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Development and Validation of a Stability-Indicating RP-HPLC Method for Simultaneous Estimation of Nirmatrelvir and Ritonavir in Tablet Dosage Form

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ABSTRACT

A rapid, accurate, and stability-indicating RP-HPLC method was developed for the simultaneous estimation of Nirmatrelvir and Ritonavir in tablet dosage form. Chromatographic separation was achieved using a Kromasil C8 column (250 × 4.6 mm, 5 µm) with a mobile phase consisting of 0.1% orthophosphoric acid and acetonitrile in the ratio of 60:40 v/v at a flow rate of 1.0 mL/min. Detection was carried out at 240 nm with a column temperature maintained at 30°C. Nirmatrelvir and Ritonavir were eluted at retention times of 2.303 min and 2.783 min respectively. The developed method was validated according to ICH guidelines for parameters including specificity, linearity, precision, accuracy, robustness, LOD, and LOQ. The %RSD values were below 2%, indicating good precision of the method. Recovery studies showed satisfactory accuracy for both drugs. The proposed method was found to be simple, reliable, economical, and suitable for routine quality control analysis of pharmaceutical formulations containing Nirmatrelvir and Ritonavir.

Key Words: Nirmatrelvir, Ritonavir, RP-HPLC, Stability-indicating method, Method validation

I. INTRODUCTION

Reliable analytical methods play an important role in maintaining the quality, safety, and effectiveness of pharmaceutical products. Analytical method validation helps confirm that the developed procedure consistently produces accurate and reproducible results during routine analysis. Stability-indicating analytical methods are especially important because they can detect changes in drug substances caused by degradation under different stress conditions. Such methods are useful in determining the stability and shelf life of pharmaceutical formulations [8,9,10,20].

Nirmatrelvir is an antiviral drug that inhibits the main protease enzyme of SARS-CoV-2 and prevents viral replication. Ritonavir is mainly used as a pharmacokinetic enhancer because it slows the metabolism of Nirmatrelvir by inhibiting CYP3A enzymes, thereby increasing its therapeutic effectiveness. The combination of these two drugs has gained significant importance in the treatment of COVID-19 infections [23,24,27].

Because of the increasing clinical use of this drug combination, there is a need for a reliable analytical method capable of estimating both drugs simultaneously in pharmaceutical dosage forms. Although several analytical methods have been reported individually for these drugs, only limited studies are available on stability-indicating RP-HPLC methods for simultaneous estimation. Therefore, the present work focuses on developing a simple and efficient RP-HPLC method suitable for routine pharmaceutical analysis [22,25,26,28].

Stability-indicating methods are particularly valuable because they enable the detection and measurement of degradation products formed under different stress conditions, including acidic, alkaline, oxidative, thermal, and photolytic environments. These studies provide a better understanding of the stability characteristics of drugs and assist in defining proper storage conditions and shelf life [14,15,20].

Among the various analytical techniques, Reverse Phase High Performance Liquid Chromatography (RP-HPLC) is widely used due to its reliability, sensitivity, and ability to provide consistent results. It is especially suitable for

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analysing multiple components in complex pharmaceutical formulations [16,17].

In view of these considerations, the present study aims to develop and validate a simple, rapid, cost-effective, and stability-indicating RP-HPLC method for the simultaneous estimation of Nirmatrelvir and Ritonavir in tablet dosage form. The method has been optimized and validated according to ICH Q2(R1) guidelines by evaluating parameters such as specificity, linearity, accuracy, precision, robustness, and sensitivity, confirming its applicability for routine quality control analysis [11,20,21].

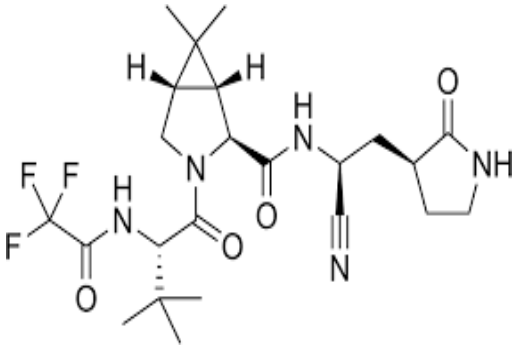

Figure 1: Structure of Nirmatrelvir

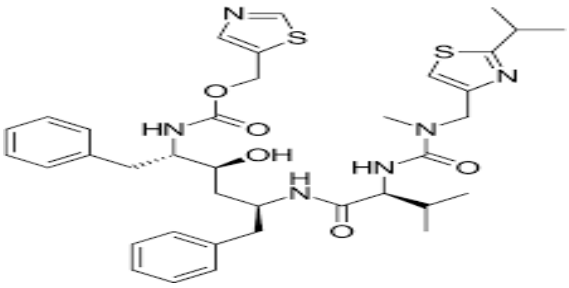

Figure 2: Structure of Ritonavir

II. Materials and Methods:

Chemicals and Reagents:

Reference standards of Nirmatrelvir and Ritonavir were obtained in pure form and used without further purification. The marketed tablet formulation containing the fixed-dose combination was procured from the local pharmaceutical market [23,24].

HPLC-grade acetonitrile was used as the organic solvent. Orthophosphoric acid (OPA) and analytical-grade reagents were utilized for preparation of the aqueous phase. Milli-Q purified water was used throughout the analysis for preparation of solutions and mobile phase.

All chemicals and solvents employed in the study were of analytical or chromatographic grade [13,19].

Instrumentation:

Chromatographic evaluation was carried out using a High-Performance Liquid Chromatography (HPLC) system comprising a quaternary solvent delivery pump, an autosampler for precise sample injection, a thermostatically controlled column compartment, and a UV–visible detector for analyte monitoring. The chromatographic signals were recorded and processed using validated data acquisition software to ensure accuracy and reliability of the results [14,18,19].

Efficient separation of the analytes was accomplished using a Kromasil C8 column (250 mm × 4.6 mm internal diameter, 5 µm particle size). The column was selected following systematic optimization experiments, as it provided satisfactory resolution, improved peak symmetry, and reproducible chromatographic performance under the optimized conditions [18,19,22].

Methodology:

Preparation of Diluent:

The diluent was prepared by combining water and acetonitrile in equal proportions (50:50 v/v). The mixture was thoroughly sonicated for approximately 10 minutes to eliminate entrapped air and dissolved gases, ensuring stable chromatographic performance. Prior to use, the solution was passed through a 0.45 µm membrane filter to remove any particulate impurities [19].

Preparation of Standard Stock Solution:

Standard stock solution was prepared by weighing appropriate quantities of Nirmatrelvir and Ritonavir and transferring them into a 100 mL volumetric flask. About 70 mL of diluent was added and the mixture was sonicated until complete dissolution was achieved. The final volume was adjusted with diluent to obtain concentrations of 150 µg/mL and 100 µg/mL respectively. The solution was filtered through a 0.45 µm membrane filter before analysis. [19],

Preparation of Working Standard Solution:

An aliquot of 1 mL from the prepared stock solution was accurately transferred into a 10 mL volumetric flask and diluted to the mark with diluent. This dilution yielded working concentrations of 15 µg/mL for Nirmatrelvir and 10 µg/mL for Ritonavir. The prepared working standard solution was utilized for system suitability testing and validation studies

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[25].

Preparation of Sample Solution:
Twenty tablets were individually weighed to determine the average tablet weight and then finely pulverized to obtain a uniform powder. A quantity of powder equivalent to 150 mg of Nirmatrelvir and 100 mg of Ritonavir was accurately weighed and transferred into a 100 mL volumetric flask .
Approximately 70 mL of diluent was added to the flask, and the mixture was sonicated for 20 minutes to facilitate complete extraction of the active pharmaceutical ingredients from the tablet matrix. After cooling to room temperature, the volume was adjusted to the mark with diluent. The solution was then centrifuged to separate insoluble excipients and subsequently filtered through a 0.45 µm membrane filter to obtain a clear sample solution.
Further appropriate dilution was carried out to achieve final concentrations corresponding to 15 µg/mL of Nirmatrelvir and 10 µg/mL of Ritonavir prior to injection into the HPLC system [25].

Preparation of Mobile Phase
The mobile phase consisted of 0.1% orthophosphoric acid and acetonitrile in the ratio of 60:40 (v/v). The mixture was degassed by sonication for 10 minutes and filtered through a 0.45 µm membrane filter prior to use [25].

Preparation of buffer:
Buffer: 0.01N Potassium dihydrogen orthophosphate: 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 then added 1ml of Triethylamine then pH adjusted to 3.5 with dil. Orthophosphoric acid solution [25].

Method Validation
As per ICH Guidelines the method was validated and the parameters like Accuracy, Precision, Specificity, Robustness, Limit of detection (LOD), Limit of Quantification (LOQ), Linearity were assessed [11,20,21].
System suitability parameters:
The system suitability parameters were determined by preparing standard solutions of Nirmatrelvir (15 ppm) and Ritonavir (10 ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined.
The % RSD for the area of six standard injections results should not be more than 2%.
Specificity
Specificity of the developed RP-HPLC method was evaluated to determine whether any excipients, impurities, or placebo components interfered with the analyte peaks. Blank and placebo solutions were injected under optimized chromatographic conditions, and no interfering peaks were observed at the retention times of Nirmatrelvir and Ritonavir, confirming the specificity of the method [11,20].
Linearity
The linearity of the method was obtained by preparation of the calibration standards of 6 different concentrations in 6 replicates. The calibration curve plots for Nirmatrelvir and Ritonavir were obtained by plotting the peak areas on the y- axis and concentrations on the X-axis over the concentration ranges of 7.5 – 22.5 µg/mL for Nirmatrelvir and 5 – 15 µg/mL for Ritonavir. The correlation coefficient should be greater than 0.99 [11,20,21].

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Accuracy
The accuracy of this method was checked by recovery experiments in which a known amount of pure standard drug was added to the sample solution and recovered in terms of peak areas of the same. The sample was spiked with the standard drug at 50%, 100%, and 150% of the test concentrations. The spiked sample was injected in triplicate. The % recovery for the spiked sample should be 98-102% [11,13,20].

Precision
Precision refers to the degree of closeness of agreement in the series of measurements resulting from the repeated sample sampling of a homogeneous sample under specified conditions. It is expressed in terms of standard deviation (SD) or relative standard deviation (RSD). Precision could be a measure of the degree of repeatability or reproducibility of the analytical method [11,20,21].

Method Precision:
Sample solutions were injected 6 times on 6 different days under optimized conditions, and their peak areas were noted The %RSD for the peak areas obtained from six standard injections should not be more than 2%.

Intermediate Precision
Sample solutions were injected 6 times on the same day under optimized conditions, and their peak areas were noted %RSD for the peak areas of the 6 replicate injection results should not be greater than 2%.

Ruggedness
The ruggedness of the method is checked by doing the experiment on different machines, by different operators, and using different columns of similar types.

Robustness
Robustness is checked by making small changes in the method, like flow rate, mobile phase ratio, and temperature. One should not find significant changes in the results, and the results should be within the range given in the ICH guidelines [11,20].

Limit of Detection and Limit of Quantitation (LOD and LOQ)
LOD is the lowest amount that can be detected, though it is not necessary that it can be quantitatively measured or estimated. It is calculated by the formula,

$LOD=3.3\sigma/S$, where σ=SD, s=slope
LOQ is the lowest amount of the analyte in the sample that can be measured or estimated with precision and accuracy.
$LOQ=10\sigma/S$, where σ=SD, s=slope

III. RESULTS AND DISCUSSION

An RP-HPLC method was successfully developed for the simultaneous estimation of Nirmatrelvir and Ritonavir in tablet dosage form. Various chromatographic conditions were tested to obtain satisfactory separation with acceptable peak symmetry and resolution.

The optimized chromatographic conditions for the estimation of Nirmatrelvir and Ritonavir were achieved by employing a Kromasil C8 column (250 mm × 4.6 mm, 5 µm). The mobile phase consisted of 0.1% orthophosphoric acid and acetonitrile in the ratio of 60:40 (v/v). The mobile phase was pumped at a flow rate of

1.0 mL/min, and detection was performed at 240 nm. The column temperature was maintained at 30°C, and the injection volume was 10 µL. The total run time for chromatographic analysis was 10 minutes. The diluent used for the preparation of standard and sample solutions was a mixture of water and acetonitrile (50:50 v/v).

Under these optimized chromatographic conditions, good chromatographic separation was achieved for both Nirmatrelvir and Ritonavir compounds. The retention time for Nirmatrelvir was found to be 2.303 minutes, while that of Ritonavir was 2.783 minutes. The peaks obtained for both compounds in the chromatogram were sharp and symmetrical.

The system suitability parameters, i.e., theoretical plate count, tailing factor, and resolution, were found to be within limits for both compounds. Hence, the chromatographic conditions for both compounds can be regarded as satisfactory [9,21].

The optimized RP-HPLC method was validated for its reliability in accordance with the guidelines of the International Council for Harmonization. All validation parameters for system suitability, specificity, linearity, precision, accuracy, robustness, limit of detection (LOD), and limit of quantification (LOQ) confirmed that the optimized RP-HPLC method is reliable and accurate for use in the estimation of Nirmatrelvir and Ritonavir in pharmaceutical formulations [11,20,21].

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Table 1. Optimized chromatographic conditions

Mobile phase	:	0.1% OPA: Acetonitrile (60:40)
Flow rate	:	1 ml/min
Column	:	Kromasil C8 (4.6 x 250mm, 5µm)
Detector wavelength	:	240 nm
Column temperature	:	30°C
Injection volume	:	10 µL
Run time	:	10min
Diluent	:	Water and Acetonitrile in the ratio 50:50

Results: Both peaks have good resolution, tailing Factor, theoretical plate count and resolution.

Observation: Nirmatrelvir and Ritonavir were eluted at 2.303 min and 2.783 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and validated.

Table 2: Linearity table for Nirmatrelvir and Ritonavir.

Nirmatrelvir		Ritonavir	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
3.75	352477	2.5	274254
7.5	724406	5	540959
11.25	1032151	7.5	827541
15	1434443	10	1094803
18.75	1781930	12.5	1376892
22.5	2108745	15	1632075

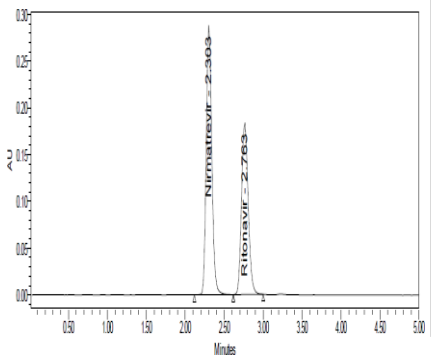

Figure 3: Optimized Chromatogram

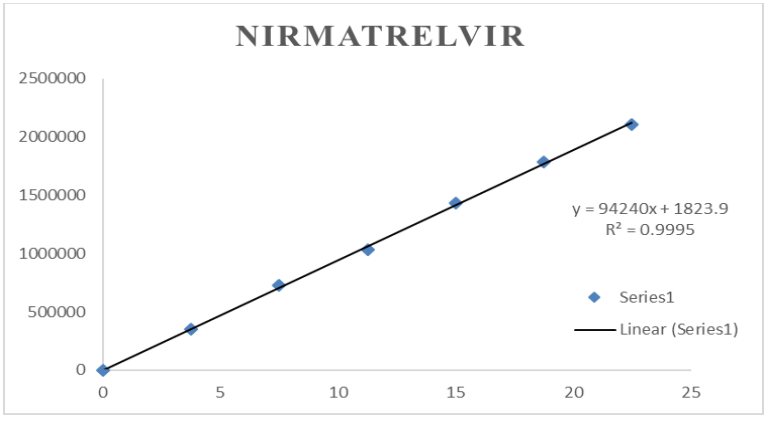

Figure 4: Calibration curve of Nirmatrelvir

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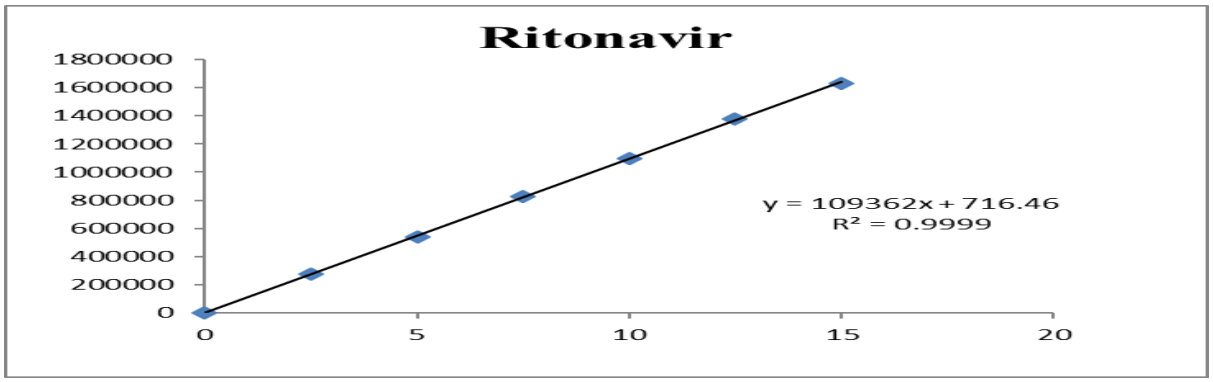

Figure 5: Calibration curve of Ritonavir

Table 3: System suitability parameters for Nirmatrelvir and Ritonavir

S no	Nirmatrelvir			Ritonavir			
Inje	RT (min)	USP Plate Count	Tailing	RT (min)	USP Plate Count	Tailing	Resolution
1	2.303	4842	1.15	2.763	4887	1.13	3.1
2	2.303	4797	1.15	2.766	4841	1.13	3.1
3	2.314	4816	1.13	2.777	4785	1.13	3.1
4	2.316	4951	1.13	2.778	4930	1.12	3.1
5	2.317	4616	1.13	2.779	4805	1.12	3.1
6	2.319	4873	1.15	2.782	4929	1.13	3.2

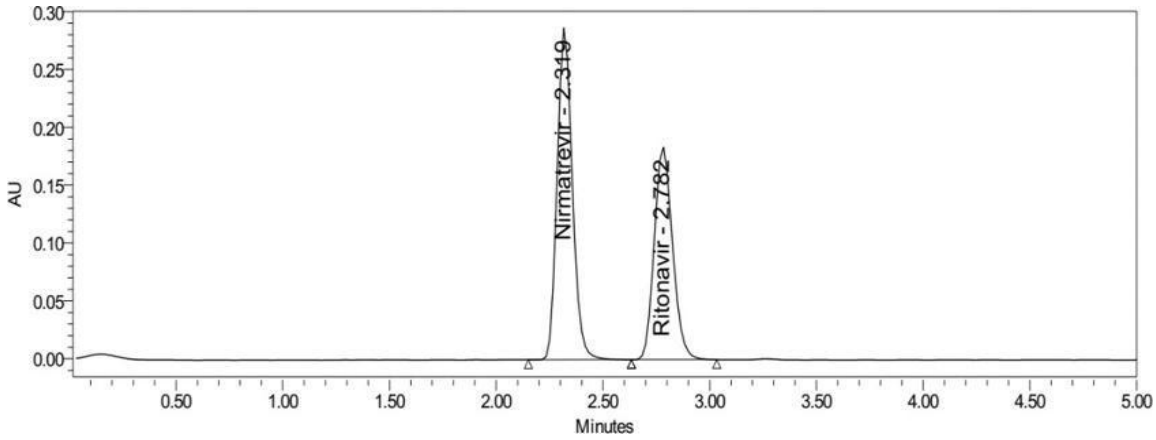

Figure 6: System suitability Chromatogram

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Precision: System Precision:

Table 4: System precision table of Nirmatrelvir and Ritonavir

S. No	Area of Nirmatrelvir	Area of Ritonavir
1.	1419602	1090699
2.	1442363	1094026
3.	1425608	1103442
4.	1412702	1098645
5.	1415829	1090155
6.	1417548	1094199
Mean	1422275	1095194
S. D	10745.4	5057.5
%RSD	0.8	0.5

Table 5: Repeatability table of Nirmatrelvir and Ritonavir

S. No	Area of Nirmatrelvir	Area of Ritonavir
1.	1413717	1092256
2.	1417831	1094219
3.	1390254	1098503
4.	1413157	1091148
5.	1390220	1097304
6.	1395996	1093189
Mean	1403529	1094437
S.D	12736.9	2895.9
%RSD	0.9	0.3

Table 6: Intermediate precision of Nirmatrelvir and Ritonavir

S. No	Area of Nirmatrelvir	Area of Ritonavir
1.	1401248	1093997
2.	1401048	1096187
3.	1409865	1096529
4.	1414345	1091644
5.	1393058	1090554
6.	1399123	1094191
Mean	1403115	1093850
S.D	7700.8	2387.7
%RSD	0.5	0.2

	Amount Spiked (µg/mL)	Amount recoverd (µg/mL)	% Recovery	Mean %Recovery
50%	7.5	7.50	99.97	99.63%
		7.49	99.84	
		7.48	99.79	
100%	15	14.96	99.71	
		14.90	99.35	
		14.93	99.55	
150%	22.5	22.43	99.71	
		22.29	99.05	
		22.43	99.67	

Table 7: Accuracy table of Nirmatrelvir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	5	4.99	99.8	99.70%
		4.99	99.8	
		5.03	100.6	
100%	10	9.94	99.4	
		9.98	99.8	
		9.95	99.5	
150%	15	14.91	99.4	
		14.90	99.4	
		14.94	99.6	

Table 8: Accuracy table of Ritonavir

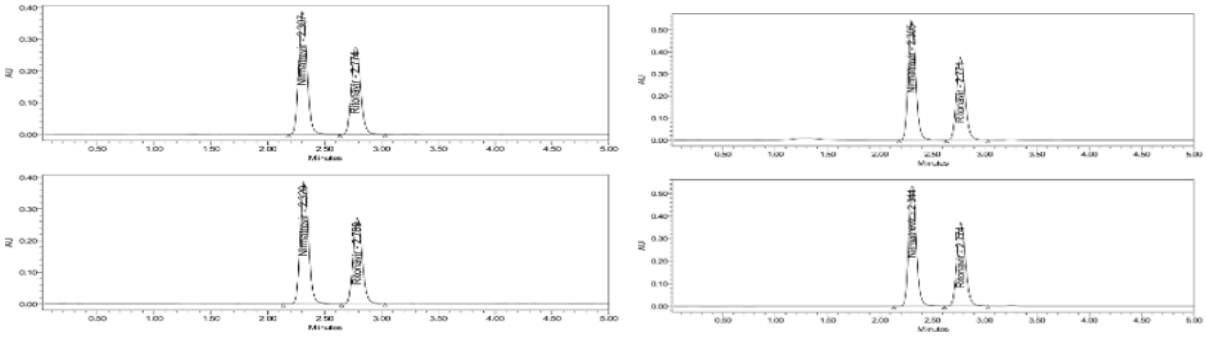

Figure 7: Accuracy 50% and 100% Chromatogram of Nirmatrelvir and Ritonavir

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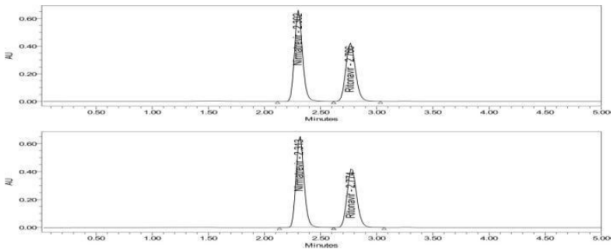

Figure 8: Accuracy 150% Chromatogram of Nirmatrelvir & Ritonavir

Molecule	LOD	LOQ
Nirmatrelvir	0.04	0.13
Ritonavir	0.01	0.02

Table 9: Sensitivity table of Nirmatrelvir and Ritonavir

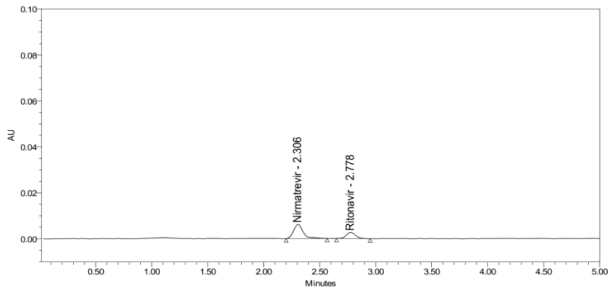

Figure 9: LOD Chromatogram of Standard

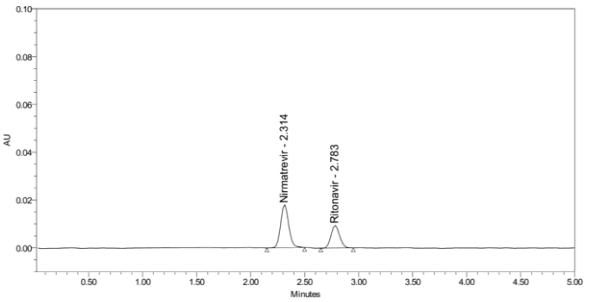

Figure 10: LOQ Chromatogram of Standard

S.no	Condition	%RSD of Nirmatrelvir	%RSD of Ritonavir
1	Flow rate (-) 0.9ml/min	0.7	0.9
2	Flow rate (+) 1.1ml/min	0.6	0
3	Mobile phase (-) 55B:45A	0.9	0.8
4	Mobile phase (+) 65B:35A	0.8	0.8
5	Temperature (-) 25°C	0.4	0.7
6	Temperature (+) 35°C	0.7	1.1

Table 10: Robustness data for Nirmatrelvir andRitonavir

S.no	Standard Area	Sample area	% Assay
1	1419602	1413717	99.30
2	1442363	1417831	99.59
3	1425608	1390254	97.65
4	1412702	1413157	99.26
5	1415829	1390220	97.65
6	1417548	1395996	98.05
Avg	1422275	1403529	98.58
Std Dev	10745.4	12736.9	0.89
%RSD	0.8	0.9	0.9

Table 11: Assay Data of Nirmatrelvir

Robustness of the developed method was evaluated by making small deliberate variations in chromatographic conditions such as flow rate (0.9 and 1.1 mL/min), mobile phase composition, and column temperature (25°C and 35°C). The samples were analyzed under these modified conditions, and the system suitability parameters were found to be within acceptable limits. The %RSD values obtained were less than 2%, indicating that the developed RP-HPLC method is robust.

Degradation studies: standards and degraded samples are injected and calculated the percentage of drug degraded in solution by applying different conditions like acid, alkali, and oxidative, photolytic, thermal and neutral analyze

Type of degradation	Nirmatrelvir			Ritonavir		
	Area	%Recovered	% Degraded	Area	%Recovered	% Degraded
Acid	1340586	94.16	5.84	1021855	93.21	6.79
Base	1362261	95.68	4.32	1042279	95.07	4.93

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Peroxide	1343040	94.33	5.67	1025372	93.53	6.47
Thermal	1393887	97.91	2.09	1065968	97.23	2.77
Uv	1402221	98.49	1.51	1076426	98.19	1.81
Water	1414868	99.38	0.62	1090370	99.46	0.54

Table 12: degradation data

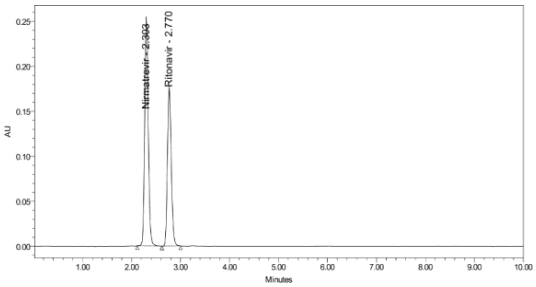

Figure 11: Acid

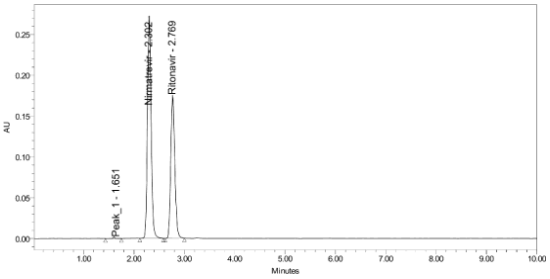

Figure 12: peroxide

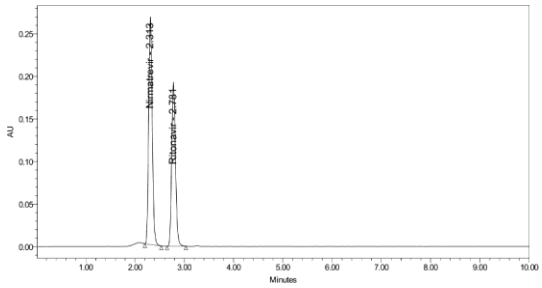

Figure 13: Thermal

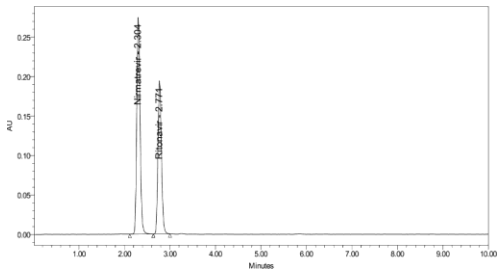

Figure 14: UV

Parameters		**Nirmatrelvir**	**Ritonavir**	**Limit**
Linearity Range (µg/ml)		3.75-22.5µg/ml	2.5-15µg/ml	R< 1
Regression coefficient		0.999	0.999	
Slope(m)		94240	109362	
Intercept(c)		1823.9	716.46	
Regression equation (Y=mx+c)		y = 94240x + 1823.9	y = 109362x + 716.46	
Assay (% mean assay)		98.58%	99.83%	90-110%
Specificity		Specific	Specific	No interference of any peak
System precision %RSD		0.8	0.5	NMT 2.0%
Method precision %RSD		0.9	0.3	NMT 2.0%
Accuracy %recovery		99.63%	99.70%	98-102%
LOD		0.04	0.01	NMT 3
LOQ		0.13	0.02	NMT 10
	FM	0.7	0.9	
	FP	0.6	0.5	
	MM	0.9	0.8	

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Robustness	**MP**	0.8	0.8	%RSD NMT 2.0
	TM	0.4	0.7	
	TP	0.7	1.1	

Table No.13: Summary Table

IV. Conclusion

A simple, Accurate, precise method was developed for the simultaneous estimation of the Nirmatrelvir and Ritonavir in Pharmaceutical dosage form. Retention time of Nirmatrelvir and Ritonavir were found to be 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 = 94240x + 1823.9, and y = 109362x + 716.46 of Ritonavir. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

This research work did not involve any human participants or animals. Hence ethical approval was not required.

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