

## RESEARCH ARTICLE

## Hello

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## NON-SPECIALIST SUMMARY

This study presents a fast, simple lab test to measure the disease-modifying antirheumatic drug baricitinib in both the plasma and aqueous humor. Using small sample volumes and one-step preparation, the LC-MS/MS method is sensitive, accurate and stable across a wide range of concentrations. Applied to adults with noninfectious uveitis, it revealed large variability in plasma concentrations and a weak correlation between plasma and aqueous humor pharmacokinetics. These findings suggest therapeutic drug monitoring of baricitinib may be warranted to promote individualizing treatment.

## 1. Introduction

Noninfectious uveitis (NIU) is a heterogeneous group of uveitis primarily caused by excessive activation of the autoimmune system, leading to inflammatory lesions in ocular tissues [1]. Damage due to NIU not only involves the eye without a specific cause but can also occur concurrently with various immune-mediated inflammatory diseases (IMIDs), such as psoriasis, Behçet's disease, sarcoidosis, and Crohn's disease [2,3]. The standard treatment strategy for NIU primarily relies on the local or systemic administration of corticosteroids and various immunosuppressive agents [4-8]. However, long-term use of these medications can lead to severe ocular complications and other systemic adverse drug reactions [9,10]. Moreover, a proportion of patients with NIU, particularly those with more severe disease, do not achieve satisfactory improvement with any of the currently available therapies [11]. Therefore, a significant unmet medical need remains for a more effective and well-tolerated NIU treatment.

The Janus kinase (JAK) superfamily, which includes JAK1, JAK2, JAK3, and TYK2, plays crucial roles in the regulation of multiple cytokine-related inflammatory pathways [12]. JAK inhibitors can effectively suppress inflammation by inhibiting the JAK-STAT signaling pathway. Given the close relationship of the pathogenesis of NIU with several autoimmune diseases, as well as the significant role of the JAK-STAT signaling pathway in inflammation and the beneficial prospects of JAK inhibitors in rheumatic diseases, it is speculated that JAK inhibitors could achieve good or even superior therapeutic outcomes in the treatment of refractory NIU [13,14].

Baricitinib is a selective and reversible JAK 1/2 inhibitor that has been approved for the treatment of several IMIDs; however, its use in NIU is still off-label. Baricitinib is a highly soluble and poorly permeable drug characterized by low or inconsistent bioavailability in different disease populations. The inter-individual variability in baricitinib pharmacokinetics (PK) was 17–26% in healthy volunteers and 41% in patients with rheumatoid arthritis [15]. In recent years, increasing evidence has supported its potential in the treatment of concomitant uveitis in many IMIDs [16-18]. However, little information is available regarding the PK profiles of baricitinib in these patients. Notably, the European Medicines Agency (EMA) has highlighted the safety risks of all JAK inhibitors in terms of major cardiovascular problems, malignancy, venous thromboembolism, serious infections, and death [19]. These findings collectively indicate that implementing clinical pharmacology studies and therapeutic drug monitoring (TDM) to balance the cost/risk/benefit of baricitinib is warranted.

The detection conditions and validation results of the quantification method are prerequisites for PK studies. Several publications have described liquid chromatographic tandem mass spectro-

metric (LC–MS/MS) methods to determine baricitinib concentrations in human or animal plasma [20–25]. However, clinical baricitinib quantification in the aqueous humor of patients with NIU remains unavailable. Furthermore, whether the linear or TDM window of baricitinib based on plasma can be extended to the aqueous humor is unclear. Therefore, this article aimed to develop a straightforward, rapid, sensitive, reproducible, and reliable method using LC–MS/MS to quantify baricitinib in both plasma and aqueous humor. This method will promote future clinical studies and rational use of baricitinib in the treatment of NIU.

## 2. Materials and Methods

### 2.1. Chemical substances and laboratory materials

Reference baricitinib (lot 22Z102-M1, purity: 98.68%) and internal standard baricitinib-*d*5 (lot 22T083-L1, purity: 98%) (Figure 1) were procured from Shanghai Zzbio Co., Ltd. (Shanghai, China). HPLC-grade methanol, acetonitrile, dimethyl sulfoxide (DMSO), ammonium acetate, and ACS-grade formic acid were obtained from Aladdin Company (Shanghai, China). The ultrapure water used in this study was produced using an Elga Purelab Flex 3 water system (ELGA, High Wycombe, UK). Blank plasma and whole blood from healthy volunteers were donated by the Phase I Clinical Laboratory of Tongji Hospital (ethical approval number: TJ-IRB20220737). Blank aqueous humor was provided by patients who underwent cataract surgery in our hospital. Written informed consent was obtained from all the patients.

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### 2.2. Preparation of Calibration Standards and Quality Controls

Accurately weighed amounts of baricitinib and baricitinib-*d*5 (IS) were dissolved in methanol:DMSO (40:60, v/v) to yield standard stock solutions of 1.0 mg/mL and 40 µg/mL, respectively. The baricitinib stock solution was diluted with acetonitrile to obtain a series of standard (4–4,000 ng/mL) and quality control (QC) working solutions (4, 12, 150 ng/mL, 3,000 ng/mL). Similarly, a 40 ng/mL IS solution was prepared by diluting the stock IS solution.

Calibration standards for baricitinib in human plasma or aqueous humor were prepared at concentrations of 0.2, 0.5, 2, 5, 10, 40, 100, and 200 ng/mL. This was achieved by mixing 20 µL of standard working solutions with 180 µL of blank plasma or aqueous humor. QC samples were independently prepared using the same method. These QC samples included 0.20 ng/mL (lower limit of quantification, LLOQ), 0.6 ng/mL (low-quality control, LQC), 7.5 ng/mL (medium-quality control, MQC), and 150 ng/mL (high-quality control, HQC). Furthermore, a dilution quality control (DQC) sample was prepared at a concentration of 400 ng/mL. Subsequently, the DQC sample was diluted with blank plasma or aqueous humor (dilution factor: 5) to produce an 80 ng/mL dilution test sample.

### 2.3. Sample Preparation

An aliquot of 20 µL IS working solution (40 ng/mL) was added to 50 µL of plasma or aqueous humor sample. The mixture was vortex-mixed for 10 s. Subsequently, an aliquot of 100 µL acetonitrile was added to facilitate protein precipitation. The mixture was then thoroughly extracted on a vortex mixer for 5 min and centrifuged at 12,000 rpm for 10 min at 4 °C. Next, 100 µL of the protein-free supernatant was carefully transferred to a new 1.5 mL plastic vial, followed by 100 µL of an acetonitrile–water solution (50:50, v/v). Finally, a 6 µL sample of the resulting mixture was injected for detection and analysis.

### 2.4. Instrumental Settings

LC-MS/MS analysis was performed using a Shimadzu LC-20A liquid chromatographic system (Shimadzu Corp., Kyoto, Japan) coupled to a QTRAP 5500 mass spectrometer (AB Sciex, MA, USA). The mass spectrometer was equipped with an electrospray ionization (ESI) source. Positive

multiple reaction monitoring (MRM) mode was used to analyze the samples. The quantitative and confirming MRM mass transitions ( $m/z$ ) were  $372.3 > 186.1$  and  $372.2 > 251.1$  for baricitinib, and  $377.2 > 186.1$  and  $377.2 > 251.1$  for IS, respectively. A simple gradient elution method was employed using an Ultimate XB-C<sub>18</sub> column (2.1 mm × 50.0 mm, 5 μm) (Welch, Shanghai, China). The mobile phase consisted of 10 mM ammonium formate in water (phase A) and 0.2% formic acid in a mixture of methanol, acetonitrile, and isopropanol (75:15:15, v/v/v phase B). The column oven and autosampler were set at 40 °C and 15 °C, respectively.

The total analysis time for each sample was 2.5 min, with a constant flow rate of 0.72 mL/min. The gradient elution program was as follows: 0 – 0.2 min: 20% B; 0.2 – 1.3 min: 20 – 88% B; 1.3 – 1.8 min: 88% B; 1.8 – 2.0 min: 88 – 20% B; 2.0 – 2.5 min: 20% B. This gradient elution program allowed for the effective separation and analysis of the compounds of interest.

The specific source and gas parameters used were as follows: collision gas set to medium; curtain gas maintained at 40 psi; ion source gas 1 set to 50 psi; ion source gas 2 adjusted to 55 psi; ion spray voltage set at 5500 V; and ion spray temperature held at 600 °C. The transitions and optimized settings are listed in **Table 1**. Data acquisition and analysis were performed using the Analyst software package (Applied Biosystems, Version 1.6.3).

MRM, multiple reaction monitoring

## 2.5. Method Validation

All method validation procedures adhered to the bioanalytical method validation guidance provided by the United States Food and Drug Administration and the European Medicines Agency [26,27].

### 2.5.1. Selectivity, specificity, and carryover

Method selectivity and specificity were assessed by analyzing and comparing the peak areas of the analytes and IS in blank matrices, which included normal blank plasma or aqueous humor with the LLOQ samples. This analysis confirmed the absence of endogenous interference peaks. Six different sources of blank matrix were used to ensure the robustness of the data. Potential carry-over effects were evaluated by comparing the signal intensities in double-blank samples after running the calibration standards at the upper limit of quantification (ULOQ, 200 ng/mL baricitinib) with the LLOQ samples five times. The impact of phospholipids in the matrix on the signal of baricitinib was evaluated by post-column infusion of the IS while an extracted blank sample or a solvent sample was being analyzed [28]. Notably, any chromatographic interference resulting from endogenous components should have a minimal impact on the accurate quantification of each compound. Baricitinib should not be eluted at a time when phospholipids are suppressing the signal of the IS. The peak area attributable to interfering components should not exceed 20% of the analyte response at the LLOQ and not more than 5% of the IS response in the LLOQ sample for each matrix. This criterion ensured reliable quantification of the target compounds in the analysis.

### 2.5.2. Linearity

Linearity was assessed across a given concentration range for all analytes. Five calibration curves were constructed using eight freshly prepared calibration standards within the concentration range of 0.2 to 200 ng/mL over three consecutive days. Linear regression analysis was performed to fit the relationship between the peak area ratio ( $y$ ) of baricitinib to IS and the nominal analytical concentration ( $x$ ). A linear regression equation using a weighting scheme of  $1/x^2$  was established. The calibration curves were considered acceptable if the accuracy of the back-calculated concentrations of the calibration standards was within ±20% for the LLOQ and within ±15% at all other levels, with at least three-quarters of the calibration points meeting this accuracy requirement.

### 2.5.3. Precision and accuracy

Precision and accuracy assays were implemented for each QC sample, including LLOQ, LQC, MQC, and HQC. To evaluate method accuracy, we calculated the percentage ratio of each QC level to the nominal concentration as the mean of measured/nominal  $\times 100\%$ . Precision was evaluated by the coefficient of variation (%CV), defined as the relative standard deviation (RSD) of each QC concentration level. Intra-run assays involved the analysis of six replicates at each QC concentration level in each analytical run. Inter-run assays were performed for each QC concentration level by pooling data from three analytical runs over three days. Accuracy (RE%) and precision (%CV) below 15% (with the LLOQ threshold set at  $\leq 20\%$ ) were deemed acceptable.

### 2.5.4. Matrix effect, recovery and dilution integrity

Extraction recovery was evaluated by comparing the QC samples (pre-addition of the analyte and IS) with the samples that were spiked with analyte post-extraction. Its performance was expressed as the percentage of the peak areas of the analyte and IS in the pre-addition QC sample versus the peak areas of the analyte and IS obtained from samples that were spiked with analyte post-extraction. Extraction recovery was assessed at low, medium, and high QC levels, with six parallel samples prepared for each.

Matrix effect assays were performed by analyzing six replicates of low, medium, and high QCs, each prepared using blank plasma or aqueous humor from six different donors, respectively. Matrix effect assays in three replicates of lipemic or hemolyzed plasma were also performed. The matrix factor (MF) was determined by calculating the ratio of peak areas obtained from samples that were spiked with analyte post-extraction to the peak areas obtained by spiking analyte into neat solutions. The IS-normalized MF (NMF) was calculated by dividing the MF of the analyte by that of the IS.

For both extraction recovery and NMF at each QC level, the accuracy should be within  $\pm 15\%$  of the nominal concentration and the precision (%CV) should not be greater than 15%.

Dilution integrity was assessed using six determinations at concentrations exceeding the maximum calibration limit. The DQC samples were prepared and further diluted with blank plasma or aqueous humor at a ratio of 1:4 (v/v). The concentration of baricitinib in the matrix-spiked DQC samples was set to 80 ng/mL, which fell within the linear range. The calculated accuracy and precision did not exceed 15%.

### 2.5.5. Stability

Analyte stability was evaluated in plasma using three levels of QC samples stored under four different storage conditions. Freeze-thaw stability was assessed by subjecting samples to three full freeze-thaw cycles (transitioning from  $-80^\circ\text{C}$  to  $25^\circ\text{C}$ ) at room temperature. Analyses were performed on QC samples stored at  $25^\circ\text{C}$  for 24 hours for short-term stability assessment. The long-term stability of the plasma samples was analyzed after simultaneous preservation at  $-20^\circ\text{C}$  and  $-80^\circ\text{C}$  for 14 and 45 days. QC samples were extracted at  $4^\circ\text{C}$  and stored in an autosampler for 24 and 48 hours to assess post-process stability. Stability was determined by comparing the average analyte concentrations to the initial nominal concentrations, with stability defined as a percentage difference within  $\pm 15.0\%$ .

## 2.6. Applicability of the Method for TDM

The utility of the validated method was assessed by analyzing plasma or aqueous humor samples from 10 patients with NIU. Inclusion criteria were as follows: (1) age between 18 and 65 years; (2) meeting the diagnostic criteria for NIU, including intermediate uveitis, posterior uveitis, or panuveitis; (3) active uveitis at day 1/baseline examination with at least one of the following: (1) active inflammatory chorioretinal lesions and/or inflammatory retinal vasculopathy; (2)  $\geq 2+$  anterior chamber cells (according to the SUN criteria); (3)  $\geq 2+$  inflammatory vitreous opacity (according to the SUN criteria). Patients with the following conditions were excluded: (1) infectious uveitis

or uncontrolled tuberculosis or hepatitis B; (2) severe glaucoma or cataract unrelated to uveitis; (3) elderly or obese individuals or those with a history of deep vein thrombosis or pulmonary thrombosis, surgery or other bedridden conditions, severe cardiovascular and cerebrovascular diseases, or uncontrolled systemic diseases such as hypertension and diabetes. All participants provided informed consent before inclusion in the study.

All patients were orally administered 4 mg baricitinib (OLUMIANT®) once daily at 10:00 AM. Whole blood was drawn into EDTA-K2 vacuum tubes immediately pre-dose (0 h), and 0.5, 1, and 1.5 h post-dose after administration of the second dose. Aqueous humor samples were drawn into sterile plastic tubes immediately before administration of the second dose (0 h). Whole blood was centrifuged at  $1800 \times g$  for 10 min, and the plasma and aqueous humor were stored at  $-70^\circ\text{C}$  until analysis. The trapezoid rule was used to calculate the area under the blood concentration–time curve over 1.5 h ( $\text{AUC}_{0-1.5}$ ), and then the median, first quartile, and third quartile of the  $\text{AUC}_{0-1.5}$  adjusted by the daily dose were calculated. Pearson correlation coefficients were used to assess the association between the concentration in aqueous humor and the concentration or  $\text{AUC}_{0-1.5}$  of plasma using R software (version 4.2.1; <http://www.r-project.org>; accessed on January 30, 2023). Statistical analysis and figure generation were performed using the tidyverse and ggplot2 packages.

### 3. Results and Discussion

#### 3.1. Optimization of Chromatography and Mass Spectrometry

A 40 ng/mL solution of baricitinib and the IS was used to determine the mass spectrometric parameters. Consistent with previous reports [14], a more stable and higher signal-to-noise intensity ratio was observed in the ESI positive-ion mode. This generated the most abundant prominent protonated molecular ions  $[\text{M}+\text{H}]^+$  at 372 and 377 for baricitinib and its isotopic IS, respectively. Both analytes produced predominant fragment ions at  $m/z$  186.1 and 251.1. Finally, we adopted  $372.2 > 186.1$  and  $377.2 > 186.1$  as the quantified mass transitions for baricitinib and IS, respectively, while the other two pairs of mass transitions were used for compound confirmation.

Because a single-step protein precipitation was sufficient to cover the sensitivity and concentration range, acetonitrile was chosen as the extracting solution due to its superior ability to yield cleaner and more efficient results than methanol. Following this step, the supernatants were reconstituted with an equivalent volume of 50% methanol containing 0.1% formic acid to further minimize the matrix effect and maximize ionization intensity.

To achieve optimal chromatographic separation, the composition of the mobile phase was systematically optimized. The aqueous phase consisted of various concentrations of ammonium formate or ammonium acetate solution, while the organic phase was evaluated in different stages. Initially, a binary mixture of methanol and acetonitrile was employed to balance the insufficient ionization efficiency associated with acetonitrile and the relatively high column pressure induced by methanol. Subsequently, the addition of a small proportion of isopropanol to this binary mixture improved peak shape by reducing tailing, yielding symmetric and sharp peaks. The final mobile phase comprised 10 mM ammonium formate and 0.2% formic acid in water as the aqueous component, and a ternary mixture of methanol, acetonitrile, and isopropanol (75:15:15, v/v/v) as the organic component.

Multiple commercially available  $\text{C}_{18}$  columns were evaluated to enhance chromatographic peak shape, peak width, and separation. The Ultimate XB- $\text{C}_{18}$  column (2.1 mm  $\times$  50.0 mm, 5  $\mu\text{m}$ ) was selected due to its favorable peak shape, narrow peak width, and cost-effectiveness. Under the aforementioned chromatographic conditions, baricitinib and the IS were effectively separated with a retention time of 1.27 min within a total running time of 2.5 min, resulting in a shorter analysis time than prior methods [20–24].

## 3.2. Method Validation

### 3.2.1. Selectivity, specificity, and carry-over

**Figures 2** and **3** display the typical MRM chromatograms of baricitinib for the double-blank, LLOQ, and patient samples from plasma and aqueous humor. These chromatograms demonstrate the absence of any endogenous substances that interfere with baricitinib and the IS. The post-column infusion assay indicated that the IS was not co-eluted with phospholipids from 1.0 to 1.3 min, demonstrating negligible influence of phospholipids in both plasma and aqueous humor. Responses attributable to interfering components in the blank plasma and aqueous humor samples were less than 3.17% and 1.75% of the corresponding LLOQ samples, respectively. Both the analyte and the IS displayed peaks at 1.27 min.

The carry-over effect was minimal, as determined by comparing the peak areas of the double-blank samples injected after the ULOQ samples with those of the LLOQ samples. The average peak areas of the carry-over blank samples were less than 0.46% and 0.33% of the LLOQ sample for baricitinib and IS in plasma, respectively, whereas the carry-over effect was less than 0.06% and 0.69% for the same compounds in aqueous humor.

### 3.2.2. Interference Among Analyte and IS

For the plasma matrix, the average peak areas of the double-blank and ULOQ samples without IS did not exceed 16% and 4% of the corresponding peak areas observed in the LLOQ samples, respectively. Similarly, these values did not exceed 14% and 3% for the aqueous humor matrix, respectively. This observation demonstrated excellent compliance with the acceptance criteria outlined by the FDA and EMA.

### 3.2.3. Linearity, LLOQ, Accuracy and Precision

The calibration curves exhibited linearity in both plasma and aqueous humor over a concentration range of 0.2 to 200 ng/mL, with determination coefficients ( $r^2$ ) higher than 0.99. The regression equations and  $r^2$  for these calibration curves are presented in **Table 2**, affirming the method's satisfactory sensitivity and excellent linearity. Furthermore, the LLOQ of baricitinib was 0.2 ng/mL in both plasma and aqueous humor, ensuring a signal-to-noise ratio exceeding the baseline by 10-fold. This stringent criterion reinforces the reliability of concentration detection. The intra- and inter-run accuracy of baricitinib in plasma and aqueous humor ranged from 102.2% to 108.5% and 101.0% to 106.8%, respectively, while the intra- and inter-run precision ranged from 3.7% to 11.7% and 1.0% to 9.0%, respectively. All results indicated that the accuracy and precision of the validated method met the determination requirements.

Accuracy presented as Mean $\pm$ SD; Precision presented as %CV.

### 3.2.4. Matrix effect, recovery and dilution intensity

The results showed that the relative extraction recovery of baricitinib at all QC levels ranged from 86.5% to 94.5% in normal plasma and from 86.2% to 92.3% in aqueous humor, while the relative extraction recovery of the IS ranged from 85.3% to 99.9% in plasma and from 94.2% to 95.9% in aqueous humor (**Table 3**). The overall %CV of the extraction recovery for all samples in both matrices did not exceed 15%, suggesting that the sample pretreatment method was reproducible and stable.

The IS-normalized MF values of baricitinib varied from 98.3% to 107.4% in normal plasma, 92.8% to 101.1% in normal aqueous humor, 103.7% to 109.6% in hemolyzed plasma, and 98.1% to 108.8% in lipemic plasma. In addition, the %CV of the IS-normalized MF values in all matrices did not exceed 15% (**Table 3**). These results indicate that the use of acetonitrile as a deproteinization solvent, together with acetonitrile–water (50:50, v/v) dilution of sample supernatants, effectively removed matrix interference.

Data presented as Mean%CV; %CV, coefficient of variation; n, number of replicates. IS, internal standard; MF, matrix factor

In terms of dilution integrity, the mean values of the calculated accuracy from six determinations diluted from the 5-fold concentration of baricitinib with normal plasma or aqueous humor were 99% and 101.3%, respectively, with corresponding precision (%CV) of 2.7% and 4.9%. Thus, the validated method was reliable and robust if the concentration range was within 5-fold of the ULOQ.

### 3.2.5. Stability

The stability results for baricitinib under various simulated conditions are presented in [Table 4](#). The results indicated that baricitinib maintained reasonable stability when left at room temperature for 24 h, kept in the autosampler for 24 h, subjected to three freeze-thaw cycles, and stored at  $-80^{\circ}\text{C}$  for 320 days in normal plasma or aqueous humor. The degradation rate of baricitinib in whole blood maintained at  $4^{\circ}\text{C}$  for 24 h was less than 4% with an RSD below 7.4%. Moreover, the accuracy and precision for the stock solution were 85.3%-105.4% and 6.4%-9.3%, respectively, when stored at  $-80^{\circ}\text{C}$  for 6 months. These findings indicate that this method is stable for processing plasma or aqueous humor samples over an extended period.

Data presented as Mean %CV; %CV, coefficient of variation; n, number of replicates.

### 3.3. Clinical Application

The plasma concentration–time profiles are shown in [Figure 4](#), and the demographic and laboratory characteristics are summarized in [Table 5](#). The plasma concentrations of baricitinib at 0 h (before the second dose) and at 0.5, 1, and 1.5 h after the second dose were 2.25 (1.79, 3.31), 30.18 (11.27, 44.02), 44.64 (30.46, 60.02), and 43.53 (36.87, 54.34) ng/mL, with %CV values of 49.6%, 81.0%, 48.6%, and 30.4%, respectively. The calculated  $\text{AUC}_{0-1.5}$  and daily dose-normalized  $\text{AUC}_{0-1.5}$  ( $\text{AUC}_{0-1.5}/\text{D}$ ) were 53.30 (40.62, 87.0) and 13.30 (10.15, 18.57) ng·h/mL/mg, respectively, with both %CV values being 51.4%. Furthermore, half of the patients did not exhibit a delayed absorption peak within 1.5 h, indicating that not all patients achieved the peak concentration within 1.0 h. This finding is inconsistent with results from earlier phase I and II clinical trials [\[17\]](#). One plausible explanation for this discrepancy is that our study, conducted in a real-world clinical setting, did not strictly require patients to take the medication on an empty stomach, and some may have taken it after a meal, despite the fact that oral bioavailability is not affected by food.

<sup>a</sup> Calculated according to the Chronic Kidney Disease Epidemiology Collaboration equation and converted to L/h;

<sup>b</sup> Represented as mean  $\pm$  SD.

The trough concentration ( $C_{\min}$ ) of baricitinib after the first dose in the anterior chamber was 0.94 (0.89, 1.16) ng/mL, with a %CV of 20.2%, which was significantly lower than the plasma  $C_{\min}$  (49.6%). Furthermore, significant correlations between the concentrations in the aqueous humor at 0 h and the plasma concentrations at 0, 0.5, 1, and 1.5 h, as well as the corresponding plasma  $\text{AUC}_{0-1.5}$ , were not observed ([Figure 5](#)). These findings indicate that drug concentrations in the anterior chamber are less susceptible to fluctuations in plasma concentrations and that it may not be feasible to predict the therapeutic efficacy of ocular diseases by monitoring drug concentrations in plasma.

### 3.4. Comparison with Previous Studies

Several methods have been established to quantify baricitinib in the plasma of rats [\[20,21\]](#) and humans [\[22-24\]](#). To date, these quantification methods have been validated by LC-MS/MS in MRM-positive mode, with three reports [\[23-25\]](#) using isotopic IS and analysis times exceeding 2.8 min. In addition, the application of microsampling technology in one pharmacokinetic study [\[23\]](#)

conducted in pediatrics, although innovative, presented high technical barriers with no superior cost-effectiveness. The latest published method by Cafaro et al. [25], which is similar to ours, requires only a small sample volume, a straightforward sample preparation protocol, and a low detection limit. However, their method did not cover both peak and trough points to verify its applicability, nor did it explore drug concentrations in aqueous humor. Furthermore, all other studies based on human plasma used liquid–liquid extraction for sample pretreatment, which is complicated and time-consuming. In summary, there are currently no reports on the determination of baricitinib in aqueous humor. This study provides improvements by using LC-MS/MS; for instance, the sensitivity was adequate without the need for complicated and time-consuming preprocessing, the analytical time was shorter, and the matrix effect was eliminated.

An important factor contributing to the success of ocular therapy is the ability of drug molecules to penetrate targeted ocular tissues at effective concentrations. Moreover, aqueous humor is an accessible site for the systemic administration of baricitinib. Given the presence of the blood–ocular barrier, baricitinib in the aqueous humor is closer to the ciliary body or choroid than that in plasma, and monitoring its PK in the aqueous humor is relatively more suitable than plasma for evaluating the exposure–efficacy relationship in patients with NIU. Nevertheless, it must be clearly recognized that aqueous humor remains a surrogate matrix. Researchers have advocated the development of baricitinib ophthalmic liposomes, as direct ocular administration may achieve better penetration into target tissues than oral administration, potentially enhancing therapeutic efficacy [29].

This study also presents some limitations. Firstly, plasma concentrations at the terminal elimination phase were not monitored during the second dosage interval. Therefore,  $AUC_{0-1.5}$  could not fully substitute for the  $AUC_{24}$  of baricitinib in plasma. Secondly, the small sample size in our study may limit the generalizability of the pharmacokinetic variability observed. In addition, variability in drug administration (fasted vs fed states) may have contributed to the observed PK variability, but was not controlled in this real-world study. Lastly, the use of Pearson correlation provides only a preliminary assessment of the relationship between plasma and aqueous humor concentrations. More robust analyses (e.g., population pharmacokinetics) would better characterize the compartmental dynamics and exposure–effect relationship in NIU cohorts but would require larger sample sizes. These limitations highlight opportunities for future research to optimize baricitinib TDM in ocular diseases.

## 4. Conclusion

This method provides a reliable and valuable reference for promoting future clinical studies and improving the therapeutic management of baricitinib in patients with NIU. It is urgent to implement population pharmacokinetic/pharmacodynamic study to establish the exposure–efficacy relationship of baricitinib in NIU cohorts.

(figure placeholder)

FIGURE 1. Chemical structure of baricitinib (A) and its isotopic internal standard (B).

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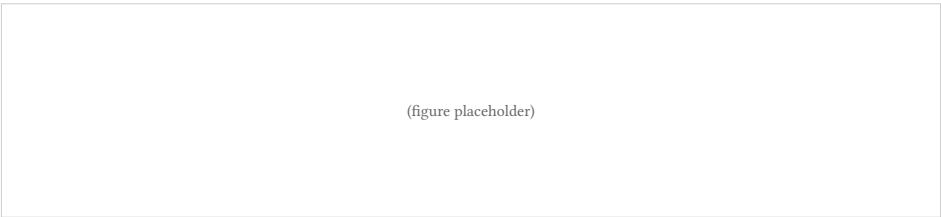


FIGURE 2. Typical MRM chromatograms of baricitinib and IS in double blank plasma, LLOQ plasma sample and plasma sample from patient (No.4) collected immediately before (0 h) the second dosage administration. MRM, multiple reaction monitoring; IS, internal standard; LLOQ, lower limit of quantification.

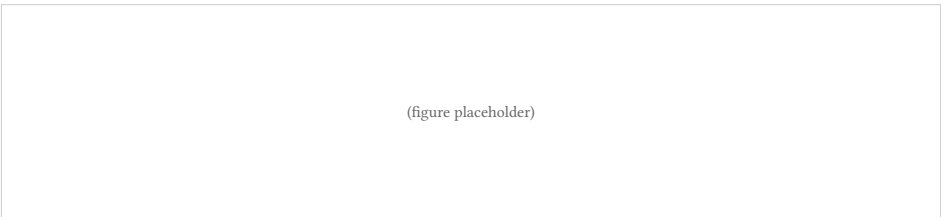


FIGURE 3. Typical MRM chromatograms of baricitinib and IS in double blank aqueous humor, LLOQ aqueous humor sample and aqueous humor sample from patient (No.4) collected immediately before (0 h) the second dosage administration. MRM, multiple reaction monitoring; IS, internal standard; LLOQ, lower limit of quantification.

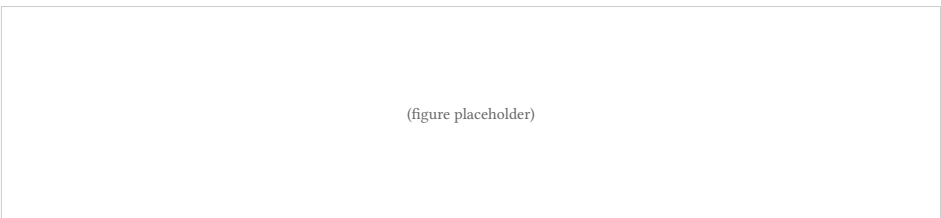


FIGURE 4. The plasma concentration versus time profiles in 10 patients with non-infectious uveitis after the second dose (baricitinib tablet, 4 mg once daily).

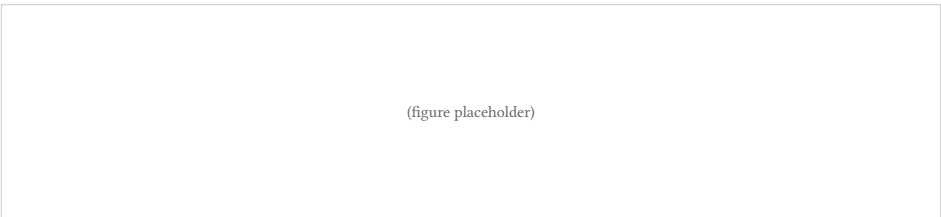


FIGURE 5. Pearson's correlation between aqueous humor and plasma. A1-3, B1, BARCI levels in plasma at 0 h (C0), 0.5 h (C0.5), 1 h (C1), and 1.5h (C1.5) versus BARCI levels in aqueous humor at 0 h. B2, BARCI AUC0-1.5 in plasma versus BARCI levels in aqueous humor at 0 h. B3, C1-3, plasma BARCI AUC0-1.5 versus plasma BARCI concentrations at 0, 0.5, 1, and 1.5 h.

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TABLE 1. Multiple reaction monitoring parameters.

| Compound       | MRM Transition (m/z) | Dwell time (msec) | DP (V) | EP (V) | CE (V) | CXP (V) |
|----------------|----------------------|-------------------|--------|--------|--------|---------|
| baricitinib    | 372.3 > 186.1        | 70                | 64     | 10     | 48     | 10      |
|                | 372.2 > 251.1        | 75                | 100    | 10     | 30     | 10      |
| baricitinib-d5 | 377.2 > 186.1        | 60                | 40     | 10     | 45     | 10      |
|                | 377.2 > 251.1        | 60                | 40     | 10     | 39     | 10      |

TABLE 2. Intra- and inter-run accuracy and precision for the quantification of baricitinib in plasma or aqueous humor by LC-MS/MS (n=6).

|                                                                            | Theoretical concentration (ng/mL) | Intra-run (n=6) |               | Inter-run (n=18) |               |
|----------------------------------------------------------------------------|-----------------------------------|-----------------|---------------|------------------|---------------|
|                                                                            |                                   | Accuracy (%)    | Precision (%) | Accuracy (%)     | Precision (%) |
| Plasma                                                                     | 0.2                               | 102.211.9       | 11.7          | 102.79.8         | 9.5           |
|                                                                            | 0.6                               | 107.79.8        | 9.2           | 108.07.1         | 6.6           |
|                                                                            | 7.5                               | 107.33.1        | 2.9           | 108.53.6         | 3.3           |
|                                                                            | 150                               | 104.93.8        | 3.7           | 105.63.6         | 3.4           |
| Regression equation $y = 0.05399 \cdot x^* + 0.00201$ , $r^{**2} = 0.9995$ |                                   |                 |               |                  |               |
| Aqueous humor                                                              | 0.2                               | 105.89.0        | 8.5           | 106.87.4         | 6.9           |
|                                                                            | 0.6                               | 103.09.2        | 9.0           | 105.78.0         | 7.6           |
|                                                                            | 7.5                               | 101.91.0        | 1.0           | 103.21.5         | 1.5           |
|                                                                            | 150                               | 104.20.5        | 0.5           | 101.04.1         | 4.1           |
| Regression equation $y = 0.0617 \cdot x^* + 0.00151$ , $r^{**2} = 0.9989$  |                                   |                 |               |                  |               |

TABLE 3. Extraction recovery and matrix effect of baricitinib in plasma or aqueous humor.

| Theoretical concentration (ng/mL) | Extraction recovery (%) |               | IS-normalized MF (%) |                            |                        |                      |
|-----------------------------------|-------------------------|---------------|----------------------|----------------------------|------------------------|----------------------|
|                                   | Normal plasma           | Aqueous humor | Normal plasma (n=6)  | Normal aqueous humor (n=6) | Hemolyzed plasma (n=3) | Lipemic plasma (n=3) |
| 0.6                               | 88.311.2                | 86.22.5       | 107.413.7            | 101.15.9                   | 105.69.1               | 108.812.7            |
| 7.5                               | 94.59.3                 | 92.34.4       | 101.26.9             | 97.46.6                    | 103.77.3               | 98.17.8              |
| 150                               | 86.57.6                 | 91.50.5       | 98.37.0              | 92.80.9                    | 109.64.1               | 105.89.4             |

TABLE 4. Stability of baricitinib in plasma or aqueous humor under different conditions (n=3).

| Theoretical concentration (ng/mL) |     | Accuracy (%)           |                              |                          |                          |                     |                                           |
|-----------------------------------|-----|------------------------|------------------------------|--------------------------|--------------------------|---------------------|-------------------------------------------|
|                                   |     | Blood at 4 °C for 24 h | at room temperature for 24 h | In auto-sampler for 24 h | Three freeze-thaw cycles | -80 °C for 320 days | Solution stability at -80 °C for 6 months |
| plasma                            | 0.6 | 106.07.8               | 109.34.5                     | 103.52.9                 | 109.710.0                | 93.94.5             | 91.36.0                                   |
|                                   | 150 | 106.71.6               | 102.62.4                     | 107.82.8                 | 111.12.4                 | 110.02.1            | 96.49.0                                   |
| Aqueous humor                     | 0.6 | -                      | 98.34.4                      | 106.12.3                 | 102.42.4                 | 99.15.6             | -                                         |
|                                   | 150 | -                      | 101.65.7                     | 103.23.0                 | 103.53.8                 | 103.2v3.0           | -                                         |

TABLE 5. Patient demographics and disease characteristics.

| Characteristic                                                 | N (%) or Median (Q1, Q3) |
|----------------------------------------------------------------|--------------------------|
| Sex (Male/Female)                                              | 4/6                      |
| Age (years)                                                    | 38.5 (32.7, 44.2)        |
| Body Weight (kg)                                               | 64.5 (60.2, 71.8)        |
| Type of uveitis                                                |                          |
| Anterior uveitis                                               | 3 (30)                   |
| Posterior and/or panuveitis (and/or intermediate)              | 7(70)                    |
| Unilateral                                                     | 2 (20)                   |
| Bilateral                                                      | 8 (80)                   |
| Aetiology                                                      |                          |
| Vogt-Koyanagi-Harada syndrome                                  | 7 (70)                   |
| Behçet syndrome                                                | 1 (10)                   |
| others                                                         | 2 (20)                   |
| Comorbidities/Complications                                    |                          |
| Glaucoma                                                       | 1 (10)                   |
| Ocular hypertension                                            | 2 (20)                   |
| Epiretinal membrane                                            | 7 (70)                   |
| Cataract                                                       | 3 (30)                   |
| Retinal ischaemia within the macula                            | 3 (30)                   |
| Retinal ischaemia within peripheral retina                     | 3 (30)                   |
| Alanine aminotransferase (ALT) (IU/l)                          | 17.0 (10.2, 25.5)        |
| Aspartate aminotransferase (AST) (IU/l)                        | 16.0 (13.2, 29.5)        |
| Estimated glomerular filtration rate <sup>a</sup> (eGFR) (L/h) | 105 (102.3, 116.8)       |
| Concomitant medication                                         |                          |
| Methotrexate                                                   | 2 (20)                   |
| Methotrexate dose (mg/week) <sup>b</sup>                       | 17.5±3.5                 |

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DATA & CODE AVAILABILITY

The data presented in this study are available on request from the corresponding author.

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The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Tongji Hospital (protocol ID: NO. TJ-IRB20210615, date

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