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Original Article

Development and Validation of a New RP-HPLC Method for the Simultaneous Estimation of Nirmatrelvir, Ritonavir and Molnupiravir in Formulated Nanosponges, Plasma Samples and its Pharmacokinetic Study

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ABSTRACT

Background: Nirmatrelvir and Ritonavir are used in combination (Paxlovid®) in order to treat COVID-19. Thus, the goal of the current study was to create an easy-to-use, accurate, and sensitive method for the preparation of nanosponges utilising High-Performance Liquid Chromatography (HPLC) to simultaneously estimate Nirmatrelvir and Ritonavir in plasma samples and prepared nanosponges. **Materials and Methods:** Nanosponges containing Nirmatrelvir and Ritonavir were prepared using ethyl-cellulose. Formulated Nanosponges are useful to increase the safety and efficacy of Nirmatrelvir and Ritonavir combinational therapy and also support Therapeutic Drug Monitoring (TDM). Patients are presently advised not to use Paxlovid® because of the challenging TDM but who are most likely to become really ill from COVID-19 infections should benefit from these nanosponges. **Results:** The proposed method's validation was conducted in accordance with ICH recommendations Q2R1, demonstrating its good linearity and accuracy, and precision. We saw from the selectivity studies that the retention durations of the medications under investigation did not exhibit any additional peaks. 89.63% w/w and 89.98% w/w, respectively, were computed as the entrapment efficiency of nanosponges for Nirmatrelvir and Ritonavir, using the established method to determine their entrapment efficiency in formed nanosponges. With correlation coefficient (R2) values of 0.998 and 0.996, respectively, the observed concentration in plasma linearity for Ritonavir was 2842 ng/mL to 24550 ng/mL and for Nirmatrelvir was 2791 ng/mL to 25414 ng/mL. **Conclusion:** The new approach showed that both medications were stable over the course of the trial and the three freeze-thaw cycles.

Keywords: RP-HPLC method, Nirmatrelvir, Ritonavir, Nanosponges, Paxlovid®, Simultaneous estimation, Plasma, Pharmacokinetic study, ICH Validation.

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INTRODUCTION

The World Health Organization (WHO) has classified the coronavirus disease 2019 (COVID-19) epidemic and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as the sixth international public health emergency. These two events have caused an unprecedented pandemic.[1] Out of the seven billion confirmed cases, 6.8 lakh COVID-19 deaths had been documented as of March 7, 2023.[2]

COVID-19 virus which has the persistent mutagenic nature encourages the continuous emergence of novel variations despite intensive vaccine development and huge immunization campaigns. For the sake of this reason, the stoppage of epedimic from spreading can be done by the development of new medications, therapeutic approaches and vaccines.[3,4] Nirmatrelvir and Ritonavir also called as an oral antiviral medication combination targets the crucial protease enzyme of SARS-CoV-2.[5,6] Based on initial safety and effectiveness results from the experiment called EPIC-HR (Evaluation of Inhibition for COVID-19 in High-Risk Patients), conducted prior to the release of the Omicron version, the therapy of COVID-19 has been approved for Nimatrelvir and Ritonavir. The oral antiviral drug targets 3-chymotrypsin-like cysteine protease (Mpro), the primary proteolytic enzyme of SARS-CoV-2, Nirmatrelvir.[6-9] Antivirals are interested in targeting Mpro because it is essential to the cycle of viral replication, transforming viral polyproteins into functional units, and since there are no known human counterparts for it. Due to this there are less chances of unintended consequences.[10,11] Against a range of corona virus

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Nirmatrelvir showed significant inhibition of Mpro activity and virus multiplication when tested *in vitro*.[7]

CY3A4 predominantly metabolises nimatrelvir. To enhance the pharmacokinetic characteristics of the medication Ritonavir, a CYP3A4 inhibitor, is given in addition to Nirmatrelvir 300 mg at a low dose.[12]

Patients with mild to intermediate coronavirus disease 2019 (COVID-19) who are not hospitalised but are at high risk of developing severe COVID-19 (e.g., hospitalisation, death) are treated with Ritonavir and Nirmatrelvir (Paxlovid®).

Notable drug-drug interactions occur when Ritonavir is present in Nirmatrelvir/Ritonavir. Ritonavir is a potent CYP3A inhibitor, which means it can increase concurrent medication plasma concentrations. This increases the risk of major side effects, some of which may be fatal, such as plasma drug instability, diminished sustained release, and toxicity.

Several CYP3A-metabolized medicines cause contraindications in patients on Nirmatrelvir/Ritonavir because of these drug-drug indications.[13]

The above mentioned limitations can be overcomed by employing the Technologies utilizing nanoscale (10-1000 nm) drug carriers by improving the rate of drug dissolution, absorption, bioavailability, and half-life of the drug in biological systems with site-specificity and sustained drug release.

Nanosponges are microscopic, spherical particles with massively porous surfaces and narrowly spaced voids. Both lipophilic and hydrophilic medications can be delivered via these particles' porous polymeric carriers.[14-16]

Ethyl Cellulose (EC), one of the many polymer types that were used to make the nanosponges, was discovered to be acceptable with low toxicity, non-toxic, biodegradable, and biocompatible.

Reticuloendothelial System (RES) and glomerular filtration are expected to remove nanocarriers produced by EC with particle sizes ranging from >200 nm to 5 µm.[14,17]

The goal of the current work was to create an easy-to-use, sensitive, and accurate High-Performance Liquid Chromatographic (HPLC) method for estimating nirmatrelvir and ritonavir simultaneously in plasma samples and formed nanosponges.

MATERIALS AND METHODS

Reagents and Chemicals

Nirmatrelvir, Ritonavir granules and Molnupiravir were obtained from the Hetero Drugs, Hyderabad. HPLC grade acetonitrile and methanol were purchased from Merck and Formic acid from Fisher Chemicals.

Instrumentation

For the chromatographic separations, a Shimadzu HPLC System (Japan) outfitted with a pump (LC-20AD), autosampler (SIL-20AC HT), column oven (CTO-10AS VP), and diode array detector (SPD-M20A) was utilised. Lab solutions software was utilised to record and integrate the chromatograms.

Chromatographic conditions

Table 1 lists the chromatographic requirements for method validation.

Preparation of Test Stock Solutions

Nirmatrelvir and Ritonavir were precisely weighed in 70% acetonitrile to provide stock solutions with concentrations of 1 mg/mL. Molnupiravir was dissolved in 70% acetonitrile to create the Internal Standard (IS) stock solution, which had a concentration of 100 µg/mL. The produced solutions were kept between 2 and 8°C in a refrigerator.[18]

Preparation of 0.1% Formic Acid (Mobile Phase -A)

St. To obtain the final 0.1% formic acid, 1 mL of analytical grade formic acid was added to 1000 mL of MQ water.[19]

Method Validation

The International Conference on Harmonisation (ICH) guidelines were followed in the validation of the RP-HPLC method.

Many validation factors were examined, such as linearity, range, accuracy, precision, specificity/selectivity, sensitivity, and system adaptability throughout the project.[20]

Preparation of nanosponges

A For the complete encapsulation of the medications, a 20 mL dichloromethane was filled with the necessary amount of medications (1:1 ratio of Nirmatrelvir and Ritonavir) and polymer ethyl cellulose (1:4). To fully dissolve the medications and polymer in the organic solvent, this dispersion was sonicated for 10 min.

To fully dissolve the medications and polymer in the organic solvent, this dispersion was sonicated for 10 minClick or tap here to enter text..[16] In order to achieve total solubility, 100 mg of polyvinyl alcohol were added to 150 mL of water and mixed for five minutes using a vortex mixer to create the aqueous phase. The creation of drug-loaded nanosponges was then achieved by adding the aforementioned organic phase to the aqueous phase drop by drop and mixing at 1000 RPM for 2-3 hr.The aforementioned nasosponges were filtered and dried for 24 hr at 40°C in a hot air oven. For additional characterisation, the dried nasosponges were gathered in vials and kept at 30°C.[16]

Calculating the efficiency of entrapment

Since it affects the release properties and, in turn, the therapeutic potency, figuring out how much medication is contained in the nanosponges is crucial. A precisely weighed quantity of nanosponges (100 mg) was dissolved in 10 mL methanol, sonicated for 15 min to break the nanosponge complex and allow for full drug solvation, then centrifuged to determine the entrapment efficiency. Following filtering, the resulting supernatant was examined using an HPLC.[21]

Pharmacokinetic Study

Healthy BALB/c mice aged 6-8 weeks and weighing 30 g were chosen for the investigation. Throughout the investigation, the mice were given an ad libitum diet of regular pellets and water. API and nanosponges containing 25 mg/kg of each medication were administered intraperitoneally at a dosage volume of 10 mL/kg. In tubes coated with EDTA, blood samples were taken at 0.25, 0.5, 1, and 4 hr intervals. The plasma was separated by centrifugation for ten minutes. Following protein precipitation with cooled acetonitrile, the resulting plasma samples were processed and HPLC analysis was performed.[22]

Plasma Linearity

The HPLC system was injected with plasma samples that included the medicines and the internal standard (2500-25000 ng/mL), and the results were noted. Plotting concentration against the matching peak area ratio allowed for the creation of the calibration curve, and least square regression analysis was used to assess linearity. For every medication, the slope value and correlation coefficient (R2) values were determined independently.[5]

Table 1: Chromatographic requirements for the validation of the HPLC method.

Instrumentation	RP- HPLC / PDA
Column	Xbridge C18 (250*4.6 mm, 5 µm)
Mobile Phase (B)	Acetonitrile
Mobile Phase (A)	0.1% Formic Acid in water
Flow rate	1 mL/min
Injection volume	20 µL
Method	Isocratic A:B (40:60)
Run Time	16 min

RESULTS AND DISCUSSION

Identification of Lambda max of Nirmatrelvir, Ritonavir, and Molnupiravir by UV-visible spectrophotometer

To determine each drug's lamda max, a UV-visible spectrophotometer was used. Figures 1, 2, and 3 display the UV-visible spectra of each medication, respectively.

Method validation

Table 2 displays the findings of the validation research carried out in compliance with ICH criteria.

System suitability

The Rt of six duplicates of the Nirmatrelvir and Ritonavir solution and the RSD of the peak areas were determined to be 1.49 (<2%) and 5.40 min and 10.19 min, respectively. For the Nirmatrelvir and Ritonavir peaks, the average NTPs were 4260.50 and 6697.00 (>2000), respectively, while the average Tailing factor (Tf) was 1.44% and 1.42% (<2%). Since every response parameter fell comfortably into the acceptable range, it was possible to use the method to test Ritonavir and Nirmatrelvir simultaneously for the planned use. Chromatograms of Nirmatrelvir, Ritonavir, and blank samples are displayed in Figures 4 and 5, respectively, while a chromatogram of Molnupiravir is displayed in Figure 6.

Linearity

As seen in Figures 7 and 8, the devised approach was linear between a range of concentrations (2.5-25 µg/mL), and the R2 values for Nirmatrelvir and Ritonavir were determined to be 0.999 and 0.9977, respectively. Similarly, Figures 9 and 10 display typical plots of Ritonavir and Nirmatrelvir in plasma samples.

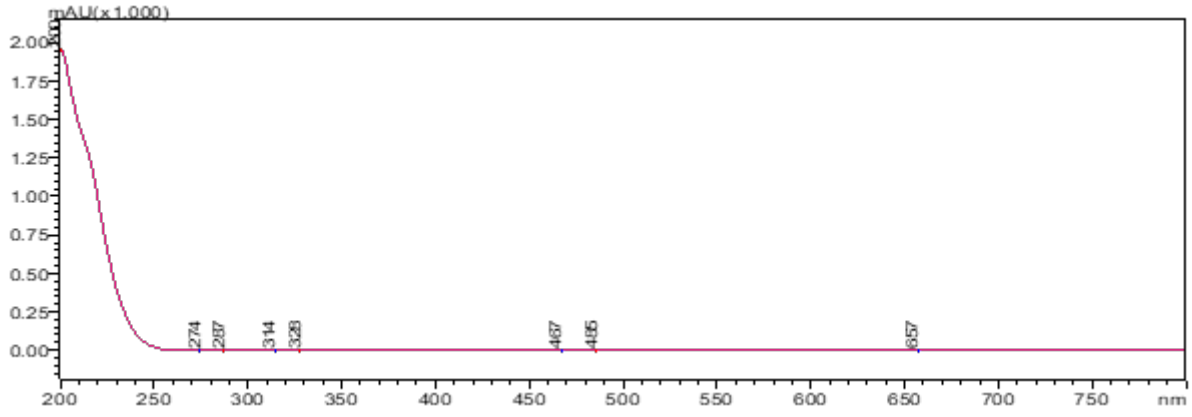

Figure 1: UV-visible spectra of Nirmatrelvir.

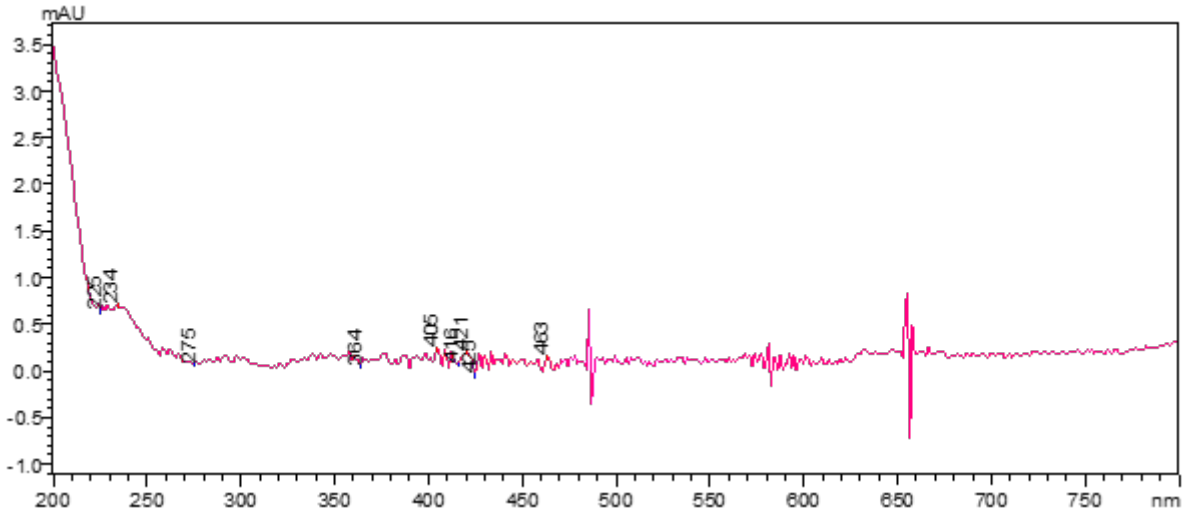

Figure 2: UV-visible spectra of Ritonavir.

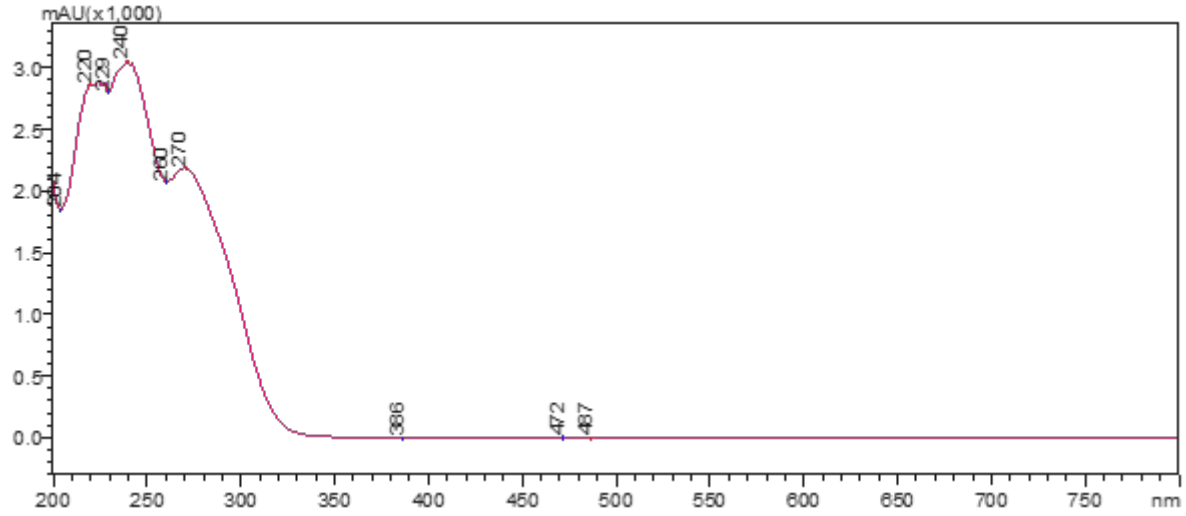

Figure 3: UV-visible spectra of Molnupiravir.

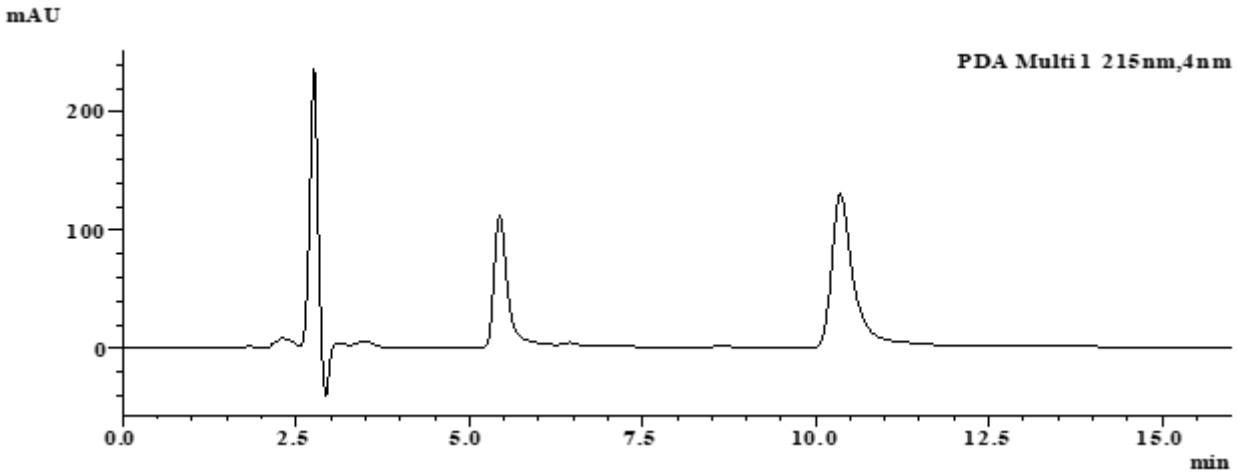

Figure 4: Chromatogram of Nirmatrelvir and Ritonavir at 215 nm.

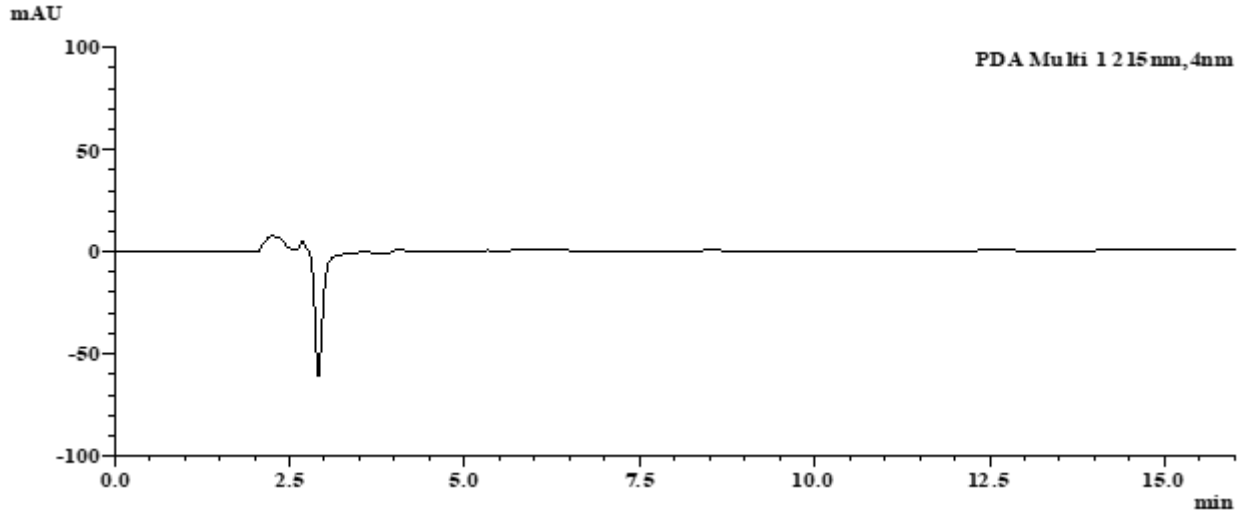

Figure 5: Chromatogram of blank sample at 215 nm.

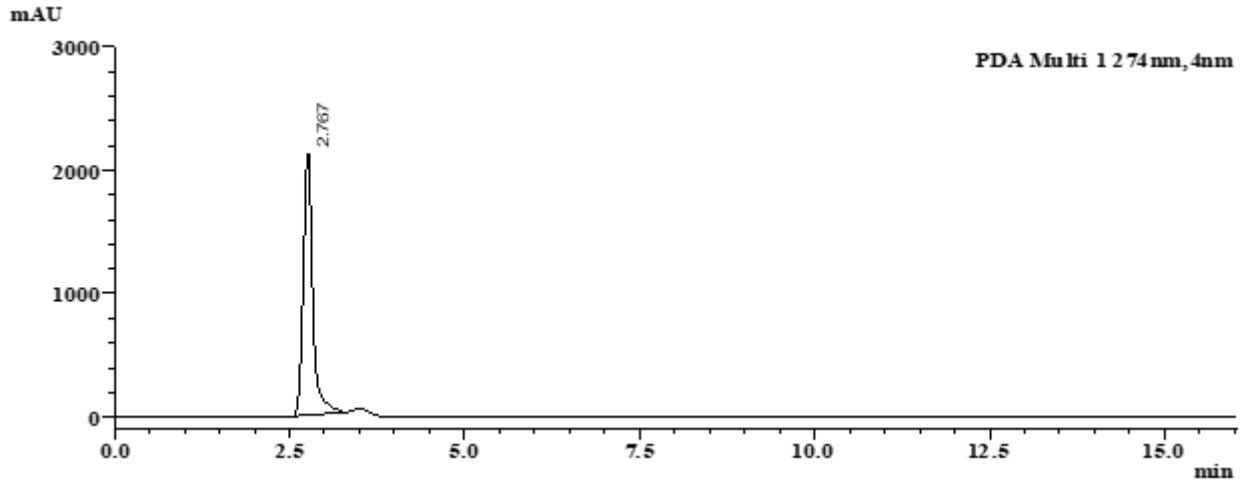

Figure 6: Chromatogram of Molnupiravir at 274 nm.

Table 2: **Results of validation parameters.**

Parameters	Retention times (Rt) in minutes		Peak Area		Number of theoretical plates (NTP)		Tailing factor (Tf)		Resolution between (Rs)
Replicates	Nirmatrelvir	Ritonavir	Nirmatrelvir	Ritonavir	Nirmatrelvir	Ritonavir	Nirmatrelvir	Ritonavir	Nirmatrelvir and Ritonavir
1	5.41	10.20	506174	100829	4224	6628	1.45	1.41	11.56
2	5.40	10.21	503170	100533	4228	6566	1.45	1.42	11.50
3	5.40	10.16	499659	100190	4287	6589	1.45	1.43	11.56
4	5.40	10.20	506908	100908	4250	6921	1.48	1.40	11.70
5	5.39	10.17	491168	103904	4307	6708	1.41	1.39	11.63
6	5.41	10.18	495120	102984	4267	6770	1.42	1.46	11.63
Mean	5.40	10.19	500366.50	101558.00	4260.50	6697.00	1.44	1.42	11.60
SD	0.01	0.02	6277.21	1510.84	32.88	133.58	0.03	0.02	0.07
CV	0.00	0.00	0.01	0.01	0.01	0.02	0.02	0.02	0.01
%RSD	0.13	0.18	1.25	1.49	0.77	1.99	1.83	1.57	0.62

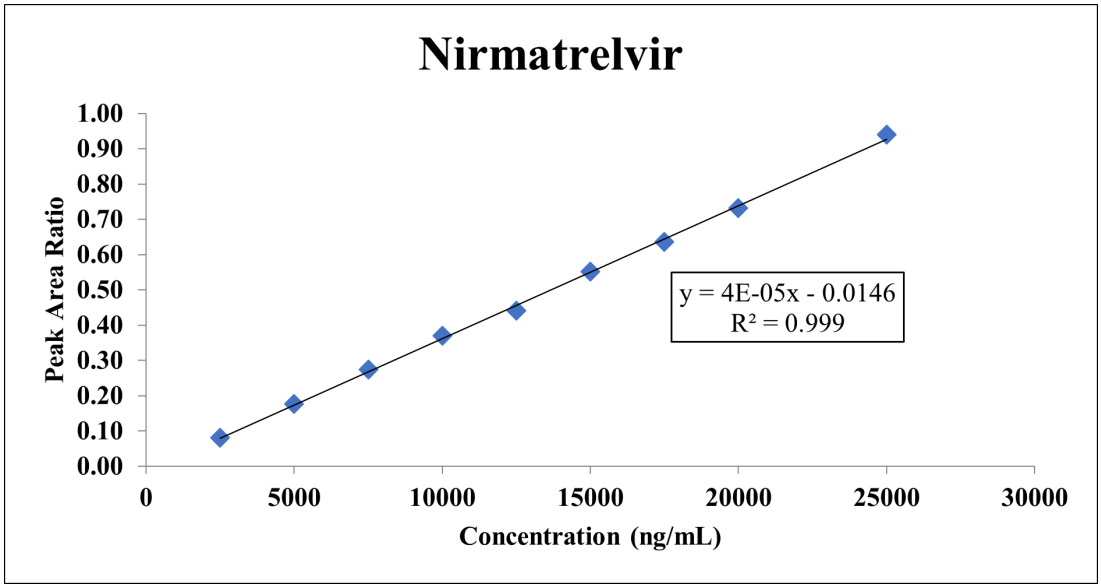

Figure 7: Standard plot of Nirmatrelvir.

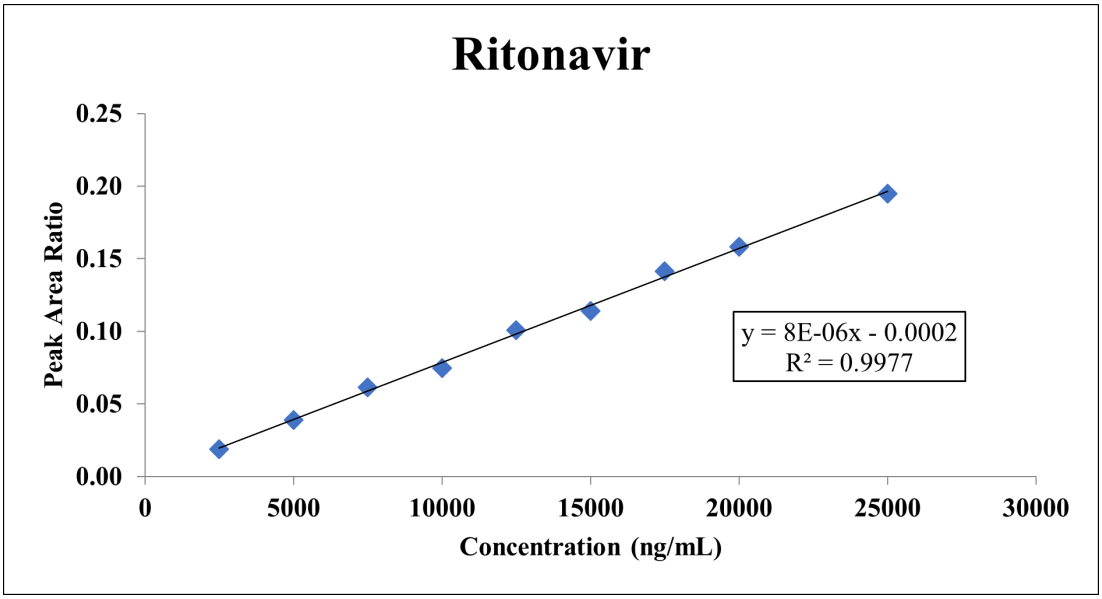

Figure 8: Standard plot of Ritonavir.

Precision and Accuracy

For both intra-day and inter-day precision, the percent RSD was less than 2%, and the mean recovery for both medications ranged from 100 to 101% (Tables 3 and 4). With LOD values for Nirmatrelvir and Ritonavir of 209.24 and 591.50 ng/mL and LOQ values of 634.05 and 1792.42 ng/mL, respectively, the method was exact and accurate, and it complies with ICH criteria. These results imply that the methodology for the TDM of both medications in human plasma is accurate and precise enough.

Robustness

Table 5 gives the reults for robustness testing. The robustness testing results presented in Table 5 provide valuable insights into the stability and reliability of the HPLC method for the analysis of Nirmatrelvir, Ritonavir, and their combination. In the method of validation, robustness testing is a critical component, which is aimed at assessing the method's ability to remain unaffected by small variations in experimental conditions. The response characteristics recorded at wavelengths of 213 nm and 217 nm for Nirmatrelvir at a concentration of 25000 ng/mL show modest differences in mean area ratio.

Nonetheless, the method's high degree of accuracy and stability is suggested by the consistently low values of the Standard Deviation (SD), Coefficient of Variation (CV), and percentage Relative Standard Deviation (%RSD).

Ritonavir has similar stability in the mean area ratio at the same concentration, with minimal changes in SD, CV, and %RSD. This constancy highlights the method's dependability in precisely

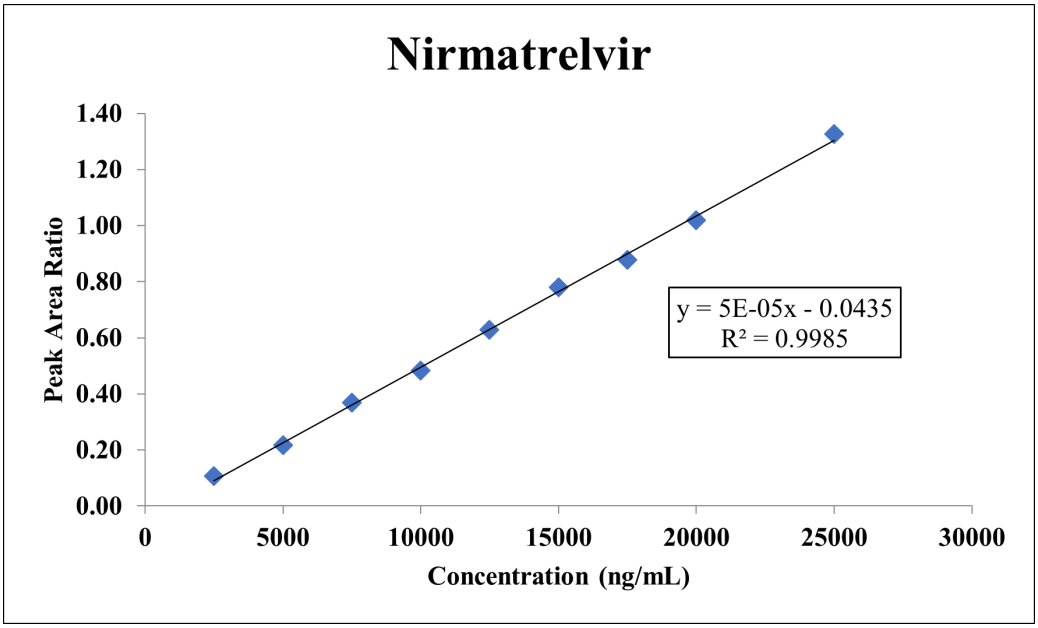

Figure 9: Standard plot of Nirmatrelvir in plasma.

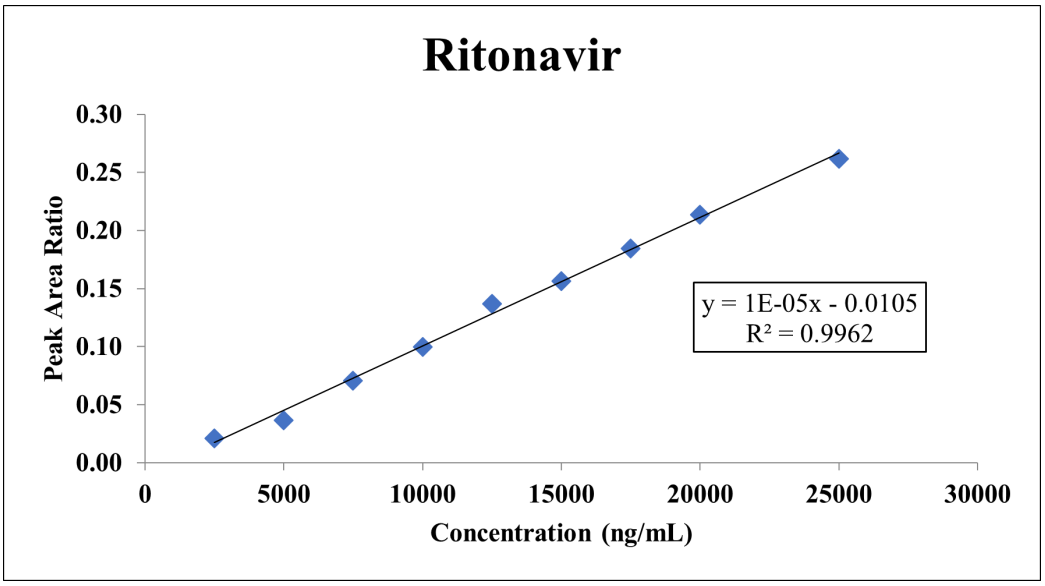

Figure 10: Standard plot of Ritonavir in plasma.

measuring Ritonavir.In addition to this, the approach shows resilience to changes in the mobile phase's composition when evaluating the combination of Nirmatrelvir and Ritonavir at various ratios.

The mean concentrations of both medicines show low fluctuations besides percentage RSD, low SD, and low CV values, showing that the method performs consistently independent of the mobile phase ratio.

Considerably, the minute differences in mean concentrations and percent RSD values between various mobile phase ratios show how crucial it is to optimise experimental settings in order to get the best possible analytical performance. The findings imply that although slight modifications to the composition of the mobile phase may affect the chromatographic behaviour, the approach is still reliable and capable of precise quantification in a variety of settings.

In general, the results of the robustness tests validate the accuracy and consistency of the HPLC technique utilised for the examination of Nirmatrelvir, Ritonavir, and their amalgamation. These results provide assurance that the approach is appropriate for routine analysis in quality control and pharmaceutical research applications, guaranteeing reliable and precise measurement of drug concentrations.

Table 3: Displaying the results of precision testing.

Interday	Nominal concentration (ng/mL)	17500	20000	25000	Grand %RSD
Day-1 (Nirmatrelvir)	Mean	17303.91	20132.19	25854.88	1.06
	SD	242.72	318.50	51.28	
	CV	0.01	0.02	0.00	
	%RSD	1.40	1.58	0.20	
Day-1 (Ritonavir)	Mean	17749.30	20235.14	25097.91	1.01
	SD	329.68	159.57	94.83	
	CV	0.02	0.01	0.00	
	%RSD	1.86	0.79	0.38	
Day-2 (Nirmatrelvir)	Mean	17445.60	20558.38	25783.37	0.75
	SD	43.11	18.45	493.88	
	CV	0.00	0.00	0.02	
	%RSD	0.25	0.09	1.92	
Day-2 (Ritonavir)	Mean	17071.67	19915.80	24568.08	1.66
	SD	209.63	360.24	476.21	
	CV	0.01	0.02	0.02	
	%RSD	1.23	1.81	1.94	
Day-3 (Nirmatrelvir)	Mean	17709.78	20755.83	25856.42	1.73
	SD	304.84	372.79	429.34	
	CV	0.02	0.02	0.02	
	%RSD	1.72	1.80	1.66	
Day-3 (Ritonavir)	Mean	17143.64	19823.50	24256.13	1.96
	SD	354.99	391.10	518.18	
	CV	0.02	0.02	0.02	
	%RSD	2.07	1.97	2.14	

Table 4: Results of accuracy testing.

Sl. No.	Concentration (ng/mL)	Mean	% Recovery
1	17500	17749.30	101.4
2	20000	20235.14	101.2
3	25000	25097.91	100.4
Grand mean			101.0
SD			0.5

Application of the method

The created technique works well for analysing medications and ready-made formulations (Nanosponges) in plasma and figuring out how they behave pharmacologically. Table 6 shows the plasma concentrations of both medications at various time intervals. Table 7 and Figures 11 and 12 show the pharmacokinetic parameters.

For every drug, the following pharmacokinetic parameters were calculated: $AUC_{0\text{-last}}$ (the area under the concentration-time curve from time zero to the last measurable concentration), $AUC_{0\text{-inf}}$ (the area under the concentration-time curve from time zero to infinity), MRT (Mean Residence Time), and C_{max} (Maximum Plasma Concentration). These values are listed in Table 7.

The C_{max} and T_{max} values for Nirmatrelvir ranged from 5355.96 ng/mL to 7887.13 ng/mL and 0.00 to 1.00 hr, respectively. Between 12802.11 and 14158.45 ng/mL and 25223.82 and 33500.57 ng/mL, respectively, were the ranges of the $AUC_{0\text{-last}}$ and $AUC_{0\text{-inf}}$ values. The range of the Mean Residence Time (MRT) was 5.3 to 7.9 hr.

Ritonavir showed comparable ranges in C_{max} values (902.03 ng/mL to 1177.12 ng/mL) and T_{max} values (0.00 to 1.00 hr). Between

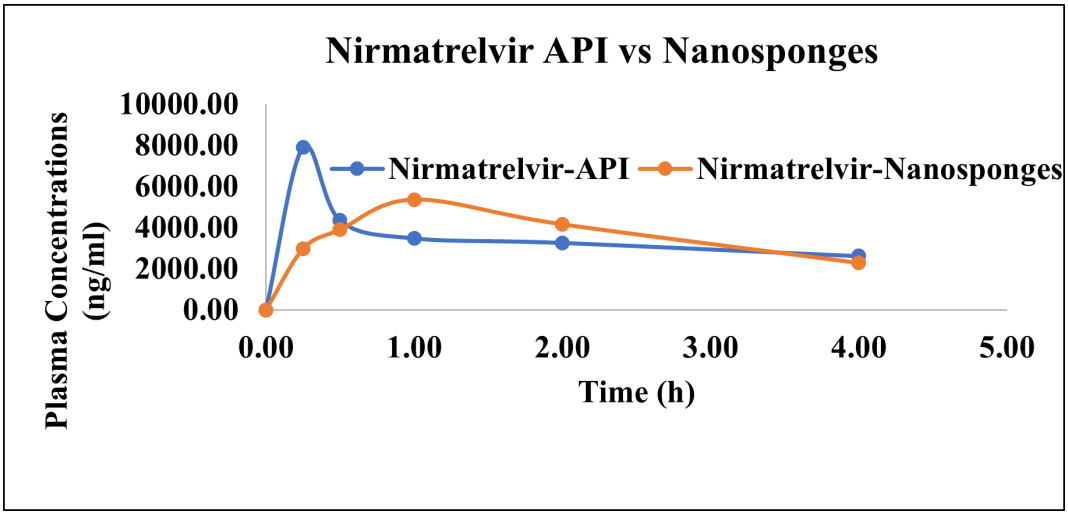

Figure 11: Nirmatrelvir API vs. Nanosponges pharmacokinetic graph in plasma.

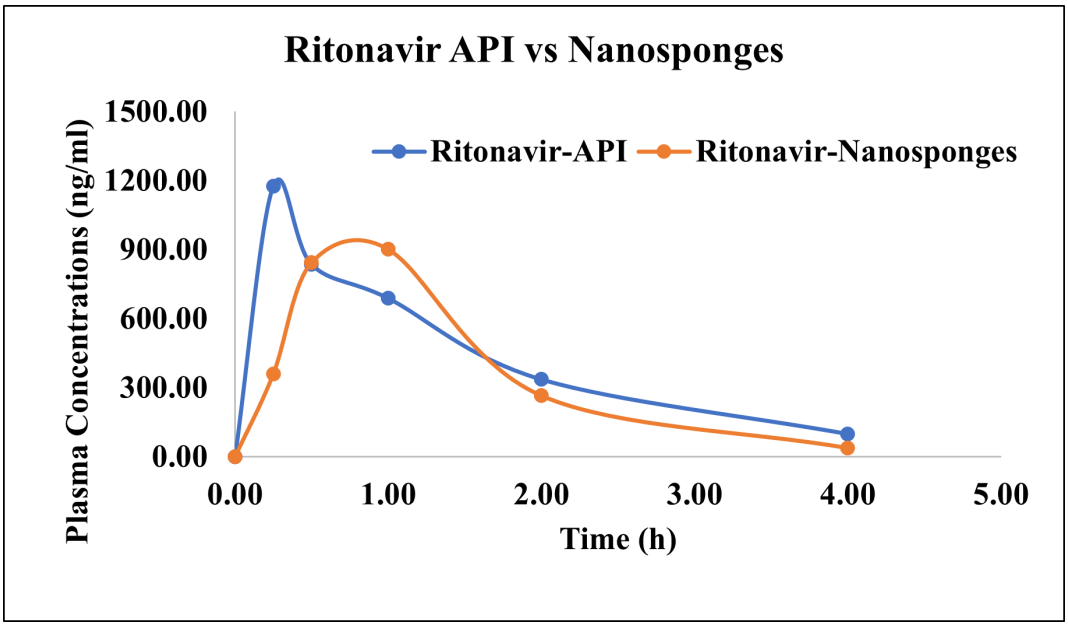

Figure 12: Ritonavir API versus nanosponges on a pharmacokinetic curve in plasma.

1509.60 and 1664.53 ng/mL and 1392.95 to 1664.53 ng/mL, respectively, were the ranges of the $AUC_{0\text{-last}}$ and $AUC_{0\text{-inf}}$ values. Between 1.4 and 1.7 hr was the range of the Mean Resident Time (MRT).

The pharmacokinetic profiles of Ritonavir and Nirmatrelvir differ significantly, according to the data, indicating variations in their processes of absorption, distribution, metabolism, and elimination. When compared to Ritonavir, Nirmatrelvir exhibits larger C_{max} and AUC values, suggesting that the two medications may have distinct rates of absorption and metabolism.

The measured T_{max} values represent that both medications are rapidly absorbed after being administered intraperitoneally, with Nirmatrelvir showing a somewhat later T_{max} than Ritonavir. The determined $AUC_{0\text{-inf}}$ values indicate prolonged exposure to both medications, with Nirmatrelvir often showing higher exposure levels than Ritonavir. The average amount of time a drug molecule spends in the systemic circulation, which reflects the kinetics of both systemic exposure and removal, is shown by the Mean Residence Time (MRT). Both Nirmatrelvir and Ritonavir appear to have relatively short residence times based on the MRT readings, suggesting quick removal or elimination processes.

Pharmacokinetic research results are displayed in Table 7.

Table 5: Robustness test results.

1. Validation parameters of Nirmatrelvir at 25000 ng/mL.		
Parameters	Responses	
Wavelengths ±2	213 nm	217 nm
Mean area ratio	1.17	0.88
SD	0.02	0.00
CV	0.02	0.00
%RSD	1.52	0.15
2. Validation parameters of Ritonavir at 25000 ng/mL.		
Parameters	Responses	
Mean area ratio	0.24	0.18
SD	0.00	0.00
CV	0.02	0.02
%RSD	1.62	1.80
3. Validation parameters of Nirmatrelvir+Ritonavir at 25000 ng/mL, 62:38 v/v mobile phase ratio.		
Parameters	Responses	
Drugs	Nirmatrelvir	Ritonavir
Mean concentration	25873.82	25648.92
SD	260.55	501.83
CV	0.01	0.02
%RSD	1.01	1.96
4. Validation parameters of Nirmatrelvir+Ritonavir at 25000 ng/mL, 58:42 v/v mobile phase ratio.		
Parameters	Responses	
Drugs	Nirmatrelvir	Ritonavir
Mean concentration	25450.82	22566.92
SD	220.55	451.83
CV	0.01	0.02
%RSD	1.70	1.57

Table 6: Nirmatrelvir and Ritonavir plasma concentrations at various intervals of time.

Plasma Concentration (ng/mL)				
Time (hr)	**Nirmatrelvir-API**	**Ritonavir-API**	**Nirmatrelvir-Nanosponges**	**Ritonavir-Nanosponges**
0.00	0.00	0.00	0.00	0.00
0.25	7887.13	1177.12	2958.26	360.37
0.50	4352.51	835.63	3897.95	844.59
1.00	3479.60	689.04	5355.96	902.03
2.00	3259.00	336.29	4159.11	266.01
4.00	2617.87	99.38	2282.96	38.08

Table 7: Pharmacokinetic results.

Study Title	Determination of Intraperitonial Pharmacokinetics of Nirmatrelvir and Ritonavir at 25 mg/kg dose administered in Balb/c Mice.			
Test System	BALB/c Mice			
Dose (mg/kg)	25 mg/kg			
Route of Administration	Intraperitonial - IP			
Bilogical Sample	Plasma			
Pharmacokinetic parameters				
Dose (mg/kg)	25.00	25.00	25.00	25.00
C_{max} (ng/mL)	7887.13	1177.12	5355.96	902.03
T_{max} (hr)	0.00	0.00	1.00	1.00
$AUC_{0\text{-last}}$ (ng*h/mL)	12802.11	1509.60	14158.45	1342.65
$AUC_{0\text{-inf}}$ (ng*h/mL)	25223.82	1664.53	33500.57	1392.95
MRT(hr)	5.3	1.7	7.9	1.4

CONCLUSION

In conclusion, our research effectively illustrates the suitability of the suggested approach for concurrent measurement of Nirmatrelvir and Ritonavir in nanosponges by the utilisation of the devised HPLC methodology. Excellent linearity, good accuracy, and precision were attained, and sufficient selectivity was maintained to get rid of any possible interferences. In particular, over the duration of the research and throughout freeze-thaw cycles, both medications showed stability. Therapeutic drug monitoring can be supported by using formed nanosponges, which offer a promising means of improving the safety and effectiveness of Nirmatrelvir and Ritonavir combinational therapy. This strategy has special promise for patients who are at high risk of developing a serious illness after contracting COVID-19 but are currently recommended not to use Paxlovid® due to difficulties with therapeutic medication monitoring.

But before this technique is widely used, further research must be done, especially in populations with conditions like hemolyzed plasma and lipemic populations. Furthermore, the utilisation of this technique in real-world clinical research samples can greatly aid in investigating the pharmacokinetics and possible interactions between drugs and meals.

The pharmacokinetic analysis carried out in BALB/c mice provides an in-depth knowledge of the intraperitoneal pharmacokinetics of Ritonavir and Nirmatrelvir, clarifying critical factors necessary to comprehend their behaviour in vivo. Future research aiming at enhancing dose schedules and therapeutic approaches will build on these findings to further the pharmacological characterisation of these medications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HPLC: High Pressure Liquid Chromatography; **RP-HPLC**: Reverse-Phase High Pressure Liquid Chromatography; **COVID-19:** Coronavirus Disease 2019; **TDM:** Therapeutic Drug Monitoring; **ICH**: International Conference on Harmonisation; **WHO:** World Health Organization; **SARS-CoV-2:** Severe Acute Respiratory Syndrome Coronavirus 2; **EC:** Ethyl Cellulose; **RES:** Reticuloendothelial System; **IS:** Internal Standard; **EDTA:** Ethylenediaminetetraacetic acid; **Tf:** Tailing factor; **NTP:** Number of theoretical plates; **RT:** Retention time; **LOD:** Limit Of Detection; **LOQ:** Limit Of Quantification; **RSD:** Relative Standard Deviation; **CV:** Coefficient of Variation; **SD:** Standard Deviation; **C_{max}:** Maximum Plasma Concentration; **AUC:** Area Under Curve; **MRT:** Mean Residence Time

SUMMARY

To summarize, our study shows that the proposed method is suitable for measuring Nirmatrelvir and Ritonavir in nanosponges simultaneously using the developed HPLC technique. Optimal selectivity was maintained to eliminate any potential interferences, and high levels of linearity, accuracy, and precision were achieved. Both drugs exhibited remarkable stability over the course of the study, even during freeze-thaw cycles. A potential way to enhance the efficacy and safety of Nirmatrelvir and Ritonavir combination treatment is via the use of formulated nanosponges, which may be used to facilitate therapeutic drug monitoring. This approach shows particular potential for individuals who are at high risk of severe disease after getting COVID-19 but are now advised

against using Paxlovid® because of problems with therapeutic drug monitoring. Pharmacokinetic analysis in BALB/c mice provides crucial insights into the intraperitoneal behavior of these drugs, guiding future research on dosing schedules and therapeutic strategies to enhance their pharmacological profile.

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