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Development and validation of a LC-MS/MS method for the simultaneous determination of simnotrelvir and ritonavir in human serum and bronchoalveolar lavage fluid

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Abstract

"Xiannuoxin" (simnotrelvir/ritonavir) is a novel anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) drug developed in China, which plays an antiviral role by inhibiting 3C-like protease ($3CL^{pro}$). At present, it has been put into clinical use, while a simple, accurate and sensitive detection method is urgently needed for the quantification of simnotrelvir/ritonavir in human serum and bronchoalveolar lavage fluid (BALF) to ensure safe and efficacious antiviral therapeutics. In this study, we developed a liquid chromatography tandem mass spectrophotometry (LC-MS/MS) method for the simultaneous determination of simnotrelvir, ritonavir and urea concentrations in human serum and BALF samples. Prior to LC-MS/MS analysis, a user-friendly, one-step pre-analytical process was conducted, followed by a rapid chromatographic run lasting 3 min. This was then succeeded by positive and negative electrospray ionization and detection using a triple quadrupole tandem mass spectrometer in the multiple reaction monitoring mode. Subsequently, the LC-MS/MS method underwent a comprehensive validation in aspects such as sensitivity (LoQs of 2.5 ng/mL, 0.1 ng/mL, and 1 μg/mL for simnotrelvir, ritonavir and urea), linearity, carryover, precision, trueness (recovery rates of simnotrelvir, ritonavir and urea were between 85 and 115%), matrix effect (within 85-115%) and stability (stable for 72 h at room temperature). The validation results demonstrated that this LC-MS/MS method was robust and reliable. Notably, we can use the urea dilution correction method to calculate the concentrations of simnotrelvir and ritonavir in epithelial lining fluid (ELF), which is of great significance for evaluating the effectiveness and safety of antiviral drug treatment.

Keywords LC-MS/MS, Simnotrelvir, Ritonavir, Urea, Serum, Bronchoalveolar lavage fluid, Epithelial lining fluid

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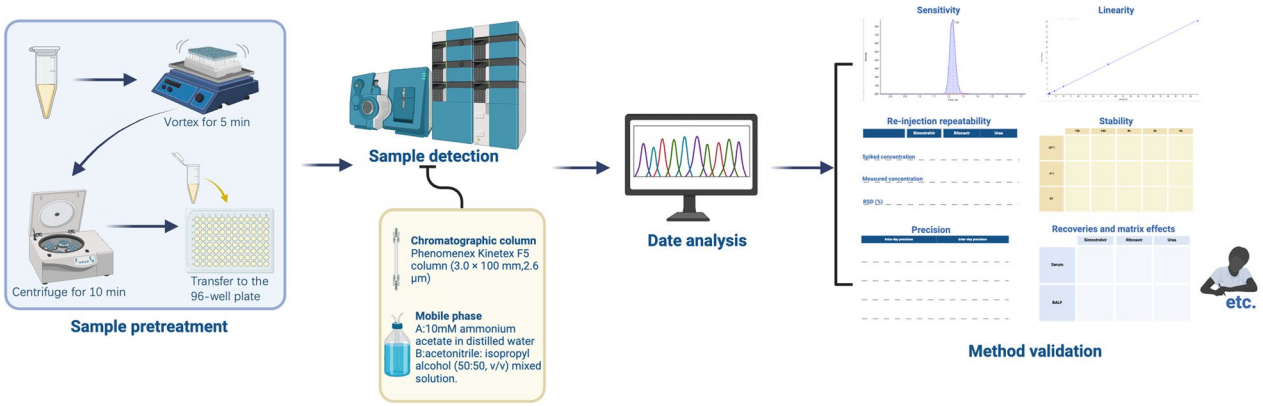
Graphical Abstract



Introduction

Since the end of 2019, almost the whole world had experienced multiple outbreaks of COVID-19, an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which can be transmitted through respiratory tract, aerosol, contact and so on [1]. During the pandemic, the infectious disease resulted in significant casualties, widespread social unrest, and substantial economic losses worldwide, posing a formidable public health threat. SARS-CoV-2 belongs to the genus *Betacoronavirus* and is an enveloped single-stranded RNA virus [2]. However, unlike other coronaviruses, SARS-CoV-2 can not only cause discomfort related to the respiratory system of infected patients, but also have certain effects on cardiovascular system, central nervous system, digestive system, urinary system and other systems, which may eventually lead to multiple organ failure [3]. Meanwhile, as the disease spreaded over time, new viral variants with stronger immune escape capabilities emerged [4]. Therefore, it is imperative to explore more effective antiviral strategies.

Among all the known RNA viruses, coronavirus stands out as the one with the most extensive genome, most of which is transcribed into mRNA, which subsequently encode proteins necessary for viral replication and gene expression [5]. In this process, the main protease (also named 3C-like protease, $3CL^{pro}$) plays an important role, which can process peptides into functional proteins and promote the replication and transcription of coronavirus [6]. Therefore, inhibition of $3CL^{pro}$ can effectively block replication and transcription of viral RNA, thus blocking viral proliferation. Based on the structural characteristics, $3CL^{pro}$ can be labeled as structurally conservative and substrate specific, which makes it an optional target for antiviral therapy [7].

"Xiannuoxin", consisting of two drug ingredients-simnotrelvir and ritonavir, mainly acts on $3CL^{pro}$ and has been approved for antiviral treatment of patients with mild to moderate COVID-19 [8]. Simnotrelvir is a kind of domestic anti-coronavirus drug with entirely intellectual property rights independently developed in China. It is metabolized by cytochrome P450 3A enzyme (CYP3A), which inhibits post-translational modification of the functional proteins of coronaviruses and blocks viral replication [9]. In previous animal studies, simnotrelvir has shown superior antiviral effects with dose-dependent characteristics, and it can significantly reduce the viral load in the lung and brain tissues of animals [9]. Although ritonavir has no direct antiviral effect, it can act as a pharmacokinetic enhancer by inhibiting CYP3A4 (one type of CYP3A), which can enhance the drug exposure and prolong the half-life of simnotrelvir, thus improving the antiviral effect [10]. Some adverse reactions have been described in patients treated with simnotrelvir/ritonavir, including gastrointestinal diseases, skin and subcutaneous tissue disorders [11], which might be associated with the inappropriate drug dosing. In order to achieve the expected efficacy and reduce the occurrence of adverse events, therapeutic drug monitoring (TDM) of simnotrelvir and ritonavir is critical for the achievement of individualized and precision medicine. Therefore, it is essential to establish a rapid and accurate method to quantify the concentrations of simnotrelvir and ritonavir in patients taking "Xiannuoxin".

Based on the PK/PD characteristics of the drugs, peripheral blood sample analysis serves as a standard method for conducting TDM. Peripheral blood samples can be classified as whole blood, serum and plasma samples, which are selected based on the drug's binding affinity to various blood components and its free state [12]. Sometimes the concentrations of antiviral drugs in the alveolar epithelium can better reflect the efficacy and safety compared to those in the blood, so the alveolar epithelial lining fluid (ELF) or bronchoalveolar lavage fluid (BALF) are also monitoring substrates. ELF refers to the interface fluid between the epithelial cells of the alveolar cavity. For pulmonary infection, whether the effective concentration of anti-infective drugs can be reached in the epithelial lining fluid affects the actual clinical efficacy and the prognosis of patients [13]. At present, bronchoalveolar lavage is the most commonly used technique to obtain BALF samples and evaluate the drug concentration in the ELF. The determination of the drug concentration in the ELF primarily depends on urea dilution correction method. Urea (CH_4N_2O) is a polar small molecule that can freely pass through the alveolar wall, so the concentration of urea in the blood is the same as that in the ELF. Therefore, it has been proposed that we can measure the drug concentration in BALF, as well as urea concentrations in serum and BALF, and then the actual drug concentration in ELF could be calculated according to urea dilution correction method [14, 15].

Currently, there is no established method for simultaneously detecting the concentrations of simnotrelvir and ritonavir. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an analytical technique that combines the separation capability of liquid chromatography with the detection power of tandem mass spectrometry. As a high-resolution and highly sensitive detection method, it is considered the standard for TDM. Sample pretreatment is critical for LC-MS/MS analysis. Several sample pretreatment techniques, including protein precipitation, solid-phase extraction (SPE), and liquid–liquid extraction (LLE), have been widely used [16]. When selecting an appropriate pretreatment method, we should take sample type, analyte characteristics, and matrix effect into account. Additionally, the entire analytical process should adhere to Green Analytical Chemistry (GAC) principles [17–19], including highly efficient and accurate analysis with minimal environmental impact by reducing hazardous substances, adopting miniaturization, and implementing sustainable technologies.

In this study, we described a method of simultaneous determination of simnotrelvir, ritonavir and urea by LC-MS/MS, which is the first method that detect the concentration of these two drugs simultaneously. It achieved the quantification of drug concentrations in serum and BALF, and the actual concentrations of simnotrelvir and ritonavir in ELF could be determined according to urea dilution correction method. This LC-MS/MS method has the characteristics of simple operation, high sensitivity, precision and trueness, and can achieve TDM of simnotrelvir and ritonavir, and thus promote the realization of precision therapy for COVID-19 patients.

Materials and methods

Chemicals, materials and reagents

Simnotrelvir, ritonavir, ritonavir-D6 and urea were purchased from Alta Scientific (Tianjin, China), simnotrelvir-D4 was purchased from Jiangsu Simcere Pharmaceutical (Jiangsu, China), and urea-$^{13}C^{15}N_2$ was purchased from Shanghai Macklin Biochemical (Shanghai, China) (Fig. 1). Ammonium acetate was purchased from Aladdin Biochemical Technology (Shanghai, China). Methanol and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dimethyl sulfoxide (DMSO) was purchased from Solarbio Science & Technology (Beijing, China). Isopropanol was purchased from Merck (Darmstadt, Germany), and distilled water of chromatographic purity was purchased from A.S. WATSON & W (Guangzhou, China). Peripheral blood samples and alveolar lavage fluid were collected from Peking University People's Hospital.

Standards, quality controls and internal standard mixture

The standard solutions of simnotrelvir (XS1-XS8), ritonavir (RS1-RS8) and urea (US1-US8) were obtained by step by step dilution using 50% methanol (Supplementary Table 1-3). Then, 10 µL simnotrelvir standard solution (XS1-XS8), 10 µL ritonavir standard solution (RS1-RS8) and 10 µL urea standard solution (US1-US8) were added into 170 µL PBS to obtain standard curve working solutions (STD1-STD8). The concentrations of simnotrelvir in the standard curve working solution ranged from 2.5 ng/mL to 5000 ng/mL, those of ritonavir ranged from 0.1 ng/mL to 1000 ng/mL, and those of urea ranged from 1 µg/mL to 1000 µg/mL.

The quality control (QC) samples (low, medium and high) were prepared independently by adding 10 µL simnotrelvir standard solution (XS3/XS5/XS7), 10 µL ritonavir standard solution (RS3/RS5/RS7) and 10 µL urea standard solution (US3/US5/US7) into 170 µL PBS. The concentrations of simnotrelvir in QC samples were 10 ng/mL (low), 100 ng/mL (medium) and 1000 ng/mL (high), the concentrations of ritonavir in QC samples were 2 ng/mL (low), 50 ng/mL (medium) and 500 ng/mL (high), and the concentrations of urea in QC samples were 5 µg/mL (low), 40 µg/mL (medium) and 400 µg/mL (high).

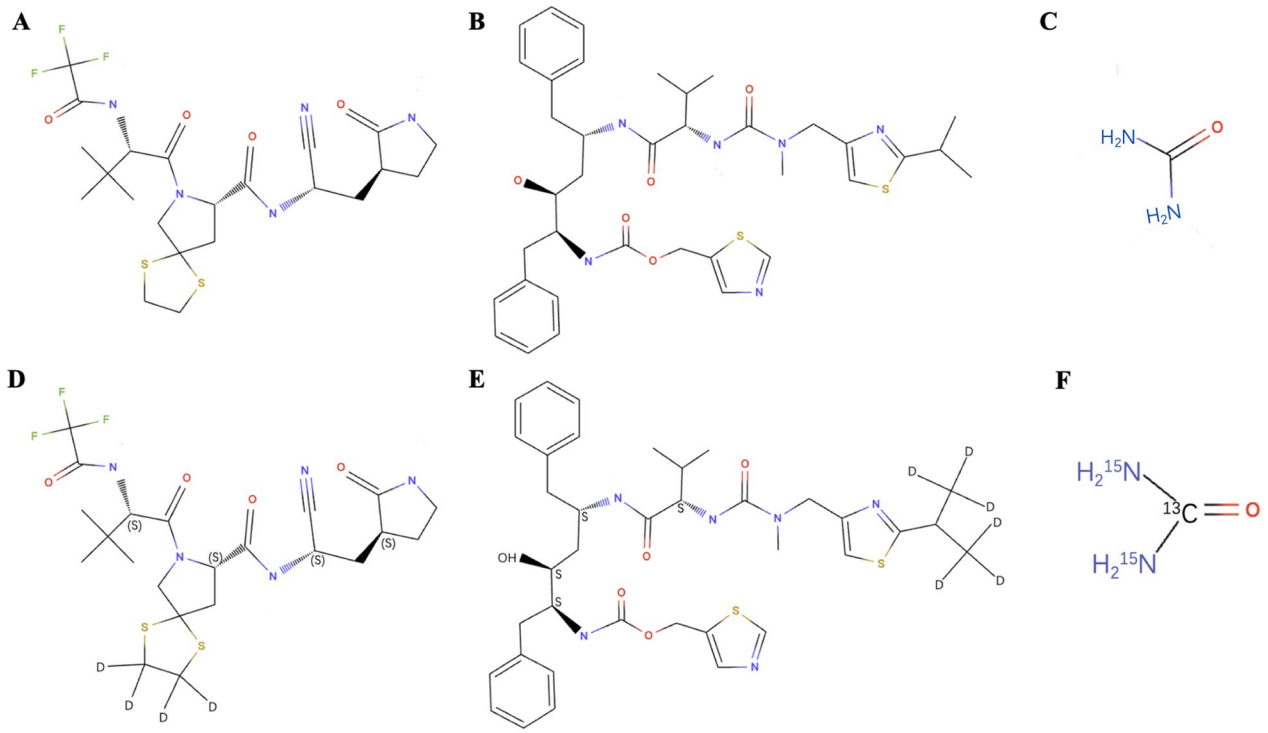


Fig. 1 Chemical structures of standard compounds. **A** Simnotrelvir; **B** Ritonavir; **C** Urea; **D** Simnotrelvir-D4; **E** Ritonavir-D6; **F** Urea -$^{13}C^{15}N_2$

The internal standard solution was prepared as follows. DMSO was used to dissolve simnotrelvir-D4 and 50% methanol was used as diluent, and the final concentration of simnotrelvir-D4 work solution was 10 μg/mL. The ritonavir-D6 stock solution was diluted with 50% methanol, and the final concentration of ritonavir-D6 work solution was 2 μg/mL. Urea-$^{13}C^{15}N_2$ was dissolved and diluted with distilled water, and the final concentration of urea-$^{13}C^{15}N_2$ work solution was 200 μg/mL. The internal standard mixture of simnotrelvir, ritonavir and urea were prepared at the ratio of 1:1:2 to obtain the final concentration of simnotrelvir-D4 at 2.5 μg/mL, ritonavir-D6 at 0.5 μg/mL and urea-$^{13}C^{15}N_2$ at 100 μg /mL.

Sample pretreatment

Prior to sample pretreatment, all serum and BALF samples to be tested were stored at − 20 °C. 200 μL standard curve working solution (STD1-STD8), QC samples (low, medium and high), serum or BALF samples were added with 20 μL internal standard mixture, and then mixed with 400 μL 100% acetonitrile for protein precipitation. Next vortexed the samples for 5 min and centrifuged them for 10 min at 13,000 rpm at 4 °C. Subsequently, 200 μL supernatant was taken and transferred to the 96-well plate for LC-MS/MS analysis.

Instrumentation

The CalQuant-S LC-MS/MS platform (CALIBRA, Hangzhou, China), which is composed of a high-performance liquid chromatography and a triple quadrupole mass spectrometer, was used for the detection of simnotrelvir, ritonavir and urea. Phenomenex Kinetex F5 column (3.0 × 100 mm, 2.6 μm) was used as the chromatographic column for separation. The column temperature was set to 40 °C and the injection volume was set to 10 μL. The mobile phase A was 10 mM ammonium acetate in distilled water, and mobile phase B was acetonitrile: isopropyl alcohol (50:50, v/v) mixed solution. The sample was mixed with 40% mobile phase A and 60% mobile phase B for isocratic elution. The elution time was 3 min and the flow rate was set at 0.5 mL/min (Supplementary Table 4). Negative electrospray ionization (ESI-) mode was selected for simnotrelvir and simnotrelvir-D4, and ESI + mode was used for ritonavir, ritonavir-D6, urea and urea-$^{13}C^{15}N_2$. The ion spray voltage was set at 4500 V. The selection of electrospray ionization (ESI) modes was based on the protonation/deprotonation characteristics of the analytes, ensuring optimal ionization efficiency and detection sensitivity. The temperature of the capillary was set at 200 °C. For the ion source, gas 1 (nitrogen, used as nebulizing gas) had a nebulizing pressure of 50 psi, and gas 2 (nitrogen, used as auxiliary gas) had a pressure of 60 psi. The curtain gas (nitrogen) had a pressure of 40 psi,

Table 1 Summary of ion pair parameters

ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	CE (volts)	DP (volts)	EP (volts)	CXP (volts)
Simnotrelvir-1	548.0	521.0	40	− 29	− 95	− 10	− 15
Simnotrelvir-2*	548.0	461.0	40	− 36	− 95	− 10	− 15
IS-Simnotrelvir-D4-1	552.1	525.1	40	− 32	− 120	− 10	− 15
IS-Simnotrelvir-D4-2*	552.1	461.0	40	− 33	− 120	− 10	− 15
Ritonavir-1*	721.3	296.1	50	25	70	10	6
Ritonavir-2	721.3	426.3	100	25	70	10	6
IS Ritonavir-D6-1*	727.5	302.0	50	26	70	10	6
IS Ritonavir-D6-2	727.5	426.4	100	30	70	10	6
Urea-1*	61.0	44.0	100	55	80	10	12
IS Urea-$^{13}C^{15}N_2$*	64.0	46.0	100	55	80	10	12

DP: Declustering Potential; EP: Entrance Potential; CXP: Cell Exit Potential; *Quantitative ion pair

and the collision gas (nitrogen) had a pressure of 9 psi (Supplementary Table 4). The multiple reaction monitoring (MRM) transitions of these analytes were optimized individually and described in Table 1. Chromatographic data was aquired by Analyst MD 1.6.3 (SCIEX, Framingham, MA, USA) and peak integration and quantification were performed by MultiQuant MD 3.0.3 (SCIEX, Framingham, MA, USA).

Method validation

Rigorous validation was conducted in accordance with *Guideline 9012: Validation of Quantitative Analysis Methods of Biological Samples in Chinese Pharmacopoeia (2020 edition)* [20] and *ICH M10 Bioanalytical Method Validation (2022 editon)* [21].

Calibration, linearity and sensitivity

The calibration curve was generated by plotting the ratio of the analyte peak area to the internal standard peak area (vertical coordinate) against the concentrations of standard curve points (horizontal coordinate), with the curve fitted using the least squares method. The linearity of simnotrelvir, ritonavir and urea was evaluated and acceptable if the coefficient of determination (R^2) for linear regression was above 0.99. The limit of quantification (LoQ) represented the lowest concentration within the dynamic range that exhibited a signal-to-noise ratio (S/N) exceeding 10. By performing 10 replicate measurements of the lowest concentration sample and calculating the coefficient of variation (CV), we deem the LoQ acceptable when the CV was below 20%.

Carryover

The carry-over effect was assessed by injecting blank samples subsequent to the injection of the highest point of the calibration curve. It was deemed insignificant if the signal intensity of the blank samples was less than 20% of the LoQ or 0.1% of the highest point of the calibration curve.

Precision

We evaluated the precision of the LC-MS/MS method by using QC samples (low, medium and high). For each concentration level of the QC samples, three replicates were analyzed a day in five consecutive days. The coefficients of variation (CVs) of intra-day and inter-day precision of the method considered were calculated. The precision was acceptable if the intra-day and inter-day CVs were all less than 15%.

Trueness

Ten serum and BALF samples were randomly selected and mixed to prepare serum and BALF matrix. For each matrix, 10 µL of low, medium and high concentration standard solutions of simnotrelvir, ritonavir and urea were added into 170 µL of the blank serum and BALF matrix, respectively. After that, the samples were subjected to the sample pretreatment and LC-MS/MS analysis process. The recovery rates of simnotrelvir, ritonavir and urea were calculated as the following formula: Recovery % = (Actual sample concentration−actual matrix concentration)/theoretical spiked concentration × 100%.

Matrix effect

Serum and BALF spiked samples (low, medium and high) were subjected to sample pretreatment and LC-MS/MS analysis as before. After three parallel measurements, the average concentration (A) of each spiked sample was obtained. The low, medium and high-level standard solutions in PBS were also detected and the average concentration (B) of each standard solution in PBS in three

parallel measurements was obtained. In addition, the blank serum and BALF matrix samples were detected at the same time, and the average concentration (C) of the blank serum and BALF matrix was obtained. The following formula was used to calculate the matrix effect of the analytes: Matrix effect %=(A spiked sample concentration-C blank matrix concentration)/B standard solution concentration×100%.

Stability and re-injection reproducibility

The stability and re-injection reproducibility of simnotrelvir were evaluated as follows. Three different levels (low, medium and high) of simnotrelvir standard solution were added into the serum and BALF matrix, respectively. The samples were placed at room temperature, 4 °C and − 20 °C for 0 h, 2 h, 8 h, 24 h and 72 h, respectively. The same pretreatment and sampling process were carried out as before. In addition, the re-injection repeatability test was also performed, that is, the injected samples were placed in the automatic sampler for 24 h and then injected again. The stability and re-injection reproducibility were acceptable if the relative standard deviation (RSD) was within 15%.

Results and discussions

Method development and optimization

The objective of the project was to develop a sensitive and rapid analytical method for quantifying simnotrelvir, ritonavir and urea in human serum and BALF. Therefore, the LC and MS conditions, such as column oven temperature, injection volume, spray voltage and collision gas, were assessed to achieve the quantification of the analytes with high sensitivity and a short retention time. Different gradients of mobile phases (10 mM ammonium acetate and 20 mM ammonium acetate) and columns [Kinetex F5 column (3.0×100 mm, 2.6 μm, Phenomenex), XBridge C18 column (2.1×100 mm, 3.5 μm, Waters) and XBridge BEH C8 column (2.1×100 mm, 2.5 μm, Waters)] were evaluated independently as well. Here, we used 10 mM or 20 mM ammonium acetate in distilled water as mobile phase A, and found that the response intensity of simnotrelvir and ritonavir decreased if mobile phase A was 20 mM ammonium acetate aqueous solution, compared with the mobile phase A being 10 mM ammonium acetate aqueous solution (Supplementary Fig. 1), indicating that 10 mM ammonium acetate in distilled water as mobile phase A provided higher sensitivity. The lower concentration of salt in the mobile phase also helped to protect the detection system, so our final analytical method used the 10 mM ammonium acetate aqueous solution as the mobile phase A.

As for the chromatographic column, the comparison results were shown in Supplementary Figs. 2 and 3. XBridge C18 column (2.1×100 mm, 3.5 μm, Waters) showed lower response intensity of simnotrelvir and urea, and the peak shape of urea was poor with high baseline, making it difficult to quantify accurately compared with the Kinetex F5 column. XBridge BEH C8 column (2.1×100 mm, 2.5 μm, Waters) was tried for chromatographic separation as well, and its performance was similar to that of XBridge C18 column. Therefore, Kinetex F5 column (3.0×100 mm, 2.6 μm, Phenomenex) could exert better sensitivity and separation efficiency.

Therefore, the method have been preliminarily established, and the total ion chromatogram of simnotrelvir, ritonavir, and urea was shown in Fig. 2.

Method validation

Calibration, linearity and sensitivity

As shown in Supplementary Fig. 4, after analyzing the calibration data, the equation of the representative calibration curve for simnotrelvir was y=1.92519e-4x+7.33450e-6 (r=0.99836), that for ritonavir was y=0.002941x+0.00144 (r=0.99982), and that for urea was y=0.02852x+0.0219 (r=0.9997). The linearity of simnotrelvir, ritonavir and urea was good when the concentration ranges were 2.5 ~ 5000 ng/mL, 0.1 ~ 1000 ng / mL and 1 ~ 1000 μg/mL, respectively. In addition, this method had great sensitivity as well, with LoQs of 2.5 ng/mL, 0.1 ng/mL, and 1 μg/mL for simnotrelvir, ritonavir and urea, and S/Ns of 159.3, 134.3, and 140.1 at LoQs, respectively (Fig. 3). In addition, the reproducibility of the lowest concentration samples was within an acceptable range (Table 2).

Carryover

The MRM chromatograms of the blank samples immediately after the upper limit of the calibration curve exhibited peak areas less than 0.1% of the upper limit of the calibration curve (Table 3), which proves that there was no obvious contamination in this method.

Precision

The precision results were displayed in Table 4. The intra-day CVs of the different QC levels ranged from 0.93% to 2.92% in serum and from 0.96% to 2.46% in BALF for simnotrelvir, from 0.63% to 1.76% in serum and from 1.16% to 5.12% in BALF for ritonavir, and from 0.35% to 2.39% in PBS for urea, respectively. The inter-day CVs of the different QC levels ranged from 2.64% to 6.43% in serum and from 6.28% to 9.18% in BALF for simnotrelvir, from 3.98% to 5.26% in serum and from 6.04% to 9.47% in BALF for ritonavir, and from 3.02% to 7.69% in PBS for urea, respectively. These results suggested that this LC-MS/MS method had great precision.

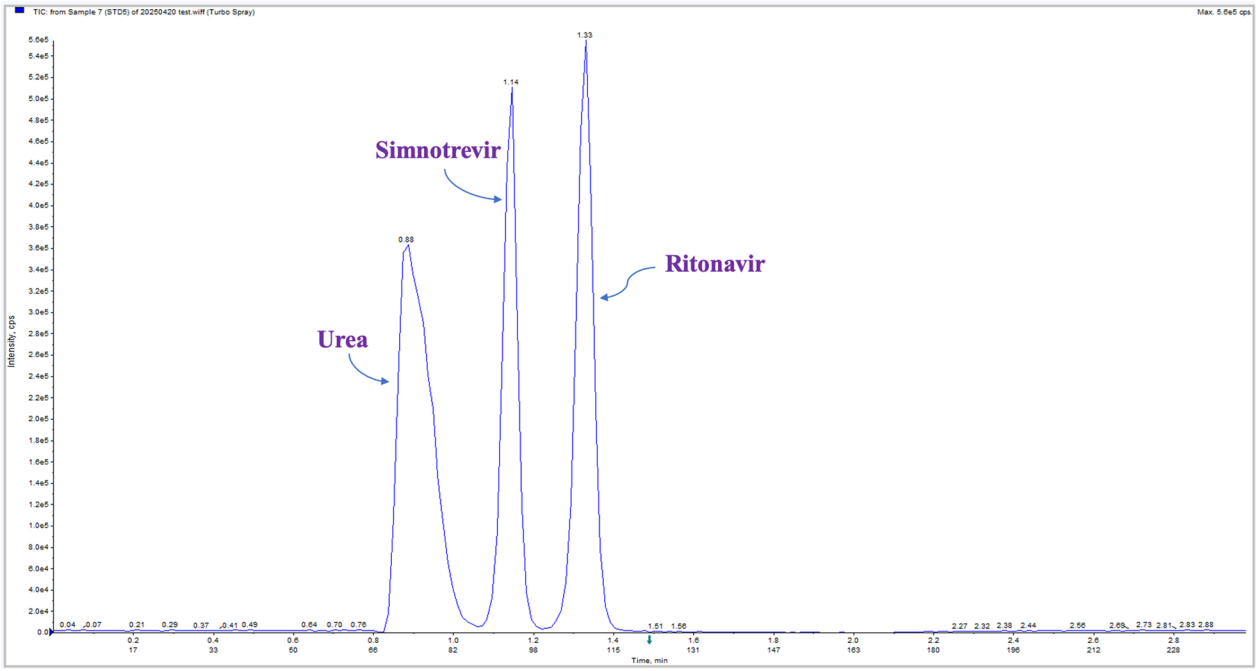


Fig. 2 The mass spectra of simultaneously detecting simnotrelvir, ritonavir, urea

Trueness

Detection trueness was evaluated by recovery rate. As shown in Table 5, the recovery rates for serum ranged from 88.31% to 91.15% for simnotrelvir, from 97.43% to 109.71% for ritonavir, and from 92.23% to 101.51% for urea, respectively. In addition, the recovery rates for BALF ranged from 92.97% to 99.08% for simnotrelvir, from 93.02% to 105.70% for ritonavir, and from 97.61% to 109.75% for urea, respectively. Collectively, these results suggested that this LC-MS/MS method had high trueness for both serum and BALF samples.

Matrix effect

The matrix effect was assessed at low, medium, and high concentration levels (Table 5). After normalization by the internal standard, the matrix effects (ME%) for serum ranged from 85.67% to 95.15% for simnotrelvir, from 103.98% to 108.93% for ritonavir, and from 95.16% to 100.93% for urea, respectively. In addition, the matrix effects for BALF ranged from 89.45% to 94.94% for simnotrelvir, from 87.10% to 98.70% for ritonavir, and from 93.46% to 99.61% for urea, respectively.

Stability and re-injection reproducibility

It has been demonstrated that both ritonavir and urea are relatively stable. Ritonavir showed good stability under the condition of − 20 °C freezing for 96 h [22], and remained stable under storage conditions of − 70 °C for one month[23] and − 80 °C for three months [24]. Similarly, the freeze through stability, benchtop stability, and long term stability of urea in human samples are all within the acceptable range of 15% [25].

Here, we determined the stability of simnotrelvir. Simnotrelvir stability was tested at low, medium and high levels in different conditions as described in Table 6. The results indicated that simnotrelvir in serum and BALF was stable for 72 h at room temperature, 4 °C and − 20 °C. In addition, autosampler extracts were stable at 4 °C for 24 h, and re-injection reproducibility was acceptable (Table 7).

Drug concentrations in ELF

Since it is not the period of the COVID-19 pandemic, it is difficult to find relevant patients infected with SARS-CoV-2 and taking the antiviral treatment of "Xiannuoxin". Compared to serum samples, the BALF samples are even more difficult to obtain. Therefore, we did not directly use relevant clinical samples in this study. In this LC-MS/MS method, concentrations of simnotrelvir, ritonavir and urea in serum and BALF were simultaneously detected, and the actual drug concentration in ELF can be calculated using Rennard's formula as follows [14, 15]:

$$V_{ELF} = V_{BALF} \times ([U]_{BALF} \div [U]_{ELF})$$

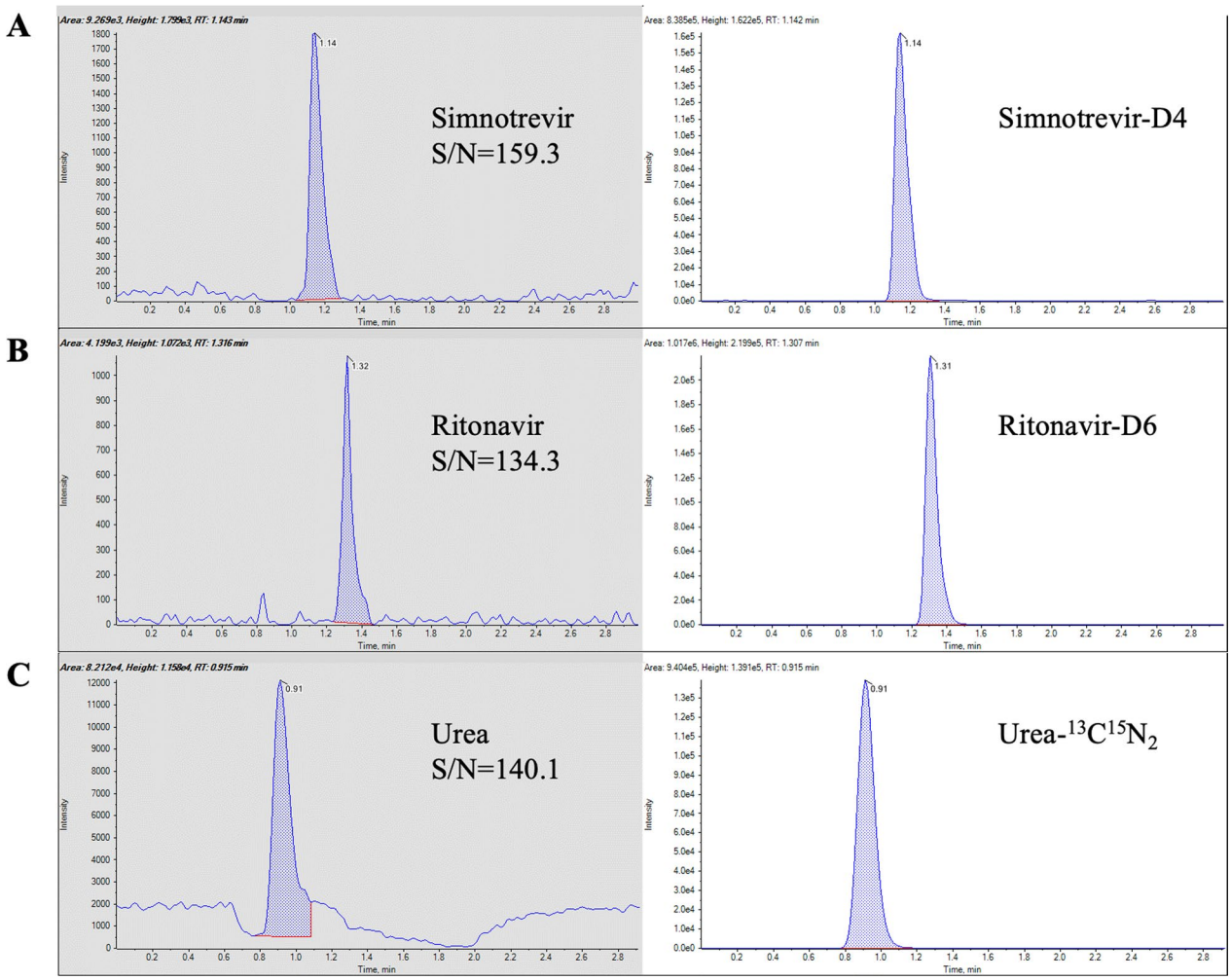


Fig. 3 The corresponding chromatograms at LOQ. **A** Simnotrelvir; **B** Ritonavir; **C** Urea

Table 2 LoQs of simnotrelvir, ritonavir and urea

	Simnotrelvir		Ritonavir		Urea
	Serum	BALF	Serum	BALF	PBS
Mean of STD1	2.496	2.585	0.113	0.113	0.990
Trueness (%)	99.82	103.38	113.40	112.70	99.04
SD of STD1	0.090	0.099	0.020	0.011	0.059
CV of STD1 (%)	3.61	3.84	17.88	10.18	5.99

here, V_{ELF} is the volume of ELF, V_{BALF} is the volume of BALF, $[U]_{BALF}$ is the urea concentration in BALF, and $[U]_{ELF}$ is the urea concentration in ELF.

The drug concentration ($[D]_{ELF}$) in ELF is calculated as follows:

$$\begin{aligned}[D]_{ELF} &= [D]_{BALF} \times (V_{BALF} \div V_{ELF}) \\ &= [D]_{BALF} \times ([U]_{ELF} \div [U]_{BALF}) \\ &= [D]_{BALF} \times ([U]_{serum} \div [U]_{BALF})\end{aligned}$$

here, $[D]_{BALF}$ is the concentration of drug in BALF, $[U]_{serum}$ is the concentration of urea in serum.

Therefore, by combining our LC-MS/MS method with the above formula, we can easily calculate the drug concentration in ELF.

Greenness profile

In line with green analytical chemistry principles, we also evaluated our established method using Click Analytical Chemistry Index (CACI) [26]. Although the LC-MS/MS method has the disadvantages of long pretreatment time, manual operation and non-miniaturization, the CACI (Supplementary Fig. 5) of our method still achieved high scores in sample size, feasibility, applications and sensitivity.

Table 3 Carryover of simnotrelvir, ritonavir and urea

	Simnotrelvir	Simnotrelvir-D4	Ritonavir	Ritonavir-D6	Urea	Urea-$^{13}C^{15}N_2$
Area (STD8)	3,575,796.32	3,561,430.22	5,378,379.90	173,121.45	11,700,979.04	400,931.55
Area (BLANK)	55.43	70.74	1913.81	62.71	2880.35	363.82
Carryover Rate (%)	0.0016	0.0020	0.0356	0.0362	0.0246	0.0907

Table 4 The detection precision of simnotrelvir, ritonavir and urea

Simnotrelvir						
Group	**Intra-day precision**			**Inter-day precision**		
	Mean	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
Serum						
Low	9.783	0.286	2.92	9.600	0.617	6.43
Medium	95.156	0.882	0.93	97.616	2.572	2.64
High	936.145	13.802	1.47	921.821	31.551	3.42
BALF						
Low	9.797	0.241	2.46	9.794	0.738	7.53
Medium	95.232	2.197	2.31	102.807	6.454	6.28
High	937.137	9.012	0.96	964.375	88.494	9.18
Ritonavir						
Group	**Intra-day precision**			**Inter-day precision**		
	Mean	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
Serum						
Low	2.016	0.013	0.63	2.117	0.111	5.26
Medium	52.781	0.686	1.30	53.909	2.186	4.05
High	530.440	9.310	1.76	534.874	21.267	3.98
BALF						
Low	1.963	0.101	5.12	2.023	0.192	9.47
Medium	48.795	0.565	1.16	51.973	3.137	6.04
High	539.699	7.301	1.35	528.737	40.204	7.60
Urea						
Group	**Intraday precision**			**Inter-day precision**		
PBS	**Mean**	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
Low	5.145	0.123	2.39	5.334	0.250	4.69
Medium	41.36	0.143	0.35	41.158	1.242	3.02
High	423.646	3.287	0.78	399.183	30.697	7.69

Comparison with other reported methods

Although there was no detection method for simnotrelvir, several different methods for ritonavir detection had been described, including microcrystalline cellulose/MOF hybrid dSPME technique for ritonavir extraction from human plasma, followed by liquid chromatography analysis [27] and a simple and rapid LC-MS/MS method for the quantification of nirmatrelvir/ritonavir in plasma of patients with COVID-19 [24]. Compared to the previous studies, our LC-MS/MS method exhibited a wider linear range and a lower LoQ, and could detect the concentration of drugs in serum and BALF simultaneously, which might streamline the TDM workflows of simnotrelvir and ritonavir, thereby advancing precision therapy for patients and potentially improving clinical outcomes.

Table 5 Recoveries and matrix effects of simnotrelvir, ritonavir and urea

Group	Simnotrelvir			Ritonavir			Urea		
	Low (10 ng/mL)	Medium (100 ng/mL)	High (1000 ng/mL)	Low (2 ng/mL)	Medium (50 ng/mL)	High (500 ng/mL)	Low (100 μg/mL)	Medium (200 μg/mL)	High (400 μg/mL)
Recoveries of serum (%)	88.31	90.45	91.15	97.43	101.44	109.71	92.23	101.51	101.12
Recoveries of BALF (%)	98.28	99.08	92.97	93.02	105.70	104	97.61	109.75	109.75
Matrix effects of serum (%)	89.51	95.15	85.67	108.93	107.04	103.98	95.43	95.16	100.93
Matrix effects of BALF (%)	89.92	94.94	89.45	98.70	98.52	87.10	98.41	93.46	99.61

Table 6 Stability of simnotrelvir in serum and BALF

	72 h		24 h		8 h		2 h			
	Serum	BALF	Serum	BALF	Serum	BALF	Serum	BALF	Serum	BALF
– 20 °C										
Low (%)	93.92	102.01	87.76	88.47	86.41	97.97	86.40	104.78	98.22	96.50
Medium (%)	89.75	102.37	92.45	96.28	91.92	102.79	92.47	102.84	95.84	102.15
High (%)	85.74	97.54	91.77	93.82	91.45	98.90	89.15	105.16	91.03	104.07
4 °C										
Low (%)	91.96	101.33	86.25	93.72	99.41	99.90	101.14	99.04	98.22	96.50
Medium (%)	106.41	94.47	92.86	98.41	95.03	105.73	89.19	102.85	95.84	102.15
High (%)	98.66	94.92	91.69	98.10	93.54	107.10	93.37	103.25	91.03	104.07
RT										
Low (%)	93.62	94.06	91.31	96.54	100.13	96.57	89.08	106.74	98.22	96.50
Medium (%)	90.17	93.46	101.44	103.93	100.30	108.86	105.76	106.86	95.84	102.15
High (%)	91.01	102.63	90.65	98.84	97.04	108.66	99.98	111.90	91.03	104.07

RT: Room Temperature; BALF: Bronchoalveolar Lavage Fluid

Table 7 The result of re-injection repeatability test

	Simnotrelvir			Ritonavir			Urea		
	Spiked concentration	Measured concentration	RSD (%)	Spiked concentration	Measured concentration	RSD (%)	Spiked concentration	Measured concentration	RSD (%)
QCL	10	9.001	5.21	2	2.26	3.02	5	4.352	5.42
QCM	100	96.754	8.13	50	51.644	2.20	40	41.545	0.85
QCH	1000	1042.691	4.85	500	531.582	0.74	400	429.946	0.44

RSD: Relative Standard Deviation

Limitations and future perspectives

Since it is not the outbreak period of COVID-19 at present, we have not been able to collect the relevant clinical medication samples. Therefore, we chose to use the spiked samples to verify the performance of this detection method. In the future, our LC-MS/MS method needs to be further verified using real clinical specimens collected from patients taking these drugs.

Conclusion

In this study, a robust and reliable LC-MS/MS method was developed for simultaneous determination of simnotrelvir, ritonavir and urea in serum and BALF, and the

drug concentrations in ELF could be calculated based on the above method. Simple protein precipitation was used for sample pretreatment and easy to operate. In addition, we also found that this method has excellent sensitivity, precision and trueness, and its stability can meet the requirements of clinical use. Our method might be capable of promoting the precision dosage of simnotrelvir and ritonavir in clinical practice.

Abbreviations

BALF	Bronchoalveolar lavage fluid
CACI	Click Analytical Chemistry Index
CV	Coefficient of variation
CYP3A	Cytochrome P450 3A enzyme
DMSO	Dimethyl sulfoxide
ELF	Epithelial lining fluid
ESI	Electrospray ionization
GAC	Green analytical chemistry
LC -MS/MS	Liquid chromatography tandem mass spectrophotometry
LLE	Liquid-liquid extraction
LoQ	Limit of quantification
ME	Matrix effect
MRM	Multiple reaction monitoring
QC	Quality control
RSD	Relative standard deviation
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
S/N	Signal-to-noise ratio
SPE	Solid-phase extraction
TDM	Therapeutic drug monitoring
$3CL^{pro}$	3C-like protease

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13065-025-01534-x.

Additional file 1: Supplementary Tables.

Additional file 2: Supplementary Fig. 1. The chromatograms of simnotrelvir-D4 and ritonavir-D6 with different mobile phases. (A) The chromatogram of simnotrelvir-D4 when the mobile phase A was 20 mM ammonium acetate aqueous solution; (B) The chromatogram of simnotrelvir-D4 when the mobile phase A was 10 mM ammonium acetate aqueous solution; (C) The chromatogram of ritonavir-D6 when the mobile phase A was 20 mM ammonium acetate aqueous solution; (D) The chromatogram of ritonavir-D6 when the mobile phase A was 10 mM ammonium acetate aqueous solution.

Additional file 3: Supplementary Fig. 2. The chromatograms of simnotrelvir, simnotrelvir-D4 in QCM samples using different chromatographic column. (A) The performance of Kinetex F5 column (3.0 × 100 mm, 2.6 μm, Phenomenex); (B) The performance of XBridge C18 column (2.1 × 100 mm, 3.5 μm, Waters); (C) The performance of XBridge BEH C8 column (2.1 × 100 mm, 2.5 μm, Waters).

Additional file 4: Supplementary Fig. 3. The chromatograms of urea, urea-$^{13}C^{15}N_2$ in QCM samples by using different chromatographic column. (A) The performance of Kinetex F5 column (3.0 × 100 mm, 2.6 μm, Phenomenex); (B) The performance of XBridge C18 column (2.1 × 100 mm, 3.5 μm, Waters); (C) The performance of XBridge BEH C8 column (2.1 × 100 mm, 2.5 μm, Waters).

Additional file 5: Supplementary Fig. 4. The representative standard curves. (A) Simnotrelvir; (B) Ritonavir; (C) Urea.

Additional file 6: Supplementary Fig. 5. The Click Analytical Chemistry Index result of the method.

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None.

Author contributions
XY, WL, YZ contributed to the method design, validation and improvement. LC and HW provided funding for the project and worked with XY, WL, YZ to complete the first draft, as well as the review and revision of the article. MJ, ZY and BC supervised the management and resource allocation of the project. CZ, LP and FZ contributed to the improvement of the project. DW participated in the establishment of method. All authors gave final approval for publication.

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Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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