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Development of a novel UHPLC-MS/MS method for quantitative analysis of pirtobrutinib in rat plasma: application to pharmacokinetic study

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Abstract

Bruton's tyrosine kinase (BTK) inhibitors play a critical role in the treatment of mantle cell lymphoma (MCL). pirtobrutinib, a new, highly selective, non-covalent BTK inhibitor, was approved by the FDA for the treatment of MCL, chronic lymphocytic leukemia (CLL), and small lymphocytic lymphoma (SLL). In this study, we established a robust and reliable method for the quantitation of pirtobrutinib in rat plasma using ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Acetonitrile and 0.1% formic acid served as the mobile phase, with zanubrutinib as the internal standard (IS). Detection ion transitions were *m/z* 480.12→294.05 for pirtobrutinib and *m/z* 472.20→ 289.96 for Zanubrutinib. The intra-day and inter-day relative standard deviation (RSD%) values of pirtobrutinib were less than 9.8% and 10.3%, respectively. Recovery and matrix effects ranged from 95.1 to 101.5% and 91.7-100.4%. In addition, the test sample stability was confirmed under various storage conditions, and this method was successfully applied to a pharmacokinetic study of pirtobrutinib at a dose of 10 mg·kg^{-1}.

Keywords Pirtobrutinib, Pharmacokinetics, UHPLC-MS/MS, Zanubrutinib

Introduction

Bruton's tyrosine kinase (BTK) is a crucial non-receptor tyrosine kinase involved in regulating B-cell (B lymphocytes) proliferation, maturation, differentiation, apoptosis, and migration [1]. Activation of the B-cell receptor (BCR) signaling pathway, in which BTK is a key component, promotes tumor survival and proliferation [2]. BTK inhibitors (BTKi) specifically block BTK activation, impeding BCR signaling and inducing tumor cell apoptosis, making BTKi a potent for treating B-cell malignancies [3].

BTK inhibitors have increasingly replaced chemotherapy for conditions such as chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) [4], their good selectivity and reduced adverse event [5]. However, earlier generations of BTK inhibitors bind to BTK covalently and irreversibly, making it difficult to achieve minimal residue disease negativity in the treatment of CLL, and leading to challenges such as occasional intolerance and drug resistance [6]. In contrast, third-generation BTKi, including Pirtobrutinib, noncovalently and reversibly bind BTK, offering a new option for CLL patients resistant to previous BTK inhibitors due to genetic mutations such as BTK C481 [7]. The non-covalent BTKi have

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demonstrated efficacy and safety in clinical trials, bringing new hope for those with previous covalent BTKi treatment resistance or intolerance [8].

Pirtobrutinib (Fig. 1A), a third-generation BTKi, is the first non-covalent and reversible BTK inhibitor, to receive FDA approval in 2023 [9] for the treatment of CLL/SLL patients who have undergone at least two prior lines of treatment, including BTKi and B-cell lymphoma-2 (BCL-2) inhibitor therapy. Unlike covalent BTKis, Pirtobrutinib exhibits highly selective inhibition effects on both wild type and C481-mutant BTK with low nM potency in vitro. Furthermore, pirtobrutinib can inhibit the autophosphorylation of Y223 in all mutant BTKs (C481S, C481T, C481R) [10]. In the Phase I/II BRUIN study [11], 323 patients received treatment with pirobrutinib with no dose-limiting toxicities during the trial. The most common grade 3 adverse event (AE) was granulocyte deficiency (10%), with 1% of patients discontinuing medication due to treatment-related AEs. Among 121 CLL/SLL patients who had previously received covalent BTKi treatment and were evaluable for efficacy, the overall response rate (ORR) of pirtobrutinib treatment was 62%. Moreover, the ORR was similar in CLL patients with previous covalent BTKi resistance, covalent BTKi intolerance, C481-mutant or wild-type BTK. In addition, in a comparison study conducted to estimate the effect of pirtobrutinib versus venetoclax (BCL-2 inhibitor) monotherapy in patients with covalent BTKi-pretreated CLL, the ORR was significantly higher for pirtobrutinib [12]. The results indicated that pirtobrutinib was well-tolerated, effective, and suitable for patients with relapsed CLL following covalent BTKi.

Pirtobrutinib has high permeability in vitro, but low aqueous solubility. To reduce the variability in oral absorption, a spray-dried dispersion tablet formulation was developed, providing consistent oral bioavailability [11]. According to the information in the package insert of JAYPIRCA®, The absolute bioavailability of pirtobrutinib after a single oral 200 mg dose is 85.5% (range 75.9–90.9%). The median time to reach peak plasma concentration (t_{max}) is approximately 2 h (0.833 to 4.15 h). The effective half-life of pirtobrutinib is approximately 19 h and the mean apparent clearance is 2.05 L/h (37.2%). In vitro, pirtobrutinib is primarily metabolized

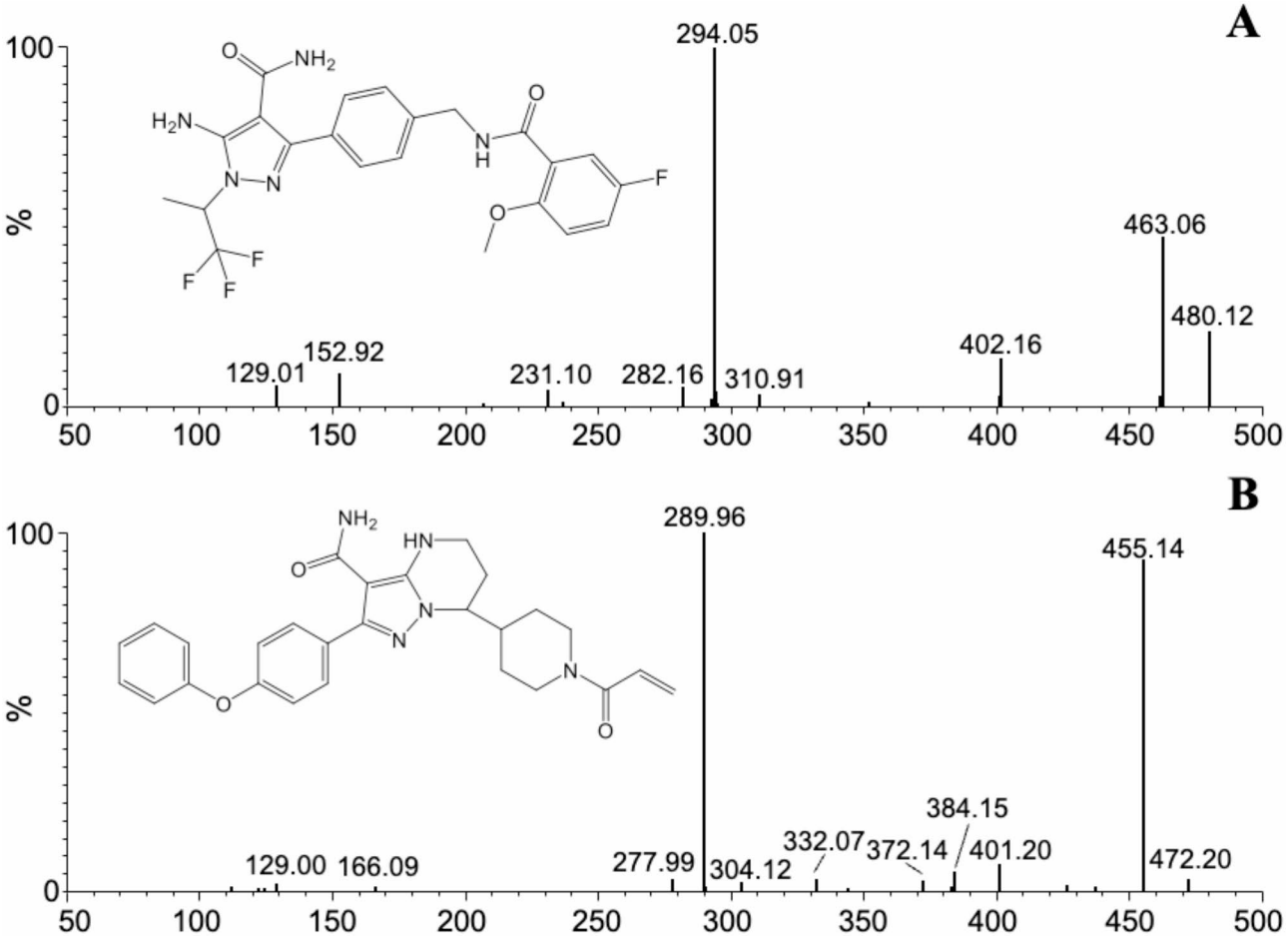

Fig. 1 Mass spectra of pirtobrutinib (**A**) and zanubrutinib (**B**) in this study

by CYP3A4 and direct glucuronidation by UGT1A8 and UGT1A9, and the human protein binding is 96%, which is independent of concentration.

Despite these advancements, there is currently no established ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) determination method for pirtobrutinib, however, pharmacokinetics of pirtobrutinib has not been reported. Considering the urgency of clinical application of this new generation anti-cancer drug and the demand of accurate quantification in plasma for therapeutic concentration monitoring, we established a sensitive and stable determination method based on UHPLC-MS/MS. In addition, this method has been successfully applied to pharmacokinetic studies of pirtobrutinib in rats administered intragastric at a dose of 10 $mg{\cdot}kg^{-1}$. This study contributes valuable data for further research on BTKis such as development of new generation BTKi drugs, drug-drug interaction studies, and therapeutic drugs monitoring in both preclinical and clinical studies.

Experimental

Chemical reagent

Pirtobrutinib (Fig. 1A) and zanubrutinib (used as internal standard, IS, Fig. 1B) were purchased from DC Chemicals company (Shanghai, China) with >99% purity. The HPLC grade acetonitrile, methanol and formic acid were provided by Fisher Scientific (Boston, MA), the dimethyl sulfoxide was purchased from J&K Scientific (Beijing, China), and the carboxymethyl cellulose sodium (CMC-Na) was provided by Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). The ultrapure water was prepared by Milli-Q ultrapure water system (Millipore, Bedford, USA).

Instrument and parameter conditions

An UHPLC chromatographic system of the SCIEX ExionLC AC system (San Diego, CA, USA) was used for separation optimization. Chromatographic separation was accomplished on a SHIMADZU Shim-pack GIST C18 column (2.1 mm×100 mm, 2 μm; Japan), the mobile phases was 0.1% formic acid and acetonitrile. Gradient elution was as follows: 0–0.5 min (40% acetonitrile) and 0.5–1.0 min (40–90% acetonitrile), 1.0–2.0 min (90% acetonitrile) and 2.0–2.1 min (90–40% acetonitrile), followed by keeping 40% acetonitrile between 2.1 and 4.0 min for equilibration. The column and auto-sampler temperature were maintained at 40 °C and 4 °C. The whole analytical assay time was 4.0 min, the flow rate was 0.4 mL/min and the injection volume was 1 μL. The retention time was 2.26 min for pirtobrutinib, and 2.25 min for IS, respectively.

A SCIEX QTRAP 6500 + triple quadrupole mass spectrometer (San Diego, CA, USA) was used for sample detection. MRM in the positive mode was used to detect pirtobrutinib and IS with ion transition of *m/z* 480.12→294.05 and *m/z* 472.20→289.96, the collision energy for both transitions was 30 V and 40 V, respectively. The ion spray voltage and temperature were set as 5000 V and 500 °C, respectively.

Calibration solution and QC sample preparation

Stock solutions of pirtobrutinib and the IS were made in dimethyl sulfoxide at a concentration of 1 mg/mL, both the pirtobrutinib working solution and the 400 ng/mL IS working solution were diluted with acetonitrile from their respective stock solutions. Calibration curve standard and quality controls (QCs) solution was prepared by ten times dilution of 5 μL corresponding working solution add to 45 μL rat blank plasma. The final concentrations of eight calibration standards samples were set to 0.5, 1.0, 5.0, 20, 100, 200, 500 ng/ mL, respectively, and the three concentrations of QC samples were 1, 25, 400 ng/mL. All solutions and samples were kept at − 20 °C before use.

Sample preparation

The samples were extracted via protein precipitation, 50 μL plasma sample, 10 μL of the IS working solution (concentration of 400 ng/mL), and 200 μL cold methanol (-20 °C) was mixed and vortexed well in an Eppendorf tube. The samples were centrifuged at 14, 000 *rpm* for 10 min at 4 °C, the supernatant was transferred to autosampler vials and injected for UHPLC-MS/MS analysis.

Method validation

To verify the applicability of the quantitative method, selectivity, sensitivity, accuracy, precision, recovery, matrix effect, stability and dilution integrity was validated. The Validation of analytical method in this study was carried out in compliance with "Bioanalytical Method Validation Guidance for Industry", which was issued by FDA in 2018 [13].

Animal experiments

Six male Sprague–Dawley rats (age: 8 weeks; weight:230 g ± 10 g) were purchased from Beijing Huafukang Biotechnology Co. Ltd (Beijing, China), the experimental animal production license number: SCXK 2024-0003. All animals were fed in a SPF conditioned environment for 1 week and before the experiment. The animal experimental protocol in this study was approved by the animal Ethics Committee of Beijing Friendship Hospital (Beijing, China). The rats were anesthetized via isoflurane inhalation prior to blood sample collection.

Euthanasia of experimental animals was executed in line with the AVMA Guidelines for the Euthanasia of Animals. After completion of the experiment, all animals

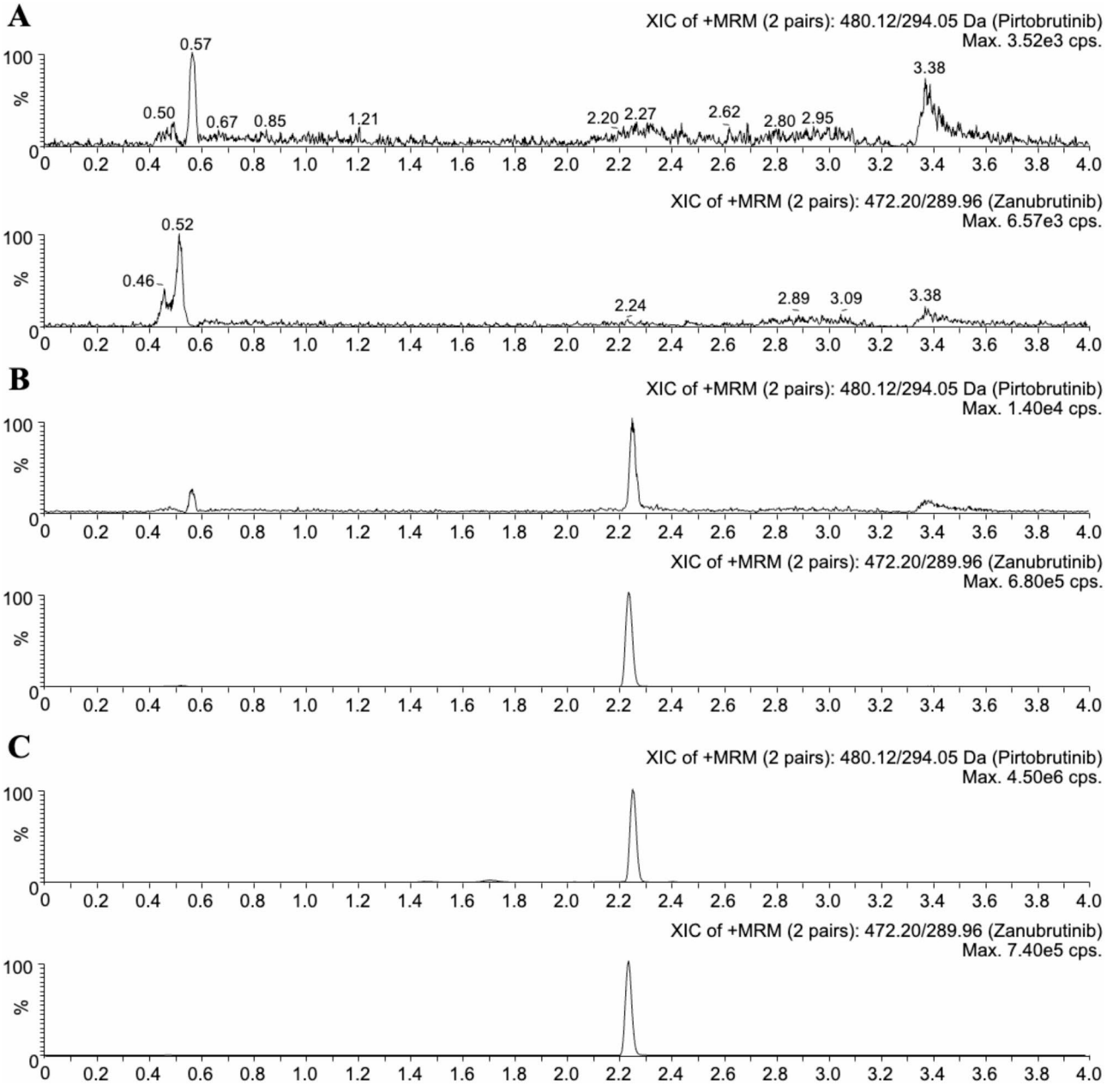

Fig. 2 Representative chromatograms of pirtobrutinib and IS in rat plasma: **A** blank plasma; **B** blank plasma spiked with analyte at LLOQ and IS; **C** plasma sample collected from a rat at 0.25 h after intragastric administration of 10 mg·kg^{-1} pirtobrutinib (the concentration is 140 ng/mL)

were euthanized with carbon dioxide, and then packed and cremated after confirming that the animals were free of life pointers.

Table 1 The precision and accuracy of pirtobrutinib in rat plasma ($n=6$)

Analyte	Concentration (ng/mL)	Intra-day		Inter-day	
		RSD %	RE %	RSD %	RE %
Pirtobrutinib	0.5	9.2	-3.5	8.9	-0.5
	1.0	6.3	-9.2	6.3	-8.4
	25	9.8	-7.4	9.9	0.6
	400	7.7	-10.3	10.3	-5.8

Pharmacokinetic studies and incurred sample reanalysis (ISR)

After fasted for 12 h, all rats were intragastrically administered 10 mg·kg^{-1} pirtobrutinib suspension, which was prepared in 0.5% carboxymethyl cellulose sodium (CMC-Na). Before administration and 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h after administration, 200 μL blood samples were collected from the ophthalmic

Table 2 Recovery and matrix effect of pirtobrutinib in rat plasma ($n=6$)

Analyte	Concentration (ng/mL)	Matrix effect (%)		Recovery (%)	
		Mean ± SD	RSD %	Mean ± SD	RSD %
Pirtobrutinib	1.0	95.1 ± 8.4	8.9	96.1 ± 12.8	13.3
	25	101.5 ± 6.0	5.9	100.4 ± 10.0	9.9
	400	97.5 ± 6.0	6.1	91.7 ± 7.2	7.8

veins and put into EDTA-K_2 tubes. Whole blood samples were centrifuged at 4000 *rpm* for 10 min at 4 °C, then, the supernatant plasma was separated and frozen at – 80 °C.

The primary pharmacokinetic parameters were calculated by non-compartmental pharmacokinetic analysis (NCA) model using Phoenix software (WinNonlin, Version 8.4, Mountain View, CA, USA), including area under the curve (AUC) from zero to last quantifiable concentration (AUC_{0-t}), AUC from zero to infinity ($AUC_{0-\infty}$), mean residence time from zero to last quantifiable concentration (MRT_{0-t}), mean residence time from zero to infinity ($MRT_{0-\infty}$), half-life of elimination ($t_{1/2}$), time to peak concentration (T_{max}) and peak concentration (C_{max}).

The ISR was evaluated in this study. Twelve incurred plasma samples selected from the time points around C_{max} and the elimination phase were reanalyzed.

Results and discussion

Method development

When using UHPLC-MS/MS for quantitative analysis of compounds, the optimization and selection of chromatographic separation conditions and mass spectrometry parameters are particularly important, as they affect the sensitivity and accuracy of quantification results [14]. Chromatographic separation mainly involves the selection of the solid phase in chromatographic column, mobile phase and gradient program. The separation performance of different chromatographic columns has been evaluated in this method, the peak of analytes exhibited appropriate width and peak shape on the Shimadzu Shim pack GIST C18 column (2.1 mm×100 mm, 2 μm), demonstrating better separation and symmetry. Meanwhile, the column temperature was investigated and set as 40 ºC, which was suitable for peak shape and width. When selecting the mobile phase, ultrapure water with 0.1% formic acid was chosen for the aqueous phase. Methanol or acetonitrile as organic phase were compared, and the results showed that acetonitrile could accelerate the elution of pirobrutinib from the chromatographic column.

Generally, a stable isotope-labeled internal standard (IS) would be better for quantitation in LC-MS/MS method, while difficult to obtain. Therefore, another BTKi analogue-zanubrutinib was chosen as the IS in this experiment, zanubrutinib has a similar chromatographic status to pirtobrutinib, which meets the requirements of the method validation in terms of specificity, matrix effects and recovery aspects. The retention time of pirtobrutinib and IS were 2.26 min and 2.25 min, the chromatographic separation of the two compounds was shown in Fig. 2.

In the optimization of mass spectrometry parameters, pirtobrutinib a good MS response in ESI positive ion mode and produced abundant product ion fragmentations in MS^2 spectrum (Fig. 1). The precursor ion of pirtobrutinib was $[M+H]^+$ at *m/z* 480, it was proposed that the product ion at *m/z* 463 was due to the loss of NH_3, the C-N bond in the middle of the molecule between amide groups and phenyl methyl group was easy to cleavage, generated the fragment ion at *m/z* 310.9, followed by the loss of NH_3, the most abundant and significant characteristic ion at *m/z* 294 was produced, then, the methyl on the phenyl group was removed and formed into fragment ion at *m/z* 282. In terms of the low noise interference and high intensity, m/z 480.12→294.05 and 472.20→289.96 were selected as ion transitions for pirtobrutinib and IS, respectively. However, due to the significant impact on response intensity of collision voltage settings, the collision voltages were carefully optimized and set to 30 eV and 40 eV for pirobrutinib and IS.

In addition, the protein precipitant process was adopted in sample pre-treatment process and the protocol was investigated under several conditions, among which three different precipitant solvents (methanol, acetonitrile, and methanol/acetonitrile (4:1, v/v)) were compared and optimized during the experiment. The results showed that cold methanol effectively extracted pirobrutinib and remove the interference from plasma, and the matrix effect and recovery rate also meet the standards.

Method validation

Specificity and sensitivity

As shown in Fig. 2, the retention time of pirtobrutinib and the IS were 2.26 min and 2.25 min. In six blank plasma samples from different rats (Fig. 2A), no interfering peaks

Table 3 Stability results of pirtobrutinib in plasma under different conditions ($n=6$)

Analyte	Concentration (ng/mL)	Room temperature, 4 h		Autosampler 4 ℃, 24 h		Three freeze-thaw		-80 ℃, 30 days	
		RSD %	RE %	RSD %	RE %	RSD %	RE %	RSD %	RE %
Pirtobrutinib	1.0	7.5	0	6.1	-6.9	4.1	-3.7	5.4	-1.8
	25	3.5	1.7	9.8	-9.4	8.5	3.3	3.7	9.4
	400	5.2	-2.6	6.8	-11.7	5.9	6.0	7.0	4.5

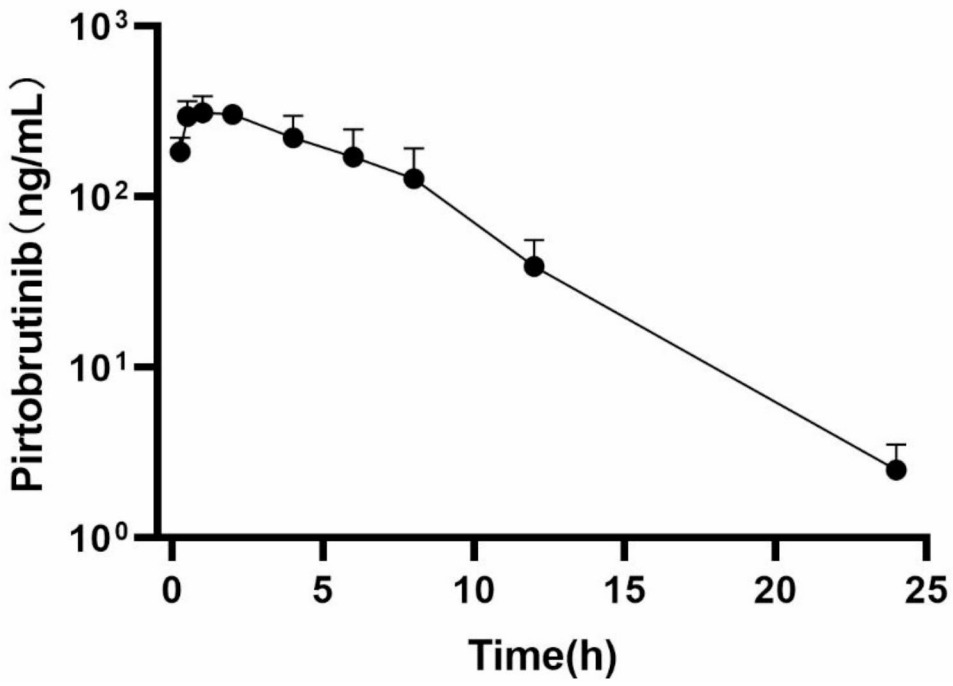

Fig. 3 Mean plasma concentration–time curves of pirtobrutinib in rats after intragastric administration of pirtobrutinib at a single dose of 10 mg·kg^{-1}. ($n = 6$)

for pirtobrutinib and IS were observed from endogenous substances. The results confirmed that the specificity and sensitivity of the optimized method complied with the bioanalytical method validation guidelines.

Calibration and LLOQ

A linear relationship was observed between pirtobrutinib concentration in rat plasma and the corresponding peak area. The representative linear regression equation of the calibration curve was $Y = 0.0305X + 0.00279$ ($r2 = 0.999$). The lower limit of quantification (LLOQ) for pirtobrutinib was 0.5 ng/ml, with an S/N ratio for pirtobrutinib significantly higher than 10 at this LLOQ level. The precision and accuracy at the LLOQ level were 9.2% and − 3.5%, respectively, meeting the requirements for quantification.

Table 4 The main pharmacokinetic parameters of pirtobrutinib in rat plasma after intragastric administration of pirtobrutinib at a single dose of 10 mg·kg^{-1}

Parameter	Pirtobrutinib
$AUC_{0\text{-}t}$ (ng/mL*h)	2341 ± 578
$AUC_{0\text{-}\infty}$ (ng/mL*h)	2352 ± 582
$MRT_{0\text{-}t}$ (h)	5.05 ± 0.923
$MRT_{0\text{-}\infty}$ (h)	5.15 ± 0.940
$t_{1/2}$ (h)	2.95 ± 0.121
T_{max} (h)	1.08 ± 0.736
C_{max} (ng/mL)	344 ± 56.6

Accuracy and precision

The precision and accuracy of pirtobrutinib were assessed by testing QC samples from low to high concentrations (1.0, 25 and 400 ng/ mL). The results were listed in Table 1, the intra- and inter-day accuracy and precision were both within ± 10.3%. indicating that the determination of pirtobrutinib in rat plasma in this method aligns well with the acceptance criteria.

Recovery and matrix effect

In this study, a simple protein precipitation method was employed for plasma extraction. As shown in Table 2, the results of the IS-normalized recovery and matrix effects were acceptable, with mean recovery for pirtobrutinib falling within the limits of 91.7–100.4% with an RSD% ≤ 13.3%. The mean matrix effect for pirtobrutinib ranged from 95.1 to 101.5% with an RSD% less than 8.9%, demonstrating that both recovery and matrix effects for this extraction procedure were acceptable.

Stability

To assess the short- and long-term stability of pirtobrutinib in rat plasma before and after sample pretreatment, four different storage conditions were tested, including room temperature for 4 h, 4 °C in autosampler after preparation, three freeze- thawing cycles (− 80 °C to room temperature), and long-term storage at − 80 °C for one month. As listed in Table 3, the stability test of the three QC samples showed that the RSD% value was

less than 9.8%, and the RE% value was less than ± 11.7%, both meeting the ± 15% stability criteria under all conditions. These results indicate that pirtobrutinib is stable in plasma under several storage conditions and handling conditions.

Dilution integrity

To evaluated dilution integrity, the concentration of the dilution QC samples was set as 800 ng/mL, with a dilution factor of 2, six replicates were analyzed within a sample analysis batch. The mean accuracy of 6 replicates was 1.67%, and the %CV was 2.95, which is consistent with the acceptance criteria in the guideline (the mean accuracy of the dilution QCs should be within ± 15% of the nominal concentration and the precision (%CV) should not exceed 15%).

Pharmacokinetic study and ISR

The developed UHPLC-MS/MS assay was successfully applied to pharmacokinetic study of pirtobrutinib in rats. The mean plasma concentration versus time curve was shown in Fig. 3, and the main pharmacokinetic parameters were summarized in Table 4. After intragastric administration, pirtobrutinib was rapidly absorbed, reaching its peak concentration at 1.08 ± 0.736 h (T_{max}). The C_{max} was 344 ± 56.6 ng/ml, with a $t_{1/2}$ of 2.95 ± 0.121 h. At the last sampling timepoint (48 h), the concentration of pirtobrutinib was below the LLOQ due to the rapid metabolization.

To our knowledge, no prior studies have reported quantitative analysis of pirtobrutinib concentrations in plasma. This study is the first to develop and validate a UHPLC-MS/MS assay for the accurate determination of pirtobrutinib, successfully applying to a pharmacokinetic study in rats. However, due to the species differences, the pharmacokinetic data of rats in this study may not fully represent pirtobrutinib's behavior in humans, further research is needed to investigate the pharmacokinetics of pirtobrutinib in human subjects.

The %difference between repeat value and initial value of twelve incurred plasma samples were less than 20%. It shows that ISR in this study meet the acceptance criteria (the percent difference should be ≤ 20% for at least 2/3 of the repeats).

Conclusions

A robust and reliable UPLC-MS/MS assay was firstly developed and validated for the quantitative determination of pirtobrutinib in rat plasma. This method was successfully applied to a pharmacokinetic study in which pirtobrutinib was intragastric administered at a dose of 10 $mg{\cdot}kg^{-1}$ in rats. The findings from this research provide a valuable reference for further preclinical and clinical studies of pirtobrutinib and other BTK inhibitors.

Author contributions
RD conceived and designed the experiments; MZ, JL and HY performed the experiments; JW and MH analyzed the data; MZ wrote the paper. All authors read and approved the final manuscript.

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Data availability
All data and material analyzed or generated during this investigation are included in this published article. The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Declarations

Ethics approval and consent to participate
According to the Guidelines of the Experimental Animal Care and Use of Laboratory Animals of The Beijing Friendship Hospital. All animal procedures and experimental protocols were approved by Institutional Animal Care and Use Committee of Beijing Friendship Hospital, Capital Medical University (Ethics approval number: 24-1003).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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