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Ultra-performance liquid chromatography tandem mass spectrometry method for the determination of AZ66, a sigma receptor ligand, in rat plasma and its application to *in vivo* pharmacokinetics

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Abstract

Methamphetamine abuse continues as a major problem in the USA owing to its powerful psychological addictive properties. AZ66, 3-[4-(4-cyclohexylpiperazine-1-yl)pentyl]-6-fluorobenzo[d]thiazole-2(3H)-one, an optimized sigma receptor ligand, is a promising therapeutic agent against methamphetamine. To study the *in vivo* pharmacokinetics of this novel sigma receptor ligand in rats, a sensitive ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) method was developed in rat plasma and validated. The developed method requires a small volume of plasma (100 μ L) and a simple liquid–liquid extraction. The chromatographic separations were achieved in 3.3 min using an Acquity UPLC BEH Shield RP₁₈ column. The mass spectrophotometric detection was carried out using a Waters Micromass Quattro MicroTM triple-quadrupole system. Multiple reaction monitoring was used for the quantitation with transitions m/z 406 $\rightarrow$ m/z 181 for AZ66 and m/z 448 $\rightarrow$ m/z 285 for aripiprazole. The method was validated over a concentration range of 1–3500 ng/mL and the lower limit of quantitation was determined to be 1 ng/mL. Validation of the assay demonstrated that the developed UPLC/MS/MS method was sensitive, accurate and selective for the determination of AZ66 in rat plasma. The present method has been successfully applied to an i.v. pharmacokinetic study in Sprague–Dawley rats.

Keywords

mass spectrometry; AZ66; pharmacokinetics; rats; method validation

Introduction

Methamphetamine (METH) is a psychomotor stimulant, neurotoxic, addictive and the second most illicit drug abused world wide (Kiyatkin and Sharma, 2009; Lai *et al.*, 2009; Sharma and Kiyatkin, 2009). Methamphetamine can be synthesized readily from over-the-counter drugs. Current surveys estimate that around 15–16 million people have abused methamphetamine (Krasnova and Cadet, 2009). The short-term stimulant effects of METH include euphoria, hyperthermia, enhanced energy, increased physical activity and decreased appetite (Krasnova and Cadet, 2009; Kaushal and Matsumoto, 2011). Repeated METH use has been reported to result in addiction, psychosis, changes in brain structure and function involving terminals of dopamine and serotonin neurons, memory loss and neurodegeneration (Ricaurte *et al.*, 1980; Sharma and Kiyatkin, 2009; Kousik *et al.*, 2012). Multiple mechanisms that contribute to the METH-induced neurotoxicity include oxidative stress owing to formation of oxygen and nitrogen reactive species, abnormal dopamine and glutamate transmission, mitochondrial dysfunction, astroglial and microglial activation and brain hyperthermia (Brown *et al.*, 2003; Thomas *et al.*, 2004; Cadet *et al.*, 2005; Sharma and Kiyatkin, 2009). However, the mechanisms underlying these neurodegenerative effects are not yet thoroughly understood. Methamphetamine enhances the synaptic levels of norepinephrine, dopamine and serotonin by reversing the transport that facilitates excess release of these monoamines and also by preventing their re-uptake (Rothman *et al.*, 2001; Kousik *et al.*, 2012). Various effects of METH have been reported to be related to its interaction with monoamine transporters. Nevertheless, METH also has an affinity for both sigma-1 and sigma-2 receptors and it interacts with sigma receptors at physiologically attainable concentrations, suggesting that they play a role in the effect of METH (Nguyen *et al.*, 2005; Kaushal *et al.*, 2011; Rodvelt *et al.*, 2011; Seminerio *et al.*, 2012b). Sigma receptors are unique proteins discovered by Martin *et al.* in 1976 (Martin *et al.*, 1976; Zeng *et al.*, 2007; Garcés-Ramírez *et al.*, 2011; Jamalapuram *et al.*, 2012). Based on the specific pharmacological and functional characteristics, sigma receptors are divided into two subtypes, sigma-1 and sigma-2 (Hellewell *et al.*, 1994; Crawford and Bowen, 2002; Kashiwagi *et al.*, 2009). The sigma-1 receptor is a 25–29 kDa protein with 223 amino acids and was cloned in 1996 (Hanner *et al.*, 1996; Monassier and Bousquet, 2002; Romieu *et al.*, 2002; Daniels *et al.*, 2006). It is localized in various organs, such as liver, heart and the gastrointestinal tract. The limbic system of brain is particularly rich in sigma-1 receptors (Crawford and Bowen, 2002; Monassier and Bousquet, 2002). The sigma-2 receptor is an 18–21 kDa protein that has not yet been cloned (Wheeler *et al.*, 2000; Kashiwagi *et al.*, 2009). Sigma receptors are distributed in systems that mediate the actions of METH such as the dopaminergic system in the brain. The activation of these receptors results in the synthesis and release of dopamine (Weiser *et al.*, 1995; Matsumoto *et al.*, 2008; Rodvelt *et al.*, 2011). At this time, there is no pharmacotherapy approved by the US Food and Drug Administration (FDA) to treat the harmful effects of METH (Kaushal and Matsumoto, 2011; Graves and Napier, 2012).

Sigma receptor antagonists are currently being investigated as potential agents for the treatment of METH abuse and toxicity. Targeting these receptors could become a promising therapeutic approach in treating addiction to METH (Kaushal *et al.*, 2011; Seminerio *et al.*,

2012b). In addition, the neurotoxic and stimulant effects of METH were shown to be attenuated in mouse by sigma receptor antagonists such as AC927 and CM156 (Kaushal *et al.*, 2011, 2012). Even though several sigma receptors antagonists have been synthesized, a primary concern with these compounds is that they are not purely selective for sigma receptors (Xu *et al.*, 2010). In addition to their affinity for sigma receptors, most of these compounds also interact with dopamine transporters, opioid receptors or *N*-methyl-D-aspartate receptors.

AZ66 {3-[4-(4-cyclohexylpiperazin-1-yl)pentyl]-6-fluorobenzo[d]thiazole-2(3*H*)-one; Fig. 1} is a synthetic piperazine derivative identified as a promising lead for the treatment of METH abuse, based on its toxicological and pharmacological data. Radioligand binding studies have shown that AZ66 has high nanomolar affinity for both subtypes of sigma receptors and is highly selective over a wide battery of CNS targets (Seminerio *et al.*, 2012b). In addition, AZ66 exhibited an appreciably longer half-life *in vitro* compared with CM156 {3-[4-(4-cyclohexylpiperazin-1-yl)butyl]benzo[d]thiazole-2(3*H*)-thione}, a precursor of AZ66 that exhibited an *in vitro* half-life of 4.62 min in rat liver microsomes. AZ66 was found to significantly attenuate convulsions in mice treated with a toxic dose of cocaine, demonstrating its anticocaine activity (Seminerio *et al.*, 2012b). After intraperitoneal administration to Swiss Webster mice, AZ66 was shown to attenuate METH-induced dopaminergic neurotoxicity and hyperthermia. AZ66 also attenuated the memory impairment caused by the repeated intake of METH in mouse (Seminerio *et al.*, 2012a).

To aid discovery programs, accurate pharmacokinetics and metabolism data must be collected as early as possible. To completely describe the pharmacokinetic behavior of drug candidates in laboratory animals and in humans, an array of bioanalytical methods are required. In the present study, a sensitive and reproducible analytical method was developed and validated for quantification of AZ66 in rat plasma using UPLC/MS/MS. The sample preparation is simple and employs a single step liquid-liquid extraction. The assay was validated for selectivity, sensitivity, linearity, recovery, matrix effect, accuracy, precision and stability. The developed and validated method was successfully utilized to study the pharmacokinetic behavior of AZ66 in male Sprague-Dawley rats, following i.v. administration.

Experimental

Chemicals and reagents

AZ66 (>99% purity as determined by HPLC) was synthesized in the Department of Medicinal Chemistry, The University of Mississippi (Oxford, MS, USA) as previously reported (Seminerio *et al.*, 2012a). Aripiprazole (99% purity), internal standard (IS), was purchased from Sigma-Aldrich (St Louis, MO, USA). Acetic acid and ammonium acetate were purchased from Sigma-Aldrich (St Louis, MO, USA). Solvents (HPLC grade) such as methanol, acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Rat plasma was obtained from Innovative Research (Peary Court Novi, MI, USA).

Calibration standards and quality control sample preparation

An accurately weighed amount (0.31 mg) of AZ66 was dissolved in water (310 μ L) to obtain a stock solution at a concentration of 1.0mg/mL. A series of working standard solutions of AZ66 were prepared at concentrations from 10 to 35,000 ng/mL by diluting the stock solution with methanol. The IS stock solution (1mg/mL) was prepared by dissolving 0.24mg of aripiprazole in 240 μ L methanol. The working standard solution (3 μ g/mL) of the IS was prepared by diluting 3 μ L of 1mg/mL solution with 997 μ L of methanol. All stock solutions were stored at -20°C . Calibration standards were freshly prepared by diluting blank rat plasma (100 μ L) with the working standard solutions to obtain final concentrations of 1, 10, 50, 100, 500, 1000, 2000 and 3500 ng/mL. Quality control (QC) samples were prepared at three concentrations of 2, 400 and 3000 ng/mL by diluting rat plasma (100 μ L) with the appropriate amounts of each working standard solution.

Sample preparation

A simple liquid–liquid extraction method was used for the extraction of AZ66 from rat plasma. Plasma samples (100 μ L) were spiked with 10 μ L of the IS and vortexed for 30 s. The samples were extracted with 800 μ L of chloroform by vortex-mixing for 10min and the resultant mixture was then centrifuged at 10,000 *g* for 10min at 4°C . The organic phase was transferred in to an eppendorf tube and evaporated to dryness in a vacuum oven at 25°C . The residue was dissolved in 100 μ L of methanol by vortex-mixing for 1 min. An aliquot of 10 μ L of sample was used for the UPLC/MS/MS analysis.

UPLC/MS/MS conditions

An Acquity UPLC (Waters Corp., Milford, MA, USA) system coupled with a tandem mass spectrophotometer was used for the quantitative analysis of AZ66. Chromatographic separations were achieved on an Acquity UPLC BEH Shield RP 18 column ($2.1 \times 150\text{mm}$). The mobile phase consisted of 10mM ammonium acetate containing 0.1% acetic acid and methanol (40:60, v/v) and pumped at a flow rate of 0.3mL/min. The sample injection volume was 10 μ L. The total run time was 3.3min.

A Micromass Quattro microTM system (Waters Corp., Manchester, UK) equipped with an electrospray ionization source (ESI) was used for the mass spectrophotometric detection. The ESI was operated in the positive ionization mode. The acquisitions of AZ66 and IS were performed using multiple reaction monitoring. The mass transitions chosen for the quantitation of the compounds were m/z 406 $\rightarrow$ m/z 181 for AZ66 and m/z 448 $\rightarrow$ m/z 285 for the IS. The optimized mass spectrophotometric parameters were: capillary, cone, extractor and RF lens voltages were set at 4.88 kV, 44 V, 3 V and 0.5 V, respectively. The source temperature and desolvation temperature were set at 100 and 250°C , respectively. The desolvation and cone gas flows were set at 500 and 60 L/h, respectively. Argon was used as the collision gas at 3.5×10^{-3} Pirani. Collision energies were set at 30 and 21 eV for AZ66 and the IS, respectively.

Validation of assay

The analytical method was validated according to the FDA guidelines for bioanalytical method validation (US Food and Drug Administration, 2001).

Selectivity and sensitivity—The selectivity of the method was assessed by comparing the chromatograms of the blank non-pooled rat plasma from six different batches with the corresponding rat plasma samples spiked with AZ66 and the IS. The lower limit of quantification (LLOQ) was defined as the lowest concentration that resulted in a signal to noise (S/N) ratio of 10. The acceptable criterion for the LLOQ was relative standard deviation (RSD) and accuracy values less than $\pm 20\%$. The LLOQ was evaluated by analyzing samples in six replicates on three consecutive days. The limit of detection (LOD) was defined as the concentration of analyte that yielded an S/N ratio of 3.

Linearity—Calibration standards were prepared by spiking blank rat plasma with standard working solutions of AZ66 to obtain standards at concentrations of 1, 10, 50, 100, 500, 1000, 2000 and 3500 ng/mL. An aliquot of 10 μ L of the IS was also added to obtain a concentration of 3 μ g/mL. The calibration curve was constructed by plotting the peak area ratios of analyte to the IS vs analyte concentrations.

Extraction recovery and matrix effect—The extraction recovery of AZ66 from rat plasma was determined at low, medium and high concentrations (2, 400 and 3000 ng/mL). The extraction recovery was calculated by comparing the peak area ratios of blank plasma samples spiked with analyte and IS before extracting the plasma, with peak area ratios of blank plasma samples to which analyte and IS were added after the extraction of blank plasma.

The matrix effect was assessed at three QC levels (2, 400 and 3000 ng/mL) by comparing the analyte/IS peak area ratios of extracted plasma samples with those of the corresponding AZ66 standard solutions prepared in methanol. This study was conducted with blank rat plasma from six different lots.

Precision and accuracy—The intra-day and inter-day precision and accuracy of the method were assessed from the QC samples at concentrations 2, 400 and 3000 ng/mL. To determine intra-day precision and accuracy, six replicates of QC samples were analyzed at all the three concentration levels on the same day. To determine the inter-day precision and accuracy all the three QC samples were analyzed on three consecutive days. The precision was expressed as relative standard deviation (RSD) and the accuracy as percentage bias. The intra- and inter-day precision and accuracy should not be more than 15% of RSD and 15% of percentage bias, respectively.

$$\text{Percentage bias} = 100 \times [(\text{measured} - \text{actual}) / \text{actual}]$$

Stability—Stability of a drug in plasma is a function of the chemical properties of the drug, the storage conditions, the matrix and the containers used for sample storage and processing.

Stability studies should be conducted at different storage conditions that might be encountered during regular sample handling and processing.

To determine the stability of AZ66, QC samples (six replicates) at concentrations of 10, 400 and 3000 ng/mL were exposed to various storage conditions. Freeze–thaw stability was investigated for three successive freeze–thaw cycles at –20 °C. For long-term stability, the samples were stored at –20 °C for 30 days. Short-term temperature stability was assessed at room temperature for 12 h. Post-operative stability was also evaluated by analyzing the processed samples stored in the auto-sampler at 25 °C for 24 h. The stability of the stock solutions of analyte and IS was tested daily for a period of 1 week at –20 °C. The concentrations of the stored samples for all the stability studies were compared with the freshly prepared standards.

Application to an intravenous pharmacokinetic study in rats

To assess the applicability of the developed method, the intravenous pharmacokinetic profile of AZ66 in rats was determined. The rat pharmacokinetic protocol was approved by the Institutional Animal Care and Use Committee of the University of Mississippi (OLAW Assurance number is A3356-01). Male Sprague–Dawley rats ($n = 6$) weighing 260–280 g were obtained from Harlan Laboratories (Indianapolis, IN, USA). The rats were fasted overnight before the administration of the compound and then had free access to water after dosing. The rats were also allowed free access to food beginning 4 h after dosing. A single i.v. dose of AZ66 was administered at a dose of 5mg/kg through the jugular-vein cannula. Blood samples were collected via the jugular-vein cannula just before dosing and subsequently at times of 2, 15, 30, 60, 120, 240, 480 min, and 12, 24, 30, 36 and 42 h after dosing. To clear the heparinized saline from the cannula, a blood volume of 0.05mL was withdrawn and a clean syringe was used to collect 0.15mL of blood sample. After each blood sampling, the cannula was flushed with 0.2 mL of a heparin-saline (10 IU/mL) solution. Blood samples were transferred into heparinized microcentrifuge tubes and centrifuged immediately at 10,000 g for 10 min at 4 °C. The plasma was separated from the blood cells, transferred to clean Eppendorf tube and stored at –20 °C until analyzed. The pharmacokinetic study samples were processed as described above. Pharmacokinetic parameters were determined by analyzing the plasma concentration-time data by non-compartmental analysis using WinNonlin 5.2 (Pharsight, Mountain View, CA, USA).

Results and discussion

Method development

AZ66 exhibited a higher mass spectrophotometric response using the ESI positive mode compared with the negative mode. The most intense analyte signal appeared at m/z of 406. Several fragment ions were observed in the product ion spectra, but the most abundant product ions were noted at m/z of 181 and 285 for AZ66 and the IS, respectively. The mass spectrophotometric parameters such as collision energy, source temperature, capillary voltage and gas flow were optimized to enhance this signal intensity. Figure 2 shows the product ion scans of $[M + H]^+$ of AZ66 and the IS. Aripiprazole was chosen as the internal

standard owing to its similar chromatographic and mass spectrophotometric characteristics to AZ66 and negligible endogenous interferences at m/z 448 (Vuppala *et al.*, 2011).

The chromatographic conditions were optimized through several trials using various columns and mobile phases to achieve good chromatographic behavior and better ionization of AZ66 and the IS. Columns such as the Acquity UPLC C₁₈ (2.1 × 50mm), C₈ (2.1 × 50mm) and Atlantis dC₁₈ (2.1 × 50mm) were examined. These columns resulted in broader peaks with 0.4–0.5 min width. In order to achieve sharper peaks we tried the Acquity UPLC HILIC (2.1 × 50mm) and BEH Shield RP 18 (2.1 × 150mm) columns. The unique bonding chemistry (the embedded carbamate group in the bonded phase ligand) of the BEH Shield RP₁₈ column enhances the peak shape for basic compounds. It resulted in the best performance with a peak width of 7–9 s. Ammonium acetate, formic acid and acetic acid were used alone or in combination at various concentrations in aqueous phase for the baseline separation of the analyte and the IS and to achieve higher ionization efficiency and the sensitivity. Ultimately, a 0.1% acetic acid solution with 10mM ammonium acetate in water and methanol (40:60, v/v) was chosen as the mobile phase. This mobile phase resulted in good peak shapes and high sensitivity (LOD 0.5 ng/mL). The retention times of AZ66 and the IS were 1.3 and 1.0 min, respectively, resulting in a total run time of 3.3 min.

Method validation

Selectivity and sensitivity—Typical chromatograms obtained from the LC/MS/MS analysis of blank rat plasma, blank rat plasma spiked with AZ66 and the IS, and a rat plasma sample obtained 1 h after the intravenous administration of AZ66 (5 mg/kg) are represented in Fig. 3. No obvious interferences from endogenous components were observed at the retention times of AZ66 and the IS. The LLOQ for AZ66 in plasma was found to be 1 ng/mL with precision (RSD) below 20% (2.2) and accuracy (percentage bias) within ±20% (2.1). A corresponding chromatogram is shown in Fig. 3(b). The LOD was defined at an S/N ratio of 3:1 (Jamalapuram *et al.*, 2012) and was found to be 0.5 ng/mL.

Linearity of calibration curve—The calibration curves for AZ66 were linear over the concentration range of 1–3500 ng/mL ($r^2 > 0.99$). The representative regression equation for the calibration curve was:

$$y = 0.0511x + 0.1682$$

where y represents the peak area ratios of AZ66 to the IS and x represents concentrations of analyte in plasma. The RSD of the slope was 0.00018 and the RSD of the intercept was 0.0031. Table 1 represents the linearity of calibration curves for AZ66.

Precision and accuracy of the assay—The results of the intra and inter-day precision and accuracy are presented in Table 2. The intra-day precisions for the three QC levels of AZ66 were 2.0, 0.1 and 0.1% (RSD) and the inter-day precisions were 3.0, 0.4 and 0.1% (RSD). The intra-day accuracies for the three QC levels were 1.7, 0.4 and 0.3% (percentage bias) and the inter-day accuracies were 6.3, 0.7 and 0.3% (percentage bias) for the all three QC concentrations. The data indicates that the precision and accuracy were within the

acceptable limits specified by the FDA where the precision (RSD) determined should not exceed 15% and accuracy (percentage bias) should be within $\pm 15\%$ of the actual value. These results suggest that the reported method is consistent and reproducible for the quantitation of AZ66 in rat plasma.

Extraction recovery and matrix effect—According to FDA guidelines, the extraction recovery of analyte and IS needs to be invariable and reproducible and need not be 100%. The extraction recoveries of AZ66 were 97.7 ± 3.4 , 98.2 ± 2.9 and $98.6 \pm 3.7\%$ for QC samples at the concentrations of 2, 400 and 3000 ng/mL, respectively. The percentage bias values for the recovery at three QC levels were 0.8, 0.6 and 1.1%. These results suggested that the recovery of AZ66 was precise and reproducible at all the three concentrations. The extraction recovery of the IS was consistent and found to be $67.5 \pm 3.8\%$.

The matrix effect of AZ66 was evaluated by analyzing QC samples at three concentration levels of 2, 400 and 3000 ng/mL. The mean matrix effect values ranged from 96.5 to 101.4%. The matrix effect of the IS was 95.4%. This indicated that the ion suppression from plasma matrix for AZ66 and IS was negligible.

Stability—To investigate the stability of AZ66 in rat plasma, QC samples at concentrations of 10, 400 and 3000 ng/mL were stored under various conditions that may be encountered during routine sample handling, processing and analysis. Table 3 summarizes the results of the freeze–thaw, short-term, long-term and postoperative stabilities of AZ66 in rat plasma. The results suggested that AZ66 was found to be stable after three freeze–thaw cycles, at room temperature for 12 h (25 °C) and at -20 °C for 30 days. The compound was also stable in reconstituted samples stored in the auto-sampler at 25°C for 24 h. AZ66 and the IS stock solutions were stable for 12 h maintained at room temperature and for a week stored at -20 °C. No loss of compound was observed at all the storage condition examined, suggesting that the plasma samples can be stored at -20 °C for a period of 30 days.

Application to an i.v. pharmacokinetic study in rats

The developed method was used to determine the plasma concentration of AZ66 in male Sprague–Dawley rats following i.v. (5 mg/kg) administration of the compound. Figure 4 shows the mean plasma concentration–time profiles of AZ66 after i.v. administration. The pharmacokinetic parameters are summarized in Table 4. After i.v. administration the peak plasma concentration ($C_{\max}$) was found to be 1.2 ± 0.2 µg/mL. AZ66 concentrations in plasma declined with a half-life of 7.9 ± 0.3 h after i.v. dosing. The volume of distribution was found to be 30.1 ± 3.6 L/kg. The half-life of the compound may be owing to the high volume of distribution and low clearance (2.6 ± 0.4).

Conclusions

A sensitive and reproducible UPLC/MS/MS method for the determination of AZ66 in rat plasma has been developed and validated. The developed method uses simple liquid–liquid extraction for sample pretreatment. This method demonstrated a short chromatographic run time, and excellent sensitivity, linearity, precision and accuracy. The calibration curve was linear over the range of 1–3500 ng/mL with an LLOQ of 1 ng/mL. Inter- and intra-day assay

variations were found to be <15%. AZ66 and the IS were successfully separated from endogenous interferences. The UPLC/MS/MS assay was applied to a pharmacokinetic study of AZ66 after i.v. administration in rats.

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Abbreviations used

ESI	electrospray ionization
METH	methamphetamine

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Figure 1.
Chemical structure of AZ66 (a) and aripiprazole (b).

Figure 2.
Product ion mass spectra of AZ66 (a) and aripiprazole (b).

Figure 3. Multiple reaction monitoring chromatograms of AZ66 and aripiprazole (IS) in rat plasma: (A) a blank plasma; (B) spiked plasma with AZ66 at 1 ng/mL and IS and 3 µg/mL; (C) rat plasma sample obtained at 1.0 h after i.v. administration at a dose of 5 mg/kg.

Figure 4.
Mean plasma concentration of AZ66 after i.v. administration at a dose of 5 mg/kg.

Table 1

Linearity of calibration curves for AZ66 in rat plasma

	Intra-day RSD (%)	Inter-day RSD (%)
y- Intercept	0.0031	0.0034
Slope	0.0002	0.0002
r^2	0.01	0.01

RSD, Relative standard deviation.

Table 2

Inter- and intra-day precision and accuracy of the assay in rat plasma

Spiked concentration (ng/mL)	Recovery	Intra-day precision and accuracy			Inter-day precision and accuracy		
	Bias (%)	Mean $\pm$ SD (ng/mL)	RSD (%)	Bias (%)	Mean $\pm$ SD (ng/mL)	RSD (%)	Bias (%)
1	-	1.1 $\pm$ 0.01	2.2	2.1	1.0 $\pm$ 0.01	2.4	4.6
2	0.8	2.0 $\pm$ 0.02	2.0	1.7	2.1 $\pm$ 0.03	3.0	6.3
400	0.6	401.4 $\pm$ 0.5	0.1	0.4	402.8 $\pm$ 1.6	0.4	0.7
3000	1.1	3008 $\pm$ 3.7	0.1	0.3	3009 $\pm$ 4.3	0.1	0.3

The precision is expressed as RSD and the accuracy as percentage bias.

Table 3

Stability of AZ66 in rat plasma at various storage conditions

Storage conditions	Concentration (ng/mL)		RSD (%)	Bias (%)
	Spiked	Measured		
Three freeze–thaw cycles	10	10.2 ± 0.7	6.9	−3.1
	400	396.1 ± 12.7	3.2	−0.6
	3000	2990 ± 42.9	1.4	0.2
Long-term for 30 days (−20 °C)	10	10.2 ± 0.4	3.9	0.3
	400	402.4 ± 7.1	1.8	−0.4
	3000	3028 ± 55.0	1.8	−0.6
Short-term for 12 h (25 °C)	10	10.2 ± 0.2	2.4	1.3
	400	401.4 ± 3.5	0.9	−0.5
	3000	3015 ± 30.6	0.9	1.0
Auto-sampler for 24 h (25 °C)	10	10.1 ± 0.2	2.0	1.7
	400	403.2 ± 18.6	4.6	0.8
	3000	3222 ± 79.4	2.5	4.1

Table 4

Noncompartmental pharmacokinetic parameters in rats after i.v. administration of 5 mg/kg of AZ66 ($n=6$)

Parameter	Mean $\pm$ SD
$t_{1/2}$ (h)	7.9 ± 0.3
$C_{\max}$ ($\mu\text{g/mL}$)	1.2 ± 0.2
V_d (L/kg)	30.1 ± 3.6
CL (L/h/kg)	2.6 ± 0.4
MRT (h)	7.6 ± 0.7

$t_{1/2}$, Elimination half-life; $C_{\max}$, peak plasma concentration; V_d , volume of distribution; CL , total clearance; MRT, mean residence time; SD, standard deviation.