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Original Article

INNOVATIVE UPLC METHOD FOR CONCURRENT QUANTIFICATION AND PHARMACOKINETIC ANALYSIS OF NIRMATRELVIR AND RITONAVIR IN RAT PLASMA

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ABSTRACT

Objective: The primary goal of this study was to develop a rapid, robust, and sensitive UPLC method for the simultaneous estimation of Nirmatrelvir and Ritonavir in rat plasma using Lopinavir as an internal standard. This method aimed to improve upon existing approaches by offering faster run times, superior sensitivity, and thorough linearity, matrix effect, accuracy and precision, recovery and stability in accordance with USFDA guidelines.

Methods: The UPLC analysis was carried out using a Waters Acquity UPLC system equipped with a PDA detector. An Acquity UPLC BEH Phenyl column (100 mm x 2.1 mm, 1.7 µm) was used for the separation process, with an isocratic mobile phase of buffer (Ammonium formate of pH-2.5 adjusted with 0.1% formic acid), flow rate 0.2 ml/min. Detection occurred at 236 nm, and the injection volume was 5 µl.

Results: Analysis was performed within 3 min, with a linear concentration range of 300-12000 ng/ml (r^2 = 0.99994±0.018) for Nirmatrelvir and 200-8000 ng/ml (r^2 = 0.99985±0.006) for Ritonavir. The extraction recovery results of Nirmatrelvir and Ritonavir were 97.17, 97.34, 97.34% and 96.80, 97.31, 96.95%, respectively and for matrix effect results were 97.18, 97.01 and 97.50, 97.58 at different QC concentration levels. Precision and recovery study results were determined within the acceptable limit.

Conclusion: This UPLC method provides a substantial improvement in terms of speed, sensitivity, and robustness, making it well-suited for high-throughput pharmacokinetic studies of Nirmatrelvir and Ritonavir.

Keywords: Nirmatrelvir, Ritonavir, UPLC, Validation, Bioanalytical method development, USFDA guidelines

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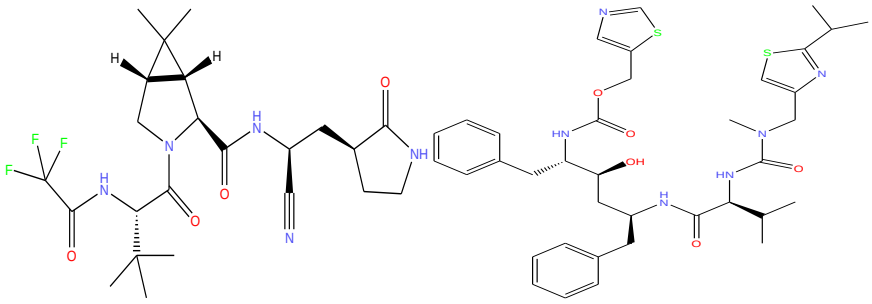
INTRODUCTION

Nirmatrelvir and Ritonavir are pivotal components in the treatment of COVID-19, particularly in the combination therapy known as Paxlovid. Nirmatrelvir is a protease inhibitor designed to target the main protease of SARS-CoV-2, essential for viral replication [1]. Ritonavir, initially developed as an HIV protease inhibitor, is used in this combination to inhibit the metabolism of Nirmatrelvir, increasing its plasma concentration and efficacy [2].

Given the critical role of these drugs during the ongoing pandemic, there is a substantial need for reliable analytical methods to quantify these drugs in biological matrices. Existing methods for estimating Nirmatrelvir and Ritonavir often involve complex procedures, longer run times, or inadequate sensitivity [3]. Traditional HPLC methods, while widely used, do not offer the speed and sensitivity required for high-throughput pharmacokinetic studies [4].

UPLC has gained prominence in bioanalytical [5] chemistry, excelling over HPLC with its enhanced speed, resolution, and sensitivity. The enhanced performance of UPLC is attributed to its ability to operate at higher pressures, allowing for the use of smaller particle-size columns, leading to better separation and faster analysis times [6]. Fig. 1 shows the chemical structures of Nirmatrelvir and Ritonavir.



(A) Nirmatrelvir **(B) Ritonavir**

Fig. 1: Structural representations of (A) Nirmatrelvir and (B) Ritonavir

According to USFDA guidelines, the current study describes a straightforward UPLC method for determining Nirmatrelvir and Ritonavir in parenteral dosage form.

Novelty and Contribution to the Analytical Field of this research introduces an innovative, rapid, and sensitive UPLC method for the concurrent estimation of Nirmatrelvir and Ritonavir in rat plasma.

Compared to previously reported methods, this UPLC method significantly reduces the analysis time to under 3 min while maintaining high sensitivity and accuracy. Lopinavir, serving as an internal standard, further enhances the reliability of the quantification process.

A literature survey identified five HPLC assay method studies [7-11], one UPLC [12], one LCMS [13] and one Bioanalytical method using HPLC [14] were available for the determination of Nirmatrelvir and Ritonavir. No bio-analytical articles were reported for determining the Nirmatrelivir and Ritonavir by using UPLC in any matrix. The developed UPLC (Ultra Performance Liquid Chromatography) method was utilized for the estimation of the combined drugs by *in vitro* method.

MATERIALS AND METHODS

Materials

Nirmatrelvir and Ritonavir were obtained from Glenmark Pharmaceuticals Ltd., Hyderabad. UPLC-grade acetonitrile, methanol, and formic acid were purchased from Merck Chemicals, Mumbai. A Milli-Q system was used to purify the water.

Equipment

The UPLC analysis was carried out using a Waters Acquity UPLC system equipped with a PDA detector. Data were processed using Empower-2 software [15, 16].

Pharmacokinetic study

Selection of animals

In order to conduct this research, 6 healthy white New Zealand rats (app. 250 g weight) were procured from Bioneeds India pvt. Ltd. in Bangalore. Six rats were used in this study Animal ethics committee (Reg. No. 1250/PO/RcBi/S/20/CPCSEA) at the institute approved the experiment protocol. The circumstances resemble those of a laboratory, and the animals have access to fresh endive, carrots, and maize. Feed for animals should be kept between 20 and 26° Celsius, with humidity between 50 and 60 %. All animals fasted for an entire day and drank water at will before being used in an experiment.

Preparation of standard solutions

Methanol was used to prepare stock solutions containing 24000 ng/ml Nirmatrelvir and 16000 ng/ml Ritonavir. These stock solutions were diluted with the mobile phase to make working solutions. The calibration standards varied from 300 to 12000 ng/ml for Nirmatrelvir and from 200 to 8000 ng/ml for Ritonavir.

Sample preparation

Rat plasma samples (200 µl) were spiked with 500 µl of internal standard (Lopinavir, 12000 ng/ml), 300 µl of acetonitrile, and vortexed for 15 min. The mixture underwent centrifugation at 5000 rpm for 15 min, followed by filtration of the supernatant through a 0.22 µm nylon filter before UPLC analysis.

Chromatographic conditions

An Acquity UPLC BEH phenyl column (100 mm x 2.1 mm, 1.7 µm) was used for the separation process, with an isocratic mobile phase of buffer (Ammonium formate of pH-2.5 adjusted with 0.1% formic acid) and acetonitrile (60:40) flowing at a rate of 0.2 ml/min. Detection occurred at 236 nm, and the injection volume was 5 µl**. Active pharmaceutical ingredients internal standards were well separated in optimized chromatographic conditions. All of the findings were within the limits. On using these optimized conditions, we get plate count and tailing values of Lopinavir, Nirmatrelvir, Ritonavir were 12485, 17362, 10150 and 1.09, 1.05, 1.12. The resolution values were 10.62, 6.37 and retention times were 1.135 min (Lopinavir), 1.973 min (Nirmatrelvir), 2.520 min (Ritonavir).

Method development

Several trials were conducted to establish a good resolution between Nirmatrelvir, Ritonavir and internal standard (Lopinavir). Phosphate and acetate buffers were used to develop the approach. But, these buffers in mobile phase has no capable of effectively separating the active ingredients and internal standard. Following that, the selected mobile phases (acetonirile and ammonium phosphate) improved the resolving power and provided better resolving power between selected drugs. To improve the separation between the peaks, acetonitrile was added to mobile phase B in varying concentrations. The RP-18 and phenyl columns were used in the development trials (table 1), but the phenyl column, 100x2.1 mm, 1.7 column coupled to the PDA detector was used to separate Nirmatrelvir, Ritonavir and internal standard. Active pharmaceutical ingredients internal standards were well separated in optimized chromatographic conditions. All of the findings were within the limits. On using these optimized conditions, we get plate count and tailing values of Lopinavir, Nirmatrelvir, Ritonavir shown in table 2. Tabulated in table 1 are the conditions of trials. Fig. 2 shows Optimized chromatogram.

Table 1: Trial conditions

Trial No.	Buffer	Mobile phase	Column	Flow rate	Observation
1	Ammonium formate of pH-2.5 with formic acid	Acetonitrile+Buffer (40+60)	Shield RP-18 100 mmx2.1 mm, 1.7µ	0.2 ml/min	Second and Third peaks were broad
2	Ammonium formate of pH-2.5 with formic acid	Acetonitrile+Buffer (50+50)	Shield RP-18 100 mmx2.1 mm, 1.7µ	0.2 ml/min	First peak plate count was not within the limit
3	Ammonium formate of pH-2.5 with formic acid	Acetonitrile+Buffer (50+50)	Phenyl column 100 mmx2.1 mm, 1.7µ	0.2 ml/min	Un known peak was formed
4	Ammonium formate of pH-2.5 with formic acid	Acetonitrile+Buffer (55+45)	Phenyl column 100 mmx2.1 mm, 1.7µ	0.2 ml/min	Less resolution between first and second peaks
5	Ammonium formate of pH-2.5 with formic acid	Acetonitrile+Buffer (60+40)	Phenyl column 100 mmx2.1 mm, 1.7µ	0.2 ml/min	This method is suitable for validation
6	Ammonium formate of pH-2.5 with formic acid	Acetonitrile+Buffer (60+40)	Phenyl column 100 mmx2.1 mm, 1.7µ	0.2 ml/min	In this method, USP tailing was less than 2, USP plate count was greater than 2000 and USP resolution was found to be greater than 2 min for all three peaks.

Table 2: Optimized chromatogram results

S. No.	Name	Mean area	Mean USP tailing	Mean USP plate count	Mean USP resolution
1	Lopinavir	46383	1.09	12485	
2	Nirmatrelvir	75133	1.05	17362	10.62
3	Ritonavir	28425	1.12	10150	6.37

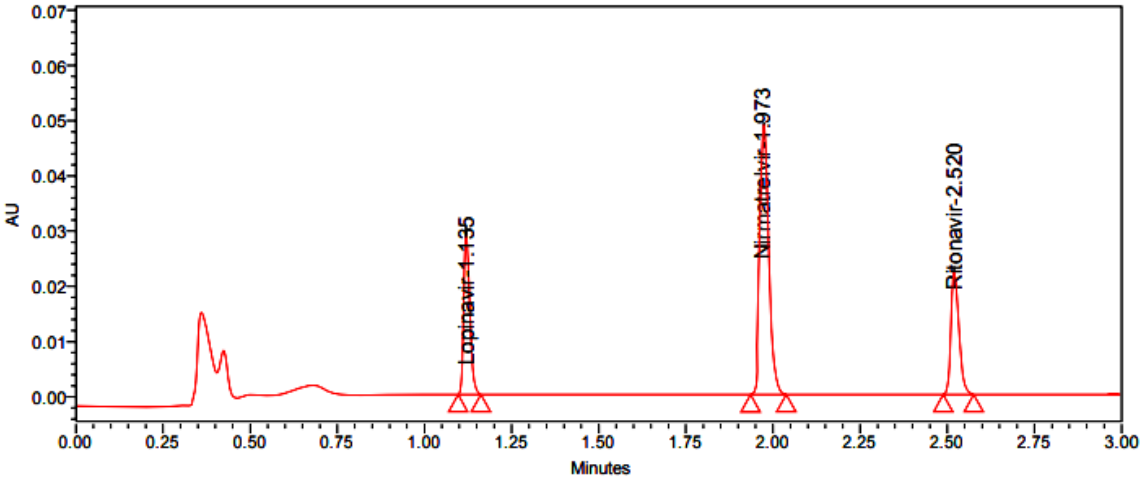


Fig. 2: Optimized chromatogram

Method validation

In line with USFDA guidelines [17, 18], the method was validated for selectivity [19, 20], matrix effect, recovery, precision, accuracy, and stability.

Selectivity

No significant interference from endogenous plasma components was observed at the retention times of the analytes and internal standard.

Matrix effect and recovery

Matrix effect was evaluated by comparing the peak areas of post-extraction spiked samples with neat standards. Recovery was calculated by comparing the peak areas of extracted samples with those of unextracted standards at equivalent concentrations.

Precision and accuracy

Intra-day and inter-day precision and accuracy were evaluated at four QC levels (LLQC, LQC, MQC, HQC). Precision (%CV) was within 15%, and accuracy was within 85-115%.

Stability

Stability was evaluated across multiple conditions, including bench-top, auto-sampler, freeze-thaw cycles, and prolonged storage.

RESULTS

Linearity

The peak area ratio of calibration standards [21, 22] was proportional to the concentration. The concentration range of Nirmatrelvir is 300-12000 ng/ml and Ritonavir is 200-8000 ng/ml. Linearity results of Nirmatrelvir and Ritonavir were shown in following table 3 and their calibration plots [23, 24] were shown in fig. 3. The calibration curves of Niramtrelvir and Ritonavir were appeared linear and Slope, intercept, coefficient of correlation were found to be 0.000269, 0.000692, 0.99994 and 0.000152, 0.001423, 0.99985.

Matrix effect

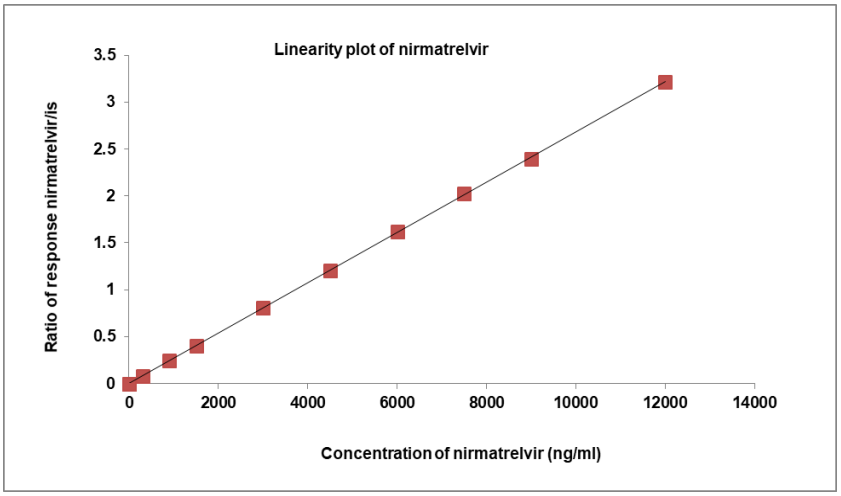
The matrix effects [25, 26] were investigated for six lots of different samples of plasma at the LQC and HQC levels were prepared from different lots of plasma (*i. e.*, a total of 36 QC samples). Percent RSD (Relative Standard Deviation) for within the signal, ion suppression/enhancement was observed as 1.0 percent for Nirmatrelvir and Ritonavir in UPLC, suggesting that under these circumstances, the matrix effect on analyte ionization is within an acceptable range of ionization. In matrix effect, LQC and HQC of Nirmatrelvir were 97.18, 97.01 and Ritonavir were 97.50, 97.58%. % CV of the both drugs at LQC level were 1.03, 2.28 and HQC level is 0.52, 0.56 respectively. It indicates that the matrix effect on the ionization of the analytes were within the suitable limit. Table 4 gives the recovery results.

Recovery

The recoveries [27, 28] for Nirmatrelvir and Ritonavir at LQC, MQC and HQC levels the results demonstrated that the bioanalytical method had good extraction efficiency. This also showed that the recovery wasn't hooked into concentration. Table 4 gives the recovery results.

Table 3: Linearity findings for nirmatrelvir and ritonavir

Linearity	Nirmatrelvir		Ritonavir	
	Conc. (ng/ml)	Area response ratio	Conc. (ng/ml)	Area response ratio
1	0	0.0	0	0.0
2	300.00	0.081	200.00	0.032
3	900.00	0.246	600.00	0.093
4	1500.00	0.402	1000.00	0.153
5	3000.00	0.806	2000.00	0.303
6	4500.00	1.209	3000.00	0.462
7	6000.00	1.620	4000.00	0.611
8	7500.00	2.022	5000.00	0.759
9	9000.00	2.396	6000.00	0.926
10	12000.00	3.212	8000.00	1.212
Slope	0.000269		0.000152	
Intercept	0.000692		0.001423	
CC	0.99994		0.99985	



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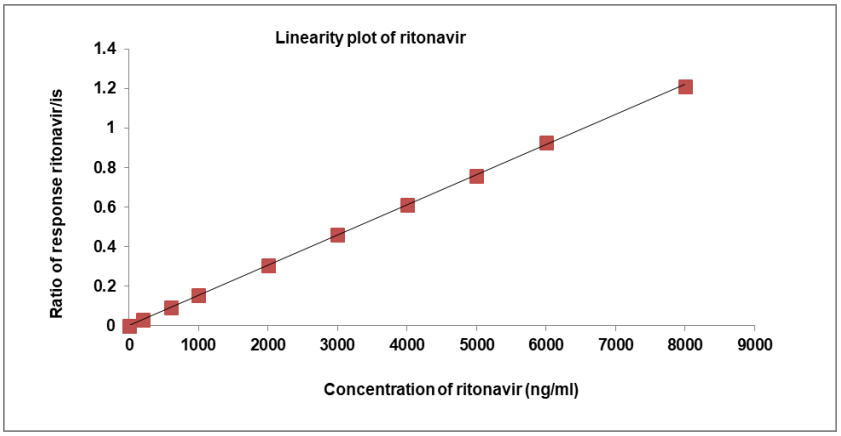

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Fig. 3: Calibration plots of (A) Nirmatrelvir and (B) Ritonavir

Table 4: Recovery results for nirmatrelvir and ritonavir

Analyte	Concentration (ng/ml)	Recovery (%)			
		Extracted		Un extracted	
		Mean	STD Dev	Mean	STD Dev
Nirmatrelvir	HQC (9000 ng/ml)	97.34%	0.00524	98.81%	0.02535
	MQC (6000 ng/ml)	97.34%	0.04528	97.62%	0.01985
	LQC (900 ng/ml)	97.17%	0.00632	97.75%	0.00847
Ritonavir	HQC (6000 ng/ml)	96.95%	0.00784	97.13%	0.06394
	MQC (4000 ng/ml)	97.31%	0.00569	97.80%	0.00089
	LQC (600 ng/ml)	96.80%	0.00125	97.73%	0.00421

(n=6)

Table 5: Precision and accuracy results

Parameter	HQC	MQC	LQC	LLQC
Nirmatrelvir				
N	6	6	6	6
Mean	111282	74260	10993	3623
SD	961.02	121.16	226.474	43.592
% CV	0.86	0.16	2.06	1.20
% mean Accuracy	98.60%	98.70%	97.40%	96.31%
Ritonavir				
N	6	6	6	6
Mean	41824	27694	4172	1374
SD	154.37	261.176	51.783	49.701
% CV	0.37	0.94	1.24	3.62
% mean Accuracy	97.91%	97.24%	97.66%	96.49%

Precision and accuracy

By pooling all individual assay results of different internal control samples, the accuracy and precision [29, 30] were calculated. It was obvious, based on the data provided, that the strategy was precise and effective. Nirmatrelvir accuracy results in quality control samples 96.31-98.70 and Ritonavir accuracy results in quality control samples 96.49-97.91. Nirmatrelvir and Ritonavir CV is<5% of total internal control samples. Table 5 shows the precision and accuracy results.

Ruggedness

The percent recoveries and percent CV of Nirmatrelvir and Ritonavir determined with two different analysts and on two different columns were within acceptable criteria in HQC, LQC and MQC samples. The percent recoveries ranged from 96.88 – 97.60% for Nirmatrelvir and 96.52%-97.44% for Ritonavir. The %CV values ranged from 0.42-1.28 for Nirmatrelvir and 0.30 – 2.19 for Ritonavir. The results proved method is rugged [31, 32].

Stability

The benchtop stability of Nirmatrelvir and Ritonavir was investigated by a stock solution prepared and stored at room temperature for 18 h. In the case of autosampler stability, the stock solution was stored for 24h in autosampler at room temperature and gave reliable stability behavior under these conditions. In order to assess freeze freeze-thaw stability, the stock arrangement was stored for 24 h at (-28±5) °C. For wet extract stability, the stock solution was stored for 18h at (2-8) °C and for dry extract stability, the stock was stored for 18h at (-20±3) °C. The short-term stability shows that the stability of drugs was stored for 7 days at (5±3) °C, and in long-term stability, the stock was stored for 28 days at (-20±3) °C and inject into the UPLC. Compare the stability results of freshly arranged stock solution with a stock solution made before 24h. % change of Nirmatrelvir and Ritonavir was 1.35% and 0.92%, respectively, which indicates that solutions are stable up to 24h. At room temperature, Nirmatrelvir and Ritonavir were stable in plasma for different conditions. It was evaluated that LQC, MQC, and HQC levels continued freezing, and defrosting of plasma specimens spiked with Nirmatrelvir and Ritonavir didn't influence its stability. It was clear from long term stability that Nirmatrelvir and Ritonavir were stable at a capability temperature of-30 °C up to 24h. The overall stability results [33, 34] of Nirmatrelvir and Ritonavir are shown in table 6.

Both drugs were stable under all tested conditions with %CV values within the acceptable range.

Table 6: Stability results

Stability	Storage condition	Conc. level	Nirmatrelvir		Ritonavir	
			Recovery (%)	Mean area±SD	Recovery (%)	Mean area±SD
Bench top stability	18 h at room temperature	LQC	97.42	10995±162.710	97.29	4156±32.221
		MQC	97.05	73022±167.355	97.07	27645±194.394
		HQC	97.87	110451±1128.98	97.82	41789±185.729
Auto sampler stability	24 h in auto sampler	LQC	96.89	10935±130.203	96.84	4137±43.709
		MQC	97.01	72989±559.579	97.82	278.59±500.50
		HQC	97.87	110451±1578.910	97.06	41464±284.91
Long term stability	28 days at (-20±3) °C	LQC	84.68	9557±24.493	84.72	3619±43.660
		MQC	85.23	64127±222.49	85.28	24288±188.504
		HQC	86.11	97179±182.45	86.59	36992±246.596
Freeze thaw stability	24h at (-28±5) °C	LQC	96.69	10912±42.838	96.68	4130±48.201
		MQC	97.85	73622±228.64	97.20	27682±148.307
		HQC	97.33	109851±1390.661	97.82	41789±169.78
Wet extract stability	18h at 2-8 °C	LQC	96.44	10884±54.851	95.51	4080±53.452
		MQC	97.46	73327±231.68	97.20	27683±205.90
		HQC	97.21	109715±205.79	96.93	41409±166.94
Dry extract stability	18h at (-20±3) °C	LQC	96.36	10875±34.679	95.63	4085±52.006
		MQC	97.65	73470±164.976	96.85	27583±286.19
		HQC	97.54	110050±629.63	97.05	41459±365.13
Short term stability	7 days at (5±3) °C	LQC	92.69	10461±57.032	92.75	3962±82.638
		MQC	94.97	71452±243.32	93.98	26764±166.02
		HQC	93.73	105784±2934.89	94.95	40562±274.01

(n=6)

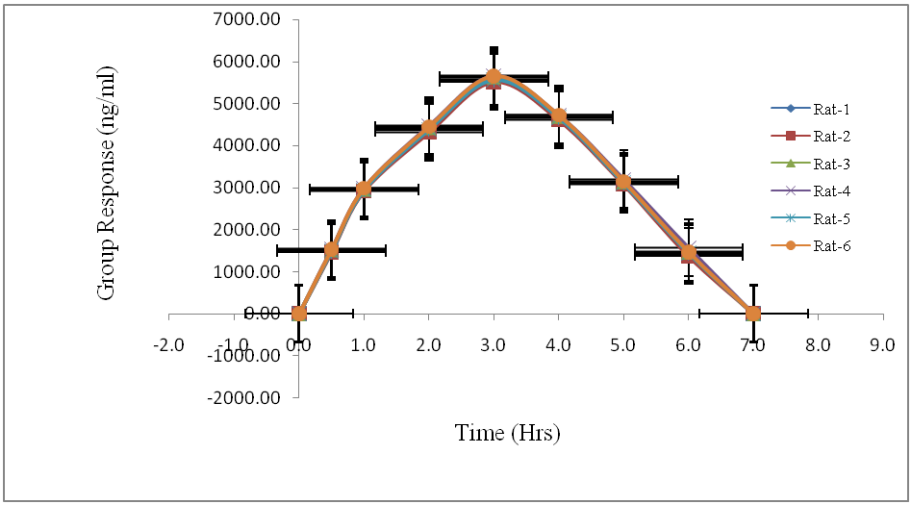
Pharmacokinetics

For investigating pharmacokinetic parameters of Nirmatrelvir and Ritonavir, market formulation dosage of 2.5 mg/1 kg Nirmatrelvir and 1.67 mg/1 kg Ritonavir was given into rat body as oral administration and to obtain mean plasma concentration-time profiles (fig. 4). Nirmatrelvir and Ritonavir demonstrate significant differences in pharmacokinetic studies after oral administration. We collected the samples from the rat body in different time periods, like 0.5, 1, 2, 3, 4, 5, 6, and 7 h from the administered time of the drugs into the rat body. After that test sample was prepared and injected into the chromatographic system to record the values. The calculated accurate bioavailability of dosage of oral administration, C_{max} after oral administration of Nirmatrelvir and Ritonavir (5597.35 and 1938.71), T_{max} (3 h and 3 h), the pharmacokinetic parameters C_{max}, T_{max}, $T_{1/2}$, AUC_{0-t}, $AUC_{0-\infty}$, were calculated and the data is shown in table 7. The $t_{½}$ values were 6 h and 6 h, respectively for Nirmatrelvir and Ritonavir. The AUC_{0-t} for Nirmatrelvir and Ritonavir were found to be 413.7 and 13959 ng-h/ml, respectively.

Table 7: Pharmacokinetic parameters of nirmatrelvir and ritonavir

Pharmacokinetic parameters	Nirmatrelvir	Ritonavir
AUC_{0-t}	413.77 ng-h/ml	13959 ng-h/ml
C_{max}	5597.354 ng/ml	1938.714 ng/ml
$AUC_{0-\infty}$	413.77 ng-h/ml	13959 ng-h/ml
t_{max}	3 H	3 H
$T_{1/2}$	6 H	6 H

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity, $AUC_{0-□}$: Area under the curve up to the last sampling time, C_{max}: The maximum plasma concentration, T_{max}: The time to reach peak concentration, $T_{1/2}$: Time the drug concentration



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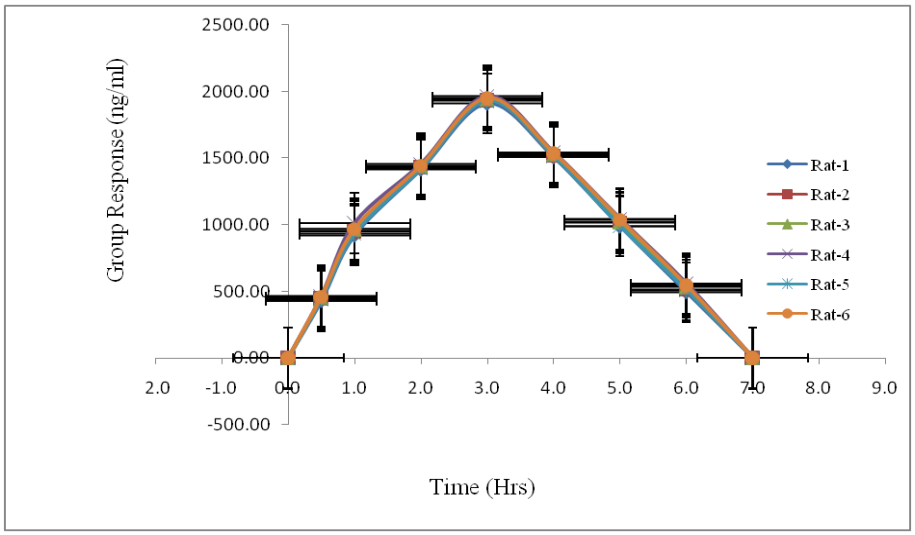

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Fig. 4: Graph depicting the time course of mean plasma concentrations of (A) Nirmatrelvir and (B) Ritonavir

DISCUSSION

The UPLC method developed for the simultaneous quantification of Nirmatrelvir and Ritonavir in rat plasma was rigorously validated and proved to be both efficient and reliable. The system suitability tests [35] demonstrated that the retention times and area ratios for both Nirmatrelvir and Ritonavir were consistent, with %CV values well within acceptable limits. Specifically, for Nirmatrelvir, the %CV for retention time was 0.97%, and for area ratio, it was 0.34%. Similarly, for Ritonavir, the %CV for retention time was 0.56%, and for area ratio, it was 0.46%.

The auto-sampler carryover effect was negligible, with carryover responses in subsequent injections being less than 15%, ensuring no significant contamination between samples. Specificity tests revealed no interfering peaks at the retention times of the analytes and the internal standard, confirming the method's ability to accurately quantify Nirmatrelvir and Ritonavir in the presence of other plasma components.

Sensitivity tests demonstrated that the method could reliably detect and quantify Nirmatrelvir and Ritonavir at their respective lower limits of quantification (LLOQ), with %CV values of 0.52% and 1.42%, respectively, and mean accuracies close to 96%. This high sensitivity is critical for pharmacokinetic studies, where low drug concentrations need to be accurately measured.

Matrix effect evaluations indicated minimal influence of plasma constituents on the analytes' ionization, with matrix factor biases within acceptable ranges and %CV values below 5% for both analytes. This ensures that the method is reliable across different plasma samples [36, 37].

Linearity tests showed excellent correlation coefficients (r^2) of 0.99994 for Nirmatrelvir and 0.99985 for Ritonavir over the concentration ranges tested, indicating that the method provides accurate and proportional responses across a wide range of concentrations. The precision and accuracy tests confirmed that the method consistently produces reliable results, with intra-day and inter-day %CV values within 15% for all quality control levels.

Nirmatrelvir and Ritonavir showed high and consistent recoveries from plasma, with mean recoveries of 97.34% and 97.31%, respectively, ensuring that the extraction procedure effectively isolates the analytes without significant loss.

Stability studies showed that both Nirmatrelvir and Ritonavir were stable across diverse conditions, including bench-top, auto-sampler, freeze-thaw, wet extract, dry extract, and long-term storage. %CV values for stability tests were within acceptable limits, confirming that the analytes remain stable throughout the sample handling and analysis process.

Pharmacokinetic studies in rats revealed that both drugs are rapidly absorbed, with peak plasma concentrations occurring around 3 h post-administration. The calculated pharmacokinetic parameters, including AUC_{0-t}, C_{max}, and $t_{1/2}$, were consistent with expected values, demonstrating the method's applicability for detailed pharmacokinetic profiling.

CONCLUSION

For the primary time new UPLC method was developed and validated for the determination of Nirmatrelvir and Ritonavir in rat plasma. Here, the described method is rugged, fast, reproducible bio analytical method. This method was validated according to USFDA guidelines. Simple and efficient method was developed and may be utilized in pharmacokinetic studies and to see the investigated analyte in body fluids.

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LIST OF ABBREVIATIONS

UPLC: Ultra Performance Liquid Chromatography, USFDA: U. S. Food and Drug Administration, HPLC: High-Performance Liquid Chromatography, LCMS: Liquid Chromatography-Mass Spectrometry, LLQC: Lower Limit of Quality Control, LQC: Lower Quality Control, MQC: Middle-Quality Control, HQC: High-Quality Control, CV: Coefficient Variance, RSD: Relative Standard Deviation

AUTHORS CONTRIBUTIONS

Gope Edward Raju collected the literature and information about the drug and carried out the research samples and prepared the manuscript. Suneetha Yaparthi supported solution preparation in analysis. Srikanth Pottendla check the data and reviewed the article.

CONFLICTS OF INTERESTS

The authors report no financial or any other conflicts of interest in this work.

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