysis (Version 6.1, Pharsight Corporation, USA), including area under the curve (AUC), maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), half-life ($T_{1/2}$), mean time residence (MRT) and clearance (CL). The oral bioavailability was calculated using the following equation: $F(\%) = (AUC_{oral} \times Dose_{intravenous})/(AUC_{intravenous} \times Dose_{oral}) \times 100\%$.

3. Results and discussion

3.1. LC/MS/MS conditions

The mass spectrometric parameters were initially optimized to achieve the best sensitivity and selectivity. The protonated molecule ion of ipatasertib and IS ($[M+H]^+$) were detected at *m/z* 458.2 and 433.1, respectively, by positive ESI ionization in Q1 scan mode. In product ion scan (shown in **Figure 1**), the most abundant product ion of ipatasertib was observed at *m/z* 387.2, which was formed by the cleavage of isopropylaminomethyl moiety. The most abundant product ion of IS was *m/z* 262.1, which was formed by the breakage of amide. The intensity of precursor-to-product ion transitions of *m/z* 458.2 > 387.2 for ipatasertib and *m/z*

433.1 > 262.1 for IS were found to be the most sensitive and therefore they were selected for MRM transitions. Corporate qualifier transitions were at *m/z* 458.2 > 72.1 for ipatasertib and *m/z* 433.1 > 280.1 for IS.

Given the complex nature of the plasma samples, an efficient pretreatment procedure is often needed to remove the proteins and the potential interferences. Protein precipitation and liquid-liquid extraction procedures were compared in the method development. Ethyl and ethyl acetate were selected as extraction solvents, both of which showed low recovery (< 75%). Protein precipitation was therefore employed for plasma pretreatment. Acetonitrile and methanol were compared and they showed comparable recoveries. Given that acetonitrile was a constituent of the mobile phase, acetonitrile was finally selected as the precipitant..

The chromatographic conditions were optimized to achieve a good resolution, symmetric peak shapes and a short run time. Four commercial columns were tested, including Agilent ZORBAX SB C_{18} column (4.6 mm × 50 mm, i.d. 5 μm), ACQUITY UPLC BEH C_{18} column (2.1 mm × 50 mm, i.d. 1.7 μm), Spursil C_{18}-EP column (2.1 × 50 mm, i.d. 3 μm) and Accucore Vanquish C_{18} column (2.1 mm × 100 mm, i.d. 1.5 μm). Spursil C_{18}-EP column (2.1 × 50 mm, i.d. 3 μm) was finally selected as it provided a better sensitivity and resolution. Compared with methanol, acetonitrile provided a better peak shape, higher sensitivity and lower potential of matrix effect. Therefore, acetonitrile-water was used as the mobile phase for the gradient elution. The addition of 0.1% formic acid in the mobile phase was found to result in a higher sensitivity.

3.2. Bioanalytical method validation

3.2.1. Selectivity and carry-over

Figure 2 displayed the representative MRM chromatograms of blank dog plasma and drug-containing plasma. Ipatasertib and IS were detected at the retention times of 0.89 and 1.22 min, respectively. There were no obvious interferences impacting the determination of ipatasertib, suggesting that the developed LC/MS/MS method is selective enough for the determination of ipatasertib. No significant peaks were detected in the blank plasma sample that was injected after ULOQ, suggesting that the present assay was free of carry-over.

3.2.2. Calibration curve and LLOQ

The developed assay was demonstrated to be linear over the concentration range of 0.3-1500 ng/mL, with correlation coefficient > 0.998 (r > 0.998). The regression equation was Y = (0.0267 ± 0.0031) X + (0.057 ± 0.0031), where Y represents the peak area ratios of analyte to IS and X means the nominal concentrations of the ipatasertib in dog plasma. The back-calculated concentrations of calibration standards were in the range of 85-115% of the nominal concentrations. The LLOQ of the assay was 0.3 ng/mL, at which the ratio of signal-to-noise was >10 with acceptable accuracy (RE% within ± 15%) and precision (RSD% <15%).

3.2.3. Accuracy and precision

As shown in **Table 1**, the intra- and inter-day RE% for ipatasertib ranged from -2.50 to 13.25%, while the intra- and inter-day RSD% was less than14.32%. All the data were within the required limits. The results suggested that the developed method was satisfactory assurance for the determination of ipatasertib in dog plasma.

3.2.4. Extraction recovery and matrix effect

The extraction recovery of ipatasertib from dog plasma was determined at three

concentrations (0.3, 80 and 1200 ng/mL). As summarized in **Table 2**, the extraction recovery of ipatasertib was more than 85.43%, with RSD less than 15%; while the extraction recovery of IS was found as 86.75%. At the same concentration levels, matrix effect of ipatasertib was 103.54, 95.74, and 99.48%, respectively. The matrix effect of IS was 96.11%. The data suggested that there were no interference effects from co-eluting substances during the ionization of the analytes.

3.2.5. Stability

Results of stability were summarized in **Table 3**. Ipatasertib in dog plasma were stable under different storage conditions. The RE% ranged from -6.67% to 10.44%, with RSD% less than 15%. The incurred sample stability testing demonstrated that the concentration of the stability samples was within 92.45-105.21% of that of T_0 samples.

3.3. Pharmacokinetic study

The validated LC/MS/MS method was successfully applied to the pharmacokinetic investigation of ipatasertib. The plasma concentration (ng/mL) *versus* time (h) profiles of ipatasertib in dog plasma following single intravenous or oral administration were described in **Figure 3**. The pharmacokinetic parameters calculated through non-compartmental analysis were summarized in **Table 4**. After intravenous administration, ipatasertib showed moderate elimination from the plasma with half-life ($T_{1/2}$) of 3.0 ± 0.7 h and clearance (CL) of 17.6 ± 2.9 mL/min/kg. The plasma exposure was 957.8 ± 145.1 ng·h/mL. Following oral administration, ipatasertib showed fast absorption with the plasma concentration at first time point (5 min) being approximately 104.5 ng /mL and reached the maximum plasma concentration 775.0 ± 92.0 ng/mL at 0.6 ± 0.3 h post-dose. The $T_{1/2}$ value (2.8 ± 0.6 h) was

comparable with that after intravenous administration. The plasma exposure was 1847.1 ± 418.5 ng·h/mL. The oral bioavailability was determined to be 19.3% in dogs, suggesting that ipatasertib may have poor gastrointestinal absorption or high first-pass metabolism.

Conclusions

A sensitive and reliable LC/MS/MS method in combination with protein precipitation procedure has been developed and validated for the determination of ipatasertib in dog plasma. The validated method displayed high sensitivity (LLOQ 0.3 ng/mL), simple sample preparation (protein precipitation), satisfactory recovery (> 85%) and short run time (2.5 min), and it was further applied to pharmacokinetic study of ipatasertib in dogs after oral and intravenous administrations. The results illustrated that oral bioavailability of ipatasertib was 19.3%.

Conflict of interest

None.

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Table 1. Inter- and intra-day precision and accuracy of ipatasertib in dog plasma (n = 6)

Nominal conc. (ng/mL)	Intra-day		Inter-day	
	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)
0.3	5.68	10.50	12.23	5.50
80	7.71	-2.50	14.32	-0.63
1200	6.25	6.55	8.29	13.25

Table 2. Matrix effect and extraction recovery of ipatasertib in dog plasma (n = 6)

Nominal conc. (ng/mL)	Extraction recovery (%)	Matrix effect (%)
0.3	92.23 ± 5.68	103.54 ± 6.88
80	88.56 ± 4.44	95.74 ± 5.74
1200	85.43 ± 5.27	99.48 ± 9.41
IS	86.75 ± 3.71	96.11 ± 5.12

Table 3. Storage stability of ipatasertib in dog plasma (n = 6)

Nominal conc. (ng/mL)	Long-term stability		Short-term stability		Three freeze/thaw cycles		Post-preparative stability	
	Found conc. (ng/mL)	(RE, %)	Found conc. (ng/mL)	(RE, %)	Found conc. (ng/mL)	(RE, %)	Found conc. (ng/mL)	(RE, %)
0.3	0.28 ± 0.07	-6.67	0.32 ± 0.02	6.67	0.29 ± 0.02	3.33	0.33 ± 0.02	10.00
80	85.36 ± 5.45	6.70	78.56 ± 10.12	-1.80	76.78 ±5.14	4.03	87.23 ± 4.87	9.04
1200	1259.23 ± 45.75	4.94	1302.25 ± 56.37	8.52	1284.32 ± 58.12	7.03	1325.28 ± 96.32	10.44

Table 4. Pharmacokinetic parameters of Ipatasertib in dog plasma after oral (10 mg/kg) and intravenous (1 mg/kg) administration

Parameter	**Oral (*n* = 4)**	**Intravenous (*n* = 4)**
$AUC_{0\text{-}t}$ (ng·h/mL)	1847.1 ± 418.5	957.8 ± 145.1
$AUC_{0\text{-}inf}$ (ng·h/mL)	1849.7 ± 420.0	959.2 ± 145.7
T_{max} (h)	0.6 ± 0.3	
C_{max} (ng/mL)	775.0 ± 92.0	1061.3 ± 213.9
$T_{1/2}$ (h)	2.8 ± 0.6	3.0 ± 0.7
CL (mL/min/kg)	93.9 ± 22.6	17.6 ± 2.9
V_d (L/kg)	22.5 ± 4.6	4.7 ± 1.1
$MRT_{0\text{-}t}$ (h)	2.4 ± 0.1	1.7 ± 0.1
$MRT_{0\text{-}inf}$ (h)	2.5 ± 0.1	1.7 ± 0.2
F (%)	19.3%	

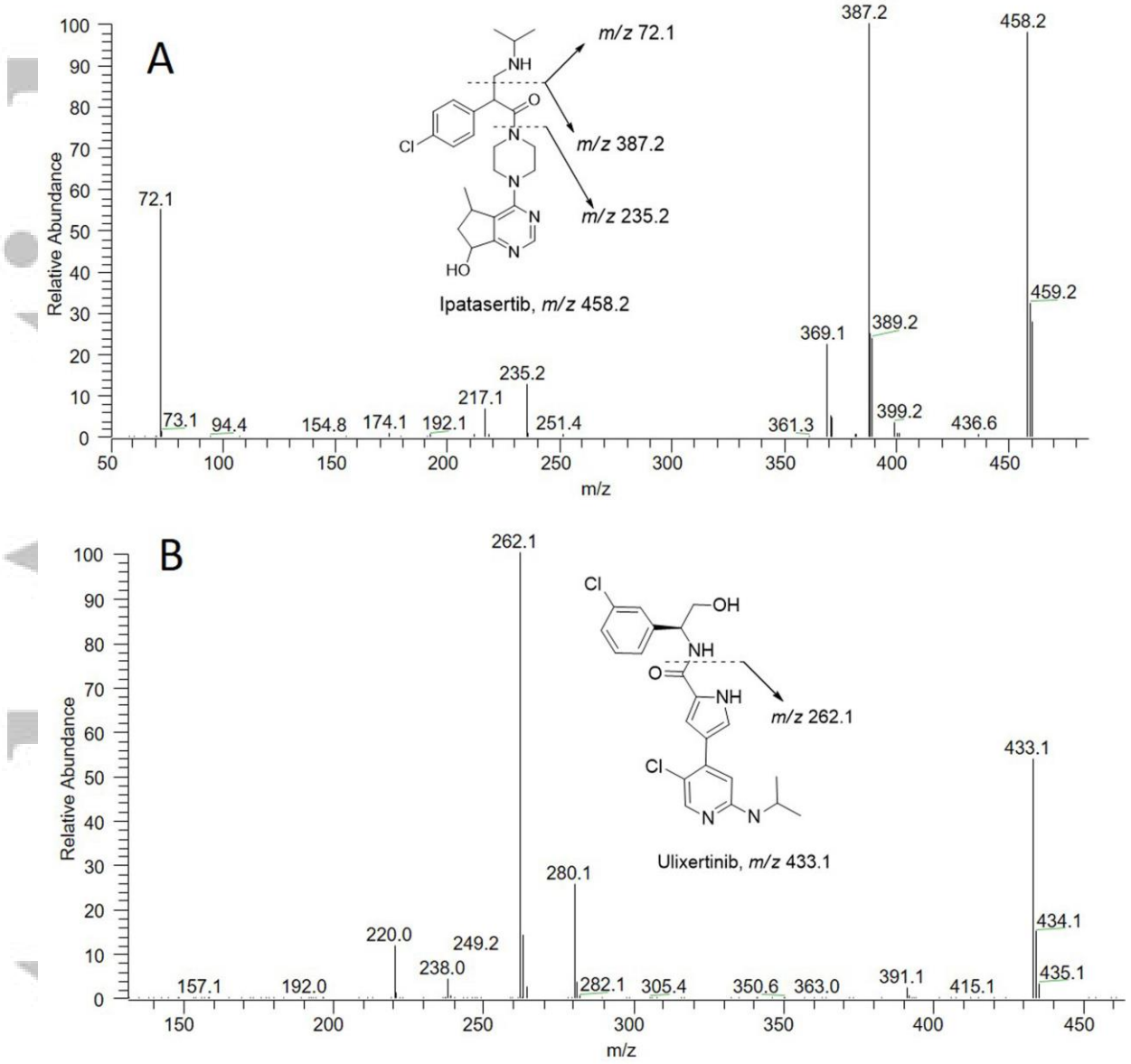

Figure 1. MS/MS spectra of ipatasertib **(A)** and ulixertinib (**B**, internal standard, IS)

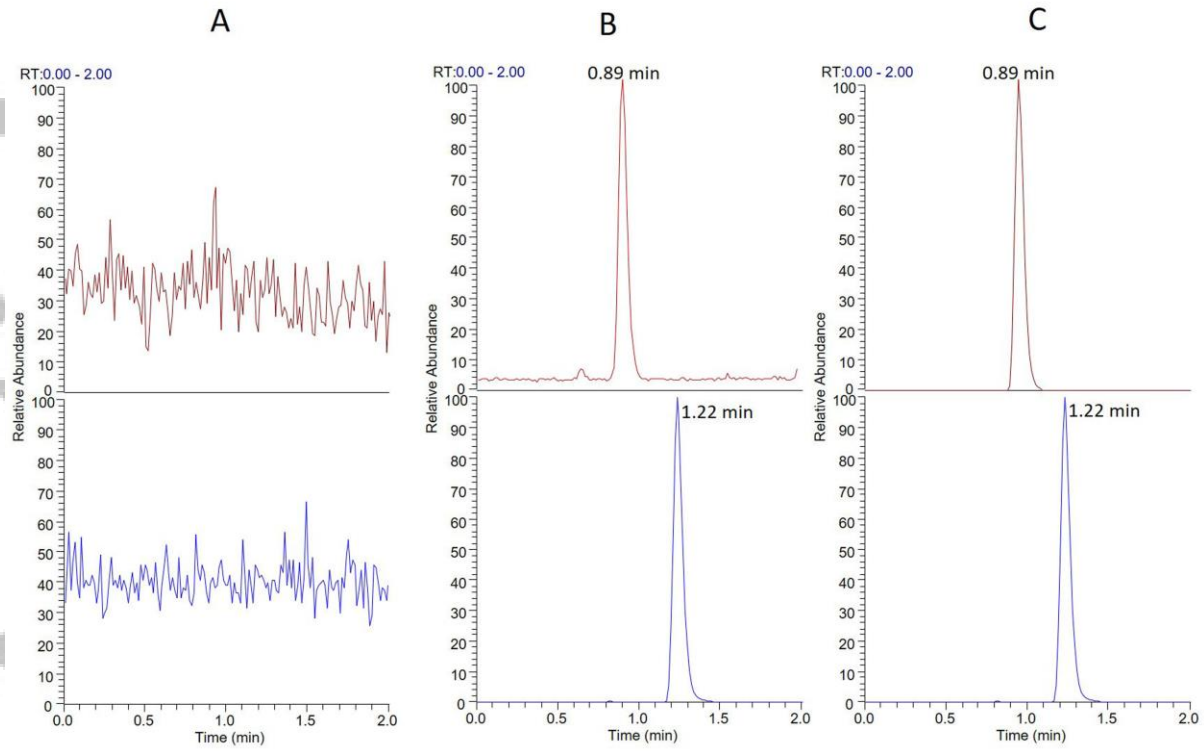

Figure 2. Representative MRM chromatograms of ipatasertib (MRM transition of *m/z* 458.2 > 387.2) and ulixertinib (MRM transition of *m/z* 433.1 > 262.1), **(A)** blank plasma sample, **(B)** blank plasma spiked with ipatasertib at LLOQ and ulixertinib, and **(C)** incurred sample collected at 1 h after oral administration of ipatasertib at a single dose of 10 mg/kg

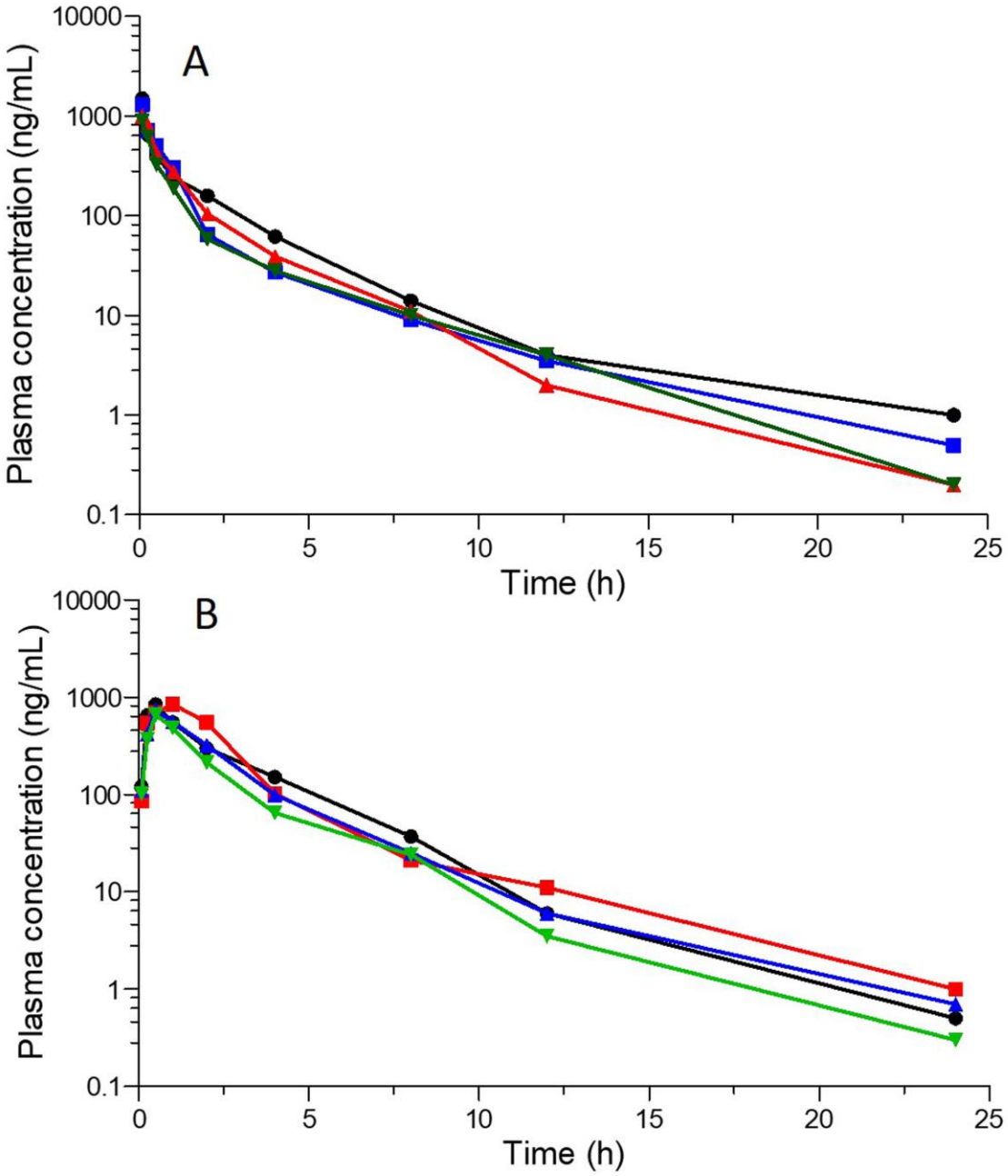

Figure 3. Plasma concentration-time curves of ipatasertib in dog plasma after intravenous (**A**, $n = 4$) and oral (**B**, $n = 4$) administration at the doses of 1 and 10 mg/kg, respectively