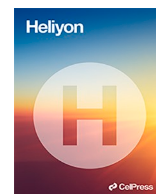
Heliyon 10 (2024) e32187

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Simultaneous measurement of COVID-19 treatment drugs (nirmatrelvir and ritonavir) in rat plasma by UPLC-MS/MS and its application to a pharmacokinetic study

Chen-Jian Zhou [a], Ya-nan Liu [b], Anzhou Wang [b], Hualu Wu [b], Ren-ai Xu [b,*], Qiang Zhang [c,**]

[a] Wenzhou Central Hospital, Zhejiang, China
[b] The First Affiliated Hospital of Wenzhou Medical University, Zhejiang, China
[c] The People's Hospital of Lishui, Zhejiang, China

A R T I C L E I N F O

Keywords:
Nirmatrelvir
Ritonavir
UPLC-MS/MS
Pharmacokinetics
rat

A B S T R A C T

PAXLOVID™ (Co-packaging of Nirmatrelvir with Ritonavir) has been approved for the treatment of Coronavirus Disease 2019 (COVID-19). The goal of the experiment was to create an accurate and straightforward analytical method using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) to simultaneously quantify nirmatrelvir and ritonavir in rat plasma, and to investigate the pharmacokinetic profiles of these drugs in rats. After protein precipitation using acetonitrile, nirmatrelvir, ritonavir, and the internal standard (IS) lopinavir were separated using ultra performance liquid chromatography (UPLC). This separation was achieved with a mobile phase composed of acetonitrile and an aqueous solution of 0.1% formic acid, using a reversed-phase column with a binary gradient elution. Using multiple reaction monitoring (MRM) technology, the analytes were detected in the positive electrospray ionization mode. Favorable linearity was observed in the calibration range of 2.0–10000 ng/mL for nirmatrelvir and 1.0–5000 ng/mL for ritonavir, respectively, within plasma samples. The lower limits of quantification (LLOQ) attained were 2.0 ng/mL for nirmatrelvir and 1.0 ng/mL for ritonavir, respectively. Both drugs demonstrated inter-day and intra-day precision below 15%, with accuracies ranging from −7.6% to 13.2%. Analytes were extracted with recoveries higher than 90.7% and without significant matrix effects. Likewise, the stability was found to meet the requirements of the analytical method under different conditions. This UPLC-MS/MS method, characterized by enabling accurate and precise quantification of nirmatrelvir and ritonavir in plasma, was effectively utilized for *in vivo* pharmacokinetic studies in rats.

1. Introduction

The respiratory pathogen fueling the Coronavirus Disease 2019 (COVID-19) pandemic is a single-stranded RNA virus belonging to the beta coronavirus family, scientifically designated as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Since its outbreak in December 2019, over 5 million people worldwide have been infected by this virus [1,2]. Because of the extraordinary

* Corresponding author.
** Corresponding author.
E-mail addresses: xra@wmu.edu.cn (R.-a. Xu), zhangqianglsrmyy@sina.com (Q. Zhang).

https://doi.org/10.1016/j.heliyon.2024.e32187
Received 20 March 2024; Received in revised form 27 May 2024; Accepted 29 May 2024
Available online 30 May 2024
2405-8440/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

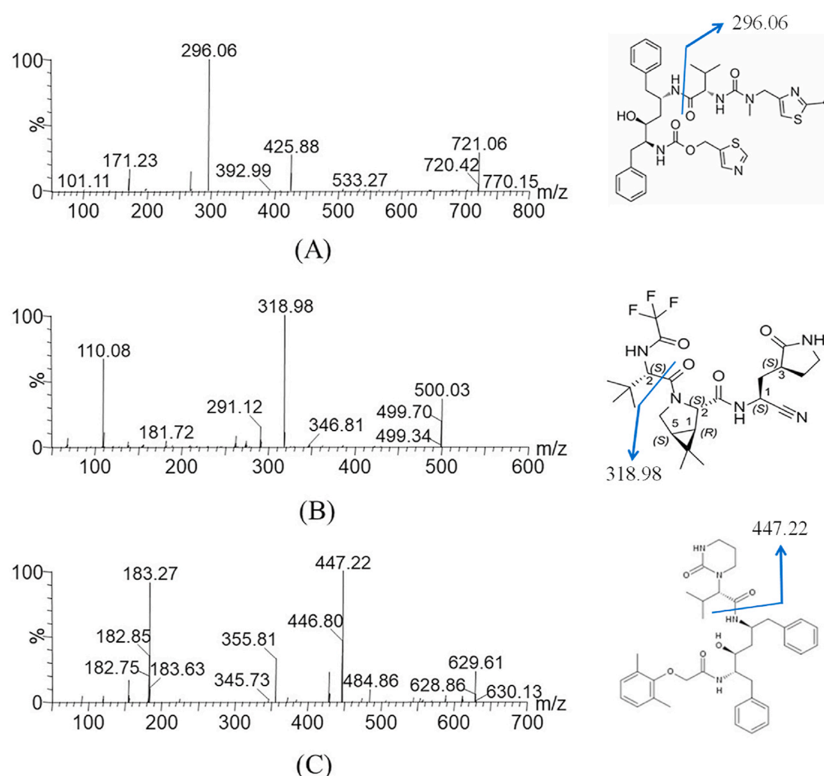


Fig. 1. Production in positive ions mode showing the production and fragmentary structure of ritonavir (A), nirmatrelvir (B), and lopinavir (IS, C) in the present study.

efforts and mutual collaboration of the international community and pharmacologists worldwide [3,4], nirmatrelvir (Fig. 1A), an oral clinical candidate for the antiviral treatment of COVID-19, is approved by the U.S. Food and Drug Administration (FDA) for the treatment of COVID-19 [5]. Moreover, it is reported that the oral antiviral drug PAXLOVID™ (nirmatrelvir co-packaged with ritonavir, Fig. 1B) significantly reduces hospitalizations and deaths in patients with COVID-19 compared with placebo [6]. It is worth mentioning that nirmatrelvir is metabolized as a substrate of the CYP3A4 enzyme, while ritonavir can inhibit the metabolism of nirmatrelvir by inhibiting the CYP3A4 enzyme, which leads to an increased plasma level of nirmatrelvir. Therefore, the co-administration of ritonavir with nirmatrelvir as an agent enhances systemic exposure concentrations and prolongs the half-life of nirmatrelvir [7].

Since nirmatrelvir and ritonavir are CYP3A4 substrates, CYP3A4 inducers or inhibitors (such as calcineurin inhibitors, azole antifungals, direct oral anticoagulants and antagonists) may affect the plasma concentration of nirmatrelvir and ritonavir, producing toxic side effects or reducing their therapeutic effect [8–10]. With the increasing severity of renal impairment, systemic exposure of nirmatrelvir in patients rises. Therefore, patients with moderate renal impairment (eGFR $\geq$ 30 to $<$ 60 mL/min) need to have the dose adjusted [11]. Moreover, there is insufficient clinical data for PAXLOVID™. Administration of PAXLOVID™ may result in unforeseen and serious adverse events not previously reported, which may lead to clinical adverse effects such as hepatotoxicity, failure of treatment, serious life-threatening effects, and the possibility of virus resistance development, especially when administered at inappropriate doses [12].

Thus, therapeutic drug monitoring (TDM) involving measuring drug levels can optimize therapeutic efficacy and minimize toxicity of PAXLOVID™. This clinical practice aims to enhance the medication's beneficial effects and reduce adverse reactions. To our knowledge, three LC-MS/MS-based bioanalytical methods have been reported that can simultaneously quantify nirmatrelvir and ritonavir in biological samples [13–15]. However, these bioanalytical methods required long running times, ranging from 4.0 min to even 10.5 min, had a limited concentration range, and showed low sensitivity ($\geq$ 10 ng/mL for nirmatrelvir and $\geq$ 2 ng/mL for ritonavir). Additionally, the trough concentration of nirmatrelvir was found to range from 1331.7 to 8352.5 ng/mL, and that of ritonavir from 53.4 to 1325.5 ng/mL, showing significant individual variation among 17 patients [16]. Moreover, compared to patients with normal renal function, the trough concentration of nirmatrelvir in critically ill patients undergoing continuous renal replacement therapy (CRRT) is approximately seven times higher, ranging from 3325.34 to 15625.46 ng/mL, and it is twice that of patients with end-stage renal disease undergoing hemodialysis [17]. As a result, these chromatography techniques fail to adequately meet the high sample throughput demands necessary for biological analysis in pharmacokinetic studies. Consequently, we sought to develop an analytical method based on ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). This method was designed to be precise and straightforward for simultaneously quantifying nirmatrelvir and ritonavir in rat plasma, and to facilitate pharmacokinetic studies in rats.

Table 1
Gradient table for chromatographic separation and optimized mass spectrometry parameters for quantification of the analytes and IS.

Gradient condition for chromatographic separation				
Time segment	Time (min)	Flow rate (μL/min)	Mobile phase	
			A%	B%
1	0	0.3	10	90
2	0.5	0.3	10	90
3	1.0	0.3	90	10
4	1.4	0.3	90	10
5	1.5	0.3	10	90
6	2.0	0.3	10	90

Mass spectrometry setting					
Analytes	Precursor ion	Product ion	CV (V)	CE (eV)	RT (min)
Nirmatrelvir	500.03	318.98	20	15	1.59
Ritonavir	721.06	296.06	20	15	1.69
IS	629.61	447.22	10	15	1.73

2. Experimental

2.1. Chemicals, materials and reagents

Nirmatrelvir, ritonavir, and lopinavir (used as an internal standard, IS, Fig. 1C) were sourced from Beijing Sunflower and Technology Development Co., Ltd (Beijing, China) with each having a purity greater than 98%. The solvents and chemicals utilized in this research, including liquid chromatography (LC) grade methanol and acetonitrile, were obtained from Merck Company (Darmstadt, Germany). LC grade formic acid was supplied by Anaqua Chemicals Supply (ACS, USA). Water of LC grade was produced using Millipore's Milli-Q purification system (Bedford, USA).

2.2. Apparatus and conditions

The experimental setup comprised an Acquity UPLC system (Waters, Milford, USA), featuring a Class I binary solvent manager for gradient elution delivery. A temperature-controlled autosampler (FTN, maintained at 10 °C) and a column oven (set to 40 °C) were incorporated. Chromatographic separation was achieved using a reverse-phase Acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm particle size), with the mobile phase consisting of acetonitrile as the organic solvent and 0.1% formic acid as the aqueous solvent. A gradient elution method was implemented, with the specific program detailed in Table 1. Throughout the analytical sequence, the flow rate was consistently maintained at 0.3 mL/min. Analyte quantification was performed on a Xevo TQ-S triple quadrupole MS/MS system (Milford, MA, USA), equipped with an electrospray ionization (ESI) source. Multiple reaction monitoring (MRM) mode was utilized for the analysis, with the MS/MS system parameters presented in Table 1. Data acquisition and system control were managed through Masslynx 4.1 software (Milford, MA, USA).

2.3. Calibration standards and QC samples preparation

The provided compounds of nirmatrelvir, ritonavir and IS were prepared as primary stock solutions of 1.00 mg/mL in methanol. Afterwards, dilution of the primary stock solution with methanol was performed to obtain the standard solutions required for the experiment. Solutions for experiment, including working solutions and stock solutions, were carefully maintained in a frozen state by storage at −80 °C in a dedicated freezing chamber. To facilitate the establishment of calibration curves for quantitative determination, various concentrations of samples were prepared by fortifying blank plasma specimens with the analytes of interest. Calibration curves were constructed by spiking blank plasma with increasing concentrations of the analytes at levels of 2.0, 5.0, 10, 50, 100, 500, 1000, 5000, 10000 ng/mL for nirmatrelvir, and 1.0, 2.5, 5.0, 25, 50, 250, 500, 2500, 5000 ng/mL for ritonavir, respectively. Quality control samples (QCs) at four concentration levels, namely lower limit of quantification (LLOQ), low (QCL), medium (QCM) and high (QCH), were prepared as described above, with concentrations of 2.0, 4.0, 800 and 8000 ng/mL for nirmatrelvir and 1.0, 2.0, 400 and 4000 ng/mL for ritonavir, respectively.

2.4. Sample processing

An additional 20 μL of IS solution (1.0 μg/mL) was pipetted into 100 μL of samples, and precipitation of plasma protein was achieved by adding 300 μL of acetonitrile. The mixtures were vortexed for 3.0 min, centrifuged for 10 min at 13000×*g* and 4 °C, and the clean supernatant (100 μL) was then pipetted into a new autosampler vial. The injection volume of each sequence was set at 1.0 μL.

2.5. Validation of method

Detailed validation was carried out to determine the reliability before the application of the newly developed UPLC-MS/MS method to pharmacokinetic analysis. In accordance with FDA guidelines, sensitivity, linearity, precision, accuracy, matrix effect and extraction recovery were systematically evaluated and stability was extensively checked [18].

Blank plasma from six different batches was examined to assess the selectivity of the method and to determine if additional interferences were present during the retention times of the analytes and IS. The quadratic, $1/x^2$, least squares linear curve fitting of each analyte was established with non-zero calibrators, and linear coefficient of determination (r^2) was predicted to be $\geq$ 0.99 for each compound. LLOQ was identified by reference to the signal-to-noise ratio (10:1), being set as the lowest concentration of the calibration curve, where the acceptable precision and accuracy of the LLOQ was $\leq \pm$20%. Using LLOQ and QC samples, the intra-day and inter-day accuracy and precision were assessed. The intra-day accuracy and precision were evaluated by analyzing five replicate samples within the same day. Conversely, the inter-day accuracy and precision were determined by measuring the samples over several consecutive days. Precision values, indicated by relative standard deviation (RSD) and accuracy values in terms of relative error (RE), were expected to be $\leq \pm$15%, except for LLOQ.

Extraction recoveries were determined by comparing the peak areas of analytes in unextracted (i.e., spiked samples prior to extraction) and extracted (i.e., spiked post-extraction samples) matrices. For each analyte, three concentrations of QCs were assessed.

Matrix effects were measured by assaying the peak ratios of analytes on two occasions (spiked in extracted blank matrix and in solvent) at three different QC levels, and were repeated five times.

Dilution integrity is essential for ensuring precise quantification when sample concentrations exceed the upper limit of quantification (ULOQ) and require dilution. This was assessed by examining five replicates of 10-fold high concentration of QC samples, following a 10-fold dilution.

The stability of the samples at room temperature was tested by leaving them on the test bench for 3 h. The prepared samples were placed in sealed autosampler bottles for 4 h at 10 °C to test the stability in the autosampler. Freeze-thaw stability was measured by examining the changes in analytes' levels after three repeated freeze-thawing. The samples were left at −80 °C for 3 weeks to test the long-term stability. All stability studies were evaluated with the calibration standard curves freshly prepared [19], and all assays were performed five times.

2.6. Animal experiments

A cohort of six male Sprague-Dawley rats (in good health, and their individual weights falling within the range of 200–220 g) was procured from the Experimental Animal Center affiliated with The First Hospital of Wenzhou Medical University, situated in Zhejiang Province, China. The animal experiments were conducted in accordance with ethical guidelines sanctioned by the Animal Care and Use Committee of The First Affiliated Hospital of Wenzhou Medical University (WYYY-IACUC-AEC-2023-029). Prior to commencing the experiment, the rats were housed in a controlled environment with clean cages for a week-long acclimation period. The ambient conditions were maintained at 25 °C and a 12-h light/dark cycle. During this time, the animals enjoyed ad libitum access to food and water. Before the day of dosing, a 12-h fasting period was performed, during which water intake remained unrestricted. Each rat was received an oral administration of a solution containing 30 mg/kg of nirmatrelvir and 10 mg/kg of ritonavir, formulated in 0.5% sodium carboxymethylcellulose. At designated time points, including pre-dose (0 h), 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 48 h post-dosing, approximately 0.3 mL of blood was drawn from the tail vein into heparinized centrifuge tubes. After centrifugation of these samples at 8000×g and 25 °C for 10 min, the supernatant was carefully transferred into fresh tubes and stored at −80 °C pending further analysis.

Pharmacokinetic parameters of nirmatrelvir and ritonavir in each rat, encompassing area under the concentration-time curve (AUC), time to reach peak plasma concentration (T_{max}), maximum plasma concentration (C_{max}), elimination half-life ($t_{1/2}$), apparent clearance ($CL_{z/F}$), and mean residence time (MRT), were analyzed through non-compartmental statistical models using the Drugs and Statistics (DAS 3.0) software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The data were presented as mean ± standard deviation (SD).

3. Results and discussions

3.1. Development of chromatography and mass spectrometric conditions

The suitable chromatographic conditions could provide high sensitivity of the analytes. The method was optimized to quantify the levels of nirmatrelvir and ritonavir with lopinavir as IS in this study. The method for purifying rat plasma was involved using the organic solvent acetonitrile, which was introduced into the system to precipitate the plasma proteins. Subsequent chromatographic separation was carried out using a UPLC BEH C18 reversed-phase column (2.1 mm × 50 mm, 1.7 μm). In line with previous experience, favorable chromatographic separations were observed using gradient elution with a mixture of acetonitrile and 0.1% aqueous formic acid in various ratios [20,21]. In this analysis, excellent separations of the analytes from endogenous impurities were realized, and the chromatographic peak shapes were symmetrical.

Organic solvent precipitation is one of the plasma protein precipitation (PPT) methods used. Compared with frequently used approaches, such as solid-phase extraction and liquid-liquid extraction, the organic solvent precipitation method is convenient, efficient and cost-effective. In addition, the protein is easily removed. The recovery of extraction with organic solvents (*e.g.* acetonitrile,

Table 2
Characteristics of related literatures for the determination of nirmatrelvir and ritonavir.

Literatures	Running time (min)	matrix	LLOQ of nirmatrelvir and ritonavir (ng/mL)
Martens-Lobenhoffer et al. [13]	10.5	human plasma	10/2.0
Liu et al. [14]	7.0	human plasma	50/10
Guyon et al. [15]	4.0	human plasma	40/10
This study	2.0	rat plasma	2.0/1.0

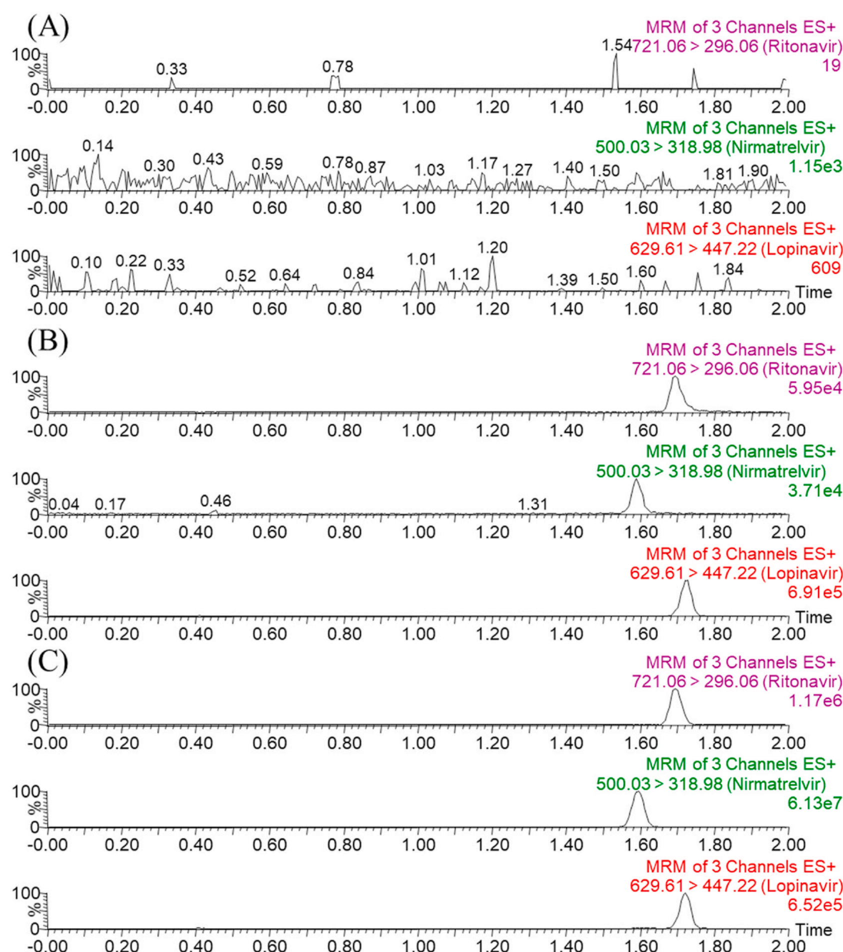


Fig. 2. Representative MRM chromatograms of nirmatrelvir, ritonavir and IS in rat sample: blank plasma (A), blank plasma spiked with standard solution at LLOQ and IS (B) and real plasma sample collected from a rat after 1.0 h oral administration of 30 mg/kg nirmatrelvir and 10 mg/kg ritonavir (C).

ethanol and methanol) was investigated in this method. After comparison, the recovery and matrix effect of sample extraction using acetonitrile as the protein-precipitating agent were superior to other solvents.

In the ion source of the mass spectrometer, the analytes were fragmented to produce positively charged $[M + H]^+$ quasimolecular ions, which were subsequently further fragmented to generate additional fragment ions shown in Fig. 1. The proposed fragmentation pathway leading to these fragment ions was described in the molecular formula of the analyte. In the MRM scans, the target fragment ions employed were m/z 500.03 → 318.98 for nirmatrelvir, m/z 721.06 → 296.06 for ritonavir, and m/z 629.61 → 447.22 for IS, as shown in Table 1. Although three LC-MS/MS-based bioanalytical methods simultaneously quantify nirmatrelvir and ritonavir in biological samples [13–15], the advantages of our study are greater sensitivity and shorter running times (Table 2). Therefore, in order to meet the high-throughput sample requirements of analysis in pharmacokinetic studies, a highly sensitive and straightforward assay has been successfully validated.

Table 3
Calibration curves for the analyses of nirmatrelvir and ritonavir in rat plasma (n = 3).

Analytes	Regression equation	r^2	Linear range (ng/mL)	LLOQ (ng/mL)
Nirmatrelvir	Y = 0.011X ± 0.005	0.999	2.0–10000	2.0
Ritonavir	Y = 0.012X ± 0.249	0.998	1.0–5000	1.0

Table 4
The accuracy and precision of each analyte in rat plasma (n = 5).

Analytes	Concentration (ng/mL)	Intra-day		Inter-day	
		RSD (%)	RE (%)	RSD (%)	RE (%)
Nirmatrelvir	2.0	9.6	−7.6	8.7	−3.5
	4.0	12.8	−1.0	11.7	11.0
	800	2.4	11.3	2.4	13.2
	8000	3.4	−4.0	2.7	−2.9
Ritonavir	1.0	13.7	5.0	13.6	9.1
	2.0	9.7	−1.1	10.3	−0.3
	400	1.7	−0.4	2.2	−0.2
	4000	3.4	−1.7	2.6	−2.5

Table 5
Recovery and matrix effect of each analyte in rat plasma (n = 5).

Analytes	Concentration (ng/mL)	Recovery (%)		Matrix effect (%)	
		Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)
	4.0	97.4 ± 9.5	9.8	101.4 ± 11.9	11.8
Nirmatrelvir	800	101.9 ± 5.8	5.7	99.8 ± 3.6	3.6
	8000	107.2 ± 5.4	5.0	98.8 ± 8.9	9.0
	2.0	93.7 ± 4.7	5.1	114.3 ± 12.0	10.5
Ritonavir	400	95.6 ± 5.3	5.6	106.0 ± 8.0	7.5
	4000	90.7 ± 5.2	5.8	108.0 ± 8.6	7.9

3.2. *Validation of the UPLC-MS/MS analysis*

3.2.1. *Fig. 2: Selectivity assessment*

To test the selectivity of this method, blank plasma (Fig. 2A), blank plasma spiked with IS, nirmatrelvir, and ritonavir (Fig. 2B), and real plasma samples from animal experiments (Fig. 2C) were compared. It was observed that the MRM mode displayed high-resolution symmetrical peaks, allowing for the differentiation of the retention times of IS, nirmatrelvir, and ritonavir. Their retention times were 1.73 min, 1.59 min, and 1.69 min, respectively. These data obviously indicated a great selectivity.

3.2.2. *Linearity and LLOQ*

The calibration curves for the two analytes in rat plasma, with ranges of 2.0–10000 ng/mL for nirmatrelvir and 1.0–5000 ng/mL for ritonavir, demonstrated excellent linearity, which was indicated by the coefficients of determination (r^2) ≥ 0.99. Table 2 provided the standard regression equations for the analytes. In these equations, Y represented the ratio of the analytes' peak areas to those of the IS, and X referred to the nominal concentration. The developed method demonstrated LLOQ at 2.0 ng/mL for nirmatrelvir and 1.0 ng/mL for ritonavir, respectively. The obtained precision and accuracy values adhered to within ±20% of the standards established by the bioanalytical method validation guidelines, as detailed in Table 3.

3.2.3. *Accuracy and precision*

Intra-day and inter-day precision and accuracy of nirmatrelvir and ritonavir were assessed at LLOQ and three concentrations (LQC, MQC and HQC), and the validated values (RSD% and RE%) should be in acceptable range. Based on the results of the methodological validation, the intra-day and inter-day precision was within 15%. The detected accuracies were within ±15%, as shown in Table 4. The accuracy and precision were conformed to the standards set forth by the FDA. The findings demonstrated that the developed UPLC-MS/MS technique, designing for the concurrent determination and measurement of plasma levels of both nirmatrelvir and ritonavir, exhibited high reproducibility.

3.2.4. *Recovery, matrix effect and dilution integrity*

The average recoveries of nirmatrelvir and ritonavir extracted from rat plasma were summarized in Table 5. With a range of 90.7%–107.2%, the extraction recoveries of QC samples were within the acceptable limits, indicating this method had a high reliability and reproducibility of the sample processing.

Table 6
Stability results of each analyte in plasma under different conditions (n = 5).

Analytes	Added (ng/mL)	Room temperature, 3 h		Autosampler 10 °C, 4 h		Three freeze-thaw		−80 °C, 3 weeks	
		RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)
Nirmatrelvir	4.0	13.2	−7.1	9.0	2.9	6.3	2.0	4.4	3.5
	800	7.2	−6.7	2.8	12.0	4.0	3.4	3.1	14.9
	8000	5.3	−13.7	1.2	−0.8	4.7	−3.6	3.3	−1.5
Ritonavir	2.0	3.1	−13.3	9.3	−3.9	4.9	−11.5	9.5	13.0
	400	8.6	−6.4	1.8	−3.0	6.1	5.1	2.7	13.4
	4000	3.5	−1.8	1.9	−3.8	6.0	12.1	7.7	6.4

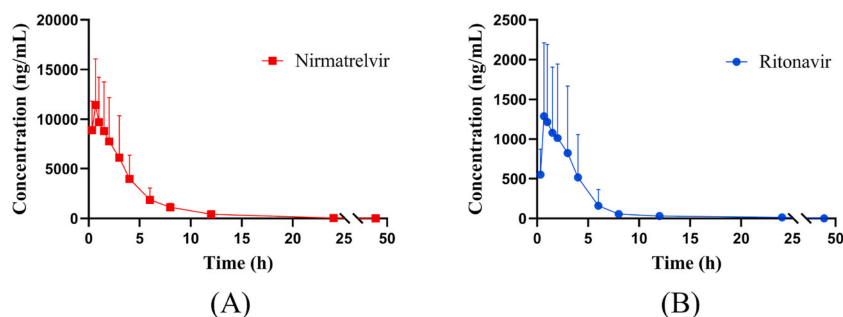


Fig. 3. Mean plasma concentration-time curves of nirmatrelvir (A) and ritonavir (B) in rats after orally administrated of 30 mg/kg nirmatrelvir and 10 mg/kg ritonavir. (n = 6, Mean ± SD).

Table 7
The main pharmacokinetic parameters of nirmatrelvir and ritonavir in rats after orally administrated of 30 mg/kg nirmatrelvir and 10 mg/kg ritonavir. (n = 6, Mean ± SD).

Parameters	Nirmatrelvir	Ritonavir
$AUC_{0\rightarrow t}$ (ng/mL•h)	44734.08 ± 18709.37	4930.57 ± 3818.61
$AUC_{0\rightarrow \infty}$ (ng/mL•h)	44749.84 ± 18697.44	4942.45 ± 3814.59
$MRT_{0\rightarrow t}$ (h)	4.40 ± 1.00	4.64 ± 2.00
$MRT_{0\rightarrow \infty}$ (h)	4.48 ± 1.01	4.87 ± 2.19
$t_{1/2}$ (h)	4.06 ± 1.15	5.63 ± 3.40
T_{max} (h)	0.89 ± 0.49	1.03 ± 0.62
$CL_{z/F}$ (L/h/kg)	0.76 ± 0.30	3.38 ± 2.37
C_{max} (ng/mL)	12019.30 ± 4006.43	1491.22 ± 907.75

AUC: area under the concentration-time curve, T_{max}: time to peak plasma concentration, C_{max}: peak plasma concentration, $t_{1/2}$: half-life, $CL_{z/F}$: clearance, MRT: mean residence time.

The matrix effects of nirmatrelvir and ritonavir in rat plasma ranged from 98.8% to 114.3% at three QCs levels (n = 5), demonstrating that this method had no obvious interference from endogenous impurities. The results were indicated in Table 5.

The dilution integrity assessment demonstrated that the accuracy and precision of samples diluted 10-fold fell within acceptable limits. This indicated that proper dilution was a viable approach for this analytical method.

3.2.5. Stability

Small variations were observed in the results of stability, with RSDs < 15%, which indicated that nirmatrelvir and ritonavir remained stable at different conditions: short-term room temperature exposure (3 h), prolonged frozen storage (−80 °C for 21 days), undergoing three freeze-thaw cycles (from −80 °C to room temperature and back), as well as temporary residence in an autosampler maintained at 10 °C for at least 4 h (see Table 6).

3.3. Animal experiments

Research on the pharmacokinetics of nirmatrelvir and ritonavir in Sprague-Dawley rats was conducted to validate the reliability and applicability of the current UPLC-MS/MS method. Fig. 3A and B respectively depicted the mean plasma concentration-time curves of nirmatrelvir and ritonavir for six rats after oral administration of 30 mg/kg nirmatrelvir and 10 mg/kg ritonavir. Table 7 listed the main pharmacokinetic parameters calculated using a non-compartmental model.

After a single oral dose of nirmatrelvir and ritonavir in Sprague-Dawley rats, they were both absorbed rapidly, peaking at C_{max}

within 0.89 ± 0.49 h and 1.03 ± 0.62 h, respectively. However, the values of T_{max} in the pharmacokinetic profile were 3.00 h and 3.98 h released by FDA, which were much longer than those of the current experiment [5]. In addition, the $CL_{z/F}$ values varied. Compared to 8.99 and 13.92 L/h/kg for nirmatrelvir and ritonavir as reported, the $CL_{z/F}$ values of the present research were 0.76 ± 0.30 and 3.38 ± 2.37 L/h/kg for rats. Moreover, the calculated plasma $t_{1/2}$ of nirmatrelvir and ritonavir were respectively 4.06 ± 1.15 h and 5.63 ± 3.40 h, similar to the pharmacokinetic properties reported by FDA, which were respectively 6.05 h and 6.15 h [5]. It was also reported that the pharmacokinetic parameters of nirmatrelvir were different between rats and monkeys [22,23]. The pharmacokinetic variation may be attributed to the limited number of rats (n = 6) and species differences in the present study by inference. Hence, for these reasons, it is necessary to conduct further investigations to probe the more accurate clinical characteristics of nirmatrelvir and ritonavir in humans.

4. Conclusions

In summary, the present study established a UPLC-MS/MS technique with reproducibility and accuracy that allowed the simultaneous quantification of nirmatrelvir and ritonavir in rats, and descriptions of the pharmacokinetic parameters in rats were presented. In addition, the newly developed analytical method was more convenient and economical than the traditional extraction method, with a run time of only 2.0 min. Consequently, the presently established UPLC-MS/MS method not only provided pharmacokinetic data, but could also serve as a methodological reference for clinical individualized administration.

Ethical approval granted and consent to participate obtained

All animals used in the study complied with the Guidelines for the Care and Use of laboratory animals approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All experimental procedures involving animals were reviewed and approved by this committee (Ethics approval number: WYYY-IACUC-AEC-2023-029) to ensure the welfare of the animals.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Chen-Jian Zhou: Project administration, Investigation, Formal analysis. **Ya-nan Liu:** Writing – review & editing, Writing – original draft, Investigation. **Anzhou Wang:** Validation, Supervision, Data curation. **Hualu Wu:** Resources, Formal analysis, Data curation. **Ren-ai Xu:** Conceptualization. **Qiang Zhang:** Writing – review & editing, Supervision, Resources, Data curation.

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.

References

[1] J. Guarner, Three emerging coronaviruses in two decades, Am. J. Clin. Pathol. 153 (2020) 420–421.
[2] A. Kumar, R. Singh, J. Kaur, S. Pandey, V. Sharma, L. Thakur, S. Sati, S. Mani, S. Asthana, T.K. Sharma, S. Chaudhuri, S. Bhattacharyya, N. Kumar, Wuhan to world: the COVID-19 pandemic, Front. Cell. Infect. Microbiol. 11 (2021) 596201.
[3] L. Zhang, Y. Liu, Potential interventions for novel coronavirus in China: A systematic review, J. Med. Virol. 92 (2020) 479–490.
[4] D.L. Heymann, N. Shindo, COVID-19: what is next for public health? Lancet 395 (2020) 542–545.
[5] Y.N. Lamb, Nirmatrelvir plus ritonavir: First approval, Drugs 82 (2022) 585–591.
[6] E. Mahase, Covid-19: pfizer's paxlovid is 89% effective in patients at risk of serious illness, company reports, BMJ 375 (2021) n2713.
[7] E.G. McDonald, T.C. Lee, Nirmatrelvir-ritonavir for COVID-19, CMAJ (Can. Med. Assoc. J.) 194 (2022) E218.
[8] J. Heskin, S.J.C. Pallett, N. Mughal, G.W. Davies, L.S.P. Moore, M. Rayment, R. Jones, Caution required with use of ritonavir-boosted PF-07321332 in COVID-19 management, Lancet 399 (2022) 21–22.
[9] R.K. Zeldin, R.A. Petruschke, Pharmacological and therapeutic properties of ritonavir-boosted protease inhibitor therapy in HIV-infected patients, J. Antimicrob. Chemother. 53 (2004) 4–9.
[10] O. Kis, K. Robillard, G.N. Chan, R. Bendayan, The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters, Trends Pharmacol. Sci. 31 (2010) 22–35.
[11] T. Lingscheid, M. Kinzig, A. Kruger, N. Muller, G. Bolke, P. Tober-Lau, F. Munn, H. Kriedemann, M. Witzenrath, L.E. Sander, F. Sorgel, F. Kurth, Pharmacokinetics of nirmatrelvir and ritonavir in COVID-19 patients with end-stage renal disease on intermittent hemodialysis, Antimicrob. Agents Chemother. 66 (2022) e0122922.
[12] B. Amani, B. Amani, Efficacy and safety of nirmatrelvir/ritonavir (Paxlovid) for COVID-19: a rapid review and meta-analysis, J. Med. Virol. 95 (2023) e28441.
[13] J. Martens-Lobenhoffer, C.R. Boger, J. Kielstein, S.M. Bode-Boger, Simultaneous quantification of nirmatrelvir and ritonavir by LC-MS/MS in patients treated for COVID-19, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 1212 (2022) 123510.
[14] C. Liu, M. Zhu, L. Cao, H. Boucetta, M. Song, T. Hang, Y. Lu, Simultaneous determination of nirmatrelvir and ritonavir in human plasma using LC-MS/MS and its pharmacokinetic application in healthy Chinese volunteers, Biomed. Chromatogr. 36 (2022) e5456.
[15] J. Guyon, M. Novion, V. Fulda, D. Ducint, M. Molimard, L. Couzi, H. Kaminski, F. Salvo, S. Bouchet, A UPLC-MS/MS method for plasma biological monitoring of nirmatrelvir and ritonavir in the context of SARS-CoV-2 infection and application to a case, J. Am. Soc. Mass Spectrom. 33 (2022) 1975–1981.

[16] F. Zhao, Z. Xiang, J. Han, J. Pan, Y. Qu, K. Fan, Z. Wu, D. Xu, Y. Yu, Z. Shen, C. Su, Simultaneous quantification of nirmatrelvir/ritonavir in human serum by LC-HRMS, J. Pharm. Biomed. Anal. 237 (2024) 115796.

[17] R. Dong, Y. Huang, X. Ling, L. Li, W. Yu, S. Jiang, High concentrations of nirmatrelvir/ritonavir in critically ill patients receiving continuous renal replacement therapy, Int. J. Antimicrob. Agents 63 (2024) 106997.

[18] (U.S. Department of Health and Human Services, Food and Drug Administration, Bioanalytical Method Validation Guidance for Industry, 2018. https://www.fda.gov/media/70858/download. (Accessed 19 June 2020).

[19] O. Awosemo, H. Neelakantan, S. Watowich, J. Ma, L. Wu, D.S. Chow, D. Liang, Development & validation of LC-MS/MS assay for 5-amino-1-methyl quinolinium in rat plasma: application to pharmacokinetic and oral bioavailability studies, J. Pharm. Biomed. Anal. 204 (2021) 114255.

[20] Y. Zhang, M. Zhu, S. Xie, X. Ye, X. Xu, Simultaneous determination of amiodarone, dronedarone, and their principal metabolites in SD rat plasma by UPLC-MS/MS and its application in pharmacokinetics, Arab. J. Chem. 14 (2021) 103300.

[21] X. Xu, S. Luo, Q. Yang, Y. Wang, W. Li, G. Lin, R.-a. Xu, Development and validation of the quantitative determination of avapritinib in rat plasma by a bioanalytical method of UPLC-MS/MS, Arab. J. Chem. 14 (2021) 103152.

[22] H. Eng, A.L. Dantonio, E.P. Kadar, R.S. Obach, L. Di, J. Lin, N.C. Patel, B. Boras, G.S. Walker, J.J. Novak, E. Kimoto, R.S.P. Singh, A.S. Kalgutkar, Disposition of Nirmatrelvir, an Orally Bioavailable Inhibitor of SARS-CoV-2 3C-Like Protease, across Animals and Humans, Drug Metab. Dispos. 50 (2022) 576–590.

[23] J.Q. Dong, M.V. Varma, A. Wolford, T. Ryder, L. Di, B. Feng, S.G. Terra, K. Sagawa, A.S. Kalgutkar, Pharmacokinetics and disposition of the thiouracil derivative PF-06282999, an orally bioavailable, irreversible inactivator of myeloperoxidase enzyme, across animals and humans, Drug Metab. Dispos. 44 (2016) 209–219.