

Validated UPLC-MS/MS method for quantification of fruquintinib in rat plasma and its application to pharmacokinetic study

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Abstract: A new, simple, and sensitive ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for quantification of fruquintinib was established to assess the pharmacokinetics of fruquintinib in the rat. The internal standard working solution was added to the plasma sample for extraction before analysis. The Acquity UPLC BEH C18 chromatography column (2.1 mm × 50 mm, 1.7 μm) was used to separated analytes under gradient elution using acetonitrile and 0.1% formic acid as the mobile phase. Positive multiple reaction monitoring modes were chosen to detect fruquintinib and diazepam (IS). The precursor-to-product ion transitions were 394.2 → 363.2 for fruquintinib and m/z 285 → 154 for IS. The current method was linear over the concentration range of 1.0–1000 ng/mL for fruquintinib with a correlation coefficient of 0.9992 or better. The matrix effect of fruquintinib and IS was acceptable under the current method. The intra- and interday precision (RSD%) and accuracy (RE%) were within 11.9% and ±13.7%, respectively. The recovery, stability, and sensitivity were validated according to the United States Food and Drug Administration (FDA) regulations for bioanalytical method validation. The analytical method had been validated and applied to a pharmacokinetic study of fruquintinib in rat.

Keywords: fruquintinib, UPLC-MS/MS, rat plasma, pharmacokinetics

Introduction

As an important hallmark of cancer development and progression, angiogenesis plays a major role in vascular remodeling, cell proliferation, cancer cell dissemination, and metastasis.^{1–4} Vascular endothelial growth factor (VEGF) secreted by tumors promotes the formation of the angiogenesis. The angiogenesises around the tumor supplied the blood flow, oxygen, and nutrients to the tumor at an advanced stage. The anti-cancer therapies targeting VEGF/vascular endothelial growth factor receptors (VEGFR) axis included neutralization of VEGF or VEGFR by monoclonal antibodies and blockage of VEGFR kinase activity with small molecule inhibitors.⁵ Due to its long half-life and high specificity, bevacizumab can sustain target inhibition and also combine with other chemotherapies. It limits bevacizumab clinical use or patients' access because of intravenous dosing, autoimmune diseases after long-term treatment, immunogenicity, and especially high cost.⁶ Therefore, an active small molecule VEGFR inhibitor by oral administration combined with chemotherapies to achieve sustained target inhibition and avoid the limitation of antibodies is highly desirable.

Small-molecule VEGFR inhibitors include first-generation sunitinib,⁷ regorafenib,^{8,9} sorafenib¹⁰ and pazopanib;¹¹ second-generation tivozanib¹² and axitinib.¹³ The less selective of these inhibitors limited their clinical use. New-generation tyrosine kinase inhibitors (TKIs) include icotinib,¹⁴ apatinib,¹⁵ famitinib,¹⁶ flumatinb,¹⁷ allitinib,¹⁸ fruquintinib,¹⁹ and selatinib,²⁰ among which icotinib and apatinib have been approved in China, while others are in clinical trials. Fruquintinib (6-(6, 7-dimethoxyquinazolin-4-yloxy) - N,2-dimethylbenzofuran-3-carboxamide, **Figure 1A**) developed by Hutchison MediPharma Ltd. (Shanghai, China) had been studied in Phase II and Phase III clinical trial for non-small-cell lung cancer (“NSCLC”) and colorectal cancer (CRC) and demonstrated highly potent efficacy and safety profile in vitro and in vivo.^{19,21–24} In 2018, fruquintinib was approved in China for treatment of CRC. It is an oral TKI with a highly selective small-molecule inhibitor of VEGFR-1, -2, and -3, thus inhibiting endothelial cell proliferation and migration.^{2,19,21}

When more than two prior systemic antineoplastic therapies have failed to treatment metastatic CRC patients, fruquintinib will be used in these patients. To the best of our knowledge, there are several LC-MS/MS methods reported for the quantitative bioanalysis of fruquintinib in plasma.^{21,25} However, these methods were not described in enough detail (eg, plasma extraction procedure, chromatography conditions, parameters of the method, etc.) for duplication in other laboratories. Furthermore, the time of sample collection

was too short, and the plasma concentration of the parent compound was still above the minimum effective concentration or lower limit of quantitation (LLOQ). Therefore, the current study is aimed to develop a new and sensitive ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for the quantification of fruquintinib and assess the pharmacokinetics of fruquintinib with sufficient time to metabolize in the rat.

Materials and methods

Chemicals and reagents

Fruquintinib (CAS NO: 1194506-26-7) was purchased from Shanghai Boylechem Co., Ltd (Songjiang District, Shanghai, China); diazepam (CAS NO: 65854-76-4) as internal standard (IS) (**Figure 1B**) and formic acid (CAS:64-18-6) were provided by Sigma-Aldrich Company (St. Louis, MO, USA); all materials were suitable for use in LC/MS or UPLC/MS laboratory testing by spiking into the matrix of choices such as urine or plasma. Acetonitrile with LC grade was supplied from Merck Company (Darmstadt, Germany), and the other reagents were all analytical grade. The water used in the laboratory was prepared by Wahaha pure water which filtered through a 0.45 µm filter. 0.1% formic acid was prepared by 1 mL formic acid diluted with the water to 1 L.

Analytical system

The Acquity UPLC BEH C18 chromatography column (2.1 mm ×50 mm, 1.7 µm) was used to separated analytes

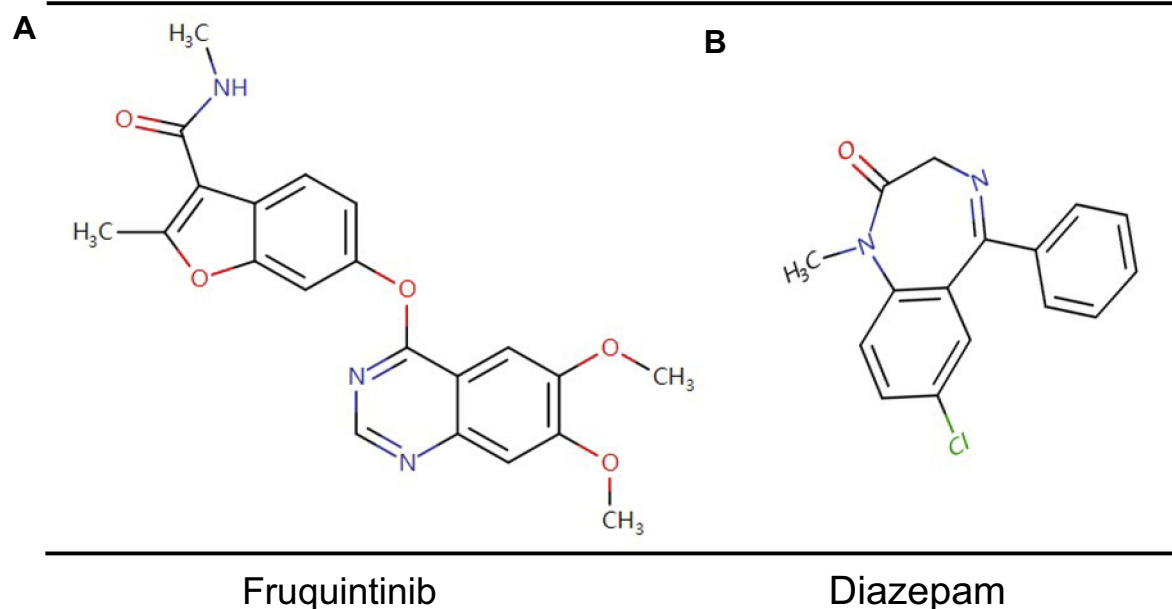


Figure 1 The chemical structures of the analytes in the present study. **(A)** fruquintinib; **(B)** diazepam.

under gradient elution using acetonitrile (mobile phase A) and 0.1% formic acid (mobile phase B) as the mobile phase. The gradient elution program was as follows: 0–0.5 mins (90% A), 0.5–1 mins (90–10% A), 1.0–2.0 mins (10% A), 2.0–2.1 mins (10–90% A), and 2.1–3.0 mins (90% A). The overall chromatographic run time was 3.0 min and the flow rate of the method was set at 0.30 mL/min. Mass spectrometric detection was performed on a XEVO TQD triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Waters Corp., Milford, MA, USA). The analytical method used positive multiple reaction monitoring mode to detect fruquintinib and diazepam (IS). The precursor-to-product ion transitions were 394.2 → 363.2 for fruquintinib and m/z 285 → 154 for IS (desolvation temperature 500°C, desolvation gas flow rate 600 L/h, the argon flow rate 150 L/h, collision 7.0 Bar). All data acquisition and instrument control were processed under the Masslynx 4.1 software (Waters Corp.).

Preparation of standard solutions and quality control sample (QC)

Fruquintinib or IS was dissolved in acetonitrile and prepared to a stock solution (1 mg/mL) which was stored at 4°C. The standard solutions were diluted to the stock solution with acetonitrile. Calibration curves standard solutions were prepared by adding blank plasma samples to the corresponding working solutions. The final concentrations of the calibration curves were as follows: 1.0, 5.0, 10, 50, 100, 500, and 1000 ng/mL for fruquintinib. The quality control sample (QC) solutions were prepared in the same manner to the following concentrations: 2.0, 80, and 800 ng/mL for fruquintinib. The above-prepared solutions were all stored at –20°C until analysis.

Plasma sample preparation

100 µL thawed plasma sample was spiked with 200 µL acetonitrile (IS 100 ng/mL) and vortex mixed for 1.0 min, and then centrifugation was done at 13,000 g for 10 mins. A clear supernatant (6 µL) after centrifugation was injected into the analytical system for analysis.

Method validation

The UPLC-MS/MS method for quantification of fruquintinib in rat plasma and its application to pharmacokinetic study was validated according to the FDA guidelines.²⁶

Blank rat plasma samples spiked with fruquintinib and IS were used to carry out selectivity. Specificity was

evaluated by the interference at the retention time of fruquintinib and IS.

A weighted ($1/x^2$) least-squares linear regression was used to assess the linearity. The current method was linear over a range of 1.0–1000 ng/mL for fruquintinib with a correlation coefficient of 0.99 or better. A deviation of ±15% for each back-calculated standard concentration was accepted, while it was ±20% for LLOQ.

The precision and accuracy of our method were evaluated by analyzing 6 replicates at 2, 80, 800 ng/mL within 1 day or on 3 consecutive days. Precision was measured as a relative standard deviation (RSD%), while the accuracy was a relative error (RE%). A deviation of ±15% for the accuracy and precision was accepted.

The recovery was calculated as the ratio of the peak area of the analyte in QCs to the post-protein precipitation at equivalent analyte concentrations (6 replicates at 2, 80, and 800 ng/mL). The matrix effects of rat plasma were assessed by comparing the ratio of the analyte concentration in the post-extracted sample to the pure standard solution at equivalent concentrations.

The stability of fruquintinib at different conditions (room temperature for 12 hrs, 4°C for 12 hrs, –80°C for 28 days, and three freeze–thaw cycles from –20°C to room temperature) was evaluated by analyzing 5 replicates at three concentration levels (2, 80, 800 ng/mL). It could be permitted when the analyte stability was between 85% and 115%.

The pharmacokinetic study in the rat

Fruquintinib dissolved in 0.5% sodium carboxymethyl cellulose (1.0 mg/mL) was orally given to eight young adult Sprague–Dawley rats (2 months, 180–220 g), and the volume of the suspension given to the animal was 1.0 mL/kg. The rat blood samples (0.3 mL) were taken via caudal vein at the following time points after drug administration: 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 9, 12, 24, and 48 hrs. 100 µL plasma was separated from whole blood after centrifuged at 10,000 g for 5 min, and then stored at –20°C before analysis. Approved by the Wenzhou Medical University Ethics Committee (SYXK 2015-0009), the study was performed under the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Noncompartmental pharmacokinetic parameters were calculated for fruquintinib.^{27–29} The pharmacokinetic parameters of fruquintinib were noted directly by DAS (Drug and statistics) software (Version 2.0,

Shanghai University of Traditional Chinese Medicine, China).³⁰

Results and discussion

The development and optimization of the method

Although liquid extraction, protein precipitation, and solid-phase extraction have been commonly used to extract analytes from plasma or serum in many studies, there are not many pieces of research to study the method for determination fruquintinib so far. It reports that liquid chromatography-tandem mass spectrometry (LC-MS/MS) had been used to determine fruquintinib in rat plasma.^{16,21,25} Due to time saving, simplicity, low matrix effects and the comparative results that we did, we adopted UPLC-MS/MS and the protein precipitation. Meanwhile, UPLC-MS/MS was available in most laboratories at present. The proper combination of mobile phases helped to achieve peak symmetry, shorten analysis time and optimize ionization. When compared to methanol-0.1% formic acid, acetonitrile-0.1% formic acid with gradient elution could provide better effects and less matrix effects. The other parameters of MS/MS conditions and chromatography had also been made optimal at the same time.

Selectivity and matrix effect

Representative chromatograms are shown in Figure 2. No evident interference was found at the elution times: fruquintinib and IS were eluted at 1.40 and 1.59 min, respectively.

The matrix effect of fruquintinib ranged from 87.4% to 95.6%, while the IS was 102.4%. Therefore, it was negligible for ion suppression or enhancement from rat plasma in the study.

Calibration curve and sensitivity

The current method was linear over a range of 1.0–1000 ng/mL for fruquintinib with a correlation coefficient of 0.9992, and the LLOQ was 1.0 ng/mL for fruquintinib. The calibration curve was $y=2.8493x+7.1062$ ($n=3$), where y =the peak area ratio of fruquintinib to IS, x =the plasma concentration of fruquintinib. The LLOQ with RSD <11.9% and RE ≤13.7% was acceptable in the present study.

Precision, accuracy, and recovery

The results in Table 1 represented the accuracies and precisions. The accuracies (RE%) of the method was within ±13.7%, while the precisions (RSD%) within 11.9%. The results demonstrated that the UPLC-MS/MS method was suited for determination fruquintinib. The recovery by protein precipitation with acetonitrile was between 82.0% and 94.4%.

Stability

The stability under different storage and process conditions (room temperature for 12 hrs, 4°C for 12 hrs, −80°C for 28 days, and three freeze–thaw cycles from −20°C to room temperature) was demonstrated and the results are summarized in Table 2 and found to be acceptable (within ±15%).

Pharmacokinetic study of fruquintinib

The current UPLC-MS/MS method was applied to the pharmacokinetic study after a single oral dose (1.0 mg/kg) of fruquintinib. The noncompartment model was used in the calculations of pharmacokinetic parameters. The pharmacokinetic parameters included $t_{1/2}$, T_{max} , CL/F, C_{max} , AUC and are shown in Table 3. The mean plasma concentration–time profile of fruquintinib after single-dose administration is presented in Figure 3.

Fruquintinib was absorbed rapidly after oral administration of 1.0 mg/kg. C_{max} was reached at 4.25 hrs after dosing, consistent with the previous study.²⁵ Because of slow elimination and low tissue distribution, the mean $t_{1/2}$ of fruquintinib was about 3.34 hrs in rat plasma. There were few studies to quantitate bioanalysis of fruquintinib in rat plasma. A study indicated that the terminal $t_{1/2}$ ranged from 1 to 2 hrs in rat.²⁵ But the time of sample collection was too short in that study, and the plasma concentration of parent compound was still above the minimum effective concentration or LLOQ. Meanwhile, the oral bioavailability of fruquintinib in the rat was 42–53% and its plasma protein binding fraction is 88–95%. The extents of CL and V_z in each species were different, which might lead to the different $t_{1/2}$ profiles. The above facts could explain the data discrepancy in C_{max} (712.28 vs 326.00 ng/mL) and $t_{1/2}$ (3.34 vs 1.40 hrs).

Conclusion

In summary, a fast, simple, sensitive, and accurate UPLC-MS/MS method was established for quantitation of fruquintinib in

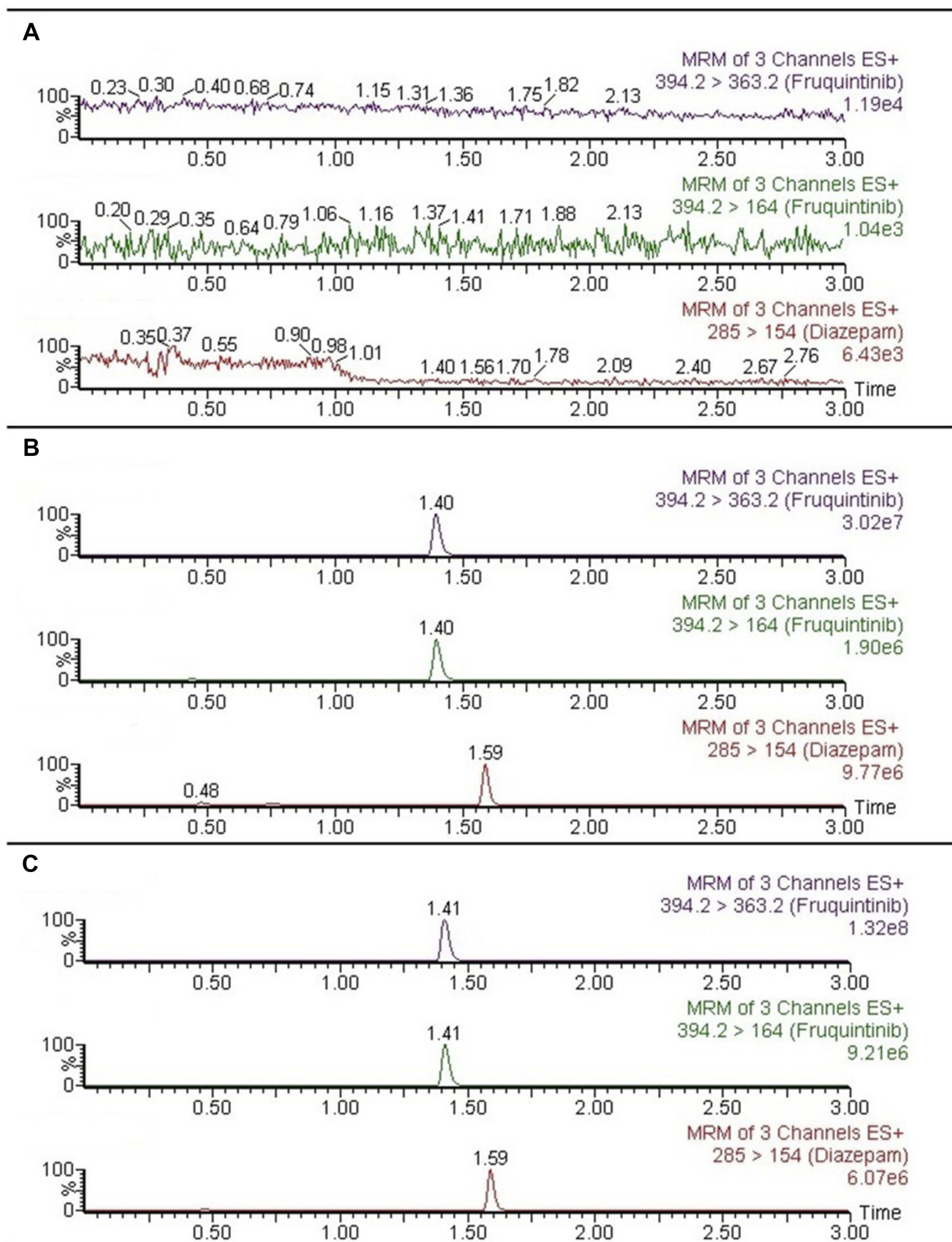


Figure 2 Representative chromatograms of blank plasma (A), blank plasma spiked with standard solution (B), and plasma sample obtained from oral administration of fruquintinib in rats (C).

Table 1 The precision, accuracy, and extraction efficiency of fruquintinib (n=6)

Concentration (ng/mL)	Precision (RSD %)		Accuracy (RE %)		Extraction efficiency (%) mean $\pm$ RSD
	Intraday	Interday	Intraday	Interday	
2	11.9	9.8	-13.0	-7.0	85.2 $\pm$ 13.1
80	6.6	2.5	11.7	13.7	94.4 $\pm$ 4.2
800	5.8	2.3	-5.1	-3.0	82.0 $\pm$ 3.5

Abbreviations: RSD, relative standard deviation; RE, relative error.

Table 2 Stability results of fruquintinib in rat plasma in different conditions (n=5)

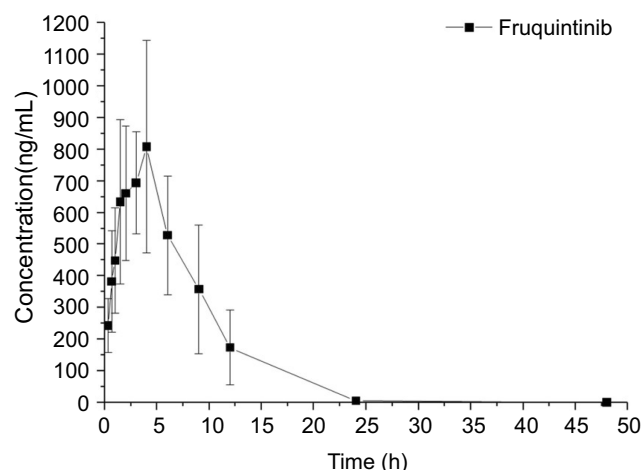
Concentration (ng/mL)	Room temperature, 12 hrs		4°C, 12 hrs		Three freeze-thaw		-80°C, 28 days	
	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)
2	12.12	3.91	9.10	7.10	11.81	-2.38	6.20	-2.38
80	4.29	-1.67	4.19	-4.21	14.00	4.66	11.91	8.42
800	1.95	2.32	0.74	6.14	9.34	5.16	8.92	3.63

Abbreviations: RSD, relative standard deviation; RE, relative error.

Table 3 The pharmacokinetic parameters of fruquintinib in rat plasma after oral administration of 1.0 mg/kg fruquintinib (n=8, mean $\pm$ SD)

Parameters	Fruquintinib
$t_{1/2}$ (hrs)	3.34 $\pm$ 1.04
T_{max} (hrs)	4.25 $\pm$ 1.17
CL/F (L/h/kg)	0.21 $\pm$ 0.12
C_{max} (ng/mL)	712.28 $\pm$ 319.40
AUC _{0-t} (ng/mL·h)	6267.54 $\pm$ 3004.66
AUC _{0-∞} (ng/mL·h)	6269.42 $\pm$ 3005.53

Abbreviations: $t_{1/2}$, half-life; T_{max} , time to reach maximum plasma concentration; CL/F, plasma clearance rate; C_{max} , the maximum plasma concentration; AUC_{0-t}, area under the systemic drug concentration-time curve from time 0 to t; AUC_{0-∞}, area under the systemic drug concentration-time curve from time 0 to infinity.

**Figure 3** Mean plasma concentration-time profiles of fruquintinib in 8 rats (mean $\pm$ SD) after oral administration of 1.0 mg/kg fruquintinib.

rat plasma and then applied to the pharmacokinetic study. This method covers a wide range of the concentration curve from 1.0 to 1000 ng/mL for fruquintinib. With small sample volume, simple extraction procedure, and fast analysis, this analytical method is applicable for large studies. It would help to elucidate the potential pharmacological actions of fruquintinib through the pharmacokinetic results.

Abbreviations

UPLC-MS/MS, ultraperformance liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantification; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; CFDA, China Food and Drug Administration; NSCLC, non-small-cell lung cancer; CRC, colorectal cancer; QCs, quality control sample; LC-MS/MS, liquid chromatography-tandem mass spectrometry; m/z, precursor-to-product ion transitions of fruquintinib.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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