Heliyon 11 (2025) e41737

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Simultaneous determination of nirmatrelvir, ritonavir, and beta-D-N4-hydroxycytidine in human plasma and epithelial lining fluid using LC-MS/MS and its clinical application to compare rates of achieving effective concentrations

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ARTICLE INFO

Keywords:
COVD-19
LC-MS/MS
Nirmatrelvir
Ritonavir
Beta-D-N4-hydroxycytidine

ABSTRACT

Currently, the trials found that the clinical efficacy of molnupiravir is lower than ritonavir-boosted nirmatrelvir. An explanation for these different efficacies in clinical treatments is still limited. The analysis method was developed and validated to simultaneously quantify nirmatrelvir, ritonavir, and beta-D-N4-hydroxycytidine (NHC) in human plasma and bronchoalveolar lavage fluid (BALF) by electrospray ionization mass spectrometry.

Our method was validated over a linear range of 30–10000 ng/mL for both matrices, with precision and accuracy within 15 % across four concentrations. Recovery rates for both analytes from plasma and BALF were between 90.7-102.2 % and 90.5–107.7 %, respectively.

The analytical method was then applied to monitor therapeutic drug concentrations in 59 plasma samples from 23 patients receiving ritonavir-boosted nirmatrelvir or molnupiravir. By setting target plasma concentrations of 292 ng/mL for nirmatrelvir and 1205 ng/mL for NHC, based on in vitro antiviral 90 % virus inhibitory concentrations (EC_{90}), the drug's molecular weight and its binding to human plasma proteins, we observed that ritonavir-boosted nirmatrelvir had substantially greater rates of achieving target plasma concentrations. Additionally, we monitored epithelial lining fluid in 4 BALF samples from 4 patients and observed that NHC exhibited higher permeability in lung tissue (approximately 20 % higher than nirmatrelvir). However, subtherapeutic antiviral concentrations of NHC were also present in epithelial lining fluid. These findings highlight the importance of considering these factors in determining the efficacy of these drugs in treating coronavirus disease 2019 (COVID-19).

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https://doi.org/10.1016/j.heliyon.2025.e41737
Received 22 July 2024; Received in revised form 28 December 2024; Accepted 6 January 2025
Available online 7 January 2025

1. Introduction

Although COVID-19 has become substantially milder, severe infections still occur, particularly in groups at high risk. Ritonavir-boosted nirmatrelvir and molnupiravir (prodrug of beta-D-N4-hydroxycytidine) are the two-leading oral COVID-19 antiviral treatments and have been used widely to accelerate SARS-CoV-2 viral clearance to treat COVID-19 [1,2]. Interestingly, the reduction rate of treatment with molnupiravir reduced the chances of hospitalization, which is lower than ritonavir-boosted nirmatrelvir in similar clinical trials [3,4]. However, an explanation for these different efficacies in clinical treatments is still limited. Only one study assessed the in-vivo antiviral activities of ritonavir-boosted nirmatrelvir and molnupiravir using the viral clearance rate as the antiviral effect measure [5]. The efficacy of antiviral agents was related to their concentrations. However, there was no study to observe their effective plasma concentrations using the rates of achieving effective concentrations and the permeability of the epithelial lining fluid in patients. Detecting the plasma or bronchoalveolar lavage fluid concentration of nirmatrelvir and NHC can provide insight into how these drugs improved therapeutic profile and reduced toxicity.

Although high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) has been used for these compounds [6–14], these results mainly focus on plasma drug concentrations that do not reflect lung tissue levels achieved in patients. The LC-MS/MS assays with positive ESI interface to detect nirmatrelvir-ritonavirin human plasma [7–9,12,13], rat plasma [10], and in the blood and brain of rats [11]. An LC-MS/MS method was published to quantify the prodrug molnupiravirand its active metabolite NHC in human plasma [14]. Another two LC-MS/MS methods were reported for NHC in human plasma [6,15]and peripheral blood mononuclear cells [15]. To our knowledge, there are no methods for quantifying BALF by LC-MS/MS and no method for simultaneously quantifying nirmatrelvir, ritonavir, and NHC. Consequently, the primary goal of the presented study was to develop and validate a method to simultaneously quantify nirmatrelvir, ritonavir, and NHC in human plasma and BALF, using protein precipitation extraction. The second goal was to compare different antiviral treatments using the rates of achieving effective concentrations and combining them with the permeability of the epithelial lining fluid.

2. Materials and methods

2.1. Instrumentation and reagents

The high performance liquid chromatography (HPLC) was a Shimadzu LC-20AD Series liquid chromatography system and included a degasser, binary pump, temperature-controlled autosampler, and temperature-controlled column compartment (Kyoto, Japan). The mass spectrometer system was an AB Sciex3200 Qtrap hybrid quadrupole linear ion trap mass with an electrospray ionization source (Applied Biosystems, MDS Sciex, Foster City, CA, USA).

Nirmatrelvir (batch no. F412320, 98 % purity) and NHC (batch no. F850476, 98 % purity), lamivudine(batch no. F676864, 98 % purity) were purchased EFEBIO Biotechnology Co., Ltd. Ritonavir (batch no. 101498–202001, 99.8 % purity) was purchased from National Institutes for Food and Drug Control. HPLC-grade formic acid was supplied by Anaqua Chemicals Supply (Eldridge Parkway, Houston, USA). HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany).

2.2. LC-MS/MS conditions

The chromatographic separation was achieved by gradient elution on a Zorbax SB-Aq C_{18} column, 5 μm particle size). A gradient mobile phase consisting of Mobile phase-A: 0.1 % formic acid solution and Mobile phase-B: acetonitrile, with a flow rate of 1.0 mL/min, and the temperature of the column set at 30 °C was employed. While reducing salt's impact in dialysate on mass spectrometry signals, utilizing a switching valve allowed the flow to be sent to waste in 0–2.8 min. The mobile phase gradient started with 88 % mobile phase A, held for 4 min, then increased in organic content to 85 % mobile phase B in 5 min. This condition was maintained for up to 10 min, followed by increasing the aqueous content back to 88 % at 10.1 min and held at the initial conditions with a total run time of 12 min.

The triple-quadrupole mass spectrometer with an electrospray source in positive ion mode was operated to detect analytes (Table 1). Multiple reaction monitoring (MRM) transitions were employed. The parameters of mass spectrometry were optimized to achieve the highest signal intensity: spray voltage, 5500 V; source temperature, 700 °C; gas 1 pressure, 65 psi; gas 2 pressure, 45psi; collision gas pressure, medium; Q1/Q3 resolution, unit; interface heater, on; The scanning time for each analyte was set to 0.61s.

Table 1
Physicochemical properties and parameters of optimized MRM method.

Compound	Transition (*m/z*);	DP (eV)	CE (eV)	EP (eV)	CXP (eV)	logP	pKa (Acidic)	t_R (min)
Nirmatrelvir	500.2 → 110.3; $[M + H]^+$	52	32	2.5	6	2.12	7.1	8.06
Ritonavir	721.4 → 296.4; $[M + H]^+$	35	27	5	8	3.9	13.68	8.48
NHC	260.1 → 128.1; $[M + H]^+$	22	15	3	3	−2	12.55	3.04
Lamivudine	230.1 → 112.1; $[M+H]^+$	45	21	2	2.9	−1.4	14.29	3.35

DP:declustering potential; CE: collision energy; EP: entrance potential; CXP: cell exit potential.
NHC:beta-d-N4-hydroxycytidine.

2.3. Sample preparation and extraction

200 μL of samples (blanks, standards, quality control (QC) samples, and clinical samples) with 10 μL of internal standard solution (IS, lamivudine, noted: subjects or patients who were taking lamivudine were excluded) were vortexed for 15 s. Subsequently, 800 μL of acetonitrile (for plasma) or 400 μL of acetonitrile for (bronchoalveolar lavage fluid) was added, and the sample was mixed and centrifuged at 4 °C at 5180×*g* for 5 min. Supernatants were transferred into the autosampler, and 10 μL injected into the LC-MS system for analysis.

2.4. Method validation

2.4.1. Specificity, calibration and linearity range

To test for specificity, the apparent response for six different sources of blank plasma at the retention time of the analytes was compared with the response at lower limit of quantification (LLOQ) or IS. The absence of interfering substances was confirmed where the responses were <20 % of each drug's lower limit of quantification and <5 % for their internal standards.

Final concentrations of calibration standards in the human plasm or the normal saline were 30, 60, 100, 200, 750, 2000, 7500, and 10000 ng/mL for nirmatrelvir, ritonavir, and NHC. QC materials in the human plasma or the normal saline were prepared at 30 (LLOQ), 60 (low), 750 (middle), and 7500 (high) ng/mL for nirmatrelvir, ritonavir, and NHC. Internal standard (lamivudine) was dissolved separately in 80:20 acetonitrile: water for a final 10,000 ng/mL concentration. Plasma concentrations were calculated with internal standards by linear regression of the peak area ratio (analyte area peak/area internal standard). Bronchoalveolar lavage fluid concentrations were calculated according to the above equation with the MRM chromatogram peak area obtained by the external standard method.

2.4.2. Accuracy and precision

Precision and accuracy were assessed by daily analysis of six replicates of four concentration QC samples levels (LLOQ, low, middle, high), and the results shown in Table 2; Intra-, inter-day accuracy and precision were within ±15 % of the nominal value; The signal-to-noise ratio of LLOQ was ≥10:1(Fig. 1).

2.4.3. Matrix effect and extraction recovery

Matrix effects were evaluated (Table 3) as follows: peak areas obtained from extracted blank matrix samples, which were afterward spiked with the respective QC sample concentrations, were compared with peak areas from samples without any matrix (diluted stock solution).

Extraction recoveries were evaluated (Table 3) as follows: the peak areas obtained from six extracted matrix samples were compared with peak areas obtained from extracted blank matrix samples, which were spiked afterward with the respective QC sample concentrations.

The spiking concentrations were identical to those of the QC samples of the low, middle, and high validation ranges. Matrix effects and extraction recoveries were expressed as the ratio of the analytical signals.

2.4.4. Stability

The stability of nirmatrelvir, ritonavir, and NHC in plasma and BALF (Two QC concentration levels) was assessed at room temperature and 4 °C over 24 h and following 3 freeze-thaw cycles spanning a period of 72h (Table 4). Furthermore, assay autosampler stability (4 °C) was assessed by re-injecting an accepted precision and accuracy batch, which had been left in the autosampler at 4 °C

Table 2
The intra-day and inter-day precision, accuracy.

Analyte	QC	Con (ng/ml)	Plasma (n = 6)		BAL (n = 6)	
			Intra-Day	Inter-Day	Intra-Day	Inter-Day
			Mean ± CV (%)	Mean ± CV (%)	Mean ± CV (%)	Mean ± CV (%)
Nirmatrelvir	LLOQ	30	32.2 ± 6.5	31.5 ± 10.3	31.4 ± 7.8	30.6 ± 12.1
	QCL	60	61.1 ± 4.5	59.5 ± 8.9	62.3 ± 5.6	61.5 ± 9.8
	QCM	750	946 ± 4.2	932 ± 6.3	953 ± 3.1	978 ± 10.3
	QCH	7500	7720 ± 3.2	7832 ± 7.5	7809 ± 3.4	7797 ± 7.7
Ritonavir	LLOQ	30	30.8 ± 5.7	33.1 ± 9.8	29.2 ± 8.3	31.4 ± 11.5
	QCL	60	63.3 ± 3.1	62.5 ± 8.0	60.7 ± 4.6	61.3 ± 13.1
	QCM	750	870 ± 3.8	900 ± 6.4	780 ± 5.9	803 ± 10.8
	QCH	7500	6827 ± 1.6	6823 ± 4.5	6956 ± 4.3	6814 ± 8.5
NHC	LLOQ	30	32.7 ± 6.4	31.4 ± 9.8	28.0 ± 9.3	30.6 ± 12.5
	QCL	60	61.2 ± 4.3	63.4 ± 12.4	59.9 ± 6.5	60.5 ± 10.6
	QCM	750	840 ± 7.3	893 ± 7.6	936 ± 9.7	919 ± 8.2
	QCH	7500	6943 ± 3.9	6903 ± 7.5	6892 ± 4.7	6906 ± 10.3

RSD; relative standard deviation, QCL; quality control law, QCM; quality control medium, QCH; quality control high, BAL; bronchoalveolar lavage fluid.

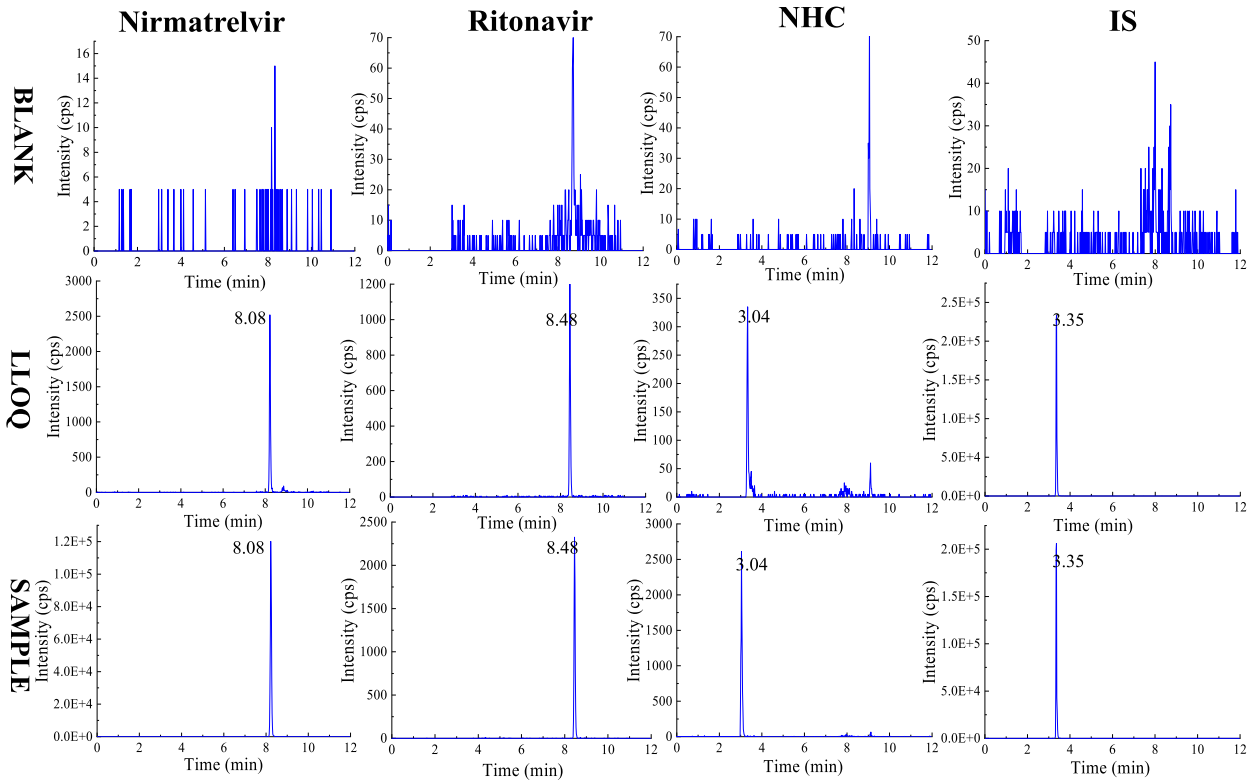


Fig. 1a. Representative LC-MS/MS chromatograms from blank, LLOQ and clinical samples of nirmatrelvir, ritonavir,beta-D-N4-hydroxycytidine (NHC) and internal standard(IS) from bronchoalveolar lavage fluid.

for 48 h. Reinjection reproducibility over 48 h was also assessed.

2.4.5. Dilution integrity

The dilution integrity experiments were conducted using samples two times the concentration of the upper limit of quantification (ULOQ). The dilution effect was verified by comparing the calculated concentration based on the dilution factor. The target accuracy of each plasma and BALF sample level was required to be 100 ± 15 %.

2.5. Clinical TDM application

2.5.1. Compare the rates of achieving effective concentrations

To compare the antiviral effects of the two drugs, we performed a screening using patients recruited to the same organ transplant unit. The experiments were approved by the Shanghai Changhai Hospital Ethics Committee (ethical batch number: CHEC 2023-100) and conducted following ethical standards, with the Declaration of Helsinki. The samples we applied for plasma and bronchoalveolar lavage fluid detection and analysis in this study were the rest of the abandoned samples after medical procedures; no medical operations beyond routine medical examination have been undertaken in the process of drawing material. All the patients signed the informed consent for medical procedures. Although, according to the Shanghai Changhai Hospital Ethics Committee, this study was exempt from informed consent, all patients provided oral informed consent. Patients were treated with nirmatrelvir/ritonavir or molnupiravir standard symptomatic treatments (Oral molnupiravir 800 mg was given twice a day; oral nirmatrelvir/ritonavir 300 mg/100 mg was given twice a day for 5 days) were included. Fifty-nine plasma samples from 23 patients were collected at pre-dose trough concentrations after administration of NMV or molnupiravir and analyzed using the established method. Based on the extracellular EC_{90} value, molecular weight, and human plasma protein binding [16], our study selected 292 ng/mL for nirmatrelvir and 1205 ng/mL for NHC as target plasma trough concentration to relate in vitro antiviral EC_{90}, which also were selected as an essential concentration for a pharmacodynamic response [4].

2.5.2. Permeability of nirmatrelvir and NHC entry into pulmonary lining fluid

The other four patients with pneumonia received oral antiviral agents (two receiving ritonavir-boosted nirmatrelvir and two receiving molnupiravir). After administering at least four doses of antiviral agents, bronchoalveolar lavage fluid and blood samples were collected at 2 h after administration, respectively. According to a previously described method [17] to collect epithelial lining fluid (ELF) from the lung lesions, an expert bronchoscopist injected three 20 mL aliquots of 0.9%saline at ambient temperature. As

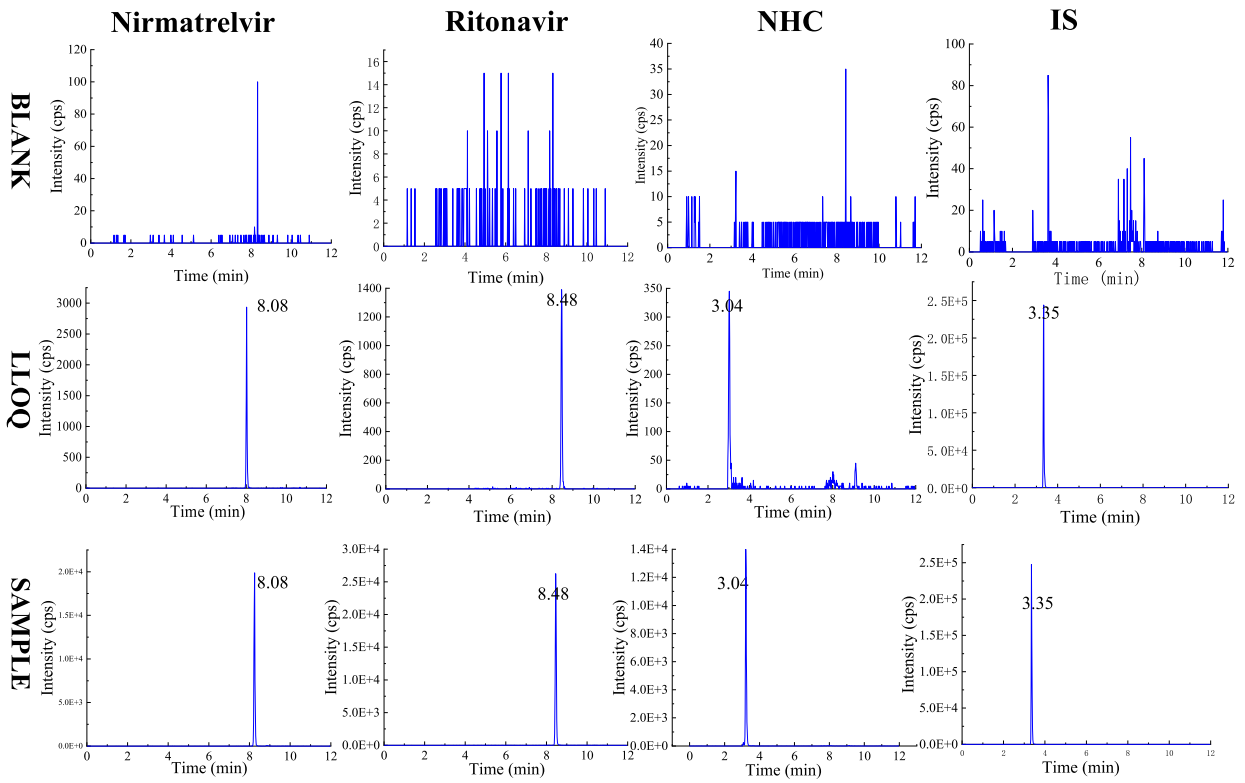


Fig. 1b. Representative LC-MS/MS chromatograms from blank, LLOQ and clinical samples of nirmatrelvir, ritonavir,beta-D-N4-hydroxycytidine (NHC) and internal standard(IS) from plasma.

Table 3
Matrix effect and extraction recovery in plasma and BAL.

Analyte	QC	Plasma (n = 6)		BAL (n = 6)	
		Extraction Recover % ± CV (%)	Matrix Effect % ± CV (%)	Extraction Recover % ± CV (%)	Matrix Effect % ± CV (%)
Nirmatrelvir	QCL	97.7 ± 4.2	101.5 ± 3.6	95.1 ± 5.6	97.1 ± 6.7
	QCM	102.2 ± 3.9	99.3 ± 4.5	93.1 ± 2.5	93.4 ± 5.1
	QCH	98.2 ± 7.5	97.1 ± 5.3	98.9 ± 7.3	98.8 ± 9.4
Ritonavir	QCL	93.7 ± 3.6	98.7 ± 4.9	97.9 ± 6.9	96.7 ± 7.5
	QCM	94.7 ± 8.7	105.5 ± 6.0	96.5 ± 7.4	99.6 ± 3.6
	QCH	90.7 ± 7.5	102.9 ± 7.0	102.7 ± 8.2	98.6 ± 6.0
NHC	QCL	92.3 ± 5.4	91.2 ± 3.3	91.7 ± 3.7	90.4 ± 8.4
	QCM	95.8 ± 6.9	92.9 ± 4.5	92.3 ± 4.2	93.0 ± 5.2
	QCH	98.2 ± 7.5	91.7 ± 8.5	90.5 ± 6.4	92.4 ± 7.5
IS		96.3 ± 8.1	90.4 ± 5.5	92.8 ± 5.5	90.1 ± 5.6

RSD; relative standard deviation, QCL; quality control law, QCM; quality control medium, QCH; quality control high.

recommended, the residence time of 0.9 % saline lavage solution in the lungs should not exceed 2 min to avoid falsely elevated concentrations of urea in the BALF, and the first recovered fluid was treated as bronchial wash and discarded. After centrifugation of the blood and BALF, plasma and BALF were analyzed immediately. Endogenous urea levels served as a dilution factor to calculate the actual concentration in the ELF.

Concentration in ELF = Concentration in BALF × Urea in serum/Urea in BALF [18]. Urea concentration in the serum and BALF were determined using a Hitachi 7600 clinical chemistry analyzer (Hitachi Ltd. Tokyo, Japan). The ratios ($R_{ELF:P}$) of nirmatrelvir or NHC were calculated using the plasma as reference (C_{ELF}/C_{plasma}) for each sample.

Table 4
The stability of each analyte under a variety of conditions.

Storage Condition			Nirmatrelvir (n = 6)				Ritonavir (n = 6)				NHC (n = 6)			
			QCL(%)		QCH(%)		QCL(%)		QCH(%)		QCL(%)		QCH(%)	
			RSD	RE	RSD	RE	RSD	RE	RSD	RE	RSD	RE	RSD	RE
Whole blood	RT	3h	3.2	0.4	3.7	4.9	7.1	−4.5	5.3	5.7	10.4	−6.3	2.7	3.2
		6h	2.0	2.5	5.3	−2.9	2.4	−1.3	7.4	10.9	11.0	−26	6.7	−20.5
		24h	3.2	0.7	5.6	4.3	1.2	4.8	3.3	7.9	7.1	−88	2.0	−58.4
	4 °C	3h	5.9	3.1	3.3	2.8	6.2	10.2	3.7	5.3	10.8	−4.7	4.2	4.4
		6h	3.2	0.5	5.9	4.1	7.6	0.5	3.5	3.1	1.8	−11.5	8.9	6.8
		24h	1.7	0.3	3.9	6.1	2.2	12.7	3.9	2.4	0.9	−16.6	3.1	−11.0
Plasma	RT	3h	3.1	5.3	1.4	9.8	1.8	4.4	1.4	0.4	13.6	10.6	7.7	−3.7
		6h	2.8	9.4	6.6	10.4	3.5	6.3	5.3	3.1	5.1	8.0	4.4	−6.8
		24h	4.0	9.7	4.7	−0.4	5.6	5.9	4.7	5.9	7.2	−28.4	13.5	−20.7
	4 °C	3h	3.2	7.4	4.0	9.7	2.3	5.4	3.3	1.6	4.1	3.9	3.0	2.1
		6h	4.1	1.5	3.7	−0.5	3.3	2.1	2.9	0.2	5.2	1.7	2.8	−3.7
		24h	6.9	7.5	5.1	6.9	5.1	6.6	3.9	2.2	7.9	−10.6	5.5	−11.1
BALF[a]	RT	3h	2.3	5.9	4.5	8.2	3.6	4.0	3.2	5.1	6.0	4.4	6.8	6.1
		6h	5.7	6.3	3.7	9.1	4.7	5.2	5.0	4.4	3.7	3.7	8.2	13.6
Autosampler stability		BAL	7.3	10.6	8.9	7.5	6.5	7.0	5.8	4.8	4.1	9.3	3.7	6.0
		Plasma	5.4	4.8	3.6	9.4	4.3	8.6	4.5	3.6	5.3	7.2	3.4	2.7
Three freeze-thaw cycles of plasma at −20 °C			6.8	−8	4.2	5.6	6.9	3.5	5.0	3.4	7.1	2.3	4.2	3.6
Plasma at −20 °C for 30 days			4.5	8.2	5.7	5.5	7.3	2.6	5.3	8.2	10.0	0.7	11.1	1.0
Plasma at −20 °C for 180 days			2.7	3.6	3.9	4.3	5.5	5.0	4.2	6.3	5.8	3.1	7.0	2.9
Plasma at −80 °C for 300 days			4.4	6.3	4.2	2.0	3.8	2.4	5.6	4.0	7.3	5.3	6.2	4.6

3. Results and discussion

3.1. Chromatographic and MS/MS conditions optimization

Simultaneous analysis using a single analytical approach for sample preparation and separation of NHC, nirmatrelvir, and ritonavir remains challenging due to the structural diversity of antiviral drugs as shown in Table 1. For example, their log P range of −2 to 3.9 covers compounds from highly hydrophilic, such as NHC, to highly lipophilic, such as ritonavir. We attempted to change chromatographic conditions using different HPLC columns, flow rates, mobile phase composition, gradient elution procedure, and column temperature. Eventually, we achieved satisfactory peak shapes for the three analytes under our experimental conditions. The column temperature was set to 30 °C instead of the relatively high column temperature (55 °C) published in the literature [12,13] for nirmatrelvir and ritonavir, thus may be improving the stability of NHC and making it more suitable for simultaneous determination of these compounds. Throughout our experiments, we observed that the AQ C_{18} column was more suitable for those drugs than HILIC and the reversed-phase C_{18} column in our trial conditions.

Here, the molecular ions were tried, i.e., the protonated $[M + H]^+$ and deprotonated $[M - H]^-$ molecules. The positive mode yielded a better MS response than the negative in our AB3200 Q_{trap}. Moreover, we observed that the signature ion fragment (*m/z*) 500.2 → 110.3 has higher fragment ion signal intensities than 500.2 → 319.3 for nirmatrelvir. For molnupiravir, the characteristic ion fragment (*m/z*) 330.5 → 128.1 showed good quantification efficiency. However, molnupiravir is unstable in biological matrices (blood, plasma, and BLAF) and rapidly converted to an active metabolite of NHC. Therapeutic drug monitoring cannot be measured immediately and hampers the implementation of molnupiravir in these analysis methods due to potential problems in the accuracy with high instability. Hence, we simultaneously quantified nirmatrelvir, ritonavir, and NHC in human plasma and bronchoalveolar lavage fluid samples by LC-MS/MS as an analytical method.

3.2. Method validation

Representative chromatograms of the blank, blank with internal standard, spiked samples containing 30 ng/mL of NHC, nirmatrelvir, ritonavir, and clinical samples are shown inFig. 1. The responses in blank plasma were always less than 5 % of the signal at the LLOQ. Interfering signals from blank plasma contributed less than 5 % of the IS signal. Selectivity meets the acceptance criteria.

There was a sufficient linear correlation between the analyte concentration and the analytical signal for all analytes in all matrices observed. The correlation coefficients (r^2) of all calibration curves were at least 0.9902 or better. The ion signal-to-noise values of LLOQ were at least >10.

Within-day as well as between-day accuracy and precision for both calibration ranges for all analytes in plasma and BALF were within the stipulated range of ±15 % of the nominal concentrations and <15 % for the respective coefficients of variation of the mean values (Table 2).

All investigated analytes in plasma or BALF can be reliably extracted by simple protein precipitation using acetonitrile resulting in good homogeneity among the entire validation range (Table 3). Recoveries and matrix effects presented were: 90.7–102.2 % and 91.2–101.5 %, respectively, (n = 6) for human plasma; recoveries and matrix effects were: 90.5–102.7 % and 90.4–99.6 %,

respectively (n = 6) for BALF.

Except for NHC, the different levels of other molecules in human plasma, whole blood, and bronchial lavage fluid were stable under different conditions. The stability of NHC needs to be attention. NHC was unstable for 6 h at room temperature, lowering the temperature or plasma separated from the whole blood can make the samples more stable. NHC demonstrated acceptable plasma stability at 4 °C for 24 h, but exceeding acceptability thresholds was observed at room temperature for 24 h. Hence, we suggest molnupiravir collection and sample processing should be placed on ice and separated as quickly as possible to reduce the loss of NHC. The results for freeze-thaw stability indicate that all analytes undergo up to three freeze-thaw cycles without impaired stability. The short-term and long-term stability data of −20 °C or −80 °C are presented in Table 4, which are stable and stored at −80 °C for up to 300 days.

For dilution integrity tests in plasma and BALF, nirmatrelvir, ritonavir, and NHC determination accuracy ranged from 97.3 % to 102.8 %, with relative standard deviation (RSD) values in the range of 5.2 %.

3.3. Clinical application

Ritonavir-boosted nirmatrelvir and molnupiravir are the two leading oral COVID-19 antiviral treatments, but their antiviral activities directly using the rates of achieving effective concentrations as the measure of antiviral effect have not been compared in patients. We tried to explore this characteristic by comparing their concentration in the plasma and assessing their permeability from the blood to the lung.

3.3.1. Compare antiviral activities using the rates of achieving effective concentrations

Our validated LC-MS/MS (liquid chromatography-mass tandem spectrometry) method was successfully applied to 59 patient samples (Fig. 2). Interestingly, we found a large part of plasma levels of nirmatrelvir (except for the first day) were above the predicted EC_{90} VS a large part of plasma levels of NHC were below the predicted EC_{90} with the continuous administration of recommended dosing regimens. We still found that many plasma levels were above EC_{90}(nirmatrelvir)on the first day of withdrawal VS plasma levels of NHC were below EC_{90} even with serial sessions.

3.3.2. The relationship between plasma and epithelial lining fluid levels

The lung is the predominant site of SARS-CoV-2 infection [19], so it is essential to investigate the relationship between plasma and epithelial lining fluid levels of nirmatrelvir or NHC in real-life treated patients. We observed that the permeability of NHC in lung tissue was higher at approximately 20 % than nirmatrelvir (Table 5). Possible reasons are that NHC is not protein bound in plasma (drug bank, https://go.drugbank.com/drugs/DB15661), making them more accessible to have higher permeability.

The NHC 90 % virus inhibitory concentrations against SARS-CoV-2 in primary human airway epithelial cultures [20] was approximately 0.5–1 μM ≈ 130–260 ng/mL. The therapeutic concentrations are potentially attained within the peak concentration at steady state but not in trough concentration for NHC fromFig. 2 and Table 5. Subtherapeutic antiviral concentration levels of NHC also exist in epithelial lining fluid. These findings may help to explain why the clinical effects of treatment with molnupiravir are lower than ritonavir-boosted nirmatrelvir in similar clinical trials.

4. Conclusion

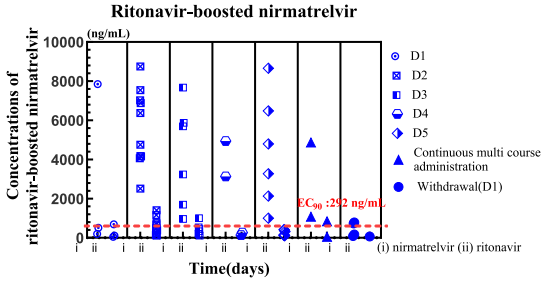
We present a validated LC-MS/MS method for simultaneously quantifying nirmatrelvir, ritonavir, and NHC in human plasm and bronchoalveolar lavage fluid. The wide range of the calibration curve (i.e., 30–10000 ng/mL) for both molecules enables a broad spectrum of applications, such as bioequivalence, clinical research, or daily routine practice. By monitoring and comparing concentrations of nirmatrelvir and NHC in plasma and epithelial lining fluid, we gain a deeper understanding of the pharmacokinetics of these compounds, which is considered a critical factor in determining their different therapeutic efficacy in treating COVID-19. Despite the very good permeability of NHC in lung tissue, the rates of achieving EC_{90} are low, which will lead to a lack effective than ritonavir-boosted nirmatrelvir.

CRediT authorship contribution statement

Wenjing Zhang: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Lin Xia:** Writing – review & editing, Validation, Investigation, Formal analysis. **Zhilong Yuan:** Visualization, Validation, Investigation, Formal analysis. **Mengdan Liu:** Validation, Investigation, Formal analysis. **Yang Jiao:** Writing – review & editing, Supervision, Resources, Conceptualization. **Zhuo Wang:** Writing – review & editing, Validation, Supervision, Funding acquisition, Formal analysis, Conceptualization.

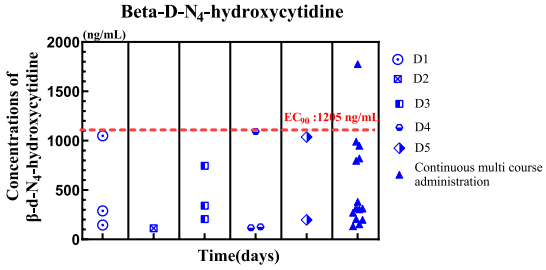
Funding

This work was supported by The Deep Blue Plan (GH145-38), The COVID-19 Intelligent Scientific Research Project(COVID-ZD-001) and Shanghai “Rising Stars of Medical Talents Youth Development Program-Youth Medical Talents” (Clinical Pharmacist Program (SHWSRS [2021]099)). All authors declare no conflicting financial interests.



a. Plasma concentrations versus time: (i) nirmatrelvir (ii) ritonavir for n = 33 blood samplings in 14 patients after multiple oral nirmatrelvir/ritonavir 300 mg/100 mg bid; with D1:between 0 and 24 h, D2: between 24 and 48 h, D3: between 48 and 72 h, D4:between 72 and 96 h, D5:between 96 and 120 h.

Fig. 2a. Plasma concentrations versus time: (i) nirmatrelvir (ii) ritonavir for n = 33 blood samplings in 14 patients after multiple oral nirmatrelvir/ritonavir 300 mg/100 mg bid; with D1:between 0 and 24 h, D2: between 24 and 48 h, D3: between 48 and 72 h, D4:between 72 and 96 h, D5: between 96 and 120 h.



b. Plasma concentrations versus time: beta-D-N4-hydroxycytidine for n = 26 blood samplings in 9 patients after multiple oral molnupiravir 800 mg bid; with D1:between 0 and 24 h, D2: between 24 and 48 h, D3: between 48 and 72 h, D4:between 72 and 96 h, D5:between 96 and 120 h.

Fig. 2b. Plasma concentrations versus time: beta-D-N4-hydroxycytidine for n = 26 blood samplings in 9 patients after multiple oral molnupiravir 800 mg bid; with D1:between 0 and 24 h, D2: between 24 and 48 h, D3: between 48 and 72 h, D4:between 72 and 96 h, D5:between 96 and 120 h.

Table 5
The permeability of nirmatrelvir and NHC in lung tissue.

Compunds	Patient No.	Urea $_{serum}$	Urea $_{BALF}$	C_{BALF} (ng/ml)	C_{ELF} (ng/ml)	C_{plasma} (ng/ml)	C_{ELF}/C_{plasma}
nirmatrelvir	1	32.5	4.6	326	2302	5960	38.62(%)
	2	20.7	3.4	750	4560	9010	50.6(%)
NHC	3	29.2	5.2	126	708	1070	66.1(%)
	4	33.4	5.1	265	1735	2690	64.5(%)

Declaration of competing interest

No potential conflict of interest was reported by the authors.

References

[1] P.R. Petit, F. Touret, J.S. Driouich, et al., Further preclinical characterization of molnupiravir against SARS-CoV-2: antiviral activity determinants and viral genome alteration patterns, Heliyon 10 (10) (2024) e30862, https://doi.org/10.1016/j.heliyon.2024.e30862.
[2] Y. Li, S.M. Lu, J.L. Wang, H.P. Yao, L.G. Liang, Progress in SARS-CoV-2, diagnostic and clinical treatment of COVID-19, Heliyon 10 (12) (2024) e33179, https://doi.org/10.1016/j.heliyon.2024.e33179.
[3] B.A. Jayk, D.S.M. Gomes, D.B. Musungaie, et al., Molnupiravir for oral treatment of covid-19 in nonhospitalized patients, N. Engl. J. Med. 386 (6) (2022) 509–520, https://doi.org/10.1056/NEJMoa2116044.

[4] J. Hammond, H. Leister-Tebbe, A. Gardner, et al., Oral nirmatrelvir for high-risk, nonhospitalized adults with covid-19, N. Engl. J. Med. 386 (15) (2022) 1397–1408, https://doi.org/10.1056/NEJMoa2118542.
[5] W. Schilling, P. Jittamala, J.A. Watson, et al., Antiviral efficacy of molnupiravir versus ritonavir-boosted nirmatrelvir in patients with early symptomatic COVID-19 (PLATCOV): an open-label, phase 2, randomised, controlled, adaptive trial, Lancet Infect. Dis. 24 (1) (2024) 36–45, https://doi.org/10.1016/S1473-3099(23)00493-0.
[6] A.S. Gouda, H.M. Marzouk, M.R. Rezk, et al., A validated LC-MS/MS method for determination of antiviral prodrug molnupiravir in human plasma and its application for a pharmacokinetic modeling study in healthy Egyptian volunteers, J. Chromatogr. B 12062022) 123363, https://doi.org/10.1016/j.jchromb.2022.123363.
[7] X. Zhu, L. Li, B. Dai, et al., A Simple and Rapid LC-MS/MS Method for the Quantification of Nirmatrelvir/Ritonavir in Plasma of Patients with COVID-19, Int. J. Anal. Chem. 20242024) 6139928, https://doi.org/10.1155/2024/6139928.
[8] Y.M. Yao, J.J. Sun, J. Chen, X.Q. Liu, H.Z. Lu, L.J. Zhang, [LC-MS/MS method for simultaneous quantification of lopinavir and ritonavir in human plasma], Yao Xue Xue Bao 45 (2) (2010) 279–282.
[9] H.M. Marzouk, A.S. Gouda, M.R. Rezk, A.M. Abdel-Megied, A validated LC - MS/MS method for determination of Molnupiravir and Favipiravir in human plasma ; Assessment of the remarkable potential synergistic effect against SARS-CoV-2 Infection : Application to a pharmacokinetic study in healthy Egyptian volunteers, Microchem J. 1972024), https://doi.org/10.1016/j.microc.2023.109815.
[10] C.J. Zhou, Y.N. Liu, A. Wang, H. Wu, R.A. Xu, Q. Zhang, Simultaneous measurement of COVID-19 treatment drugs (nirmatrelvir and ritonavir) in rat plasma by UPLC-MS/MS and its application to a pharmacokinetic study, Heliyon 10 (11) (2024) e32187, https://doi.org/10.1016/j.heliyon.2024.e32187.
[11] W.H. Lee, Y.Y. Kung, C.K. Sun, et al., The anti-COVID-19 drug nirmatrelvir crosses the blood–brain barrier and exhibits herb-drug pharmacokinetic interactions with Scutellaria baicalensis formulations, Heliyon 10 (15) (2024) e34820, https://doi.org/10.1016/j.heliyon.2024.e34820.
[12] C. Liu, M. Zhu, L. Cao, et al., Simultaneous determination of nirmatrelvir and ritonavir in human plasma using LC-MS/MS and its pharmacokinetic application in healthy Chinese volunteers, Biomed. Chromatogr. 36 (11) (2022) e5456, https://doi.org/10.1002/bmc.5456.
[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 12122022) 123510, https://doi.org/10.1016/j.jchromb.2022.123510.
[14] A. Amara, S.D. Penchala, L. Else, et al., The development and validation of a novel LC-MS/MS method for the simultaneous quantification of Molnupiravir and its metabolite β-d-N4-hydroxycytidine in human plasma and saliva, J. Pharm. Biomed. Anal. 2062021) 114356, https://doi.org/10.1016/j.jpba.2021.114356.
[15] T.L. Parsons, L.A. Kryszak, M.A. Marzinke, Development and validation of assays for the quantification of beta-D-N(4)-hydroxycytidine in human plasma and beta-D-N(4)-hydroxycytidine-triphosphate in peripheral blood mononuclear cell lysates, J. Chromatogr. B 11822021) 122921, https://doi.org/10.1016/j.jchromb.2021.122921.
[16] M. Saleh, M. Hirasawa, M. Sun, B. Gulave, J. Elassaiss-Schaap, E. de Lange, The PBPK LeiCNS-PK3.0 framework predicts Nirmatrelvir (but not Remdesivir or Molnupiravir) to achieve effective concentrations against SARS-CoV-2 in human brain cells, Eur. J. Pharm. Sci. 1812023) 106345, https://doi.org/10.1016/j.ejps.2022.106345.
[17] S.I. Rennard, G. Basset, D. Lecossier, et al., Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution, J. Appl. Physiol. 60 (2) (1986) 532–538, https://doi.org/10.1152/jappl.1986.60.2.532, 1985.
[18] K. Pocino, A. Minucci, R. Manieri, G. Conti, D. De Luca, E.D. Capoluongo, Description of an automated method for urea nitrogen determination in bronchoalveolar lavage fluid (BALF) of neonates and infants, J. Lab. Autom. 20 (6) (2015) 636–641, https://doi.org/10.1177/2211068214567147.
[19] P. Zhou, X.L. Yang, X.G. Wang, et al., A pneumonia outbreak associated with a new coronavirus of probable bat origin, Nature 579 (7798) (2020) 270–273, https://doi.org/10.1038/s41586-020-2012-7.
[20] T.P. Sheahan, A.C. Sims, S. Zhou, et al., An orally bioavailable broad-spectrum antiviral inhibits SARS-CoV-2 in human airway epithelial cell cultures and multiple coronaviruses in mice, Sci. Transl. Med. 12 (541) (2020), https://doi.org/10.1126/scitranslmed.abb5883.