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Research Article

HPLC-BASED METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF NIRMATRELVIR AND RITONAVIR IN COMBINATION THERAPY

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ABSTRACT:
A simple, Accurate, precise method was developed for the simultaneous estimation of the Nirmatrelvir and Ritonavir in pharmaceutical dosage form. Chromatogram was run through Kromasil C18 250 x 4.6 mm, 5µ. Mobile phase containing Buffer 0.1% OPA : Acetonitrile taken in the ratio 60:40 was pumped through column at a flow rate of 1.0ml/min. Buffer used in this method was 0.1% OPA. Temperature was maintained at 30°C. Optimized wavelength selected was 240 nm. Nirmatrelvir and Ritonavir were eluted at 2.303 min and 2.783 min respectively. %RSD of the Nirmatrelvir and Ritonavir were and found to be 0.8 and 0.5 respectively. %Recovery was obtained as 99.63% and 99.70% for Nirmatrelvir and Ritonavir respectively. LOD, LOQ values obtained from regression equations of Nirmatrelvir and Ritonavir were 0.04, 0.13 and 0.01, 0.02 respectively. Regression equation of Nirmatrelvir is y = 92901x + 3504.3, and y = 116867x + 4632.7 of Ritonavir.

Key Words: Nirmatrelvir and Ritonavir, Rp Hplc, Validation.

INTRODUCTION

Nirmatrelvir is an oral protease inhibitor with emergency use authorization for the treatment of mild-to-moderate COVID-19.[1] Ritonavir is an inhibitor of the HIV protease that disrupts the HIV reproductive cycle. It has been demonstrated to have beneficial effects when used in combination regimens with low-dose ritonavir and other protease inhibitors, despite the fact that it was first created as an independent antiviral drug. It comes in liquid and capsule form and is currently more frequently used as a supplement to other protease inhibitors.[2] Both drugs were inhibitors of SARS-CoV-2 3CLPRO, but nirmatrelvir has the advantage of being orally bioavailable.[3] Paxlovid, a co-packaged medication that contains both nirmatrelvir and ritonavir, was given an emergency use authorisation by the FDA to treat specific patients with mild-to-moderate COVID-19.[4] Nirmatrelevir is Chemically known as (1R,2S,5S)-N-[(1S)-1-cyano-2-[(3S)-2-oxopyrrolidin-3-yl]ethyl]-3-[(2S)-3,3-dimethyl-2-[(2,2,2-trifluoroacetyl)amino]butanoyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexane-2-carboxamide [5] and Ritonavir known as 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[[(2S)-3-methyl-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino]butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate. [6] Clinical trials have demonstrated the efficacy of nirmatrelvir-ritonavir in reducing the risk of COVID-19-related hospitalization or death. A pivotal study reported an 89% reduction in such risks among high-risk, non-hospitalized adults treated within five days of symptom onset.[7] However, recent investigations into the use of nirmatrelvir-ritonavir for treating post-acute sequelae of SARS-CoV-2 infection (PASC), commonly known as Long COVID, have not demonstrated significant improvements in symptoms compared to placebo.[8]

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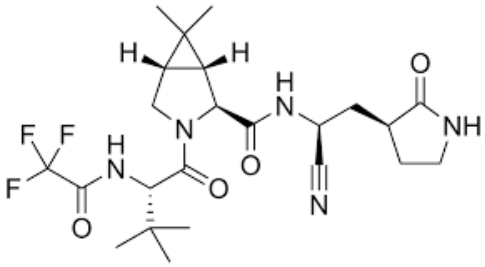

Figure 1: Structure of Nirmatrelvir

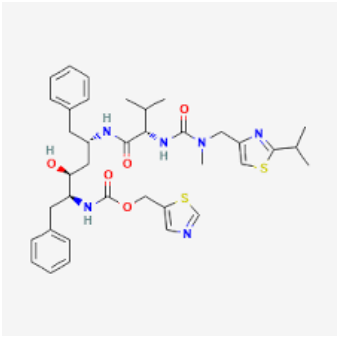

Figure 2: Structure of Ritonavir

Extensive literature research has unearthed a multitude of recorded analytical procedures, including the discovery of more economically efficient ways. Nevertheless, there is currently no documented approach for calculating stability studies. Hence, a reliable and cost-effective approach is suggested for assessing the stability of Nirmatrelvir, Ritonavir, and their medicinal dose form using RP-HPLC [9- 17]must be validated and developed as per ICH guidelines

MATERIALS AND METHODS: Spectrum pharma Research Solution with Nirmatrelvir and Ritonavir pure drugs (API) gift samples and Combination Nirmatrelvir and Ritonavir tablets (Paxlovid). The chemicals and buffers utilized in this estimation were obtained from Rankem, an Indian supplier.

Instrumentation: The development and method validation were conducted using a WATERS HPLC, specifically the model 2695 SYSTEM, equipped with a Photo diode array detector. The system also included an automated sample injector and the Empower 2 software.

Objective: In order to fulfill ICH standards, we need to design and test an HPLC technique that can detect Ritonavir and Nirmatrelvir in pharmaceutical formulations at the same time.

Table 1: Chromatographic Conditions

Mobile phase	Acetonitrile and 0.1% OPA (40:60 v/v)
Flow rate	1 ml/min
Column	Kromasil C18 (4.6 x 150mm, 5µm)
Detector wave length	236 nm
Column temperature	30°C
Injection volume	10mL
Run time	5.0 min
Buffer	OPA

Buffer Preparation: 0.01N Potassium dihyrogen Ortho phosphate: Accurately weighed 1.36gm of Potassium dihyrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then added 1ml of Triethylamine then PH adjusted to 3.5 with dil. Orthophosphoric acid solution.

API Preparation:

Preparation of Standard stock solutions: Accurately weighed 7.5mg of Nirmatrelvir, 5mg of Ritonavir and transferred to 50ml flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (150µg/ml of Nirmatrelvir and 100µg/ml Ritonavir)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (15µg/ml of Nirmatrelvir and 10µg/ml of Ritonavir)

Formulation Preparation:

Preparation of Sample stock solutions: 10 tablets were taken and calculated each tablet average tablet and equivalent to 150 mg and 100mg Was taken Then 20ml acetonitrile was added, sonicated for 25 min and made up to mark and was centrifuged for 20 min. Then the supernatant was collected and filtered using 0.45 µm filters using (Millipore, Milford, PVDF) (300µg/ml of Nirmatrelvir and 200µg/ml of Ritonavir).

Preparation of Sample working solutions (100% solution): 0.5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (15µg/ml of Nirmatrelvir and 10µg/ml of Ritonavir).

System suitability parameters: Nirmatrelvir (15 ppm) and Ritonavir (10 ppm) standard solutions were prepared, injected six times, and metrics such as peak tailing, resolution, and USP plate count were measured in order to evaluate the system suitability parameters. The region of six standard injection results should have an RSD of no more than 2%.

Specificity: Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific.

Table 2: System suitability results

S no	Nirmatrelvir				Ritonavir				
Inj	**RT**	**area**	**Plate Count**	**Tailing**	**RT**	**area**	**Plate Count**	**Tailing**	**RS**
1	2.450	1383808	7365	1.06	2.890	1173975	8251	1.03	3.1
2	2.452	1407465	7232	1.06	2.890	1193654	8290	1.03	3.1
3	2.458	1404263	7216	1.05	2.893	1190059	7968	1.02	3.0
4	2.458	1409059	7442	1.05	2.893	11959	7988	1.02	3.0
5	2.463	1409746	7379	1.03	2.903	1188744	8254	1.02	3.1
6	2.464	1410060	7272	1.06	2.904	1198070	7952	1.04	3.0
Mean		**1404067**				**1190077**			
Std ev		**10149.9**				**8629.4**			
RSD		**0.7**				**0.7**			

The % RSD for the peak areas of Nirmatrelvir and Ritonavir obtained from six replicate injections of standard solution was within the limit.

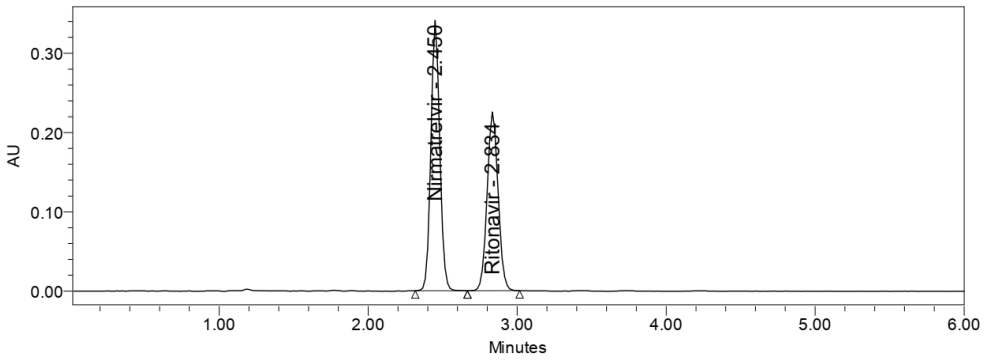

Figure 3: system suitability Chromatogram

Specificity: Checking of the interference in the optimized method. And no interference was observed so, it is specific.

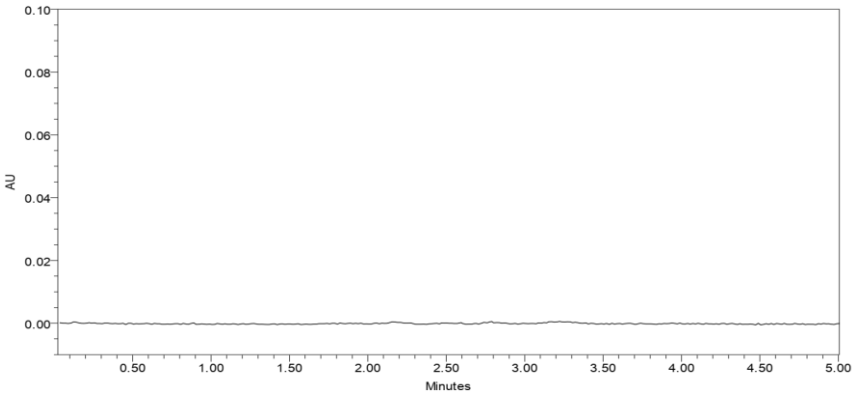

Figure.4 Specificity of Nirmatrelvir and Ritonavir

Linearity:

Calibration data is given in table 4 and regression data in table 5 and calibration curve in figure

Table 4: Calibration data of Nirmatrelvir and Ritonavir

Nirmatrelvir		Ritonavir	
Conc (µg/mL)	**Peak area**	**Conc(µg/mL)**	**Peak area**
0	0	0	0
3.75	366371	2.5	292242
7.5	702753	5	591197
11.25	1042900	7.5	887655
15	1372352	10	1188661
18.75	1750633	12.5	1456863
22.5	2105508	15	1751328

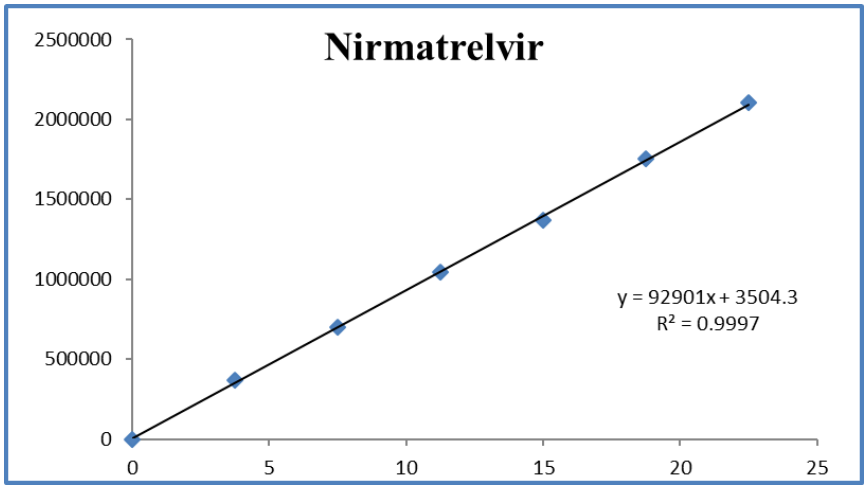

Figure 5 Calibration curve of Nirmatrelvir

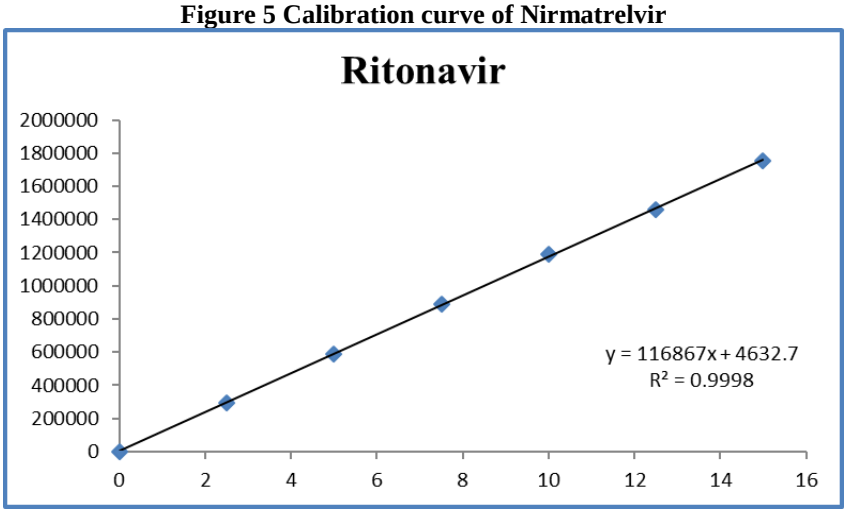

Figure 6 Calibration curve of Ritonavir

Table 5: regression data

Parameter	Nirmatrelvir	Ritonavir
Conc range (µg/mL)	3.75 – 22.5	2.5 – 15
Regression Equation	y = 92901x + 3504.3	y = 116867x + 4632.7.
Co-relation	0.999	0.999

Accuracy:

Recovery data shown in table 6

Table 6: recovery data of Nirmatrelvir and Ritonavir

	Nirmatrelvir			Ritonavir		
% Level	**Amount Spiked (µg/mL)**	**Amount recovered (µg/mL)**	**% Recovery**	**Amount Spiked (µg/mL)**	**Amount recovered (µg/mL)**	**% Recovery**
50%	7.5	7.47	99.56	5	4.99	99.79
		7.49	99.91		4.96	99.24
		7.47	99.60		4.97	99.32
100%	15	14.85	99.02	10	9.96	99.57
		14.86	99.10		9.96	99.56
		14.88	99.22		9.94	99.37
150%	22.5	22.35	99.35	15	14.90	99.31
		22.38	99.47		14.92	99.45
		22.37	99.42		14.91	99.43
% recovery	99.41			99.47		

Method Precision: The precision of the method was determined by analyzing a sample of Nirmatrelvir and Ritonavir and shown in table.

Table 7: method Precision

S. No	Area of Nirmatrelvir	Area of Ritonavir
1.	1405425	1180868
2.	1401866	1191049
3.	1403686	1190302
4.	1399153	1185571
5.	1392919	1187101
6.	1394278	1187889
Mean	**1399555**	**1187130**
S.D	**5077.6**	**3678.0**
%RSD	**0.4**	**0.3**

From the above results, the % RSD of method precision study was within the limit for Nirmatrelvir and Ritonavir.

Robustness: Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.0ml/min), mobile phase minus (40B:60A), mobile phase plus (50B:50A), temperature minus (27°C) and temperature plus(33°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Table 8: Robustness data for Nirmatrelvir and Ritonavir.

Parameter	Optimized condition	Used condition	Nirmatrelvir	Ritonavir
			Obtained %RSD	
Flow rate (±0.1ml/min)	1ml/min	0.9ml/min	0.5	0.7
		1.1 ml/min	0.4	0.6
MP (5%v/v)	60:40	55:45	0.5	0.5
		65:35	0.2	0.2
Column temp. (±3^{0}c)	30^{0}c	27 ^{0}C	0.6	0.9
		33 ^{0}C	0.3	0.4

Sensitivity:

Table 9: sensitivity of Nirmatrelvir and Ritonavir

Molecule	LOD	LOQ
Nirmatrelvir	0.10 µg/ml	0.3 µg/ml
Ritonavir	0.05 µg/ml	0.14 µg/ml

Force Degradation Studies: table shows degradation conditions and table 10 shows the obtained degraded data and chromatogram in figure.

Table 10: Degradation conditions

Stress condition	Solvent	Temp(^{0}C)	Exposed time
Acid	2N HCL	60^{0}c	60 mins
Base	2N NAOH	60^{0}c	60 mins
Oxidation	20% H_2O_2	60^{0}c	60 mins
Thermal	Diluent	105^{0}c	6 hours
Photolytic	Diluent	-	-
Hydrolytic	Water	60^{0}c	60 mins

Table 12: Degradation data

Conc of degradation study	Nirmatrelvir		Ritonavir	
	% drug Undegraded	% drug degraded	% drug Undegraded	% drug degraded
2N HCl, 60 min	93.69	6.31	93.49	6.51
2N NaOH, 60min	93.40	6.60	93.65	6.35
Oxidative, 60 min	96.76	3.24	95.88	4.12
Thermal, 1 hr	99.09	0.91	98.74	1.26
Photo, 6 hr	99.51	0.49	99.50	0.50
Neutral, 1 hr	99.59	0.41	99.92	0.08

Acid degradation chromatogram

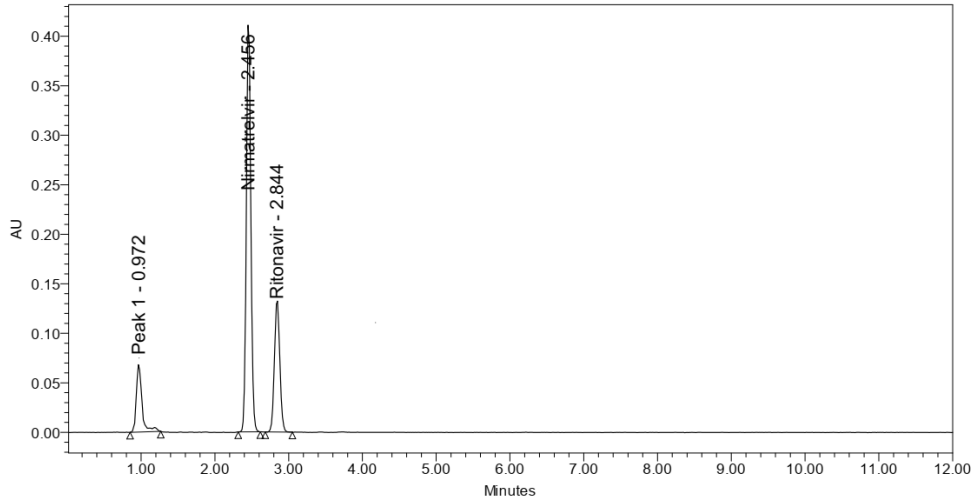

Fig 7 Acid

Base degradation chromatogram

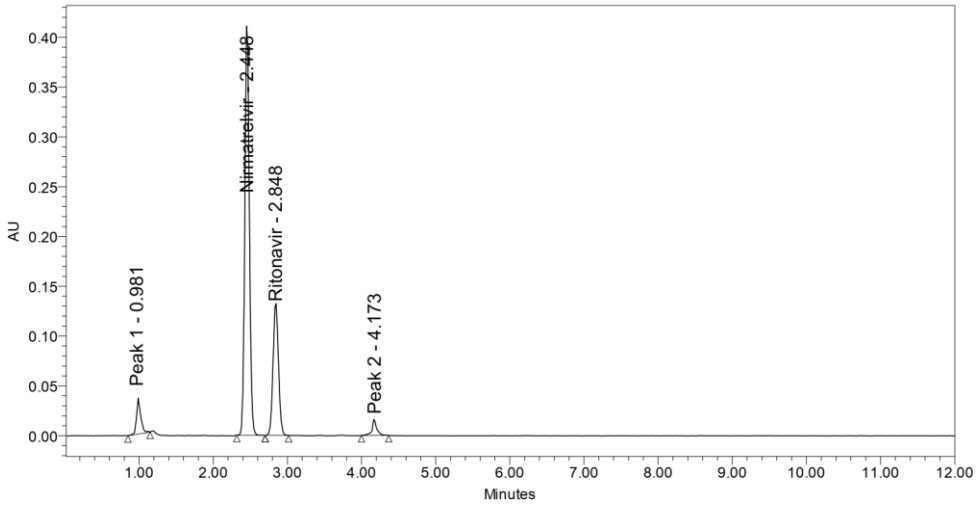

Fig 8 Base

Peroxide degradation chromatogram

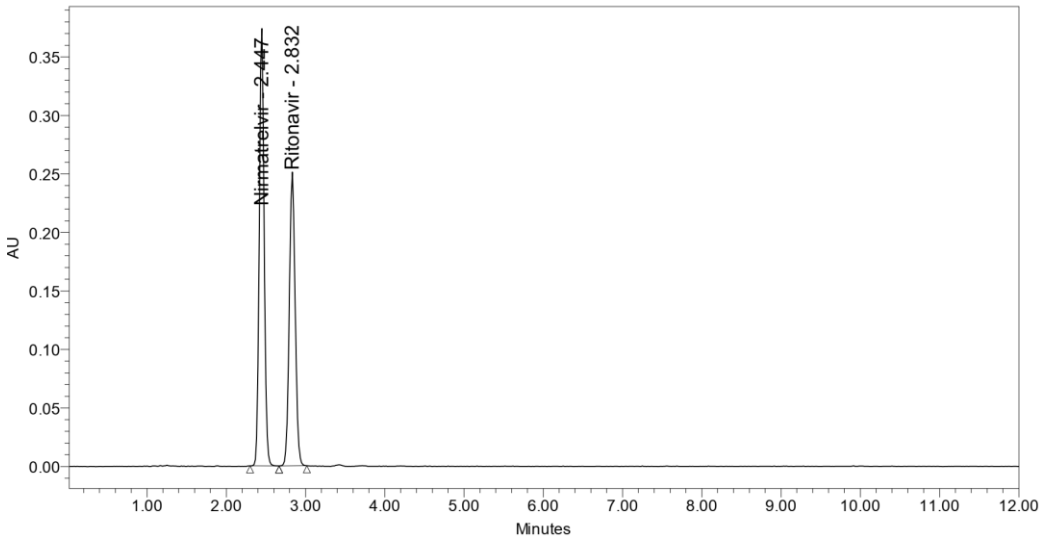

Fig 9 Peroxide

Thermal degradation chromatogram

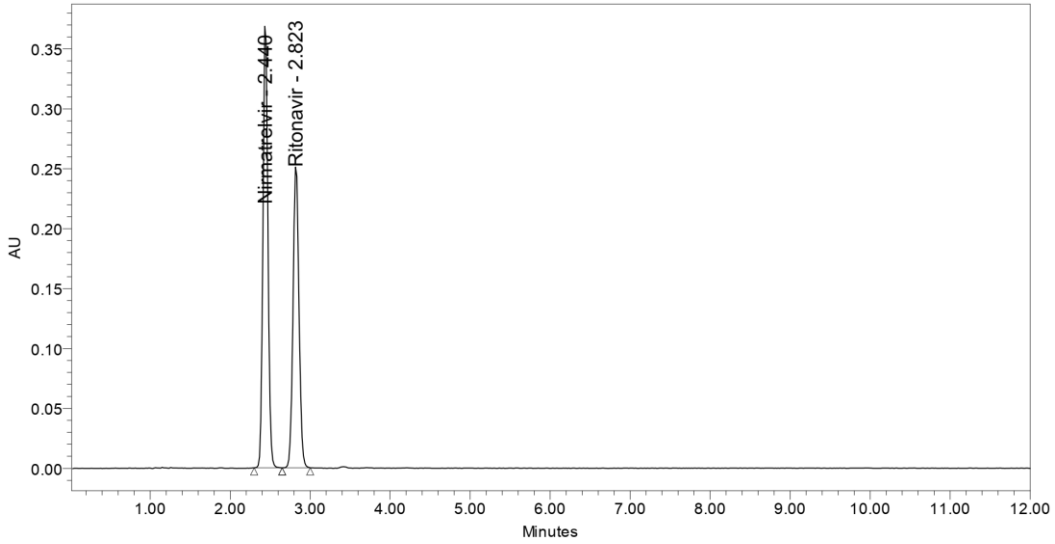

Fig 10 Thermal

UV degradation chromatogram

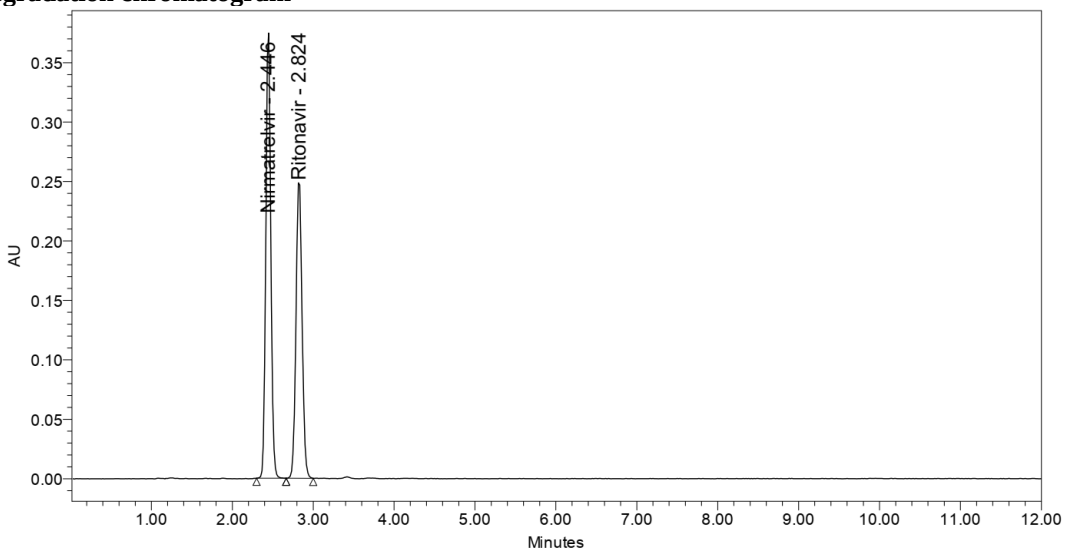

Fig 11 UV

Water degradation chromatogram

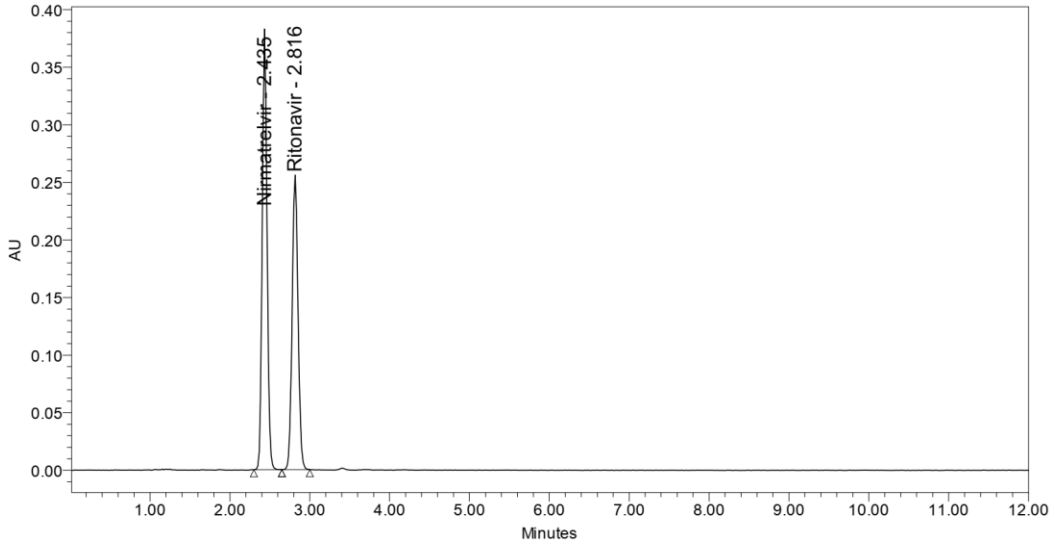

Fig 12 Water

Assay: Average % Assay for Nirmatrelvir and Ritonavir obtained was 99.58% and 99.65% respectively.

Table 13: Assay data

	Nirmatrelvir			Ritonavir		
S.no	**Std Area**	**Sample area**	**% Assay**	**Std Area**	**Sample area**	**% Assay**
1	1383808	1405425	100.00	1173975	1180868	99.13
2	1407465	1401866	99.74	1193654	1191049	99.98
3	1404263	1403686	99.87	1190059	1190302	99.92
4	1409059	1399153	99.55	1195959	1185571	99.52
5	1409746	1392919	99.11	1188744	1187101	99.65
6	1410060	1394278	99.20	1198070	1187889	99.72
Avg	**1404067**	**1399555**	**99.58**	**1190077**	**1187130**	**99.65**
Stdev	**10149.9**	**5077.6**	**0.36**	**8629.4**	**3678.0**	**0.31**
%RSD	**0.7**	**0.4**	**0.36**	**0.7**	**0.3**	**0.31**

CONCLUSION:

The study's findings will be very helpful in evaluating the quality of reasonably priced drugs that contain Ritonavir and Nirmatrelvir. This could be as a result of the study's straightforward sample preparation method, which required little mobile phase and a brief analytical period. The results of evaluating two medications combined in a single dosage demonstrated that the recently created analysis technique was almost entirely successful.

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REFERENCES:

1. Drugbank: Nirmatrelevir
2. Drugbank: Ritonavir
3. Pfizer Press Release: Pfizer Initiates Phase 1 Study Of Novel Oral Antiviral Therapeutic Agent Against SARS-COV-2.
4. FDA Emergency Use Authorization Fact Sheet: Paxlovid (nirmatrelvir and ritonavir) co-packaged for oral use.
5. PubChem: Nirmatrelevir
6. PubChem: Ritonavir
7. Hammond, J., et al. (2022). Oral Nirmatrelvir for High-Risk, Nonhospitalized Adults with Covid-19. The New England Journal of Medicine.
8. https://time.com/6986441/paxlovid-does-not-treat-long-covid/?utm_source=chatgpt.com
9. Palaniappan Ilayaraja et al., Novel stability indicating HPLC method for the quantification of Nirmatrelvir in bulk drugs, Elsevier, Volume 196, January 2024, 109707
10. S. Pallavi et al., Development and validation of a new RP-UPLC method for the simultaneous estimation of nirmatrelvir and ritonavir in bulk and copacked tablet dosage forms, RJPT, Vol 16, Issue 9, 2023
11. Mohamed S Imam et al., Adjusted green HPLC determination of nirmatrelvir and ritonavir in the new FDA approved co-packaged pharmaceutical dosage using supported computational calculations, Sci Rep, 2023 Jan 4;13(1):137
12. Santosh Kumar Gandhi et al., Development and Validation of RP-HPLC Method for the simultaneous determination of Nirmatrelvir and Ritonavir in bulk and pharmaceutical formulation, Research Journal of Chemistry and Environment 27(4):120-127, March 2023
13. Joris Guyon et al., A UPLC-MS/MS Method for Plasma Biological Monitoring of Nirmatrelvir and Ritonavir in the Context of SARS-CoV-2 Infection and Application to a Case, J Am Soc Mass Spectrom, 2022 Oct 5;33(10):1975-1981.
14. Srinivas Reddy Jitta et al., Development and Validation of RP-HPLC Method for Quantification of Total, Free and Entrapped Ritonavir in Lipid Nanocarriers and Drug content of Film Coated Fixed Dose Formulation, Indian Journal of Pharmaceutical Education and Research, 2022; 56(3s):s547-s558
15. Avinash Birajdar et al., Development and Validation of RP-HPLC method for the Estimation of Ritonavir in API and tablet Formulation, RJPT, 14(10): October 2021
16. Andrew J Ocque et al., Development and validation of a UPLC–MS/MS method for the simultaneous determination of Paritaprevir And Ritonavir In Rat Liver, Bioanalysis Vol. 8, No. 13 2016.
17. Veera Venkata Satyanarayana Peruri et al., A RP-HPLC Method for the Estimation of Ritonavir in Pharmaceutical dosage forms, Journal of Pharmacy Research 2011,4(9),3049-3051.