

ARP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR
SIMULTANEOUS DETERMINATION OF NIRMATREL VIR AND
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ABSTRACT

For the quantitative measurement of Ritonavir and Nirmatrelvir in pharmaceutical dosage form, an easy quick, accurate, sensitive, and repeatable reverse phase high performance liquid chromatography approach has been devised. Ritonavir and Nirmatrelvir were separated chromatographically using a Waters Alliance-e2695 using a Luna Phenyl Hexyl column and a mobile phase that contained 40:60% v/v acetonitrile:ammonium acetate pH-3.0/OPA. A photodiode array detector operating at room temperature was used to measure the 1.0 ml/min flow rate by absorption at 266 nm. For Ritonavir and Nirmatrelvir, the number of theoretical plates and tailing factors were NLT 2000 and should not exceed 2, respectively. Every measurement's relative standard deviation of peak areas is consistently smaller than 2.0. ICH principles were followed in the validation of the suggested approach. For the quantitative analysis of Ritonavir and the stability research of Nirmatrelvir, the approach was determined to be straightforward, affordable, appropriate, exact, accurate, and reliable.

KEYWORDS: Ritonavir, Nirmatrelvir, RP-HPLC, Method Validation, and Tablet dosage form.**1. INTRODUCTION**

In pharmaceutical research and quality control, analytical technique development is essential to guaranteeing the consistency, safety, and effectiveness of pharmaceutical products. Reverse phase high-performance liquid chromatography (RP-HPLC) is a popular analytical method for quantitative pharmaceutical analysis because of its excellent resolution, sensitivity, accuracy, repeatability, and adaptability for complicated drug compositions. RP-HPLC techniques are widely used in pharmaceutical dosage form impurity profiling, stability testing, dissolution investigations, and routine quality control.^[1,2] To determine the dependability and appropriateness of the created technique for its intended use, analytical methods must be validated in accordance

with International Council for Harmonisation (ICH) principles.^[3]

Nirmatrelvir is a novel antiviral drug chemically described as (1*R*,2*S*,5*S*)-*N*-[(1*S*)-1-cyano-2-[(3*S*)-2-oxopyrrolidin-3-yl]ethyl]-3-[(2*S*)-3,3-dimethyl-2-[(2,2,2-trifluoroacetyl)amino]butanoyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexane-2-carboxamide, having the molecular formula $C_{23}H_{32}F_3N_5O_4$ and molecular weight 499.53 g/mol. It acts as a SARS-CoV-2 main protease inhibitor and is widely used in the treatment of COVID-19 infections by inhibiting viral replication.^[4,5] Ritonavir is chemically known as 1,3-thiazol-5-ylmethyl *N*-[(2*S*,3*S*,5*S*)-3-hydroxy-5-[(2*S*)-3-methyl-2-[[methyl-[(2-propan-2-yl-1,3-thiazolyl)methyl]carbonyl]amino]butanoyl]amino]-

1,6-diphenylhexan-2-yl]carbamate. It possesses the molecular formula $C_{37}H_{48}N_6O_5S_2$ with molecular weight 720.95 g/mol. Ritonavir is an antiretroviral protease inhibitor commonly used as a pharmacokinetic enhancer to increase the therapeutic effectiveness of co-administered antiviral agents through inhibition of cytochrome P450 enzymes.^[6,7]

A significant therapeutic approach for the treatment of COVID-19 infections is the fixed-dose combination of Ritonavir and Nirmatrelvir.^[8] Therefore, quality control and regulatory compliance depend on accurate and trustworthy simultaneous estimate of these medications in pharmaceutical dose forms. Only a few analytical techniques for the simultaneous estimation of Nirmatrelvir and Ritonavir have been described, according to a study of the literature.^[9,10] Many of the techniques that have been reported include complicated mobile phase compositions, prolonged retention durations, or a lack of thorough validation data.

Therefore, in accordance with ICH Q2(R1) validation criteria, the current work was conducted to design and verify a straightforward, quick, accurate, precise, inexpensive, and reliable RP-HPLC technique for the simultaneous measurement of Nirmatrelvir and Ritonavir in tablet dosage form. To determine the proposed method's appropriateness for regular pharmaceutical analysis, it was further assessed for system suitability, specificity, linearity, precision, accuracy, robustness, limit of detection, and limit of quantification.

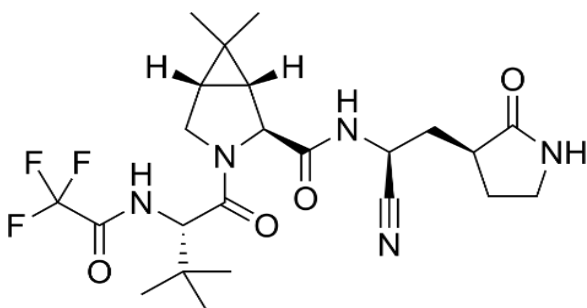


Figure 1: Molecular structure of Nirmatrelvir.

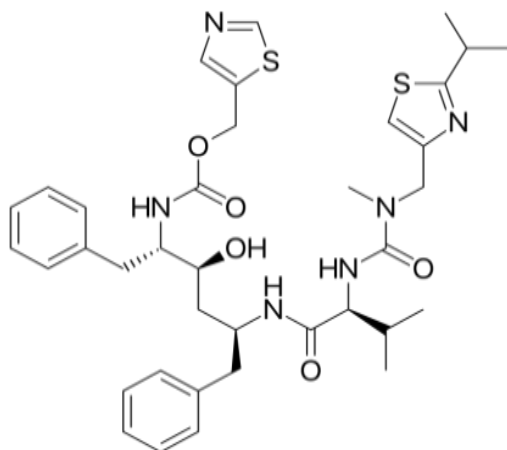


Figure 2: Molecular structure of Ritonavir.

2. MATERIALS AND METHODS

2.1 Materials

Instrumentation

The chromatographic analysis was carried out using a Waters Alliance e2695 HPLC system equipped with Empower 2.0 software, quaternary pump, autosampler, and photodiode array (PDA) detector. A Eutech pH meter was used for pH adjustment of buffer solutions. Analytical weighing was performed using a Sartorius analytical balance. UV spectral analysis was carried out using a Shimadzu UV-1700 UV-Visible spectrophotometer. Sonication was performed using a Unichrome UCA 701 ultrasonicator. All glassware such as pipettes, beakers, and burettes used during the experimental work were of Borosil make.

Chemicals and Reagents

Working standards of Ritonavir and Nirmatrelvir were procured from Zydus Cadila. HPLC grade acetonitrile and orthophosphoric acid were obtained from Rankem. HPLC grade Milli-Q water was prepared in-house and used throughout the study. Ammonium acetate of analytical reagent grade was used for preparation of buffer solution.

2.2 METHODOLOGY

2.2.1 Preparation of Solutions

Preparation of Buffer Solution

A quantity of 1.15 g of ammonium acetate was accurately weighed and dissolved in 1000 mL of HPLC grade water. The pH was adjusted to 3.0 using orthophosphoric acid. The prepared buffer solution was filtered through a 0.45 μ m membrane filter and degassed by sonication.

Preparation of Mobile Phase

The mobile phase was prepared by mixing acetonitrile and ammonium acetate buffer (pH 3.0 adjusted with OPA) in the ratio of 40:60 (% v/v). The solution was filtered through a 0.45 μ m membrane filter and degassed before use.

Preparation of Diluent

The mobile phase was used as the diluent throughout the study.

Preparation of Standard Solution

Accurately weighed 50 mg of Ritonavir and 75 mg of Nirmatrelvir working standards were transferred into a 100 mL volumetric flask. About 70 mL of diluent was added and sonicated to dissolve completely. The volume was then made up to the mark with diluent.

Further, 5 mL of the above stock solution was transferred into a 50 mL volumetric flask and diluted up to the mark with diluent to obtain a final concentration of 50 μ g/mL of Ritonavir and 75 μ g/mL of Nirmatrelvir.

Preparation of Sample Solution

Tablet powder equivalent to 102.5 mg of Ritonavir and 144 mg of Nirmatrelvir was accurately weighed and transferred into a 100 mL volumetric flask. Diluent was added and sonicated for 30 min to ensure complete extraction of drugs. The volume was made up to the mark with diluent and centrifuged for 30 min. The solution was filtered through a 0.45 µm syringe filter.

Further, 5 mL of the filtrate was transferred into a 50 mL volumetric flask and diluted up to the mark with diluent.

2.2.2 Determination of Detection Wavelength

The standard drug solution containing Ritonavir and Nirmatrelvir was scanned in the wavelength range of 200–400 nm using a PDA detector against blank mobile phase. Both drugs exhibited an isosbestic point at 266 nm, which was selected as the detection wavelength for simultaneous estimation.

Chromatographic Conditions

Chromatographic separation was achieved using a Luna Phenyl Hexyl column (250 mm × 4.6 mm, 5 µm particle size). The optimized mobile phase consisted of acetonitrile and ammonium acetate buffer (pH adjusted to 3.0 with orthophosphoric acid) in the ratio of 40:60 (% v/v). The flow rate was maintained at 1.0 mL/min with an injection volume of 10 µL. Detection was carried out at 266 nm using a PDA detector under ambient column temperature. The total run time was 10 min.

Method Development and Optimization

Several chromatographic trials were carried out using different mobile phase compositions and chromatographic conditions to obtain optimum separation with acceptable system suitability parameters. The optimized chromatographic conditions were shown in Table 1.

Table 1: Optimized Chromatographic Conditions.

Parameter	Optimized Condition
Column	Luna Phenyl Hexyl (250 × 4.6 mm, 5 µm)
Mobile Phase	Acetonitrile : Ammonium acetate buffer (40:60 v/v)
Flow Rate	1.0 mL/min
Detection Wavelength	266 nm
Injection Volume	10 µL
Run Time	10 min
Temperature	Ambient
Retention Time	Ritonavir: 2.1 min; Nirmatrelvir: 3.2 min

2.3. Method Validation

The developed RP-HPLC method for simultaneous estimation of Ritonavir and Nirmatrelvir was validated according to International Council for Harmonisation (ICH) Q2(R1) guidelines with respect to system suitability, specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ).^[11]

System Suitability

System suitability studies were performed by injecting six replicates of standard solution into the chromatographic system prior to sample analysis. Parameters such as retention time, theoretical plates, tailing factor, resolution, and percentage relative standard deviation (%RSD) of peak areas were evaluated to verify the performance of the chromatographic system.^[11,12] The acceptance criteria for system suitability were that the tailing factor should not exceed 2.0, theoretical plates should not be less than 2000, resolution between adjacent peaks should be greater than 2.0, and %RSD for peak areas should not exceed 2.0%.

Specificity

Specificity of the developed method was evaluated by comparing chromatograms obtained from blank, standard, and sample solutions in order to determine any possible interference at the retention times of Ritonavir and Nirmatrelvir.^[11,13] The absence of interfering peaks

confirmed the specificity of the developed RP-HPLC method.

Linearity

Linearity of the method was established by preparing calibration solutions at six concentration levels ranging from 12.5–75 µg/mL for Ritonavir and 18.75–112.5 µg/mL for Nirmatrelvir. Each concentration level was injected into the HPLC system and corresponding peak areas were recorded. Calibration curves were constructed by plotting concentration versus peak area, and regression analysis was carried out to determine the correlation coefficient (r^2).^[11,14] The method was considered linear when the correlation coefficient was not less than 0.999.

Accuracy

Accuracy of the developed method was determined by recovery studies using the standard addition method at 50%, 100%, and 150% concentration levels.^[11,12] Known quantities of standard drugs were added to pre-analyzed sample solutions and analyzed in triplicate. The percentage recovery values were calculated, and the method was considered accurate when the recoveries were found within the acceptable range of 98–102%.

Precision

Precision of the method was evaluated in terms of system precision, method precision, and intermediate

precision.^[11,14] System precision was determined by repeated injection of standard solution, whereas method precision was assessed by analyzing six independent sample preparations under the same operating conditions. Intermediate precision was evaluated on different days. The precision of the method was expressed as percentage relative standard deviation (%RSD), and values not exceeding 2.0% indicated good precision of the developed method.

Robustness

Robustness of the method was evaluated by introducing deliberate minor variations in chromatographic conditions such as flow rate and mobile phase composition.^[11,13] The effect of these changes on system suitability parameters and assay values was studied to determine the reliability of the method under normal usage conditions.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the response (σ) and slope of the calibration curve (S) according to ICH guidelines.^[11]

$$\text{LOD} = 3.3 \times \sigma / S$$

$$\text{LOQ} = 10 \times \sigma / S$$

The LOD and LOQ values obtained for Ritonavir were 0.15 $\mu\text{g/mL}$ and 0.5 $\mu\text{g/mL}$, respectively, while for Nirmatrelvir the values were found to be 0.225 $\mu\text{g/mL}$ and 0.75 $\mu\text{g/mL}$, respectively. The obtained values indicated adequate sensitivity of the developed RP-HPLC method.

2.4. DEGRADATION STUDIES

Preparation of stock: Accurately weigh and transfer 102.5mg of Ritonavir and 144mg Nirmatrelvir sample into a 100 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Acid degradation

Pipette 5 ml of the a fore mentioned solution was added to a 50 ml vacuum flask, followed by 1 ml of 1N HCl. The vacuum flask was then maintained at 60°C for 1 hour before being neutralised with 1 N NaOH and diluted to 50ml with diluent. Filter the solution using 0.22 micron syringe filters and transfer to bottles.

Alkali degradation

Pipette 5 ml of above solution into a 50ml volumetric flask and add 1ml of 1N NaOH was added. Then, the volumetric flask was kept at 60°C for 1 hour and then neutralized with 1N HCl and make up to 50ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Thermal degradation

Ritonavir and Nirmatrelvir sample was taken in petridish and kept in Hot air oven at 105°C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Peroxide degradation

Pipette 5 ml above stock solution was added to a 50 ml vacuum flask, 1 ml of 3 percent w/v hydrogen peroxide was added to the flask and the volume was built up to the mark using diluent. The vacuum flask was then maintained at 60°C for 1 hour. After that, the vacuum flask was left at room temperature for 15 minutes. Filter the solution using 0.22 micron syringe filters and transfer to bottles.

Reduction degradation

Pipette 5ml of above-stock solution was added to a 50ml vacuum flask, 1ml of 10% Sodium bisulphate was added to a flask and the volume was built up to the required volume with diluent. The vacuum flask was then maintained at 60°C for 1 hour. After that, the vacuum flask was left at room temperature for 15 minutes. Filter the solution using 0.22 micron syringe filters and transfer to bottles.

Photolytic degradation

Ritonavir and Nirmatrelvir sample was placed in sun light for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Hydrolysis degradation

Pipette 5ml of above-stock solution was added to a 50ml vacuum flask, 1ml of HPLC grade water was added to a flask and the volume was built up to the required volume with diluent. The vacuum flask was then maintained at 60°C for 1 hour. After that, the vacuum flask was left at room temperature for 15 minutes. Filter the solution using 0.22 micron syringe filters and transfer to bottles.

3. RESULTS & DISCUSSION

3.1 Method Development

3.1.1 Determination of Detection Wavelength

The wavelength selection for simultaneous estimation of Ritonavir and Nirmatrelvir was carried out by scanning the standard drug solutions in the wavelength range of 200–400 nm using a PDA detector against the mobile phase as blank. The UV spectra of both drugs exhibited an isosbestic point at 266 nm, indicating equal absorptivity of both analytes at this wavelength. Therefore, 266 nm was selected as the optimum detection wavelength for the developed RP-HPLC method as it provided adequate sensitivity and suitable response for both drugs.

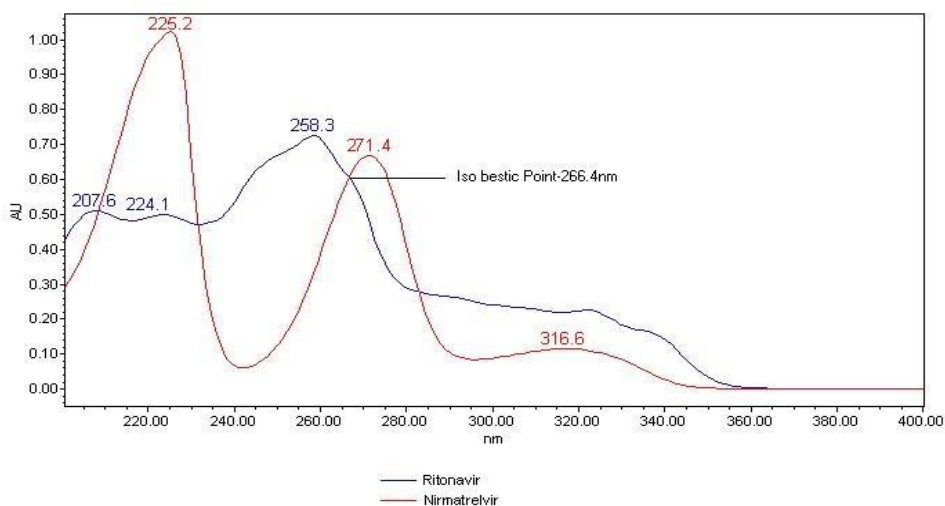


Figure 3: PDA spectra of Ritonavir and Nirmatrelvir.

3.1.2 Optimized Chromatographic Conditions

Final optimization was achieved using a Luna Phenyl Hexyl column (250 mm × 4.6 mm, 5 μm) with a mobile phase consisting of Acetonitrile and Ammonium acetate buffer pH 3.0 adjusted with OPA in the ratio of 40:60 v/v. The developed chromatographic method produced sharp, symmetrical, and well-resolved peaks for both analytes with acceptable retention times and excellent system suitability parameters.

The retention times of Ritonavir and Nirmatrelvir were found to be 2.124 min and 3.211 min, respectively. Theoretical plate counts were significantly higher than the acceptable limit of 2000, and tailing factors were below 2.0, indicating good column efficiency and peak symmetry. The chromatographic resolution between the two peaks was satisfactory, confirming adequate separation of both analytes.

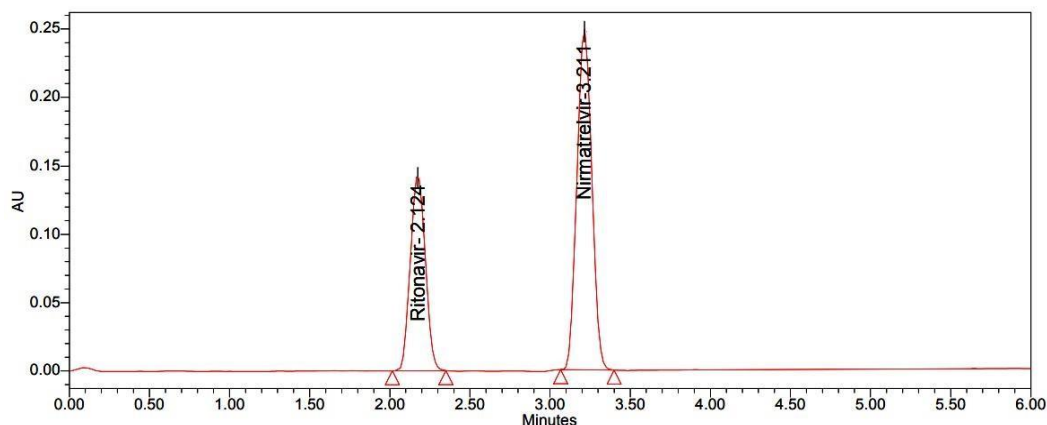


Figure 4: Optimized Chromatogram.

Table 2: Results of Optimized Chromatographic Conditions

S.No	Name of the drug	Retention Time	Area	USP Plate Count	USP Tailing	USP Resolution
1	Ritonavir	2.124	1282087		0.86	9597
2	Nirmatrelvir	3.211	1837754	5.84	1.02	12567

The optimized chromatographic conditions fulfilled all system suitability requirements. Therefore, the developed RP-HPLC method was considered suitable for further validation studies according to ICH guidelines.

3.2 METHOD VALIDATION

The developed RP-HPLC method for simultaneous estimation of Ritonavir and Nirmatrelvir was validated according to ICH Q2(R1) guidelines with respect to system suitability, specificity, assay, linearity, accuracy,

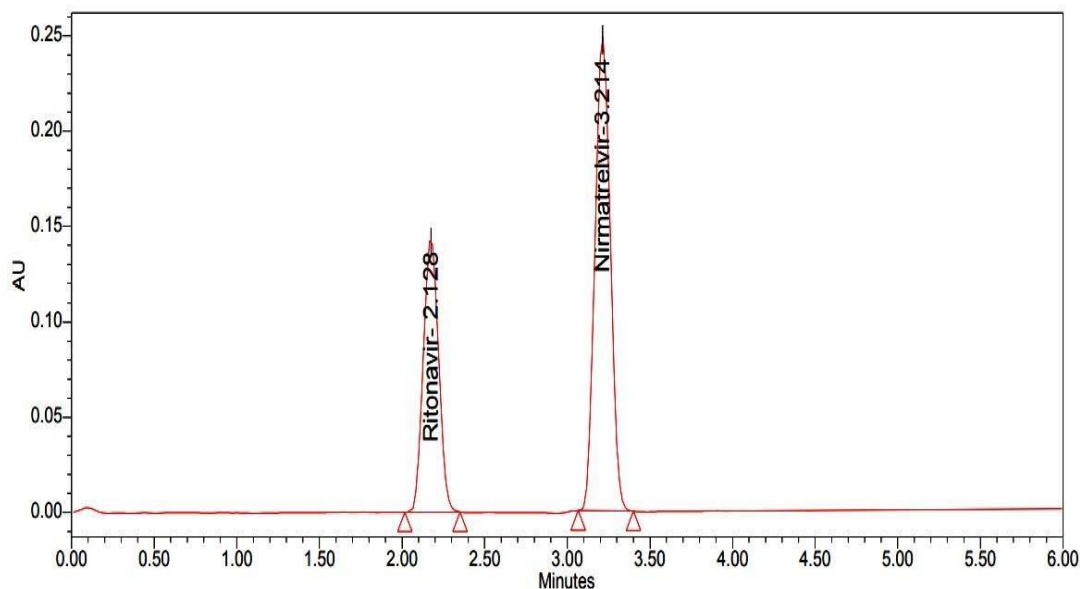
precision, robustness, limit of detection (LOD), and limit of quantification (LOQ).

System Suitability

System suitability studies were carried out by injecting six replicate injections of standard solution into the chromatographic system. Parameters such as retention time, theoretical plate count, tailing factor, resolution, and percentage relative standard deviation (%RSD) were evaluated to verify the performance of the developed method.

Table 3: System suitability parameters for Ritonavir & Nirmatrelvir

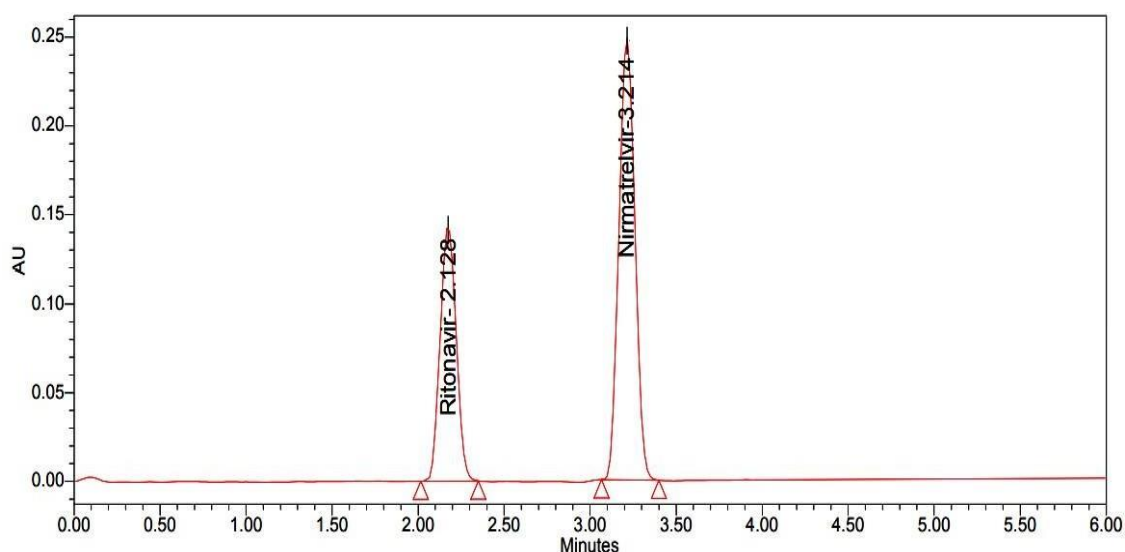
Parameter	Ritonavir	Nirmatrelvir
Retention time (min)	2.128	3.214
USP Plate count	9597	12563
USP Tailing factor	0.86	1.02
USP Resolution	—	5.81
%RSD	0.34	0.35

**Figure 5: Chromatogram of standard solution.**

The obtained system suitability results demonstrated excellent chromatographic performance with satisfactory retention, good peak symmetry, high column efficiency, and acceptable resolution between analyte peaks. The theoretical plate counts for both drugs were greater than 2000, tailing factors were below 2.0, and resolution was greater than 2.0, confirming that the developed method complied with ICH acceptance criteria.

Assay of Marketed Formulation

The developed RP-HPLC method was successfully applied for quantitative estimation of Ritonavir and Nirmatrelvir in tablet dosage form. The assay results indicated that the drug contents were within acceptable limits of labeled claim, demonstrating suitability of the method for routine pharmaceutical analysis.

**Fig. 6: Chromatogram of Assay-1.**

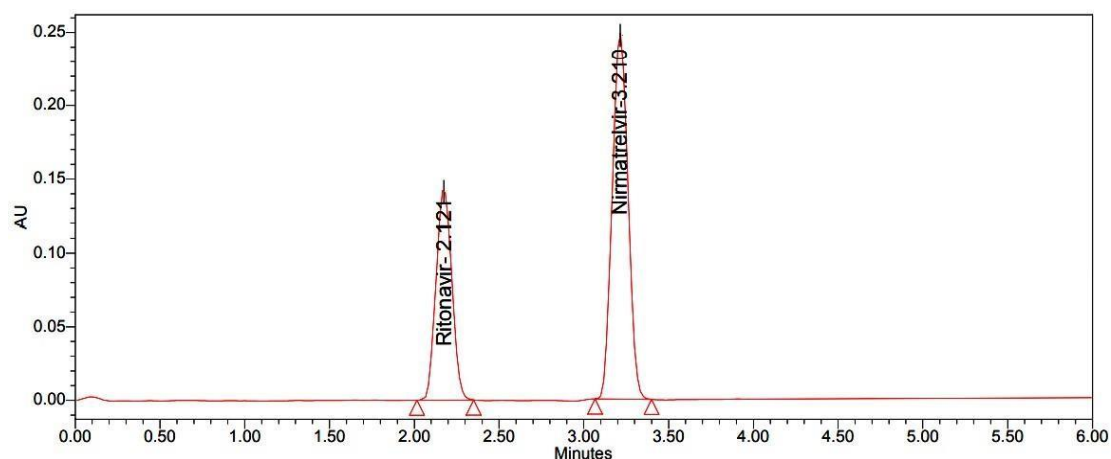


Fig. 7: Chromatogram of Assay-2.

Table 4: Assay Results of Ritonavir and Nirmatrelvir.

Drug	Avg sample area (n=5)	Std. Conc. (µg/ml)	Sample Conc. (µg/ml)	Label amount (mg)	Std purity	Amount found (µg/ml)	% assay
Ritonavir	1279401	50	50	100	99.8	49.77	99.5
Nirmatrelvir	1831224	75	75	150	99.9	74.86	99.8

The assay results confirmed that the developed method was accurate, reproducible, and suitable for routine quality control analysis of pharmaceutical dosage forms containing Ritonavir and Nirmatrelvir.

Specificity

Specificity of the developed method was evaluated by comparing chromatograms of blank, placebo, and standard solutions to determine any interference at the retention times of Ritonavir and Nirmatrelvir.

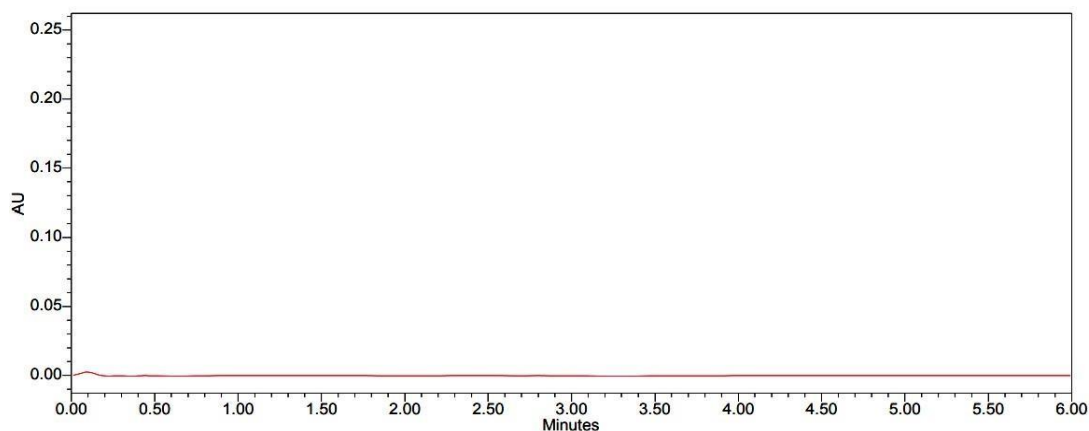


Figure 8: Chromatogram of blank.

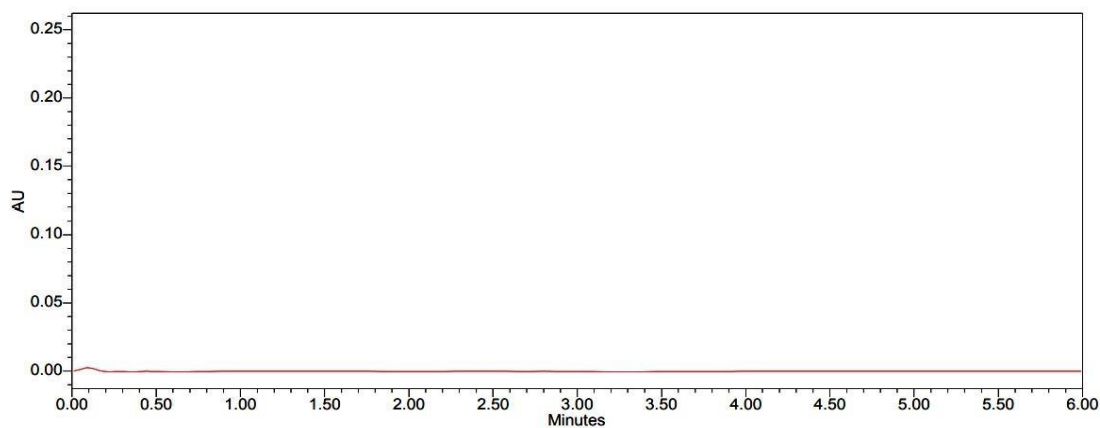


Figure 9: Chromatogram of placebo.

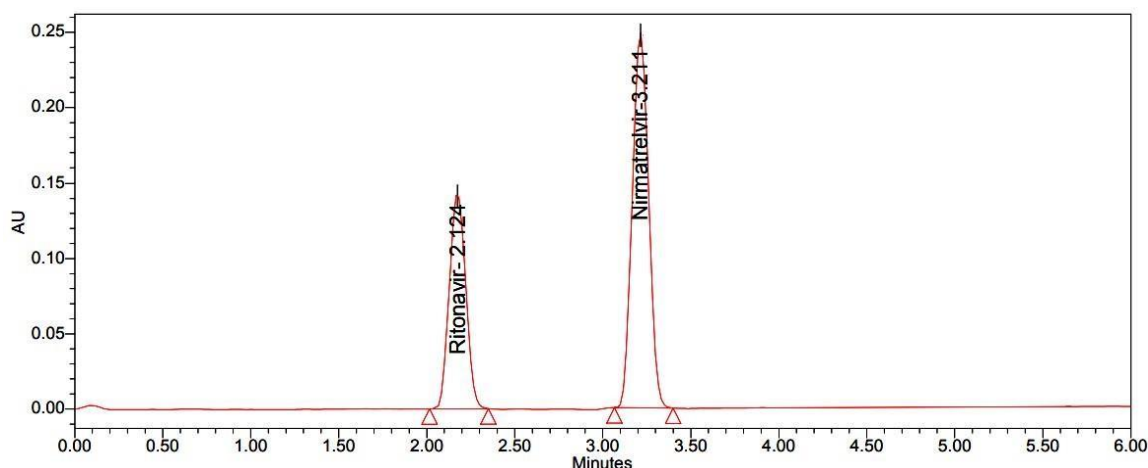


Figure 10: Optimized chromatogram.

No interfering peaks were observed at the retention times of Ritonavir (2.124 min) and Nirmatrelvir (3.211 min) in blank and placebo chromatograms. The obtained results demonstrated that the developed RP-HPLC method was highly specific for simultaneous estimation of both analytes without interference from excipients or solvent components.

Linearity

Linearity of the developed RP-HPLC method was evaluated by analyzing six concentration levels ranging from 12.5–75 µg/mL for Ritonavir and 18.75–112.5 µg/mL for Nirmatrelvir. Each concentration level was injected in duplicate and calibration curves were constructed by plotting concentration against peak area.

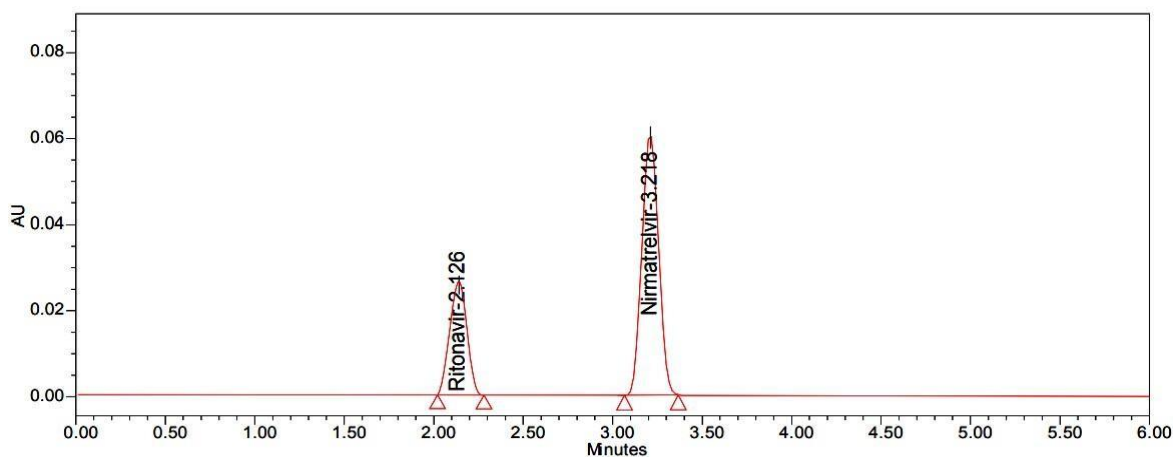


Figure 11: Chromatogram of Linearity-25%.

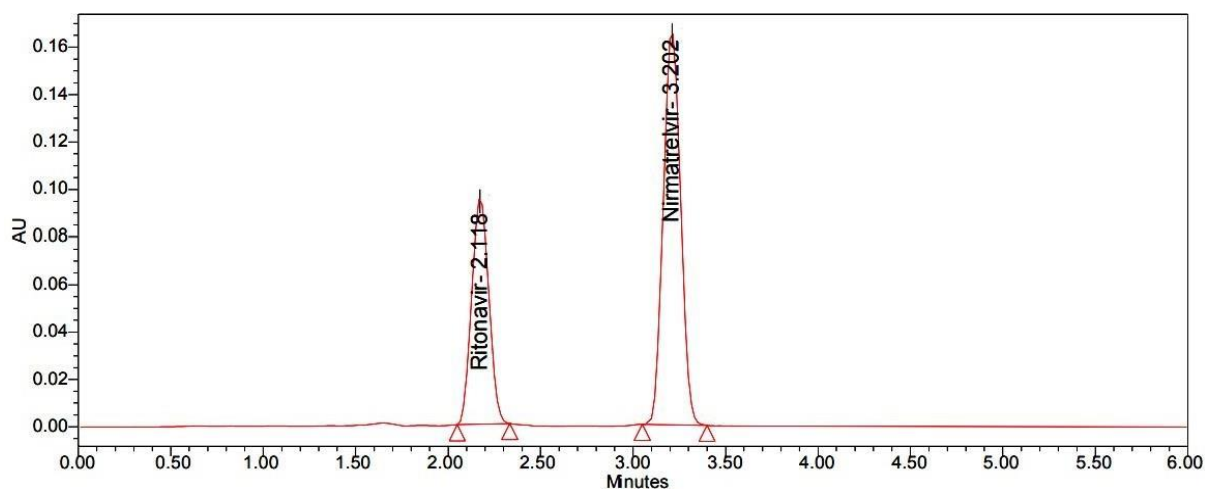


Figure 12: Chromatogram of Linearity-50%.

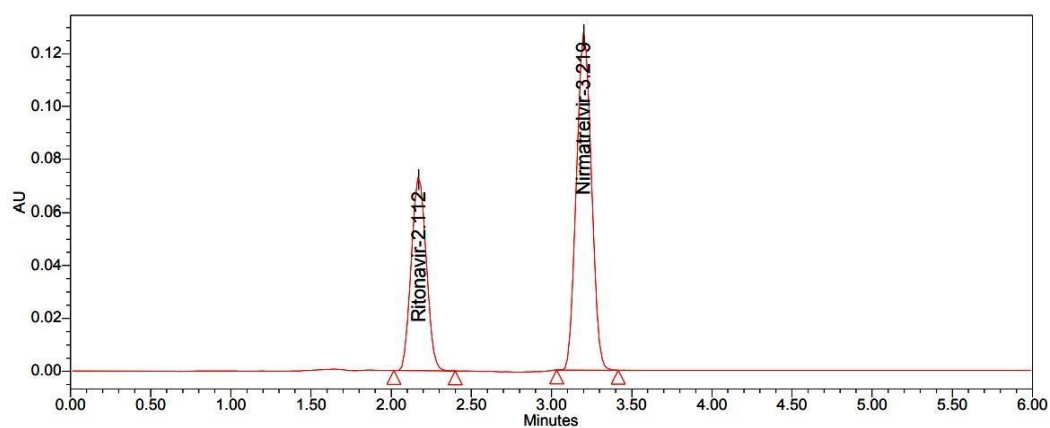


Figure 13: Chromatogram of Linearity-75%.

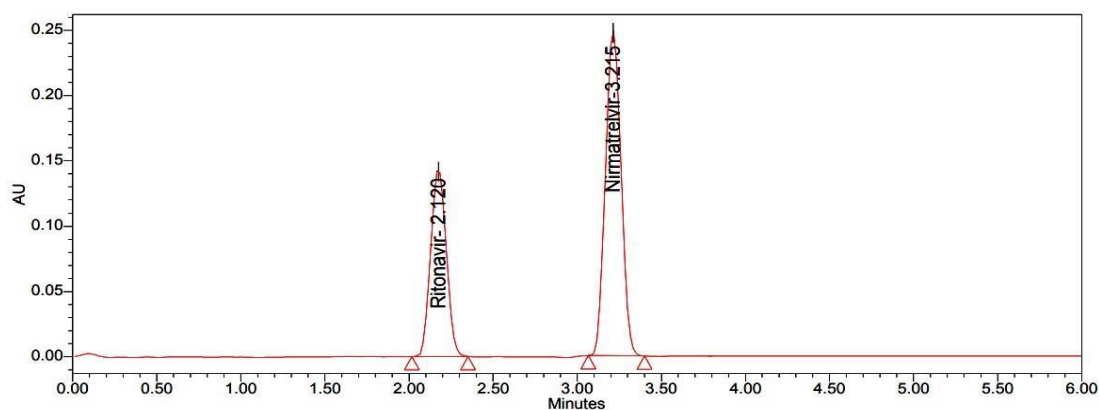


Figure 14: Chromatogram of Linearity-100%.

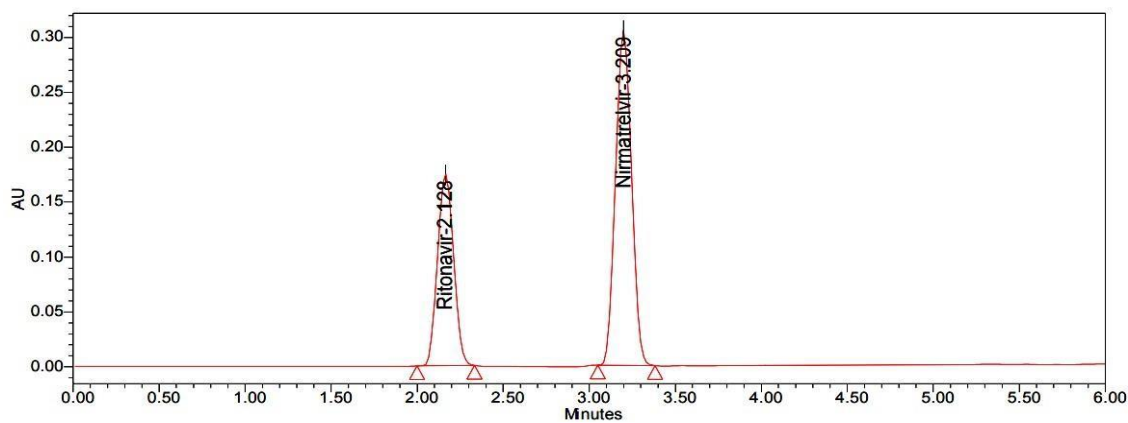


Figure 15: Chromatogram of Linearity-125%.

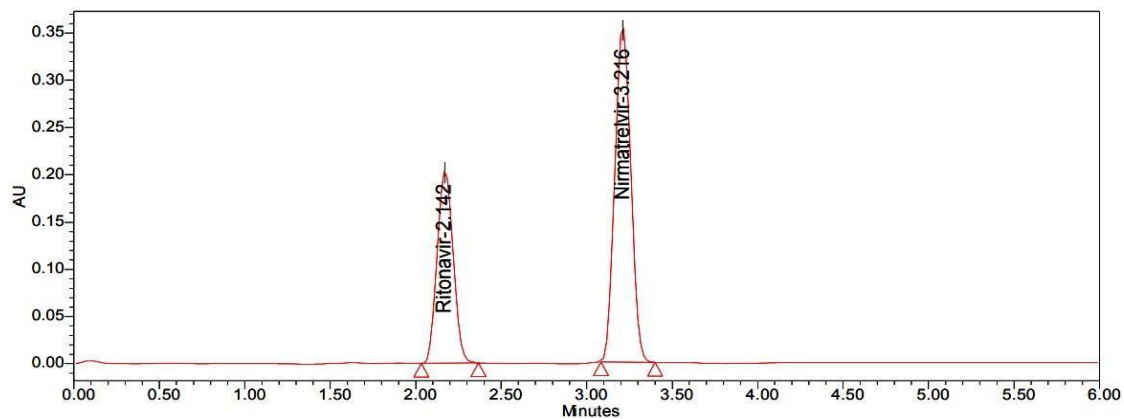


Figure 16: Chromatogram of Linearity-150%.

Table 5: Linearity Results for Ritonavir and Nirmatrelvir.

Ritonavir Conc (µg/mL)	Peak Area	Nirmatrelvir Conc (µg/mL)	Peak Area
12.50	346894	18.75	451715
25.00	633878	37.50	933987
37.50	908996	56.25	1377973
50.00	1267065	75.00	1824341
62.50	1575456	93.75	2247487
75.00	1891457	112.50	2762107
Regression equation	$y = 25041.95x + 7176.36$	Regression equation	$y = 24320.42x + 3063.68$
Correlation coefficient (R²)	0.99959	Correlation coefficient (R²)	0.99980

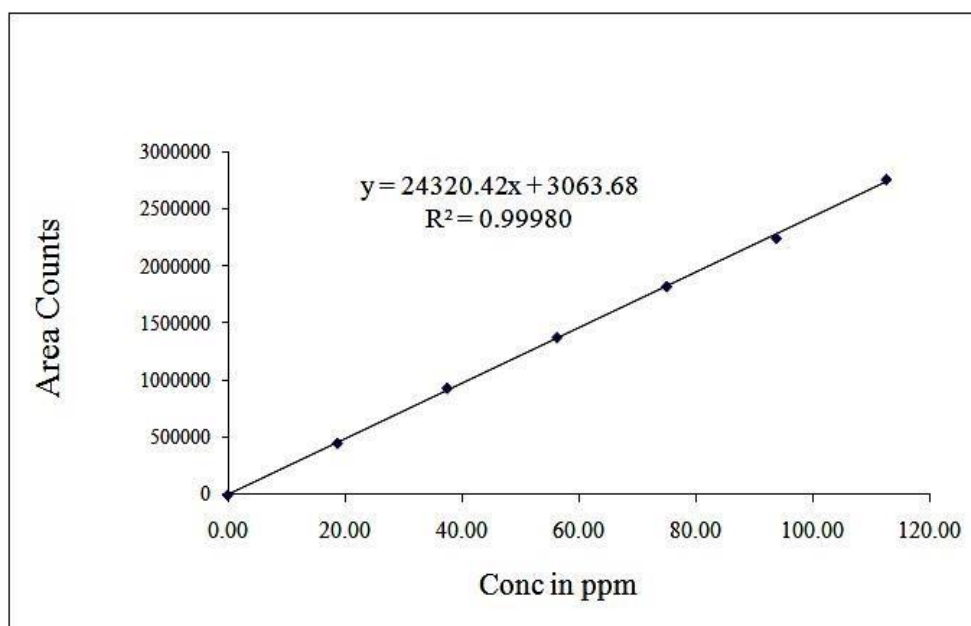


Figure 17: Calibration curve for Nirmatrelvir.

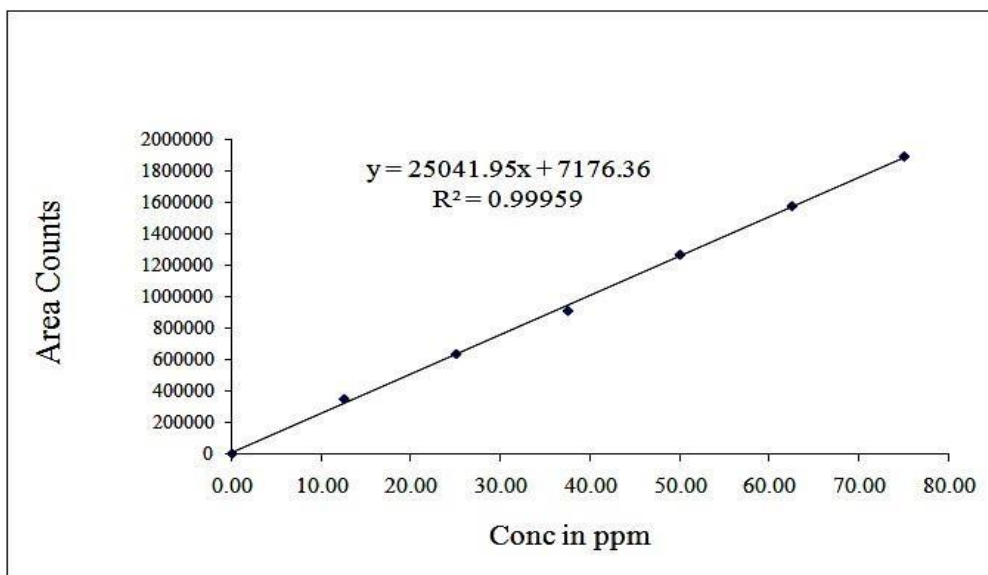


Figure 18: Calibration curve for Ritonavir.

The calibration curves showed excellent linearity over the selected concentration ranges with correlation coefficient values greater than 0.999 for both analytes. The obtained regression data indicated a strong linear relationship between concentration and peak area, confirming the suitability of the method for quantitative analysis.

ACCURACY

Accuracy of the developed method was determined by recovery studies using the standard addition technique at 50%, 100%, and 150% concentration levels.

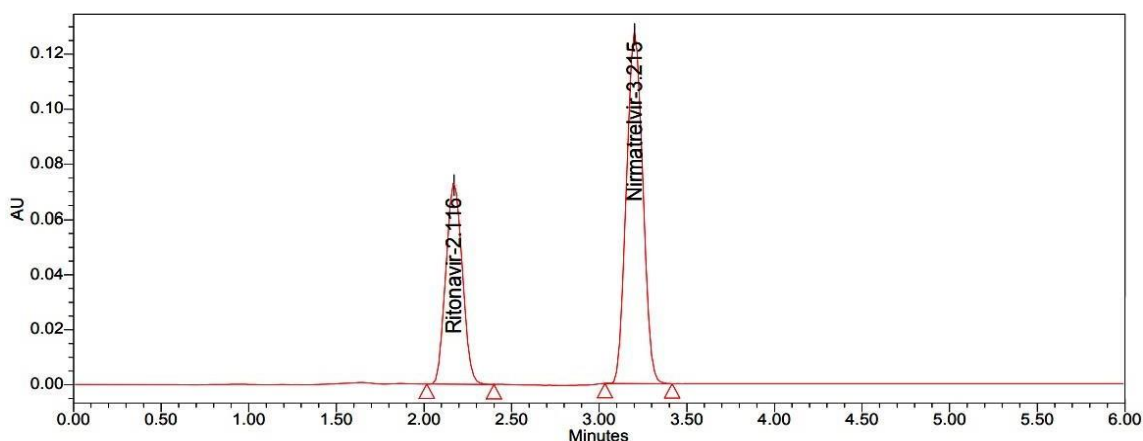


Figure 19: Chromatogram of Accuracy 50%.

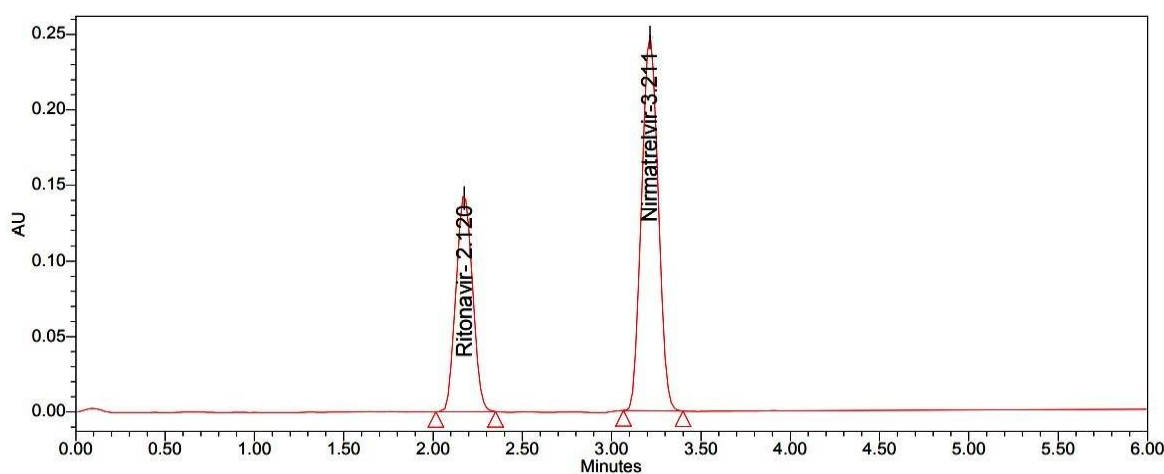


Figure 20: Chromatogram of Accuracy 100%.

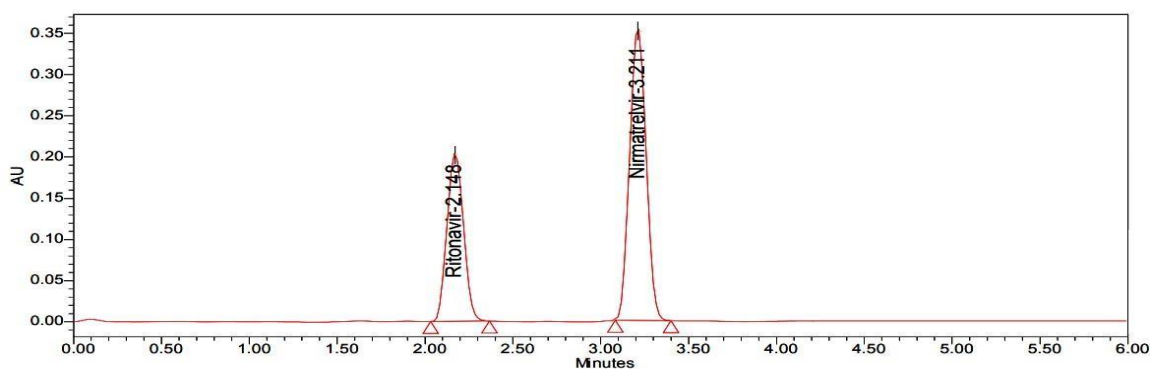


Figure 21: Chromatogram of Accuracy 150%.

Table 6: Accuracy results of Ritonavir by RP-HPLC method.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	637441	25	24.79	99.2	99.5
100%	1294821	50	50.37	100.7	
150%	1902365	75	74.0	98.7	

Table 7: The Accuracy results for Nirmatrelvir by RP-HPLC method.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	918139	37.5	37.53	100.1	100.3
100%	1854035	75.0	75.79	101.1	
150%	2745321	112.5	112.22	99.8	

The percentage recoveries obtained for both drugs were within the acceptable range of 98–102%, indicating good accuracy of the developed RP-HPLC method. The results also confirmed the absence of interference from formulation excipients during analysis.

PRECISION

System Precision:

System precision was evaluated by six replicate injections of standard solution under the same chromatographic conditions.

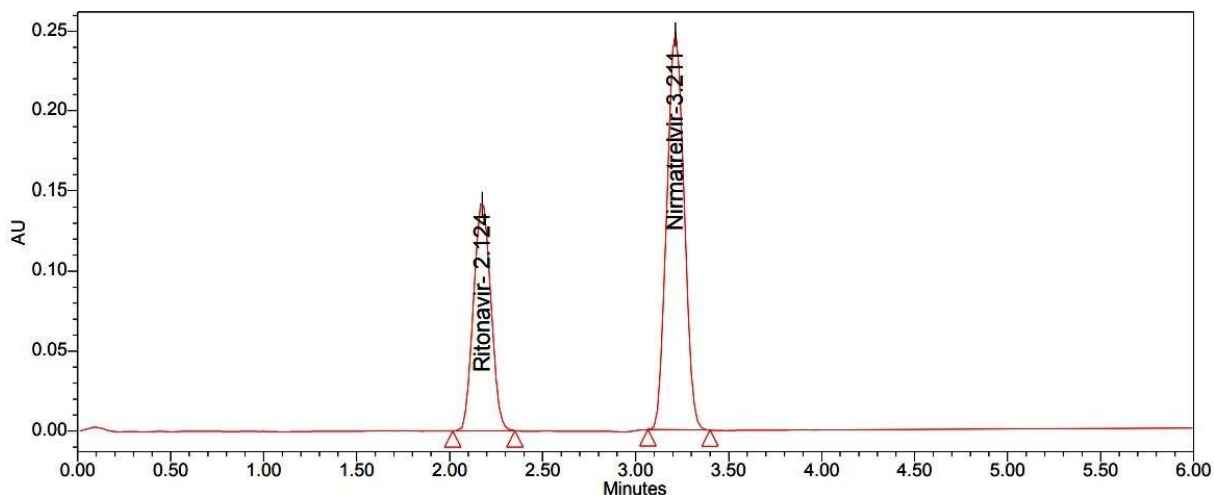


Figure 22: System precision chromatogram-1.

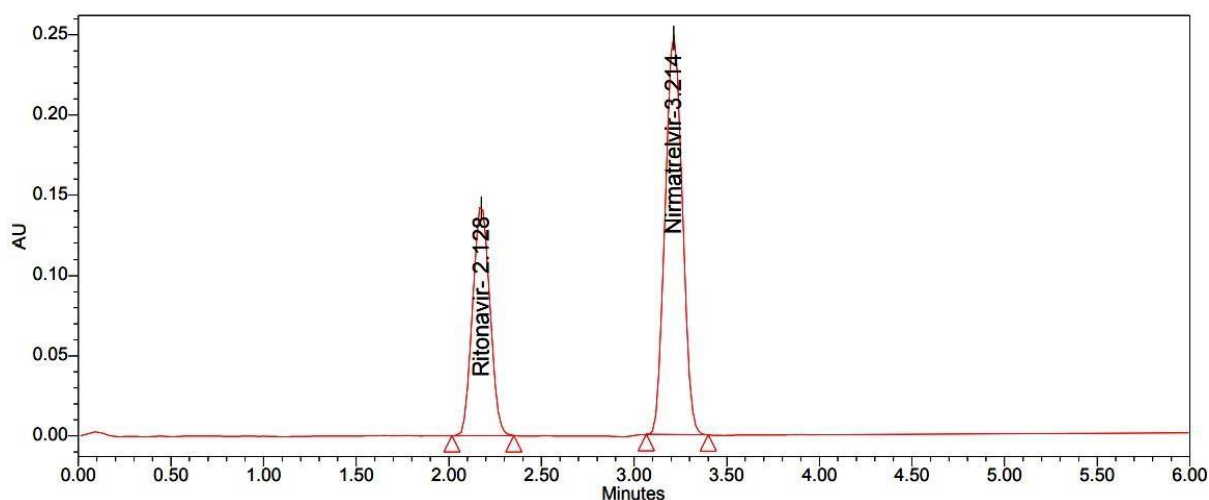


Figure 23: System precision chromatogram-2.

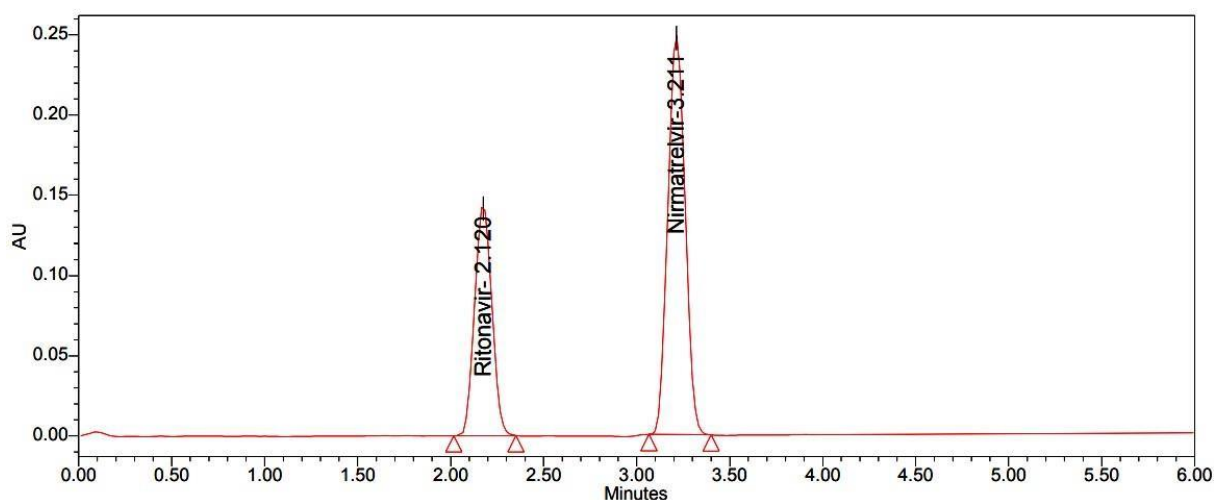


Figure 24: System precision chromatogram-3.

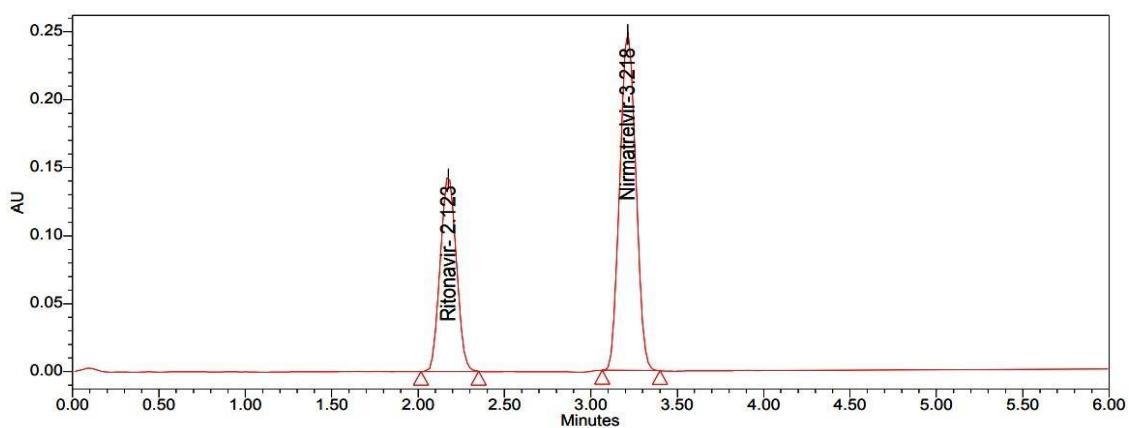


Figure 25: System precision chromatogram-4.

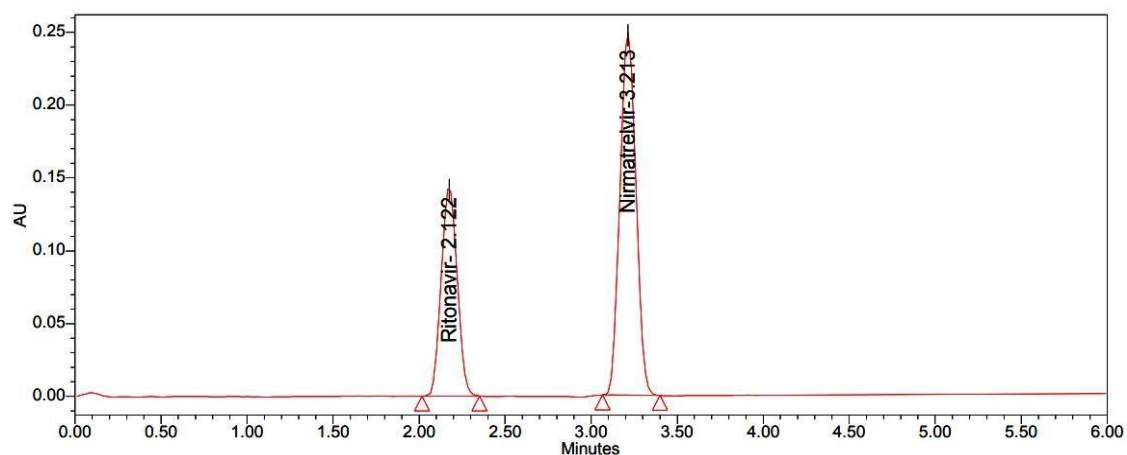


Figure 26: System precision chromatogram-5.

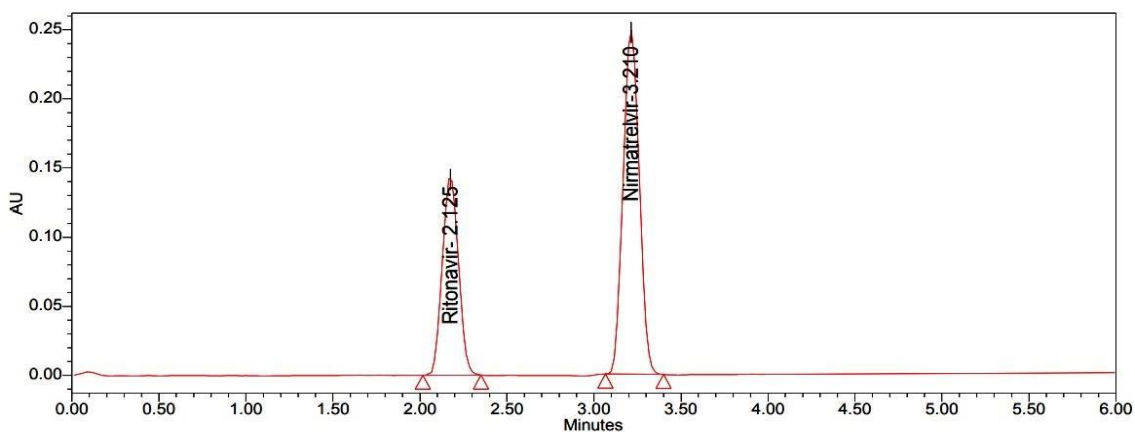


Figure 27: System precision chromatogram-6.

Table 8: System precision results of Ritonavir & Nirmatrelvir.

S. No	Concentration Ritonavir (µg/ml)	Area of Ritonavir	Concentration of Nirmatrelvir (µg/ml)	Area of Nirmatrelvir
1.	50	1282087	75	1837754
2.	50	1286345	75	1836268
3.	50	1288296	75	1827592
4.	50	1291238	75	1828449
5.	50	1278961	75	1833596
6.	50	1285673	75	1844785
Mean		1285433		1834741
S.D.		4378.74		6392.19
%RSD		0.34		0.35

The %RSD values obtained for both analytes were less than 2.0%, demonstrating excellent performance and repeatability of the chromatographic system.

Method Precision

Method precision was evaluated by analyzing six independently prepared sample solutions at the same concentration level.

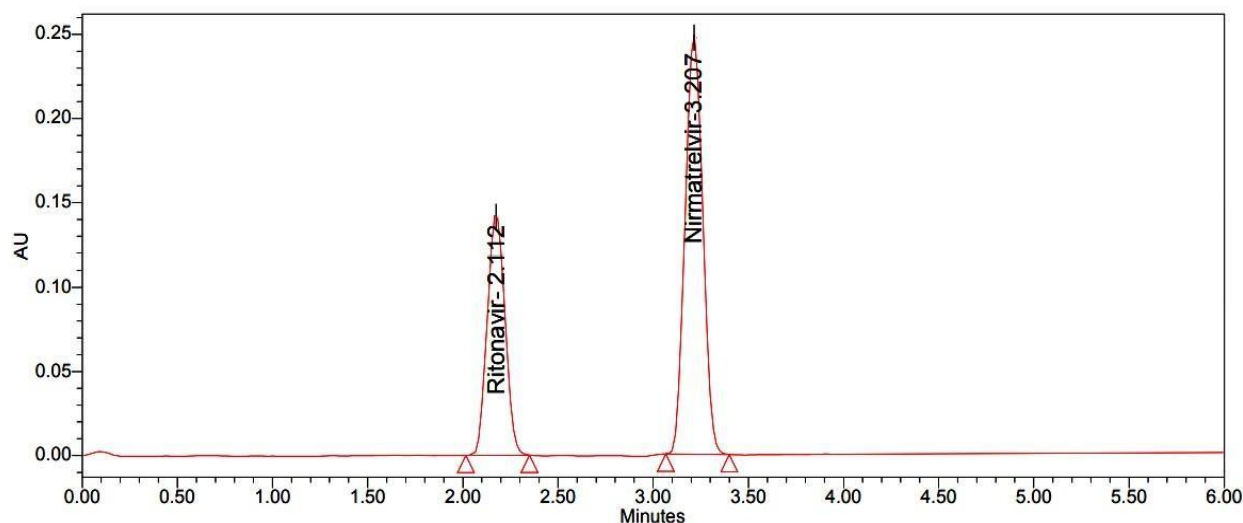


Figure 28: Repeatability chromatogram-6.

Table No. 9: Method Precision for Ritonavir & Nirmatrelvir by RP-HPLC method.

S. No.	Area for Ritonavir	Area for Nirmatrelvir
1	1276798	1819274
2	1274326	1809553
3	1293870	1864189
4	1264856	1829510
5	1280661	1844115
6	1274795	1813067
Average	1277551	1829951
Standard Deviation	9546.812	20919.848
%RSD	0.75	1.14

The %RSD values for Ritonavir and Nirmatrelvir were found to be 0.75% and 1.14%, respectively, indicating satisfactory repeatability and precision of the developed analytical method.

Intermediate precision (Day - Day Precision)

Intermediate precision was evaluated by performing the analysis on two different days under the same experimental conditions.

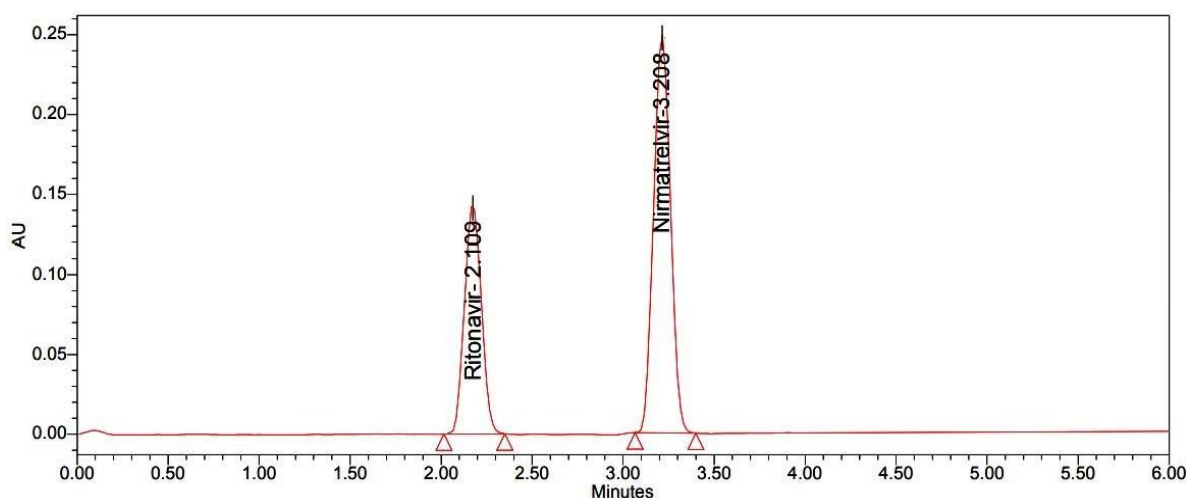


Figure 29: Inter day precision chromatogram-6 (Day-1).

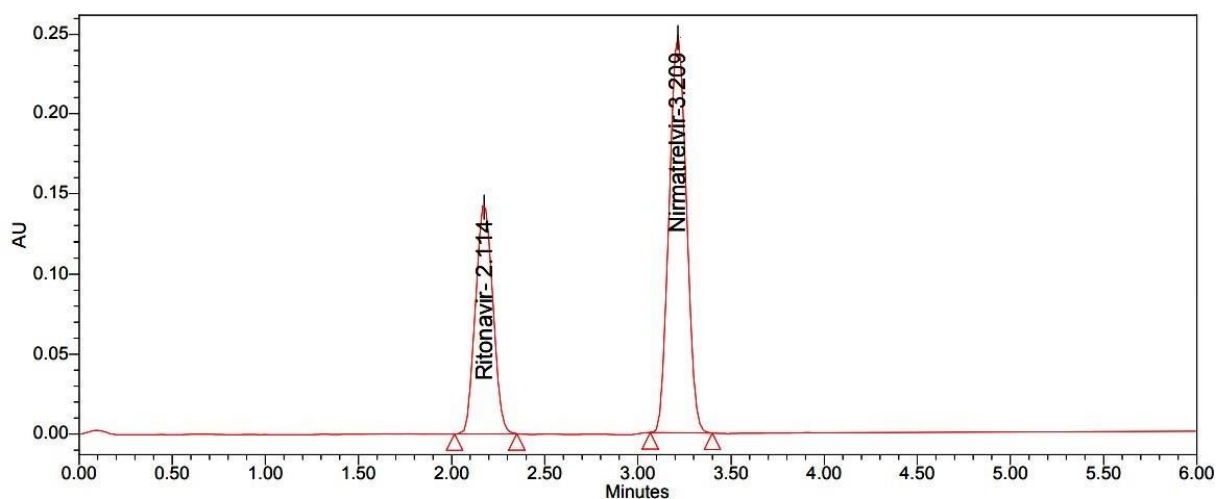


Figure 30: Inter day precision chromatogram-6 (Day-2).

Table No. 10: Intermediate Precision (Day variation) for Ritonavir and Nirmatrelvir by RP-HPLC Method.

S. No.	Area for Ritonavir		Area for Nirmatrelvir	
	Day-1	Day-2	Day-1	Day-2
1	1277865	1284522	1821234	1826854
2	1293672	1280359	1811328	1840198
3	1295429	1292647	1825384	1815200
4	1281430	1276231	1813784	1829631
5	1264778	1269830	1819128	1834658
6	1299872	1288623	1852354	1831046
Average	1285508	1282035	1823869	1829598
Standard Deviation	13237.674	8338.572	14846.512	8424.884
%RSD	1.03	0.65	0.81	0.46

The %RSD values obtained during inter-day precision studies were below 2.0%, confirming good intermediate precision and reproducibility of the developed RP-HPLC method.

ROBUSTNESS

Robustness studies were performed by making deliberate small variations in chromatographic conditions such as flow rate and mobile phase composition to evaluate the reliability of the method.

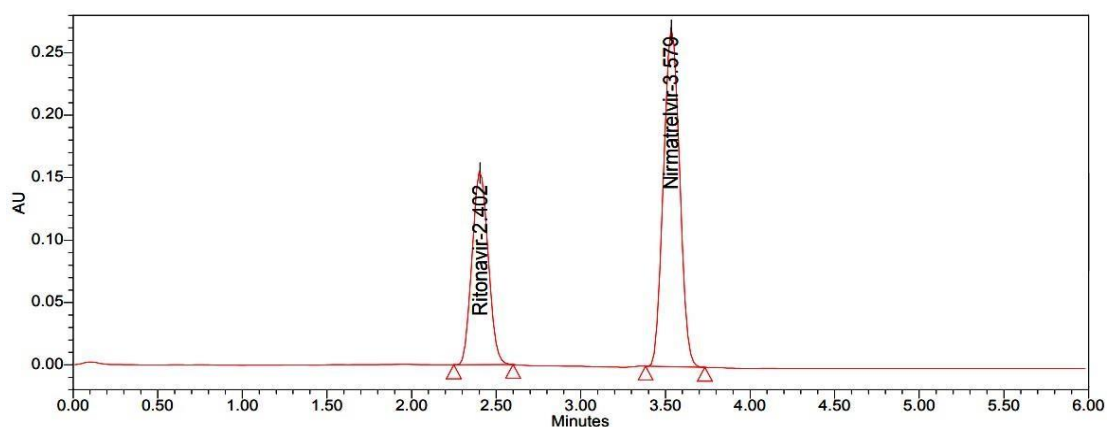


Figure 31: Chromatogram for less flow rate (0.9 ml).

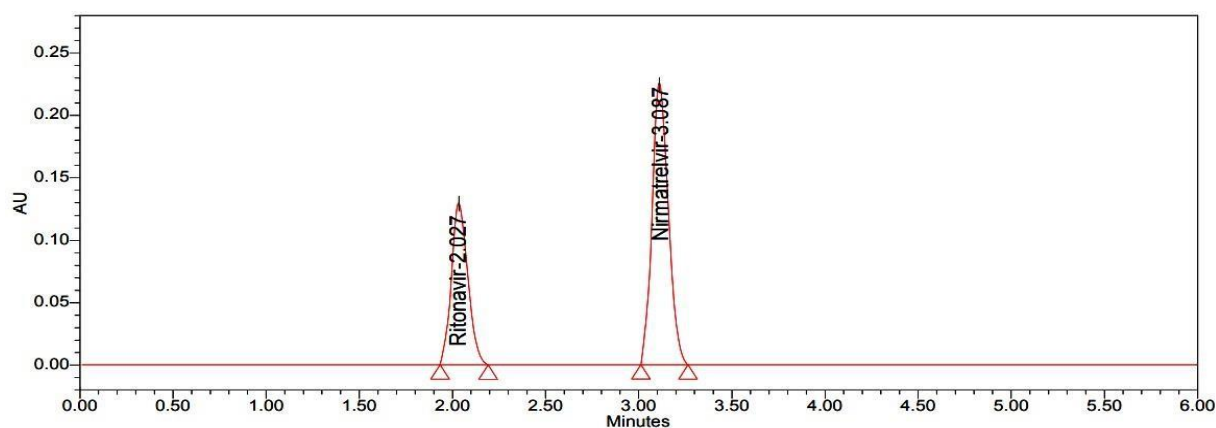


Figure 32: Chromatogram for more flow rate (1.1mL).

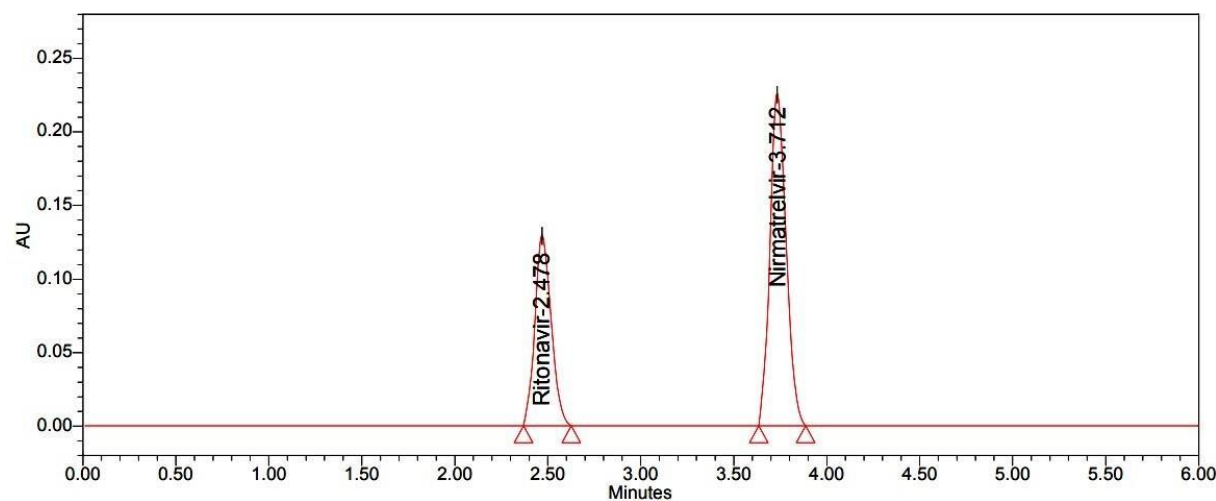


Figure 33: Chromatogram for less Organic Phase (36:64).

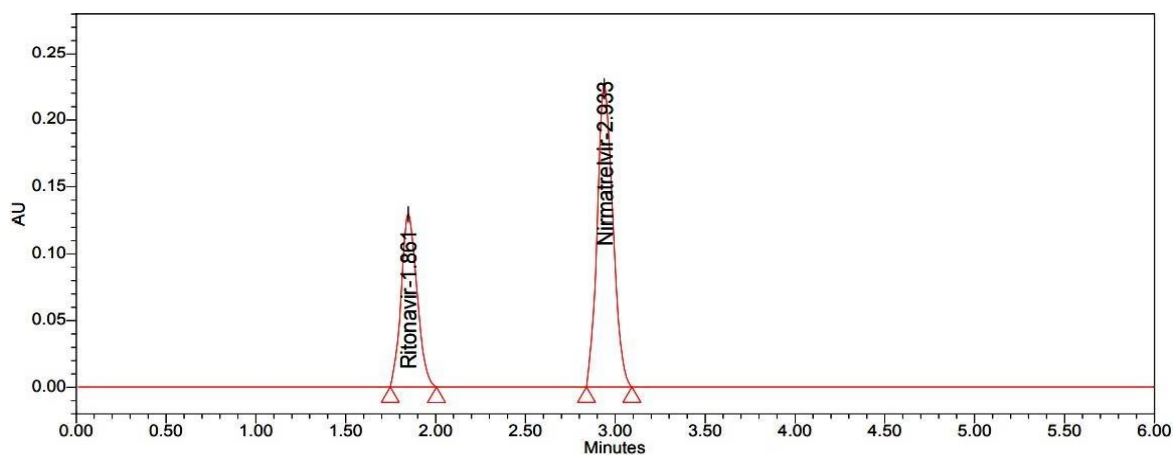


Figure 34: Chromatogram for more Organic Phase (44:56).

Table No. 11: Robustness results of Ritonavir by RP-HPLC.

Parameter	Ritonavir					
	Condition	Retention time(min)	Peak area	Resolution	Tailing	Plate count
Flow rate Change (mL/min)	Less flow (0.9ml)	2.402	1156238		0.93	9624
	Actual (1ml)	2.124	1282087		0.86	9597
	More flow (1.1ml)	2.027	1376298		0.84	9542
Organic Phase change (V/V)	Less Org (36:64)	2.478	1057424		0.89	9640
	Actual (40:60)	2.128	1286345		0.84	9527
	More Org (44:56)	1.861	1465025		0.79	9513

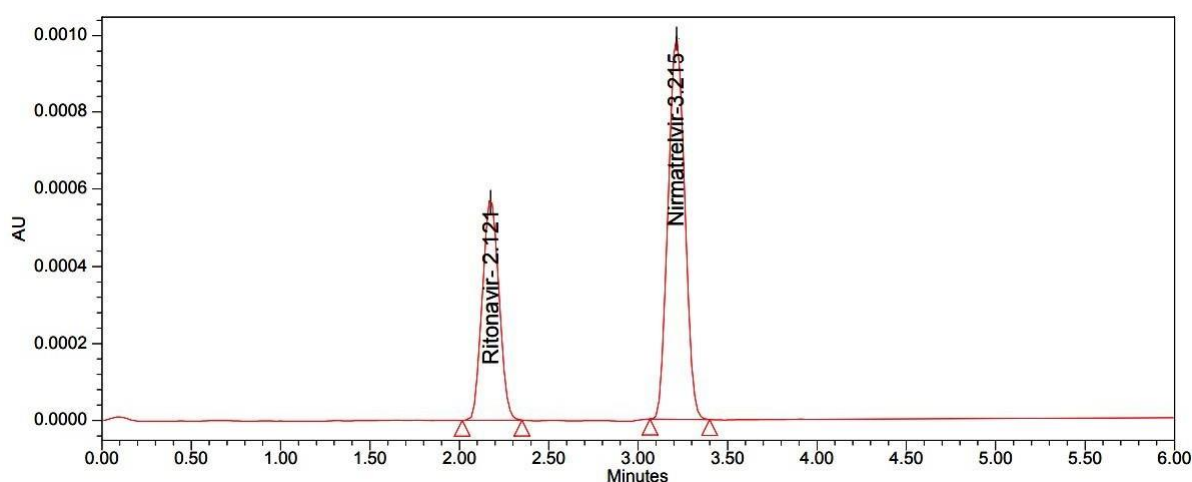
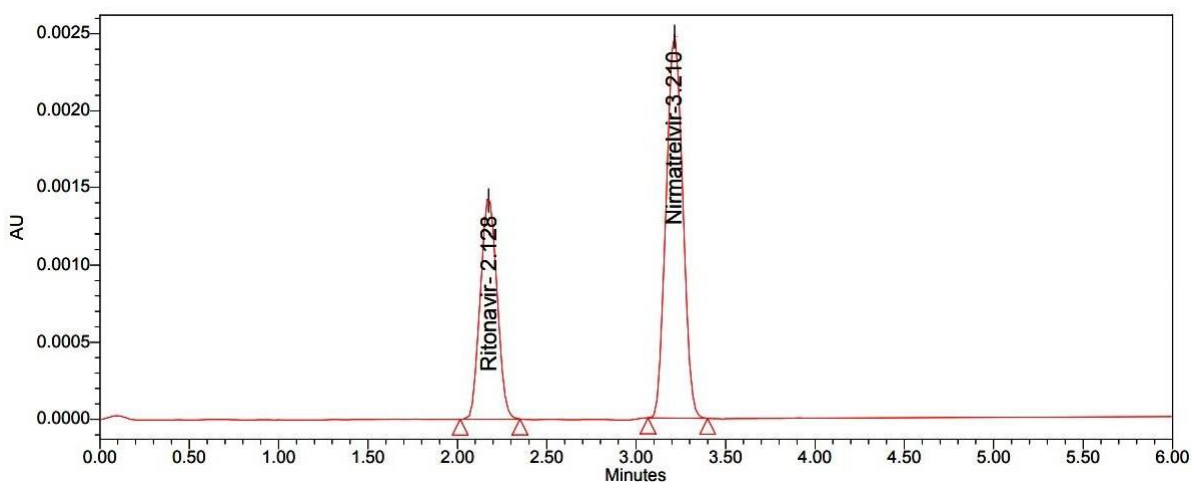
Table No. 12: Robustness results of Nirmatrelvir by RP-HPLC.

Parameter	Nirmatrelvir					
	Condition	Retention time (min)	Peak area	Resolution	Tailing	Plate count
Flow rate Change (mL/min)	Less flow (0.9ml)	3.579	1652641	5.35	1.05	12589
	Actual (1ml)	3.211	1837754	5.84	1.02	12567
	More flow (1.1ml)	3.087	2006982	5.26	0.97	12510
Organic Phase change (V/V)	Less Org (36:64)	3.712	1590138	6.18	1.09	12566
	Actual (40:60)	3.214	1836268	5.85	1.03	12515
	More Org (44:56)	2.933	2194138	5.42	1.00	12487

Minor variations in chromatographic conditions did not produce any significant effect on retention time, peak area, plate count, tailing factor, or resolution. All system suitability parameters remained within acceptable limits, confirming robustness of the developed method.

LOD and LOQ

The sensitivity of the developed RP-HPLC method was evaluated in terms of limit of detection (LOD) and limit of quantification (LOQ).

**Figure 35: Chromatogram for LOD.****Figure 36: Chromatogram for LOQ.****Table 13: LOD and LOQ Results.**

Name of drug	LOD(µg/ml)	s/n	LOQ(µg/ml)	s/n
Ritonavir	0.600	3	2.000	10
Nirmatrelvir	0.018	3	0.060	10

The obtained LOD and LOQ values indicated good sensitivity of the developed RP-HPLC method for

detection and quantification of Ritonavir and Nirmatrelvir at low concentration levels.

3.3 Degradation Studies

Different degradation studies are done and results are tabled below:

Table No. 14: Forced Degradation results for Ritonavir and Nirmatrelvir.

Results: % Degradation results	Ritonavir					Nirmatrelvir				
	Area	% Assay	% Degradation	Purity Angle	Purity Threshold	Area	% Degradation	% Assay	Purity Angle	Purity Threshold
Control	1285165	100	0	1.124	6.028	1832478	100	0	0.324	5.128
Acid	1147642	89.3	10.7	1.122	6.067	1604271	87.5	12.5	0.315	5.116
Alkali	1140854	88.8	11.2	1.106	6.014	1613122	88.0	12.0	0.347	5.156
Peroxide	1105743	86.0	14.0	1.108	6.068	1566248	85.5	14.5	0.326	5.163
Reduction	1114523	86.7	13.3	1.114	6.034	1618571	88.3	11.7	0.322	5.141
Thermal	1153257	89.7	10.3	1.127	6.061	1627201	88.8	11.2	0.361	5.105
Photolytic	1262638	98.2	1.8	1.139	6.055	1786232	97.5	2.5	0.329	5.123
Hydrolysis	1280152	99.6	0.4	1.157	6.027	1813016	98.9	1.1	0.321	5.167

CONCLUSION

A simple, rapid, precise, accurate, and stability-indicating RP-HPLC method was successfully developed and validated for the simultaneous estimation of Ritonavir and Nirmatrelvir in pharmaceutical tablet dosage form. Chromatographic separation was achieved using a Luna Phenyl Hexyl column with a mobile phase consisting of acetonitrile and ammonium acetate buffer (pH 3.0 adjusted with orthophosphoric acid) in the ratio of 40:60 v/v at a flow rate of 1.0 mL/min and detection wavelength of 266 nm.

The developed method showed satisfactory system suitability parameters with good peak symmetry, high theoretical plate count, acceptable resolution, and short retention times for both analytes. Validation studies performed according to ICH Q2(R1) guidelines demonstrated excellent specificity, linearity, accuracy, precision, robustness, sensitivity, and reproducibility of the method. The calibration curves exhibited excellent

linearity with correlation coefficient values greater than 0.999 for both drugs. Recovery studies confirmed the accuracy of the method with percentage recoveries within the acceptable range of 98–102%, while precision studies showed %RSD values below 2%, indicating good repeatability and reproducibility.

The developed RP-HPLC method was found to be sensitive with low LOD and LOQ values, making it suitable for quantitative analysis of Ritonavir and Nirmatrelvir at low concentration levels. The assay results confirmed the applicability of the method for routine quality control analysis of combined pharmaceutical dosage forms.

Overall, the proposed analytical method is economical, reliable, and suitable for routine simultaneous estimation of Ritonavir and Nirmatrelvir in bulk and pharmaceutical formulations in quality control laboratories.

Comparative results to the existing methods

Parameter	Jens Lobenhoffer et al.	Martens-Mohamed S. Imam et al.	K. Chiranjeevi et al.	Present Work
Drugs	Nirmatrelvir, Ritonavir	Nirmatrelvir, Ritonavir	Ritonavir	Ritonavir, Nirmatrelvir
Column	C18	C18	Symmetry C18 (4.6 × 100 mm, 3.5 µm)	Luna Phenyl Hexyl (250 × 4.6 mm, 5 µm)
Mobile Phase	Gradient elution	Ethanol:Water (80:20)	Buffer:ACN (50:50)	ACN:Ammonium acetate buffer pH 3.0 (40:60)
Detection	LC-MS/MS	UV, 215 nm	UV, 239 nm	UV, 266 nm
Flow Rate (mL/min)	NR	1.0	1.0	1.0
Retention Time (min)	NR	NIR: 4.9; RIT: 6.8	RIT: 5.1	RIT: 2.124; NIR: 3.211
Linearity	NIR: 10–10000 ng/mL; RIT: 2–2000 ng/mL	1–20 µg/mL	50–150 µg/mL	RIT: 12.5–75 µg/mL; NIR: 18.75–112.5 µg/mL
R ²	0.98899	0.99887	0.98999	RIT: 0.99959; NIR: 0.99980
LOD	0.650	0.693	0.711	RIT: 0.600 µg/mL; NIR: 0.018 µg/mL
LOQ	LLOQ reported	0.090	0.090	RIT: 2.000 µg/mL; NIR: 0.060 µg/mL
Accuracy	≤15% (≤20% at LLOQ)	98.3%	Recovery studies performed	RIT: 99.5%; NIR: 100.3%
Precision	≤15% RSD	0.85%	Validated	RIT: 0.75%; NIR: 1.14%
Robustness	NR	NR	Validated	Robust
Specificity	NR	Baseline separation	Validated	No interference
System Suitability	Acceptable	Acceptable	Acceptable	Within ICH limits
Major Findings	Therapeutic drug monitoring in plasma	Green HPLC method	Ritonavir assay in dosage forms	Rapid, accurate, precise and robust simultaneous method

CONFLICTS OF INTEREST

NO

FUNDING

No funding required

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