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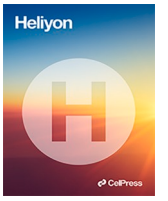

Research article

UPLC-MS/MS analysis of axitinib and pharmacokinetic application in beagle dogs

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ABSTRACT

Objective: To develop a method for determining the concentration of axitinib in beagle dog plasma and utilize this method to investigate the pharmacokinetics of orally administered axitinib in beagle dogs.

Methods: Plasma samples were processed using acetonitrile precipitation and analyzed by UPLC-MS/MS with sunitinib as an internal standard (IS). Chromatographic separation was achieved on a Waters Acquisition UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm) with a gradient elution of acetonitrile and 0.1 % formic acid. Mass spectrometry uses an electrospray ion source for positive ion detection in a multiple reaction monitoring mode. The monitored ion transitions for axitinib and sunitinib were *m/z* 387 → 355.96 and *m/z* 399.3 → 282.96, respectively. Six beagle dogs were administered 0.33 mg/kg of axitinib orally, and venous blood samples were collected at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h post-dose for pharmacokinetic analysis.

Results: The assay demonstrated a linear range of 0.5–100 ng/mL ($r^2 = 0.9992$), and the lower limit of quantification was up to 0.5 ng/mL. Precision, as assessed by relative standard deviation (RSD), was within 8.64 % for both intraday and interday variability. The relative error (RE) for precision from −2.77 %–1.20 %. The recovery rate of the analytes exceeded 85.28 % and the matrix effect was approximately 100 %. Plasma samples maintained stability under various conditions, including room temperature storage for 12 h, processed on an automatic sampler at 4 °C for 6 h, three freeze-thaw cycles, and long-term storage at −80 °C for 60 days. Pharmacokinetic parameters were determined using DAS 2.0 software, revealing a half-life ($T_{1/2}$) of 6.05 h and an area under the curve ($AUC_{(0 \rightarrow \infty)}$) of 97.13 ng h/mL for axitinib.

Conclusions: The UPLC-MS/MS method developed in this study offers high specificity, rapid analysis, high recovery, excellent linearity, and minimal plasma volume requirements, making it well-suited for pharmacokinetic and drug interaction studies in beagles dogs.

1. Introduction

Renal cell carcinoma (RCC) is a common renal malignancy originating in the parenchymal urinary system and accounts for approximately 85 % of all types of renal malignancies. RCC consists primarily of 70 % clear renal cell carcinoma, 10 % renal papillary cell carcinoma, 5 % renal chromophobe cell carcinoma, and 15 % other types of RCC [1]. Over recent years, the incidence rate of RCC has been on the rise. The GLOBOCAN 2020 global cancer statistics project that in 2020, there will be an estimated 430,000 new cases of

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RCC diagnosed and approximately 180,000 deaths annually, placing RCC 14th in the global ranking for incidence of malignant tumors, lower than cancers in the urinary system and bladder, and 15th in terms of mortality. The age-standardized incidence rate for RCC stands at 6.1 per 100,000 males and 3.2 per 100,000 females. Correspondingly, the age-standardized mortality rate is 4.6 per 100,000 for males and 1.8 per 100,000 for females [2].

The precise origins of RCC remain elusive, and its onset is related to factors such as genetics, smoking, and obesity. In recent years, data have shown that the incidence of RCC in East Asia has been on the rise year by year with changes in lifestyle and the increase in systemic diseases such as obesity and hypertension [3]. RCC has the propensity to metastasize to distant sites via the bloodstream, with the lungs, bones, and brain being common destinations for these errant cells. When distant metastasis of tumor cells occurs, it indicates that the patient's condition has deteriorated and the survival is low [4]. The onset of RCC often insidious, with the majority of cases detected incidentally through imaging studies. By the time of diagnosis, many individuals have already progressed to the later stages of the disease [5].

Patients diagnosed with RCC select suitable therapeutic approaches by considering the clinical stage of the disease and the individual's capacity to tolerate treatment. At the beginning of the twentieth century, RCC began to enter the era of targeted therapy. A suite of such targeted agents, including Sorafenib, Sunitinib, and Opdivo, have been instrumental in combating metastatic RCC. These targeted therapies have revolutionized the treatment landscape for patients with advanced RCC, significantly enhancing both the therapeutic outcomes and the overall survival rates for those afflicted with this condition.

Axitinib is a tyrosine kinase inhibitor(TKI), belonging to the second-generation vascular endothelial growth factor receptor (VEGFR) inhibitor, which can inhibit VEGFR1, 2, and 3 [6]. In January 2012, axitinib was approved in the United States and was first listed worldwide. Axitinib distinguishes itself from first-generation TKIs through its heightened specificity for VEGFR, which translates to a more favorable safety profile and a reduced incidence of adverse reactions. Its tolerability is enhanced, thus expanding the therapeutic window for patients [7]. With its superior selectivity, axitinib effectively suppresses the activity of vascular growth factor receptors. Clinically, axitinib is mainly used in adult patients with advanced RCC who have previously received a tyrosine kinase inhibitor or failed cytokine therapy.

Several studies have indicated that different doses of axitinib treatment should be formulated for different patients, and a personalized treatment dose titration should be performed to achieve better treatment outcomes for patients [8]. Additionally, while axitinib shares a comparable adverse reaction profile with other tyrosine kinase inhibitors, its side effects are generally less severe and more manageable. To optimize therapeutic outcomes and mitigate adverse effects, it is essential to delve into research that tailors the ideal dosage and selects suitable treatment strategies, taking into account the individual patient's circumstances [9]. Therefore, the development of accurate quantitative analysis techniques for axitinib is imperative, which will help to study its pharmacokinetic profile and potential drug-drug interactions, paving the way for the investigation of personalized therapeutic strategies for axitinib.

Axitinib is metabolized primarily by the liver, primarily through metabolism by cytochrome P450 (CYP) 3A4 isozymes, partially through oxidative metabolism by CYP2C19 and CYP1A2, and through glycosidation by uridine diphosphate glucuronosyltransferase (UGT) 1A1. The major circulating axitinib metabolites, glycosidic acid and sulfoxide, are inactive [10]. In previous pharmacokinetic studies, we observed that axitinib is rapidly absorbed by the body after oral administration, with peak plasma concentrations reached within 2–6 h after administration in the fed state. The rate and extent of absorption was significantly enhanced when administered on an empty stomach, with an absolute bioavailability of approximately 58 % [11]. Based on these findings, the recommended dosing of axitinib is twice daily at approximately 12-h intervals, with an initial dose recommendation of 5 mg and subsequent increases to 7 mg or 10 mg as needed [12]. In addition, population pharmacokinetic studies further revealed a linear relationship between the dose of axitinib and its maximum plasma concentration as well as the area under the plasma concentration-time curve. Notably, the study also identified possible differences in the pharmacokinetic properties of axitinib between races. In patients with moderate hepatic dysfunction, the area under the plasma concentration-time curve from zero to infinity was two times higher than in subjects with normal liver function. In contrast, acitretinib plasma exposures in patients with mild hepatic dysfunction and elderly patients were not significantly different from those with normal hepatic function, and no dose adjustment was required [13].

Methods for the determination of axitinib in human and mouse plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been reported, but these methods are deficient in sensitivity and specificity, and the long sample processing time and

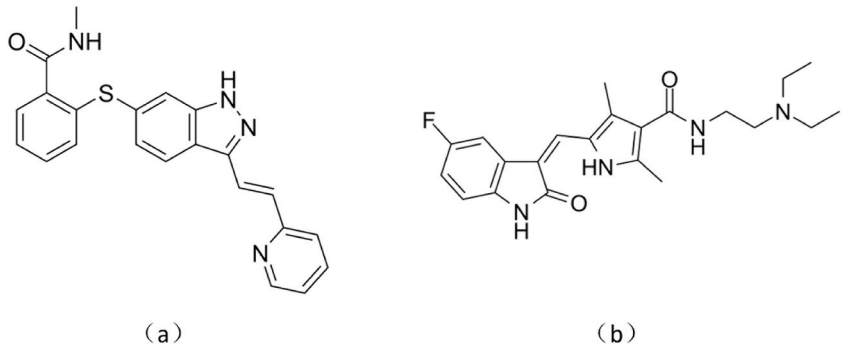

Fig. 1. Chemical structure. (a) Axitinib; (b) Sunitinib.

large injection volume make it difficult to adapt to the needs of large-scale analysis [14–16]. In addition, none of these methods have been applied to complete pharmacokinetic studies. Although ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) has attracted attention due to its advantages of short detection time, high precision and good separation, relatively limited studies have been conducted on the application of UPLC-MS/MS for the determination of axitinib in biological samples. Consequently, the objective of this study was to develop a simple, efficient, precise and rapid UPLC-MS/MS assay for the determination of acitretinib concentration in beagle dog plasma, and to further apply it to in vivo pharmacokinetic studies in beagle dogs. The establishment of this method is expected to improve the efficiency of sample processing and reduce the injection volume, thus meeting the needs of large-scale analysis and providing strong support for the pharmacokinetic and drug interaction studies of axitinib.

2. Instruments and materials

2.1. Drugs and reagents

Axitinib (Fig. 1(a)) and Sunitinib (Fig. 1(b)), both with a high degree of purity exceeding 98.0 %, have been procured from Shanghai Macklin Biochemical Technology Co., Ltd; Chromatographic grade methanol, acetonitrile, and formic acid were obtained from Tianjin Kemiou Chemical Reagent Co., Ltd.

2.2. Instruments

The UPLC instrument was Waters ACQUITY series (Milford, Massachusetts, USA), the mass spectrometer was the XEVO TQD triple quadrupole mass spectrometer (Waters, USA) with electrospray ionization (ESI) source; Masslynx 4.1 workstation (waters, USA); Ultra-pure water instrument from Sichuan Youpu Ultra Pure Water Instrument Co., Ltd; XPR analytical balance from Mettler Toledo International Limited; The −86 °C ultra-low temperature refrigerator from Zhongke Meiling Low Temperature Technology Co., Ltd.

2.3. Experimental animals

Six healthy adult Beagle dogs, ♀♂ Half each, with a body weight of 10–12 kg, from the Animal Experiment Center of the School of Basic Medicine and Forensic Medicine, Henan University of Science and Technology. Feeding conditions are as follows: temperature: 16–28 °C, humidity: 40–70 %. Experimental Animal Quality Certificate No.: SCXK (HUBEI) 2021-0020.

3. Experimental methods

3.1. Preparation of the solution

Accurately weigh 10 mg of axitinib and 10 mg of sunitinb into two 10 mL volumetric flasks. Dissolve in methanol to make a standard stock solution at a concentration of 1 mg/mL. Store in a refrigerator at 4 °C for future use. Prior to use, the standard working solution was diluted with methanol to a concentration of 100, 10, 1 and 0.1 ng/mL.

3.2. Chromatographic conditions

Mobile phase was 0.1 % aqueous solution of formic acid (A) - acetonitrile (B), the gradient elution procedure was as follows: 0.00–0.50min acetonitrile 10 %, 0.50–1.00min acetonitrile 10 % → 90 %, 1.00–2.00min acetonitrile 90 %, 2.00–2.10min acetonitrile 90 % → 10 %, 2.10–3.00min acetonitrile 10 %; The chromatographic column was the Waters Acquisition UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm), with a column temperature maintained at 45 °C and a flow rate of 0.4 mL/min; The temperature of the autosampler was set to 4 °C; The injection volume was 2.0 μL each time.

3.3. Mass spectrometry conditions

The ion source was an electrospray ion source, which is detected by positive ion method. Axitinib and IS were detected by multiple reaction monitoring (MRM) method. The desolvent was nitrogen at a flow rate of 1000 L/h and a temperature of 500 °C; The collision gas was argon at a flow rate of 0.1 mL/min; The capillary voltage was 1 kV; The collision energies (CE) of Axitinib and IS were 20V and 18V, respectively. The quantitative monitoring of Axitinib was m/z 399.30 → 282.96, and IS was m/z 387.00 → 355.96.

3.4. Plasma sample processing method

100 μL of plasma sample was accurately taken into a 1.5 mL EP tube, 20 μL of IS solution was added, vortexed and mixed, and 250 μL of acetonitrile was added. The three mixtures were vortexed for 1 min and centrifuged at 10,142×g for 5 min at 4 °C. The supernatant was placed into an injection vial with a built-in cannula, and the injection volume was set to 2 μL. UPLC-MS/MS quantitative analysis was performed.

3.5. Validation of the methodology

The validation study followed the "Guidelines for the validation of quantitative methods for the analysis of biological samples" in the four parts of the 2020 edition of the Chinese Pharmacopoeia.

3.5.1. Sample selectivity

Beagle blank plasma, blank plasma samples containing 0.5 ng/mL axitinib and IS, and beagle plasma samples after oral administration of axitinib were processed according to the plasma sample processing methods in Section 3.4. After processing, UPLC-MS/MS was performed to determine the presence of endogenous substances or other interfering signals in the region where the drug peaks appeared.

3.5.2. Linear relationship and lower limit of quantification

Take the standard working solution of axitinib, make gradient dilution, prepare plasma control solution with concentrations of 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL, and then test it according to the methods in section 3.4. The least squares method was used to perform linear regression operation on the peak area ratio (axitinib/IS) and the corresponding concentration to plot the standard curve and derive the standard curve equation. The lowest concentration of the standard curve is the lower limit of quantification (LLOQ).

3.5.3. Precision

Prepare low, medium and high concentrations (1, 10, and 75 ng/mL) of axitinib plasma control solution parallel 6 groups. The assay was performed on the same day according to the method in section 3.4, and the intraday precision and accuracy were calculated; The same procedure was performed on 3 consecutive days, and interday precision and accuracy were calculated and expressed as RSD (%) and RE (%).

3.5.4. Recovery rate

Plasma control solutions of low, medium and high concentrations of axitinib (1, 10, and 75 ng/mL) were prepared in 6 parallel groups. The plasma was processed and assayed according to the method in section 3.4, and the peak area was recorded as A1; Another blank plasma of beagle dog was processed, and the plasma was prepared by adding axitinib to the supernatant at low, medium and high concentrations (1, 10, and 75 ng/mL), and was injected for assay, and the peak area was recorded as A2. Recovery rate was calculated using A1/A2.

3.5.5. Matrix effect

Plasma control solutions of axitinib at low, medium and high concentrations (1, 10, and 75 ng/mL) were prepared from blank plasma in 6 groups in parallel, processed, and then injected into the sample for detection, and the peak area was recorded as A1; At the same time, a pure solution with the same final concentration as that of the plasma control was prepared and injected directly into the sample, and the peak area was recorded as A2. The matrix effect (ME) was calculated by A1/A2.

3.5.6. Stability

Plasma control solutions were prepared at low, medium, and high concentrations (1, 10, and 75 ng/mL) of axitinib. The plasma concentrations of axitinib were determined to study its stability after 12 h at room temperature, 6 h on an autosampler at 4 °C, repeated freeze-thawed 3 times (−20 °C to 25 °C), and freezing in a refrigerator at −80 °C for 60 days, respectively.

3.6. Pharmacokinetic applications

All beagle dogs are first raised in an experimental kennel on a normal diet for at least 14 days. The night before the experiment is fasted, but water is not prohibited. Axitinib was completely dissolved in 0.5 % carboxymethylcellulose sodium. A solution of 15 mg of axitinib per kilogram was administered orally to six beagles. Approximately 1.5 mL of venous blood was drawn from the medial cephalic vein of the forelimb or the lateral saphenous vein of the hindlimb at time points of 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h and placed in a 1.5 mL EP tube containing heparin. The blood samples were then centrifuged for 15 min (4 °C, 5000 rpm) and frozen at −80 °C.

Plasma concentrations of axitinib were assayed using the methods described in Sections 3.1 to 3.3; The main pharmacokinetic parameters C_{max}, T_{max}, $T_{1/2}$, CLz/F and AUC were calculated using a statistical moment method in the DAS (Drug And Statistics, version 2.0) program. All data are presented as mean ± standard deviation (SD).

4. Results and discussion

4.1. Development and optimization of the method

The UPLC-MS/MS technique, renowned for its minimal sample injection volume, exceptional selectivity, heightened sensitivity, and expedited analysis duration, has become a staple in pharmacokinetic research for drug concentration determination in blood. In the realm of LC-MS/MS, the judicious selection of a mobile phase is pivotal for achieving optimal separation, superior chromatographic performance, and for extending the operational lifespan of the pump. It is imperative for the mobile phase in mass

spectrometry to be devoid of non-volatile components. Water, methanol, and acetonitrile are commonly used mobile phases. Compared to water and methanol, acetonitrile improves the ionization of the sample, enhance the intensity of the signal and also improves the peak shape. Additionally, to achieve higher sensitivity, 0.1 % formic acid can be added to the mobile phase. Therefore, the final mobile phase used in this study was 0.1 % formic acid and acetonitrile.

In this experiment, sunitinib was ultimately selected as the IS due to its favorable peak profiles and comparable retention times with axitinib under the established chromatographic and mass spectrometric conditions, coupled with the absence of any cross-interference between the two compounds. The aim of this study was to investigate the sensitivity of ESI (electrospray ionization) technique for the detection of axitinib and IS in positive and negative ion modes. It was found that positive ion mode was superior to negative ion mode in terms of mass response. The full-scan mass spectra in the positive ion mode are shown in Fig. 2(a and b), from which it was found that axitinib had the most significant protonated parent and daughter ion signals at *m/z* 387 and 355.96, whereas the IS had equally abundant protonated parent and daughter ion signals at *m/z* 399.3 and 282.96. Accordingly, we identified the mass spectral transition pairs for axitinib and the IS as *m/z* 387 → 355.96 and *m/z* 399.3 → 282.96, respectively.

Furthermore, plasma sample processing is an important step in the establishment of methodology. Improved pre-treatment not only reduces the assay matrix and improves sensitivity and selectivity, but also protects the assay instrument from contamination and prolongs its lifetime [17]. This research endeavored to explore a variety of plasma sample processing techniques. Among the liquid-liquid extraction methods tested, a multitude of extractants were evaluated, yet they invariably yielded suboptimal recovery rates. The acetonitrile extraction approach emerged as a standout, as it not only effectively precipitates proteins but also boasts superior extraction efficiency for both axitinib and IS. Consequently, this study opted for the acetonitrile precipitation method to prepare plasma samples, leveraging its ability to yield cleaner extracts and thus ensuring the accuracy and reliability of subsequent analytical measurements.

4.2. Methodological validation

4.2.1. Sample selectivity

The typical chromatogram after detection is shown in Fig. 3(a)–(c). Endogenous substances in plasma have no significant interference with the detection of axitinib, indicating that the specificity of this method is good; The peak times of axitinib and IS were 1.14 min and 1.20 min, respectively.

4.2.2. Linear relationship and lower limit of quantification

As can be seen from Table 1, the linearity of axitinib plasma drug concentration was good over the concentration range of 0.5–100 ng/mL ($r^2 \geq 0.99$). The standard curve equation is: $y = 0.014x+0.0027$($r^2 = 0.9992$). The nadir on the axis of the standard curve was 0.5 ng/mL, which was the LLOQ for axitinib. Relative precision was within 10 %, and accuracy ranged from −1.33 % to 2.83 %.

4.2.3. Precision

The results are shown in Table 1. The RSD of intraday precision and interday precision of axitinib were within 8.64 %, and the precision of RE was between −2.77 % and 1.20 %, indicating that the method is relatively reliable and meets the methodological requirements.

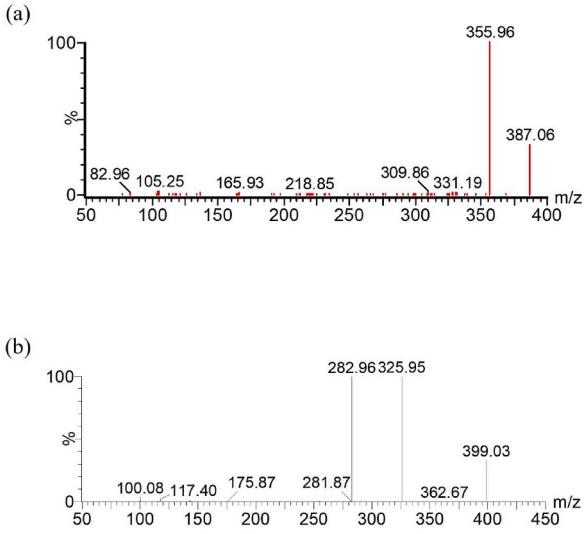

Fig. 2. The Mass spectra of axitinib (a) and sunitinib (IS, b).

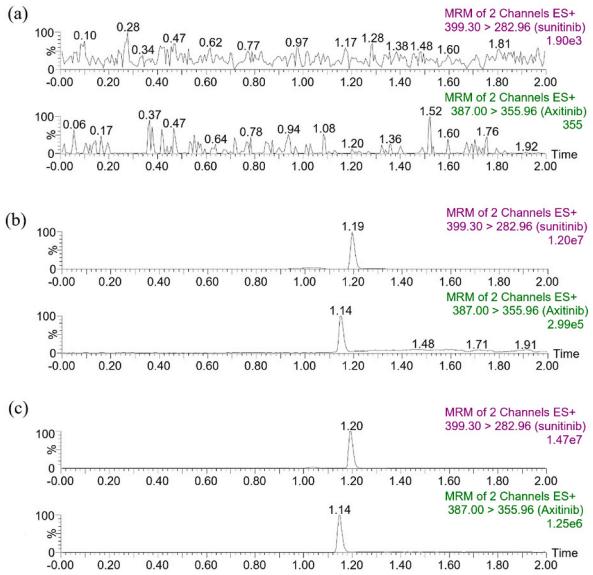

Fig. 3. Representative chromatogram. (a) blank plasma; (b) blank plasma samples spiked with axitinib 0.5 ng/mL (LLOQ) and IS; (c) Plasma samples from Beagle dogs 1.5 h after oral administration of 0.33 mg/kg axitinib.

Table 1
Precision and accuracy of axitinib in plasma of 6 beagle dogs.

Added(ng/mL)	Intraday			Interday		
	Found(ng/mL)	RSD(%)	RE(%)	Found(ng/mL)	RSD(%)	RE(%)
0.5	0.49 ± 0.04	9.03	−1.33	0.51 ± 0.03	5.45	2.83
1	1.00 ± 0.09	8.64	0.33	0.99 ± 0.05	4.55	−0.94
10	9.86 ± 0.64	6.45	−1.38	9.72 ± 0.22	2.28	−2.77
75	74.09 ± 2.22	3.00	−1.22	75.90 ± 1.49	1.96	1.20

4.2.4. Recovery rate and matrix effect

The results are shown in Table 2. The average recoveries rate for axitinib were 84.72 %–93.56 %, which met the methodological requirements. The ME of axitinib ranged from 97.49 % to 101.51 %, indicating that the beagle plasma matrix did not significantly interfere with the detection of axitinib.

4.2.5. Stability

The results are shown in Table 3. The results showed that the RSD of the three samples concentrations were less than 7 % under four different storage conditions, indicating that axitinib has good stability in beagle plasma.

4.3. Pharmacokinetic parameters

During this experiment, no poisoning or abnormal behavior was observed in all beagles. The plasma drug concentration-time profile and key pharmacokinetic parameters for the beagles post-administration are depicted in Fig. 4 and Table 4. According to the data presented in Table 4, axitinib was rapidly absorbed in beagle dogs after oral administration, reaching peak plasma concentrations within 2.17 ± 0.41 h, The half-life ($T_{1/2}$) was determined to be 6.05 ± 1.85 h, a result that is in good agreement with data from previous pharmacokinetic studies of axitinib. These results further confirm the validity of the previous study data [18].

Table 2
Recovery and matrix effect of axitinib in plasma of 6 beagle dogs.

Added(ng/mL)	Recovery(%)	RSD(%)	Matrix effect(%)	RSD(%)
1	84.72 ± 4.10	4.84	99.04 ± 4.27	4.31
10	89.16 ± 3.73	4.18	101.51 ± 3.89	3.83
75	93.56 ± 2.04	2.18	97.49 ± 5.69	5.84

Table 3
Stability of axitinib in plasma of 6 beagle dogs under different conditions.

Added(ng/mL)	Room temperature, 12h		Autosampler 4 °C, 6h		Three freeze-thaw		−80 °C, 60days	
	RSD(%)	RE(%)	RSD(%)	RE(%)	RSD(%)	RE(%)	RSD(%)	RE(%)
1	3.52	0.83	3.73	0.83	5.98	−0.33	6.99	−2.67
10	2.69	−1.58	2.35	−0.53	3.10	−5.98	4.66	−3.87
75	1.46	−2.29	2.17	−2.86	2.01	−5.64	2.52	−5.60

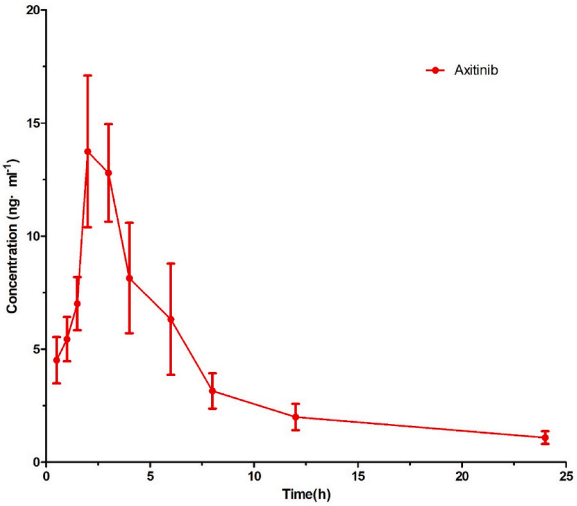

Fig. 4. Plasma drug concentration time curve of six beagle dogs after a single oral administration of 0.33 mg/kg of axitinib.

Table 4
The main pharmacokinetic parameters of axitinib in six beagle dogs (Mean ± SD).

Parameters	Unit	Axitinib
C_{max}	ng/mL	14.05 ± 3.20
T_{max}	h	2.17 ± 0.41
$T_{1/2}$	h	6.05 ± 1.85
CLz/F	L/h/kg	3.65 ± 1.12
$AUC_{(0\rightarrow t)}$	ng·h/mL	88.38 ± 20.56
$AUC_{(0\rightarrow \infty)}$	ng·h/mL	97.13 ± 25.96

5. Conclusions

In summary, this study established the first quantitative analysis method for the determination of axitinib in plasma of beagle dogs. The optimized method is stable, specific, with fast detection time, high recovery rate, good linearity, and less plasma required for the experiment. The method is suitable for monitoring therapeutic drug levels and studying drug interactions with axitinib.

CRediT authorship contribution statement

Hengyu Bi: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yixin Wang:** Conceptualization. **Xiaochen Ding:** Data curation. **Jiahang Li:** Formal analysis. **He Qi:** Funding acquisition. **Xiangjun Qiu:** Writing – review & editing.

Ethical approval

This study has obtained ethical approval and complies with the regulations of the Ethics Committee of the Animal Laboratory of Henan University of Science and Technology (Approval No. 202307002).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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