METHOD DEVELOPMENT ANDVALIDATION OF RITONAVIR AND NIRMATRELVIR IN TABLET DOSAGE FORM BY USING RP-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

The current investigation was pointed at developing and progressively validating novel, simple, responsive and stable RP-HPLC method for the measurement of active pharmaceutical ingredients of Ritonavir and Nirmatrelvir and their related substances. A simple, selective, validated and well-defined stability that shows gradient RP-HPLC methodology for the quantitative determination of ritonavir and nirmatrelvir. The chromatographic strategy utilized Column of Dikma Spursil C_{18} (4.6 x 150mm, 3µm), using isocratic elution with a mobile phase of 0.1 percent Formic acid and Acetonitrile (65:35). A flow rate of 1 ml/min and a detector wavelength of 253 nm utilizing the PDA detector were given in the instrumental settings. Using the impurity-spiked solution, the chromatographic approach was streamlined. Validation of the proposed method was carried out according to an international conference on harmonization (ICH) guidelines. LOD and LOQ for the two active ingredients and their impurities were established with respect to test concentration. The calibration charts plotted were linear with a regression coefficient of R^2=0.999, which means the linearity was within the limit. Recovery, specificity, linearity, accuracy, robustness, ruggedness was determined as a part of method validation and the results were found to be within the acceptable range. The proposed method to be fast, simple, feasible and affordable in RS condition. During stability tests, it can be used for routine analysis of production samples and to verify the quality of drug samples during stability studies.

Keywords:

Ritonavir, Nirmatrelvir , RP-HPLC , Development , Validation.

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INTRODUCTION:

Ritonavir[1] is an antiretroviral drug from the protease inhibitor class used to treat HIV infection and AIDS. Ritonavir is frequently prescribed with Highly Active Anti-Retroviral Therapy, not for its antiretroviral action, but as it inhibits the same host enzyme that metabolizes other protease inhibitors. This inhibition leads to higher plasma concentrations of these latter drugs, allowing the clinician to lower their dose and frequency and improving their clinical efficacy. It has the structural formula and shown in (Fig. 1).

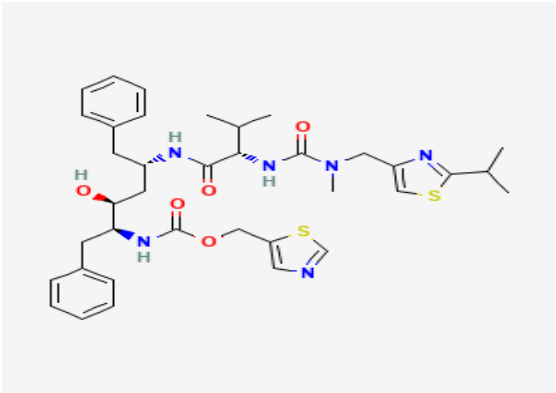


Fig: 1 Structure of Ritonavir

The chemical name of Ritonavir is (5S, 8S, 10S, 11S) - 10- hydroxy- 2- methyl- 5- (1-methylethyl) - 1- [2- (1-methylethyl) - 4-thiazolyl] - 3, 6-dioxo- 8, 11- bis (phenylmethyl)-2, 4, 7, 12- etraazatridecan- 13-oic acid 5-thiazolyl methyl ester. It is official in Indian Pharmacopoeia[2] and United States Pharmacopoeia[3]. From the literature survey, it was found that Ritonavir estimated by analytical methods such as Reversed Phase High Performance Liquid Chromatographic (RP-HPLC) method [4-10], LC-MS[11] and HPTLC method[12]. Nirmatrelvir is an orally bioavailable, peptidomimetic inhibitor of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) main protease (Mpro; 3C-like protease; 3CL protease; 3CLpro; nsp5 protease), with potential antiviral activity against SARS-CoV-2 and other coronaviruses. Upon oral administration, nirmatrelvir selectively targets, binds to, and inhibits the activity of SARS-CoV-2 Mpro. This inhibits the proteolytic cleavage of viral polyproteins, thereby inhibiting the formation of viral proteins including helicase, single-stranded-RNA-binding protein, RNA-dependent RNA polymerase, 20-O-ribose methyltransferase, endoribonuclease and exoribonuclease. This prevents viral transcription and replication.[13]

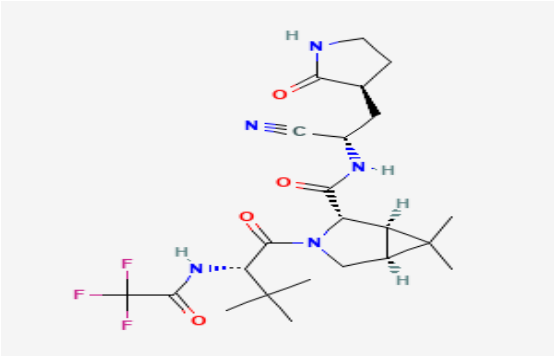


Fig: 2 Structure of Nirmatrelvir

MATERIALS AND METHODS:

Chemicals

Acetonitrile, HPLC-grade Formic acid, water, were purchased from Merck India Ltd, Mumbai, India. APIs of Ritonavir, Nirmatrelvir standards were procured from Hetero Labs, Hyderabad.

The instrumentation

Waters alliance liquid chromatography (model 2695) monitored with empower 2.0 data handling system and a detector of PDA was used for this study [14-17].

Method optimization

Initially the mobile phase tried was methanol: Ammonium acetate buffer and Methanol: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to Formic acid with buffer (pH 3.0), Acetonitrile in proportion 65: 35 v/v respectively. UV spectrum of 10 µg/ml Ritonavir and Nirmatrelvir in diluents in methanol was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 253nm. At this wavelength both the drugs show good absorbance. The developed HPLC method was utilized for the estimation of the combined drugs by *in vitro* method[18].

Validation procedure

The analytical parameters such as system suitability, precision, specificity, accuracy, linearity, robustness, LOD, LOQ, forced degradation and stability were validated according to ICH Q2 (R1) guidelines[19-27].

Preparation of buffer and mobile phase:

Preparation of 0.1% Formic acid buffer:

Pipette out 1ml of Ortho Phosphoric Acid was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.0 with triethylamine.

Preparation of mobile phase:
Accurately measured 650 ml (65%) of above buffer and 350 ml of Acetonitrile HPLC (35%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:
The Mobile phase was used as the diluent

Preparation of the Ritonavir & Nirmatrelvir standard & sample solution:
Standard Solution Preparation:
Accurately weigh and transfer 100 mg of Ritonavir and 300 mg of Nirmatrelvir working standards into a 25ml clean dry volumetric flask add about 15mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.3ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:
Accurately weigh 10 tablets of Ritonavir crush in mortar and pestle and transfer equivalent to 100 mg (i.e. 170 mg) ofRitonavir25 mL clean dry volumetric flask add about 15mL of Diluent and sonicate it up to 30 mints to dissolve it completely and make volume up to the mark with the same solvent.Then it is filtered through 0.44 micron Injection filter. (Stock solution of Ritonavir Sample)

Accurately weigh 10 Tablets of Nirmatrelvir powder crush in mortar and pestle and transfer equivalent to 300 mg of Nirmatrelvir (i.e. 380 mg) 25 mL clean dry volumetric flask add about 15 mL of Diluent and sonicate it up to 30 mints to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.44 micron Injection filter. (Stock solution of Nirmatrelvir Sample)

Further pipette 0.3 ml of each stock solutions of Ritonavir and Nirmatrelvir into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:
Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for Ritonavir and Nirmatrelvir peaks and calculate the %Assay by using the formulae.

RESULTS AND DISCUSSION
The main analytical challenge during the development of a new method was to separate active Pharma ingredients from their impurities. In order to provide a good performance, the chromatographic conditions were optimized.

System suitability
In System, suitability injecting standard solution and reported USP tailing and plate count values are tabulated in table 1.

System suitability Parameter	Acceptance criteria	Drug Name	
		Ritonavir	Nirmatrelvir
USP Plate Count	NLT 2000	2940.49	3415.94
USP Tailing	NMT 2	1.87	1.84
USP Resolution	NLT 2	-	6.06

Table 1: Results of system suitability

LINEARITY
Accurately weigh and transfer 100 mg of Ritonavir and 300mg of Nirmatrelvir working standard into a 25mL clean dry volumetric flask add about 15mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).
Preparation of Level – I:
0.1 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.
Preparation of Level – II:
0.2ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.
Preparation of Level – III:
0.3 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.
Preparation of Level – IV:
0.4 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent
Preparation of Level – V:
0.5 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

Procedure:
Inject each level into the chromatographic system and measure the peak area.Plot a graph of peak area versus concentration (on X-axis

concentration and on Y-axis Peak area) and calculate the correlation coefficient

Table 2: Results of Linearity

S. No	Linearity Level	Ritonavir		Nirmatrelvir	
		Concentration	Area	Concentration	Area
1	I	40	711032	120	1679174
2	II	80	953150	240	2328033
3	III	120	1224790	360	3082729
4	IV	160	1498943	480	3688703
5	V	200	1859124	600	4398800
Correlation Coefficient			0.999		0.999

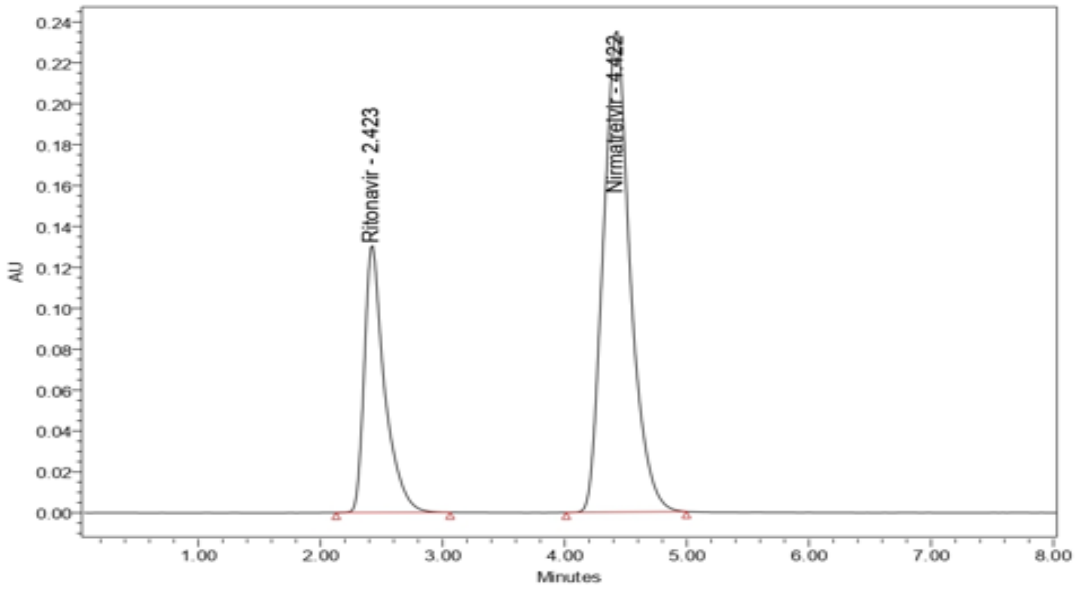
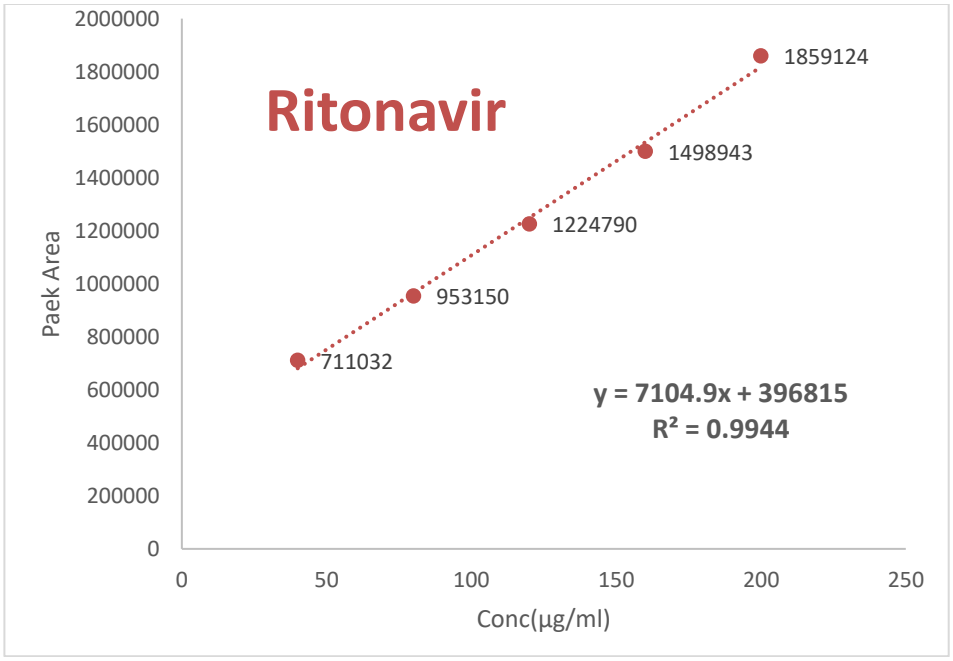


Fig. 3: Chromatogram of Linearity



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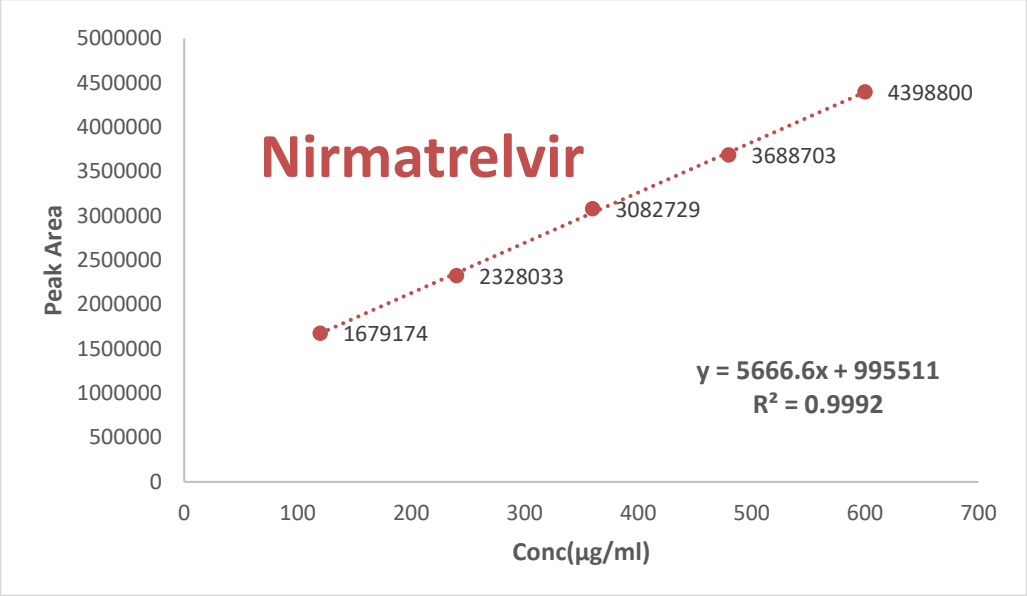

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Fig. 4: Calibration plots of (A) Favipiravir (B) Oseltamivir

Accuracy

In this method, Accuracy was conducted in triplicate by analyzing active pharma ingredient sample solution spiked with known amounts of all the impurities at three kinds of concentration levels of 50, 100 and 150% of each at a specified limit. For all impurities, percentage recoveries were measured and found to be within the limit. The accuracy and reliability of the developed method were established. The percentage recovery values were found to be in the range of 98-102% for Ritonavir and Nirmatrelvir. The results are given in table 3.

Table 3: Results of Accuracy

S. No.	% Level	Ritonavir % recovery	Nirmatrelvir % recovery
1	50	99.93	99.84
2	100	99.60	99.70
3	150	100.54	100.45
Mean		100.02	100.00

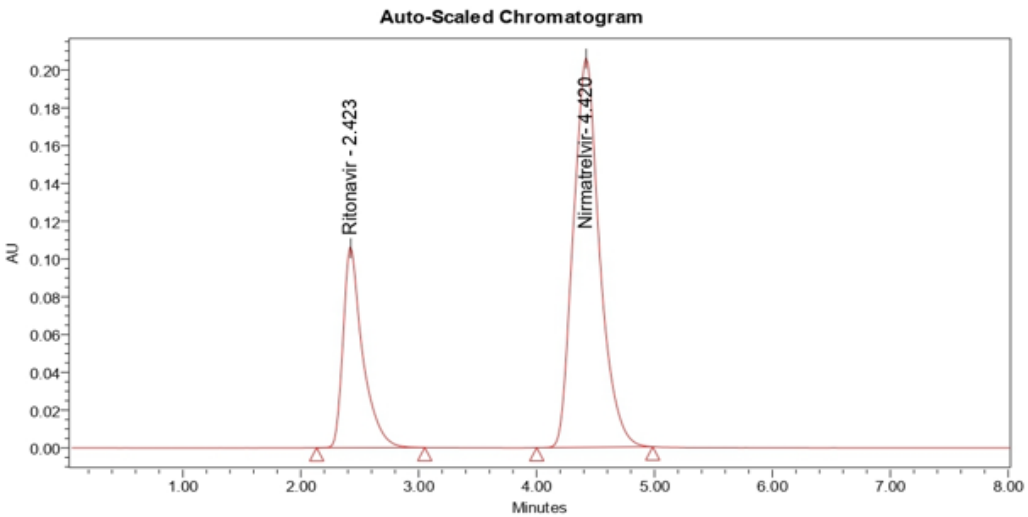


Fig. 5: Chromatogram of Accuracy

Precision

The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Table 4: Results of Precision

Injection	Area for Ritonavir	Area for Nirmatrelvir
Injection-1	1216988	3181200
Injection-2	1214954	3176524
Injection-3	1215705	3175392
Injection-4	1211677	3172805
Injection-5	1213640	3177606
Injection-6	1215077	3170928
Average	1214673.5	3175742.5
Standard Deviation	1828.1	3627.6
%RSD	0.2	0.1

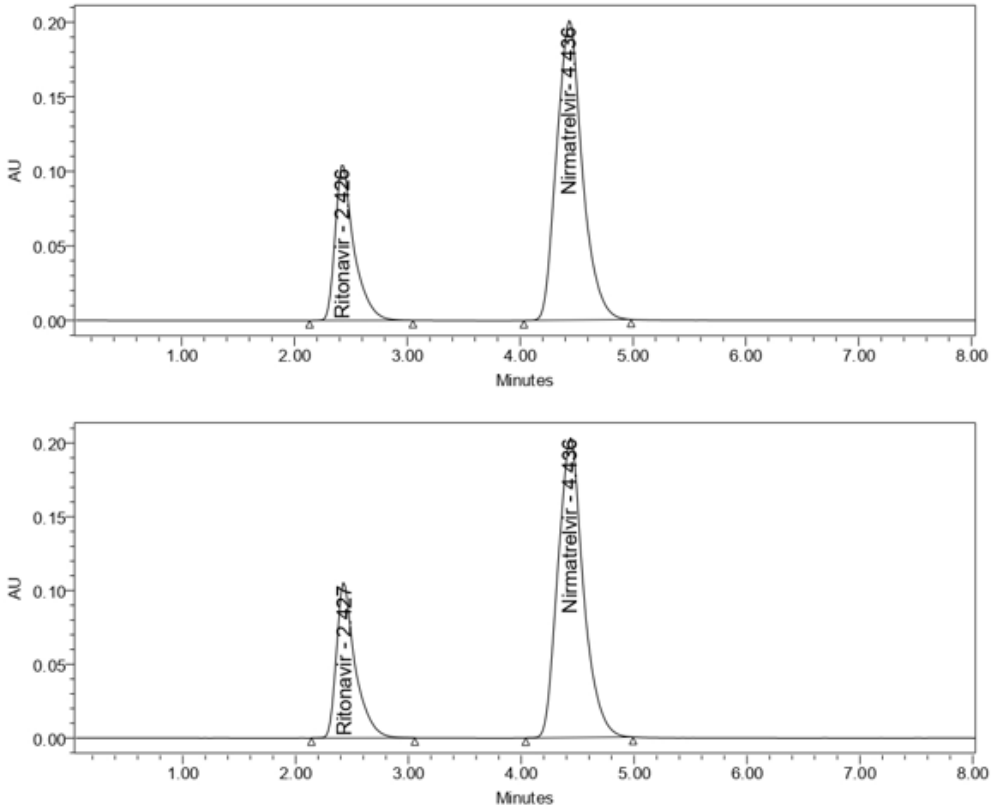


Fig. 6: Chromatogram of Precision

Intermediate Precision/Ruggedness

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day. The standard solutions prepared in the precision were injected on the other day, for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Table 5: Results of Ruggedness

Injection	Area for Ritonavir	Areafor Nirmatrelvir
Injection-1	1216459	3174077
Injection-2	1215928	3172410
Injection-3	1214728	3173476
Injection-4	1220833	3180298
Injection-5	1217919	3178180
Injection-6	1216522	3176344
Average	1217064.8	3175797.5
Standard Deviation	2113.9	3034.9
%RSD	0.2	0.1

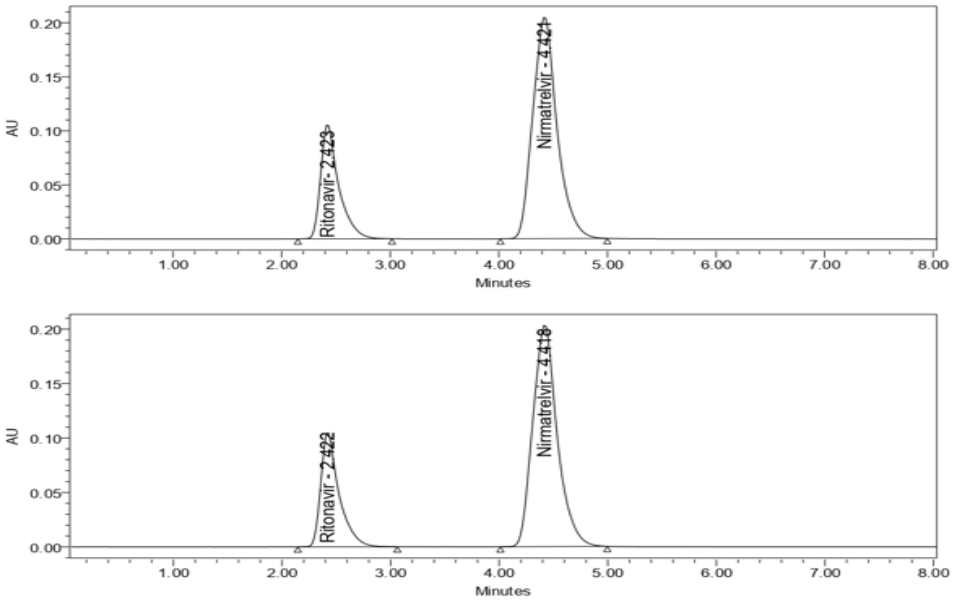


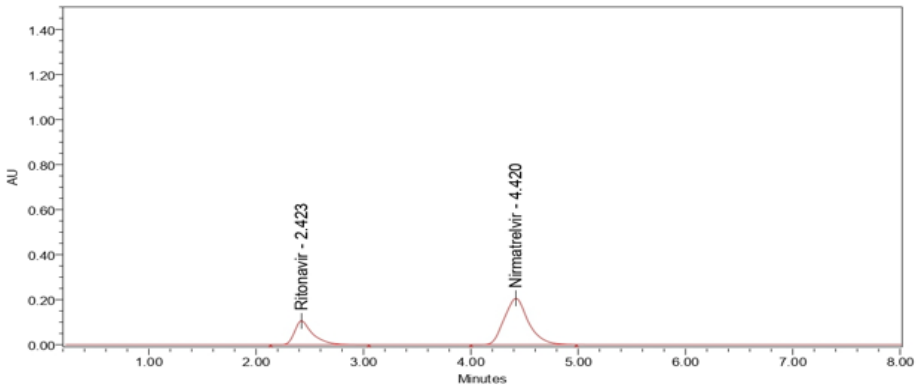
Fig. 7: Chromatogram of Ruggedness

LOD and LOQ

The LOD concentrations for Ritonavir are 0.23 µg/ml and s/n values is 3 and Nirmatrelvir 0.37 µg/ml and s/n value 3.02. The LOQ concentration for Ritonavir 0.78 µg/ml and their s/n values are 9.98 and Nirmatrelvir 1.22 µg/ml and s/n value is 10. The method is validated as per the US FDA guidelines[28].

Table 6: Results of LOD&LOQ

Ritonavir				Nirmatrelvir			
LOD		LOQ		LOD		LOQ	
Concentration	s/n	Concentration	s/n	Concentration	s/n	Concentration	s/n
0.23µg/ml	3.0	0.78µg/ml	9.98	0.37µg/ml	3.02	1.22µg/ml	10.00



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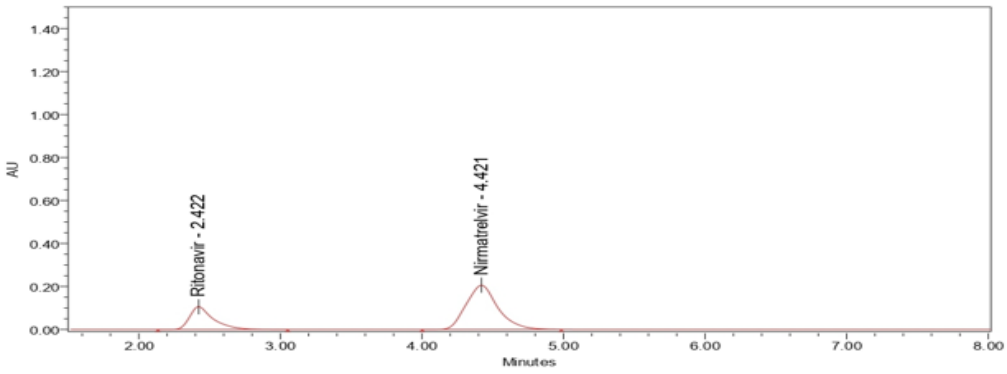

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Fig. 8: Chromatogram of (A) LOD and (B) LOQ

Robustness

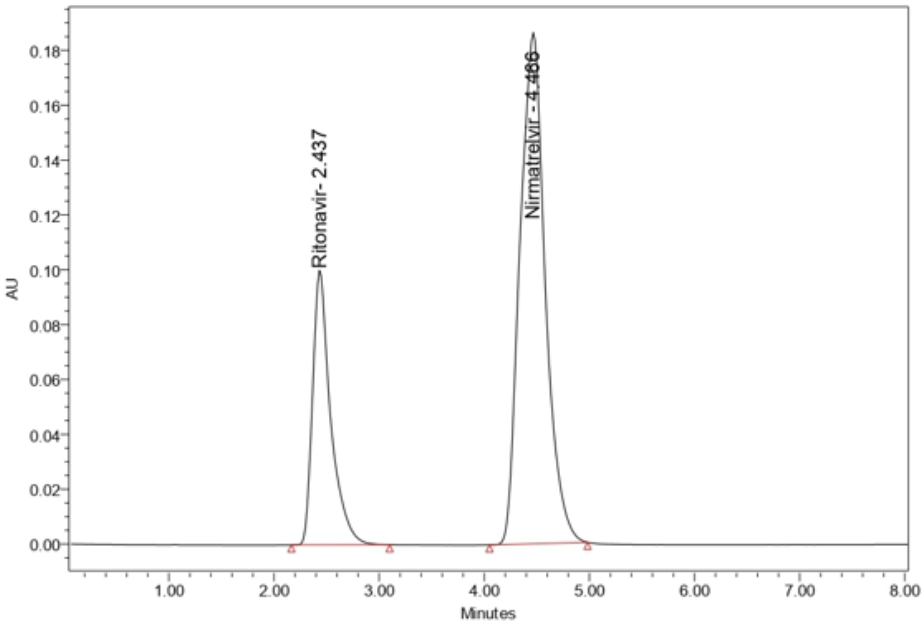
The conditions of the experiment were designed to test the robustness of the established system intentionally altered, such as flow rate, mobile phase in organic percentage in all these varied conditions. Robustness results for Ritonavir and Nirmatrelvir found to be within the limit and results are tabulated in table 8 and 9.

Table 8: Results of Robustness

S.No	Flow Rate (ml/min)	System Suitability Results (Ritonavir)		System Suitability Results (Nirmatrelvir)	
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing
1	0.9	9085.09	1.12	8731.46	1.21
2	1.0	9009.7	1.47	8509.7	1.47
3	1.1	9015.51	1.40	8549.3	1.12

Table 9: Results of Robustness

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results (Ritonavir)		System Suitability Results (Nirmatrelvir)	
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing
1	10% less	9025	1.4	8743.64	1.26
2	*Actual	9047	1.4	8709.7	1.47
3	10% more	9085.7	1.5	8887.28	1.2



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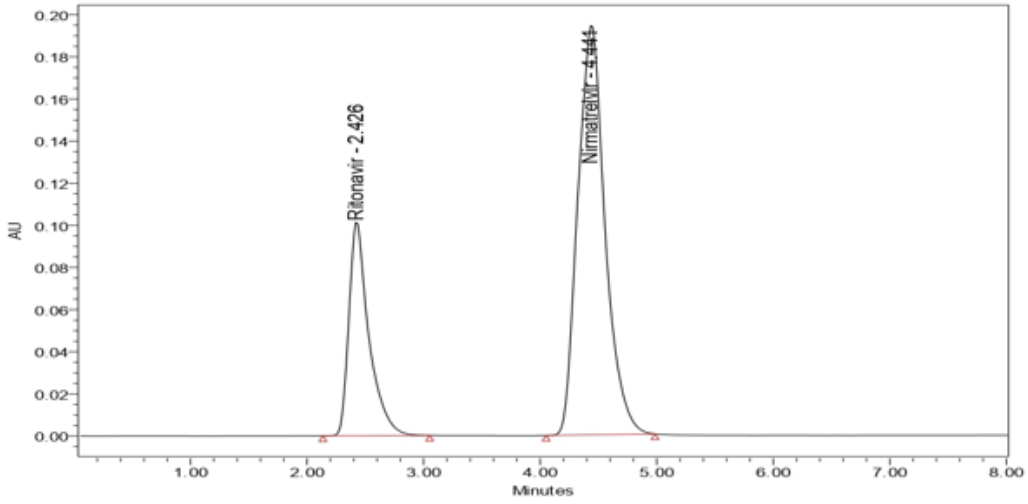

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Fig. 9: Chromatograms of (A&B) Robustness

DEGRADATION STUDIES:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Ritonavir and Nirmatrelvir using the proposed method[29].

Preparation of stock:

Accurately weigh and transfer 100 mg of Ritonavir and 300 of Nirmatrelvir working standard into a 25ml clean dry volumetric flask add about 15mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Hydrolytic degradation under acidic condition

Pipette 0.3 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition

Pipette 0.3ml of above solution into a 10ml volumetric and add 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Thermal induced degradation

Ritonavir and Nirmatrelvir sample was taken in petridish and kept in Hot air oven at 110^0 C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Oxidative degradation

Pipette 0.3ml above stock solution into a 10ml volumetric flask and 1ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Photo degradation:

Pipette 0.3 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24hrs and the volume was made up to the mark with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

Table 10: Results of Degradation studies

Sample Name	Ritonavir		Nirmatrelvir	
Standard	**% Degraded**	**% Degraded**	**Area**	**% Degraded**
Acid	1202885	100	3160974	100
Base	1155543	3.94	2901376	8.21
Peroxide	1159844	3.58	3006026	4.90
Thermal	1150954	4.32	3009639	4.79
Photo	1160629	3.51	2905892	8.07
Standard	1162114	3.39	3011197	4.74

CONCLUSION

We present in this article simple, selective, validated and well- defined stability that shows gradient RP-HPLC methodology for the quantitative determination of Ritonavir and Nirmatrelvir. All the products of degradation formed during the stress conditions and the related active pharma ingredients are well separated and peaks were well resolved from each other and separate with an appropriate retention time indicating that the proposed method to be fast, simple, feasible and affordable in RS condition. Therefore the developed method during stability tests, it can be used for routine analysis of production samples and to verify the quality of drug samples during stability studies.

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Conflict of Interest

No conflict of Interest.

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