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Research Article

Development and Validation of an HPLC Method for the Simultaneous Detection of Nirmatrelvir and Ritonavir in Pharmaceutical Formulations

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ABSTRACT:
The simultaneous estimation of the Nirmatrelvir and Ritonavir in pharmaceutical dosage form. Chromatogram was run through Discovery C18 250 x 4.6 mm, 5m. Mobile phase containing Buffer KH2PO4: Acetonitrile taken in the ratio 65:35 was pumped through column at a flow rate of 0.9 ml/min.. Temperature was maintained at 30°C. Optimized wavelength selected was 242 nm. Nirmatrelvir and Ritonavir were eluted at 2.251 min and 2.820 min respectively. %RSD of the Nirmatrelvir and Ritonavir were and found to be 0.2 and 0.3 respectively. %Recovery was obtained as 99.33% and 99.49% for Nirmatrelvir and Ritonavir respectively. LOD, LOQ values obtained from regression equations of Nirmatrelvir and Ritonavir were 0.09, 0.26 and 0.07, 0.20 respectively. Regression equation of Nirmatrelvir is y = 40331x + 7783.3, and y = 47638x + 4515.3 of Ritonavir.

Key Words: Nirmatrelvir, Ritonavir, RP HPLC, Validation.

INTRODUCTION

Nirmatrelvir and ritonavir combination is used to treat mild to moderate coronavirus disease 2019 (COVID-19) in non-hospitalized patients who are at high risk for progression to severe COVID-19[1] Nirmatrelvir (PF-07321332) is an orally bioavailable 3C-like protease (3CLPRO) inhibitor that is the subject of clinical trial NCT04756531. 3CLPRO is responsible for cleaving polyproteins 1a and 1ab of SARS-CoV-2.[2] Without the activity of the SARS-CoV-2 3CLPRO, nonstructural proteins (including proteases) cannot be released to perform their functions, inhibiting viral replication.[2,3,4] it is also written as (1R,2S,5S)-N-[(1S)-1-cyano-2-[(3S)-2-oxopyrrolidin-3-yl]ethyl]-3-[(2S)-3,3-dimethyl-2-(2,2,2-trifluoroacetamido)butanoyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexane-2-carboxamide,[5] Nirmatrelvir is administered alongside ritonavir, a potent inhibitor of CYP3A enzymes, in order to inhibit its metabolism and increase plasma nirmatrelvir concentrations[6] Ritonavir is a protease inhibitor with activity against Human Immunodeficiency Virus Type 1 (HIV-1) and its written as (1,3-thiazol-5-yl)methyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl]carbamate[7] While ritonavir is not an active antiviral agent against hepatitis C virus (HCV) infection, it is added in combination therapies indicated for the treatment of HCV infections as a booster. Ritonavir is combined with other drugs to treat coronavirus disease 2019 (COVID-19) in patients at risk for progressing into a severe form of the disease, such as nirmatrelvir.[8] Coadministration of Ritonavir allows twice-daily oral dosing of Nirmatrelvir, enabling sustained plasma concentrations above the viral inhibitory threshold[9] The fixed-dose combination, commercially known as Paxlovid, has demonstrated remarkable clinical benefits, including reduced hospitalization and mortality in high-risk COVID-19 patients when administered within five days of symptom onset [10].

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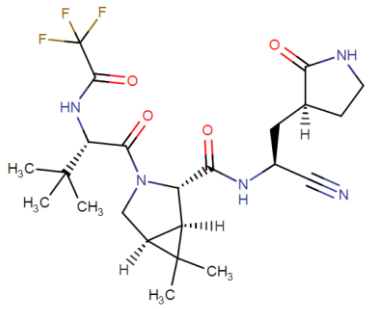

Figure 1: Structure of Nirmatrelvir

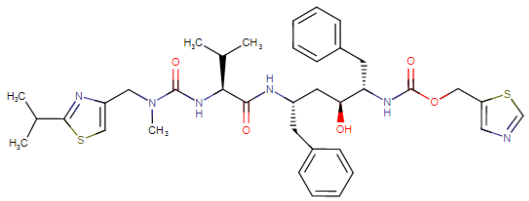

Figure 2: Structure of Ritonavir

Extensive literature research has unearthed a multitude of recorded analytical procedures, including the discovery of more economically efficient ways. Nevertheless, there is currently few 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 [11 - 14] must be validated and developed as per ICH guidelines

Materials and Methods: Spectrum pharma Research Solution provided 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 KH_2PO_4 (35:65 v/v)
Flow rate	0.9 ml/min
Column	Discovery C18 (4.6 x 250mm, 5µm)
Detector wave length	242 nm
Column temperature	30°C
Injection volume	10 mL
Run time	10.0 min
Buffer	KH_2PO_4

Buffer Preparation: 0.01N KH2PO4 Buffer: Accurately weighed 1.36gm of Potassium dihydrogen 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 (4.8-pH).

API Preparation:

Preparation of Standard stock solutions: Accurately weighed 15mg of Nirmatrelvir, 10mg 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. (300µg/ml of Nirmatrelvir and 200µg/ml Ritonavir). From this 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (30µg/ml of Nirmatrelvir and 20µg/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 in 500ml vf Then 400ml 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). from this 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (30µg/ml of Nirmatrelvir and 20µg/ml of Ritonavir).

System suitability parameters: Nirmatrelvir (30 ppm) and Ritonavir (20 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.

Precision: To demonstrate the process and system accuracy, a standard solution with a known concentration was made, and six repeated injections were given to guarantee the consistency of the recommended method.

By creating six functional sample solutions and injecting them six times, intermediate precision was also achieved. After measuring the area, the mean, standard deviation, and percentage RSD were computed. The outcomes, which were below the 2% threshold, were favorable.

Linearity: To assess the drug's linearity, serial dilutions from 25%- 150% were made. A graph was utilized to highlight the link between peak area response and medicine concentration. It was determined to be linear at the prescribed medication concentration. Dilution were as follows.

25 µg/mL: Take 0.25 mL of stock solution and dilute to 10 mL
50 µg/mL: Take 0.5 mL of stock solution and dilute to 10 mL
75 µg/mL: Take 0.75 mL of stock solution and dilute to 10 mL
100 µg/mL: Take 1.0 mL of stock solution and dilute to 10 mL
125 µg/mL: Take 1.25 mL of stock solution and dilute to 10 mL
150 µg/mL: Take 1.5 mL of stock solution and dilute to 10 mL

Accuracy: Accuracy was performed in triplicate for various concentrations equivalent to 50%, 100% and 150% of the standard amount were injected into the HPLC system per the test procedure. Dilution were as follows.

50 µg/mL: Take 0.1 mL of stock solution and dilute to 10 mL
100 µg/mL: Take 0.2 mL of stock solution and dilute to 10 mL
150 µg/mL: Take 0.3 mL of stock solution and dilute to 10 mL

Sensitivity

Limit of detection and Limit of Quantification

LOD and LOQ were computed from the average slope and standard deviation from the calibration curve as per ICH recommendations. The LOD and LOQ can be calculated using the response's standard deviation and the slope of the calibration curve.

Assay

The assay and % purity were calculated for brand Paxlovidwith label claim Ritonavir 10g and Nirmatrelvir 10mg. The observed value was compared with that of standard value without interference from the excipients used in the tablet dosage form.

Degradation studies:

Oxidation: To 1 ml of stock solution of Ritonavir and Nirmatrelvir, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60^0C. For HPLC study, the resultant solution was diluted to obtain 30µg/ml & 20µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies: To 1 ml of stock s solution Ritonavir and Nirmatrelvir, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0C. The resultant solution was diluted to obtain 30µg/ml & 20µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies: To 1 ml of stock solution Ritonavir and Nirmatrelvir, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 30µg/ml & 20µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies: The standard drug solution was placed in oven at 105°C for 1 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 30µg/ml & 20µg/ml ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo Stability studies: The photochemical stability of the drug was also studied by exposing the 200µg/ml Ritonavir &300µg/ml Nirmatrelvir solution to UV Light by keeping the beaker in UV Chamber for 1days or 200 Watt hours/m2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain30µg/ml & 20µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies: Stress testing under neutral conditions was studied by refluxing the drug in water for 1hrs at a temperature of 60ºC. For HPLC study, the resultant solution was diluted to 30µg/ml & 20µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Table 2: System suitability results

	Nirmatrelvir			Ritonavir			
Inj	**RT (min)**	**USP Plate Count**	**Tailing**	**RT (min)**	**USP Plate Count**	**Tailing**	**Resolution**
1	2.253	4794	1.13	2.805	4839	1.14	3.2
2	2.255	4749	1.13	2.806	4793	1.14	3.2
3	2.259	4768	1.12	2.810	4737	1.14	3.2
4	2.259	4903	1.12	2.812	4882	1.14	3.2
5	2.260	4664	1.12	2.812	4853	1.13	3.2
6	2.262	4825	1.13	2.815	4881	1.13	3.2

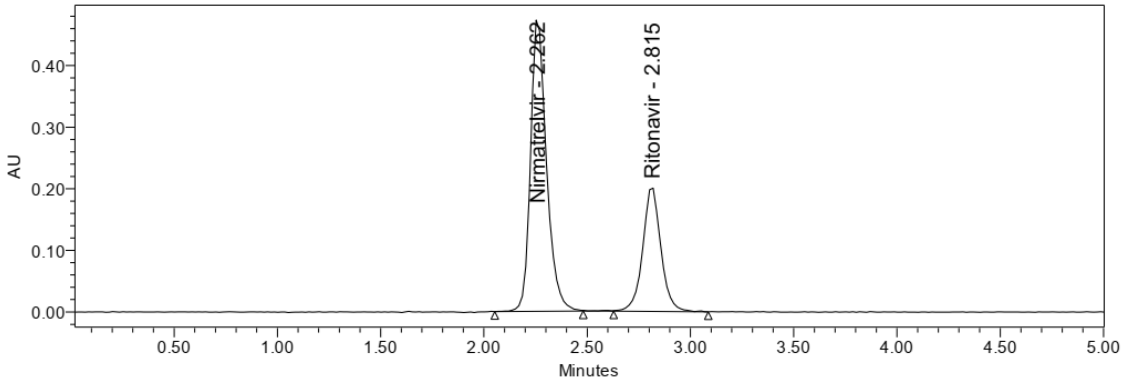

Figure 3: System suitability Chromatogram

Table 3: Specificity data

Sample name	Retention time	Area	Plate count	Tailing	Resolution
Nirmatrelvir	2.110	126598	2318	1.2	
Ritonavir	2.614	1157823	2327	1.35	2.4

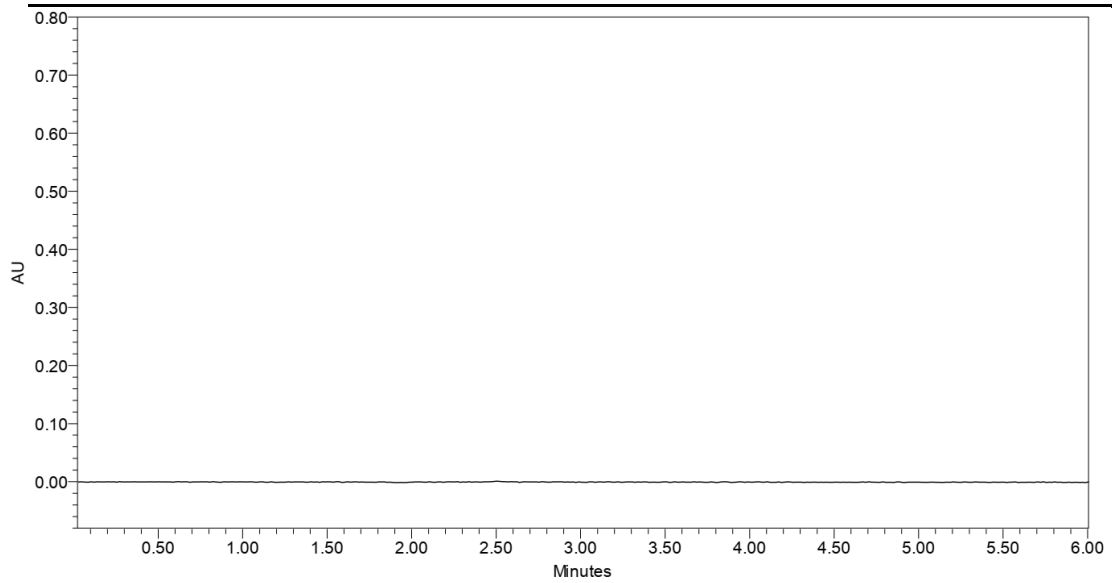

Figure.4 Specificity of Nirmatrelvir and Ritonavir

Linearity:

Calibration data is given in table 4 and regression data in table 5 and calibration curve in figure 5, 6

Table 4: Calibration data of Nirmatrelvir and Ritonavir

Nirmatrelvir		Ritonavir	
Conc (µg/mL)	**Peak area**	**Conc(µg/mL)**	**Peak area**
0	0	0	0
7.5	309437	5	242121
15	619095	10	482858
22.5	926345	15	721001
30	1221916	20	962465
37.5	1503835	25	1201495
45	1825944	30	1423653

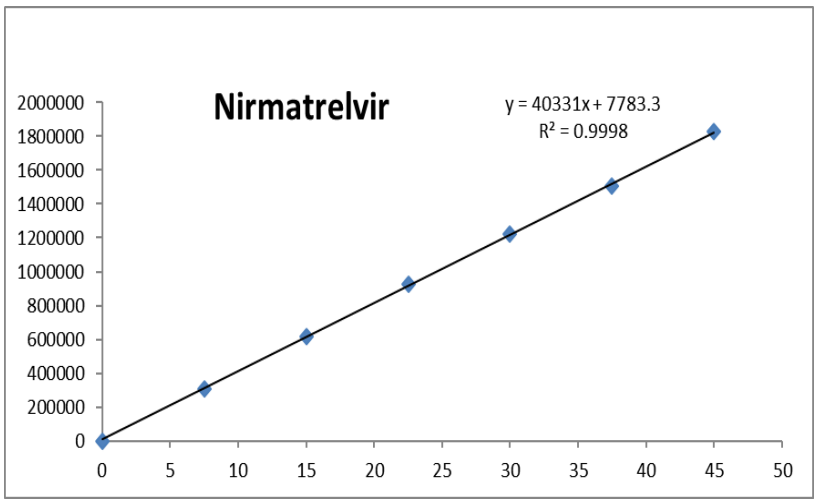

Figure 5 Calibration curve of Nirmatrelvir

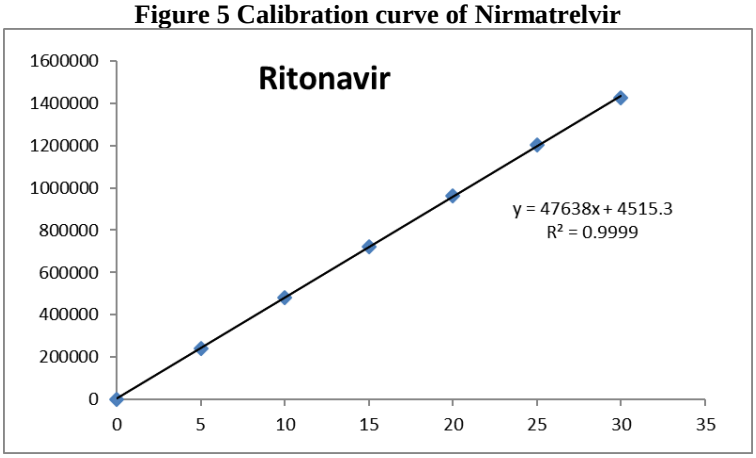

Figure 6 Calibration curve of Ritonavir

Table 5: regression data

Parameter	Nirmatrelvir	Ritonavir
Conc range (µg/mL)	7.5 – 45 µg/ml	5-30 µg/ml
Regression Equation	y = 40331x + 7783.3	y = 47638x + 4515.3
Co-relation	0.999	0.999
LOD	0.09 µg/ml	0.26 µg/ml
LOQ	0.07 µg/ml	0.20 µg/ml

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**
		14.99	99.91		9.96	99.61
50%	15	14.96	99.73	10	9.93	99.28
		14.94	99.60		9.96	99.64
		29.79	99.30		19.87	99.33
100%	30	29.31	97.68	20	19.99	99.96
		29.76	99.21		19.84	99.21
		44.90	99.79		29.86	99.53
150%	45	44.74	99.42	30	29.82	99.39
		44.69	99.31		29.84	99.46
% recovery		**99.33**			**99.49**	

System precision was performed and the data was shown in table 7

Table 7: System precision of Nirmatrelvir and Ritonavir

S. No	Area of Nirmatrelvir	Area of Ritonavir
1.	1239684	962729
2.	1241940	961367
3.	1236301	962449
4.	1235288	962784
5.	1236549	966110
6.	1239603	957317
Mean	1238228	962126
S.D	2567.7	2845.2
%RSD	0.2	0.3

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

Method Precision: The precision of the method was determined by analyzing a sample of Nirmatrelvir and Ritonavir and shown in table 8.

Table 8: method Precision

S. No	Area of Nirmatrelvir	Area of Ritonavir
1.	1236211	959724
2.	1232683	956167
3.	1230682	956551
4.	1238940	963486
5.	1232679	954066
6.	1228567	953298
Mean	1233294	957215
S.D	3749.8	3805.8
%RSD	0.3	0.4

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

Robustness: Robustness conditions like Flow minus (0.8ml/min), Flow plus (1.0ml/min), mobile phase minus (45A:55B), mobile phase plus (35A:65B), 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 9: Robustness data for Nirmatrelvir and Ritonavir.

Condition	%RSD of Nirmatrelvir	%RSD of Ritonavir
Flow rate (-) 0.8ml/min	0.3	0.5
Flow rate (+) 1.0ml/min	0.2	0.5
Mobile phase (-) 40A:60B	0.5	0.2
Mobile phase (+) 30A:70B	0.4	0.7
Temperature (-) 27°C	0.6	0.6
Temperature (+) 33°C	0.3	0.9

Force Degradation Studies: table 11 shows degradation conditions and table 10 shows the obtained degraded data and purity plot chromatogram in figure 8, 9.

Table 10: degradation conditions

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

Table 11: degradation data

Conc of degradation study	Nirmatrelvir		Ritonavir	
	% drug Undegraded	% drug degraded	% drug Undegraded	% drug degraded
Acid	96.09	3.91	96.40	3.60
Base	96.70	3.30	96.44	3.56
Peroxide	97.49	2.51	97.50	2.50
Thermal	98.27	1.73	99.48	0.52
UV	99.61	0.39	99.55	0.45
Water	99.87	0.13	99.69	0.31

Acid degradation chromatogram

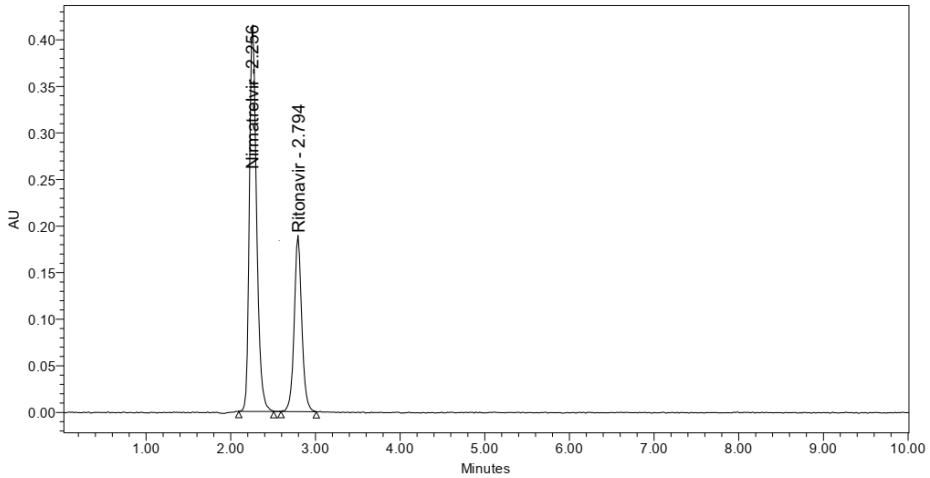

Fig 7 Acid

Base degradation chromatogram

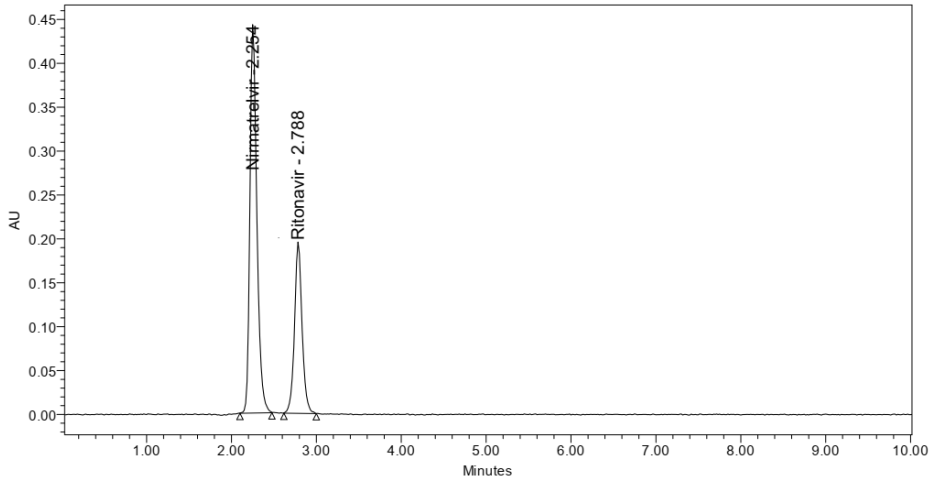

Fig 8 Base

Peroxide degradation chromatogram

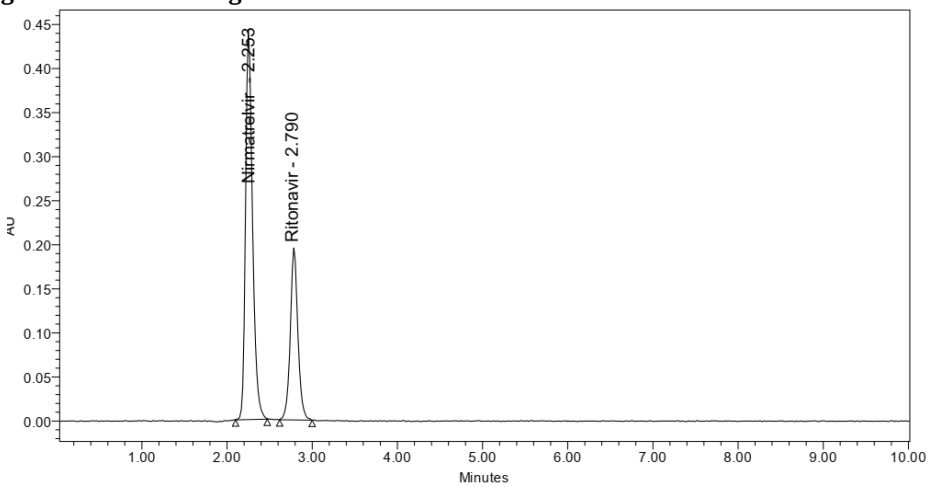

Fig 9 Peroxide

Thermal degradation chromatogram

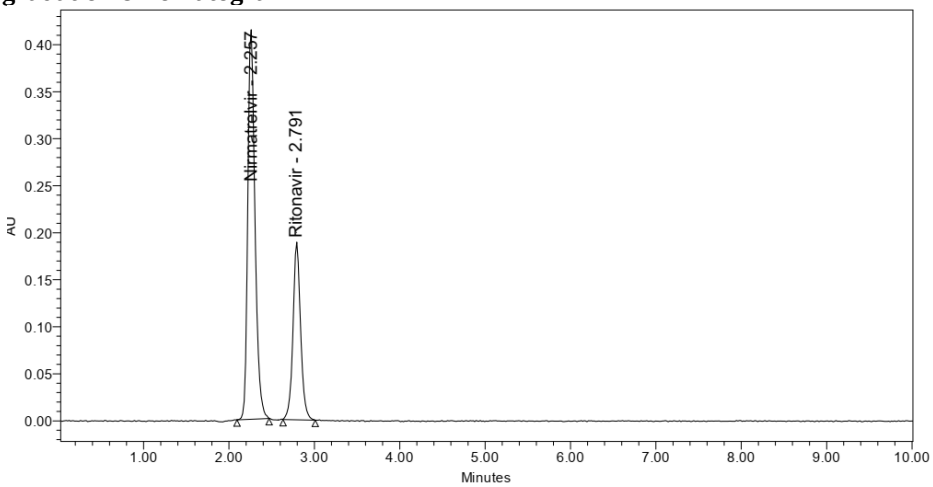

Fig 10 Thermal

UV degradation chromatogram

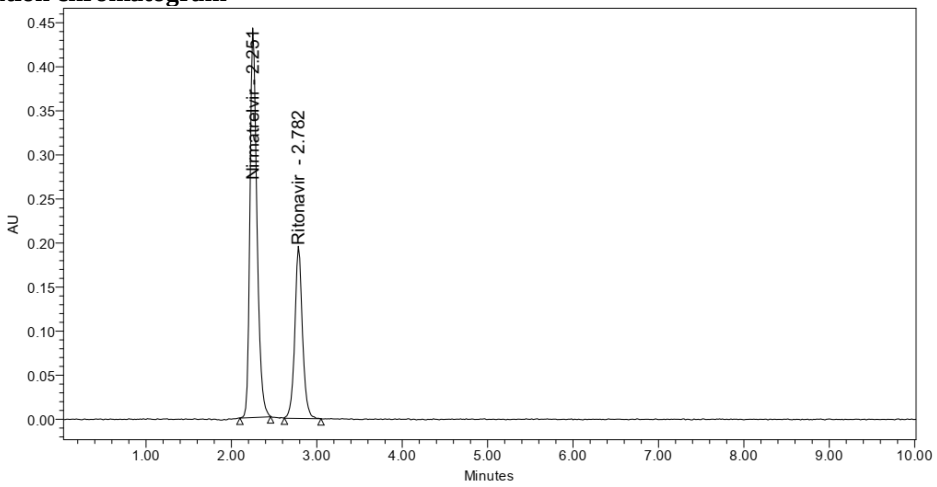

Fig 11 UV

Water degradation chromatogram

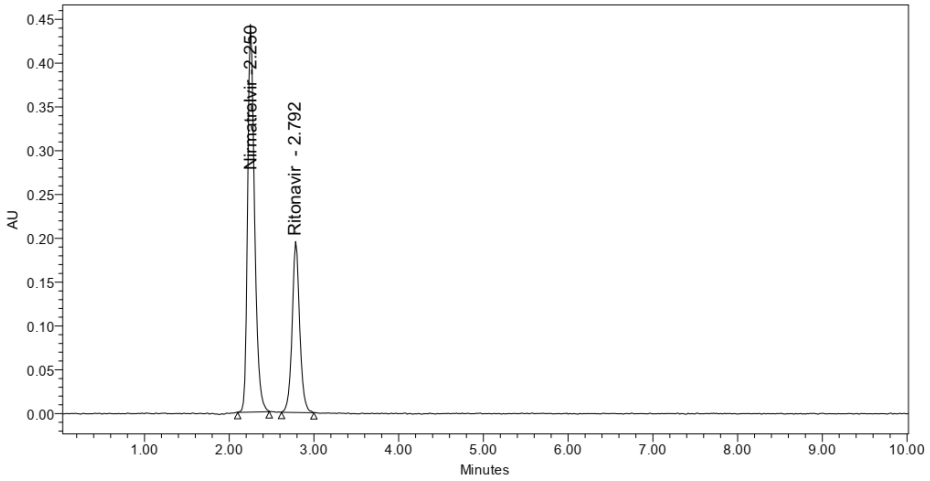

Fig 12 Water

Assay: Paxlovid, bearing the label claim Nirmatrelvir 10mg, Ritonavir 10mg. Assay was performed with the above formulation. Average % Assay for Nirmatrelvir and Ritonavir obtained was 99.29% and 99.40% respectively.

Table 13: assay data

Formulation	Label claim(mg)	% Assay*
Paxlovid	Ritonavir 100mg.	99.29%w/w
	Nirmatrelvir 150mg	99.40%w/w

CONCLUSION:

All system suitability requirements were met by the good separation of both analytes with well-resolved retention durations made possible by the adjusted chromatographic settings. The method's linearity, accuracy, precision, robustness, and sensitivity within the investigated concentration ranges were confirmed by validation parameters. The method's dependability and repeatability are shown by the low percentage of RSD values and good recovery rates. As a result, this RP-HPLC technique is appropriate for regular quality control examination, stability investigations, and the creation of Nirmatrelvir and Ritonavir combination formulations.

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