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Research Article

Estimation of Nirmatrelvir and Ritonavir using Stability Indicating RP- HPLC Method

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A simple, Accurate, precise method was developed for the simultaneous estimation of the Nirmatrelvir and Ritonavir in dosage form. Chromatogram was run through Acquity UPLC STD HSS C18 (100 x2.8mm, 2µ. Mobile phase containing Buffer Na2HPO4: Methanol was taken in the ratio of 35:65 was pumped through column at a flow rate of 0.3 ml/min. Buffer used in this method was 0.01N Kh2po4. Temperature was maintained at 30°C. Optimized wavelength selected was 245 nm. Retention time of Nirmatrelvir and Ritonavir were found to be 0.787 min and 1.058 min. %RSD of the Nirmatrelvir and Ritonavir were and found to be 0.5 and 0.4 respectively. %Recovery was obtained as 100.29% and 100.34% for Nirmatrelvir and Ritonavir respectively. LOD, LOQ values obtained from regression equations of Nirmatrelvir and Ritonavir were 0.33ppm, 0.03ppm and 1.02ppm, 0.08ppm respectively. of Nirmatrelvir is y = 114686x + 13045, and of Ritonavir is y = 112957x + 6810.8, 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.

Keywords: Nirmatrelvir, Ritonavir, RP-UPLC.

INTRODUCTION

The use of new or updated analytical processes used for stability and to enable testing of commercial pharmaceutical substances and products is known as analytical method development, and it is governed by the ICH Q14 standards. The oral combination drug nirmatrelvir/ritonavir, commonly known as ritonavir-boosted nirmatrelvir, is used to treat corona virus disease 2019 (COVID-19). It is made up of ritonavir, an inhibitor of cytochrome P450 (CYP) 3A, and nirmatrelvir, a protease inhibitor that targets the primary protease of the severe acute respiratory syndrome corona virus 2 (SARS-CoV-2). Nirmatrelvir is scientifically defined 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 and Ritonavir is defined 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. The US Food and Drug Administration (FDA) approved nirmatrelvir /ritonavir's usage in the treatment of COVID-19 on December 22, 2021, under an Emergency usage Authorization (EUA). An examination of the literature reveals that there are few ways available that use UV spectroscopic techniques to produce nirmatrelvir. Many of the methods are mainly concerned with estimating bulk and pharmaceutical dosage forms of ritonavir and nirmatrelvir. There aren't many techniques for quantifying Ritonavir and Nirmatrelvir at the same time using HPLC-DAD, HPTLC, and LC-MS/MS, respectively. Additionally, certain techniques have been created that make use of acids, highly concentrated buffers, extended run times, elevated temperatures, and a wide number of solvents. As a result, the techniques created are straightforward, affordable, and tailored for Nirmatrelvir. The pharmacological profile and

chemical structure of Nirmatrelvir Ritonavir are displayed in Figure no 1, 2 and table 1 and 2. The literature survey5-13 reveals that a few analytical methods for the determination of combination of Nirmatrelvir and Ritonavir in dosage forms by HPLC, UPLC.

Nirmatrelvir:

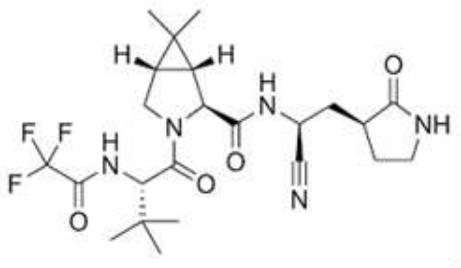

Figure 1. Structure of Nirmatrelvir

Table 1. Drug Profile of Nirmatrelvir

CAS Number	:	2628280-40-8
IUPAC Name	:	(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
Molecular Weight	:	Average: 499.535
Molecular Formula	:	C23H32F3N5O4
Category	:	Anti-viral Medication

Ritonavir

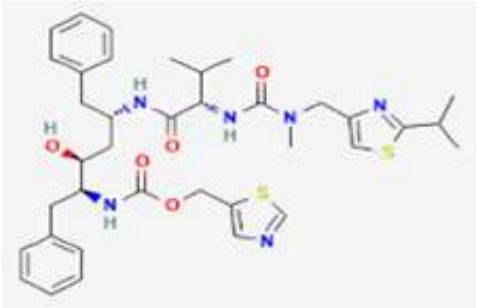

Figure 2: Structure of Ritonavir

Table 2. Drug profile of Ritonavir

CAS Number	:	155213-67-5
IUPAC Name	:	(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
Molecular Weight	:	Average: 720.944
Molecular Formula	:	C37H48N6O5S2
Category	:	Protease Inhibitors

MATERIALS AND METHODS

2.1 Chemicals:

Nirmatrelvir and Ritonavir pure drugs (API), Combination Nirmatrelvir and Ritonavir tablets (Paxlovid), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dehydrogenate ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem

2.2 Instrument:

Electronics Balance-Denver, pH meter -BVK enterprises, India, Ultrasonicator-BVK enterprises, UPLC instrument used was of WATERS Acquity UPLC SYSTEM with Auto Injector and Acquity TUV detector. Software used is Empower 3, UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz was be used for measuring absorbance of Nirmatrelvi and Ritonavir solutions.

3. Preparation

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50

Buffer: 0.01N Potassium dihyrogen Ortho phosphate

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

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

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

Preparation of Sample stock solutions: 10 tablets were taken and calculated each tablet average tablet and equivalent to 150 mg and 100mg was taken, then 20ml acetonitrile was added, sonicated for 25 min and made up to mark to yield 1100 & 500µg/ml. It was centrifuged for 20 min. Then the supernatant was collected and filtered using 0.45 µm filters using (Millipore, Milford, PVDF) (300µg/ml of Nirmatrelvir and 200µg/ml of Ritonavir).

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

3.1 Validation

System suitability parameters: The system suitability parameters were determined by preparing standard solutions of Nirmatrelvir (300ppm) and Ritonavir (100ppm) 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: 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.

3.2 Precision:

Preparation of Sample stock solutions: 10 tablets were taken and calculated each tablet average tablet and equivalent to 150 mg and 100mg was taken, then 20ml acetonitrile was added, sonicated for 25 min and made up to mark to yield 1100 & 500µg/ml. It was centrifuged for 20 min. Then the supernatant was collected and filtered using 0.45 µm filters using (Millipore, Milford, PVDF) (300µg/ml of Nirmatrelvir and 200µg/ml of Ritonavir).

Preparation of Sample working solutions (100% solution): 0.5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (15µg/ml of Nirmatrelvir and 10µg/ml of Ritonavir). The Precision were determined by preparing Sample solutions of Nirmatrelvir (15) and Ritonavir (10ppm) and the solutions were injected six

times and The % RSD for the area of six standard injections results should not be more than 2%.

3.3 Linearity:

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

Level	ml
25 %	0.25 ml from Pipette
50 %	0.5 ml from Pipette
75 %	0.75 ml from Pipette
100 %	1.0 ml from Pipette
125 %	1.25 ml from Pipette
150 %	1.5 ml from Pipette

3.4 Accuracy:

Preparation of Sample stock solutions: 10 tablets were taken and calculated each tablet average tablet and equivalent to 150 mg and 100mg was taken, then 20ml acetonitrile was added, sonicated for 25 min and made up to mark to yield 1100 & 500µg/ml. It was centrifuged for 20 min. Then the supernatant was collected and filtered using 0.45 µm filters using (Millipore, Milford, PVDF) (300µg/ml of Nirmatrelvir and 200µg/ml of Ritonavir

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

Level	ml
50 %	1.5 ml from Pipette
100 %	2.0 ml from Pipette
150 %	2.5 ml from Pipette

3.5 Forced Degradation Oxidation:

To 1 ml of stock solution of Nirmatrelvir and Ritonavir, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 600c. For HPLC study, the resultant solution was diluted to obtain 15µg/ml& 10µg/ mlsolutionand10µlwereinjected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:

To 1 ml of stock solution Nirmatrelvir and Ritonavir, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 600c. The resultant solution was diluted to obtain 15µg/ml& 10µg/ml solution and 10µl 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 Nirmatrelvir and Ritonavir, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 600c. The resultant solution was diluted to obtain 15µg/ml& 10µg/ml solution and 10µl 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 placedinovenat105°C for1h to study dry heat degradation For UPLC study, the resultant solution was diluted to 15µg/ml& 10µg/ml solution and 10µl 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 312.5µg/ml Nirmatrelvir & 125µg/ml Ritonavir solution to UV Light by keeping the beaker in UV Chamber for 1days or 200- Watt hours/m2 in photo stability chamber. For UPLC study, the resultant solution was diluted to obtain 15µg/ml& 10µg/ml solutions and 10µl 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 refluxingthedruginwaterfor1h r s at a temperature of 60º. For HPLC study, the resultant solution was diluted to 15µg/ml& 10µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

RESULTS

4.1 Optimized Chromatogram

Nirmatrelvir and Ritonavir were eluted at 0.787 min and 1.058 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

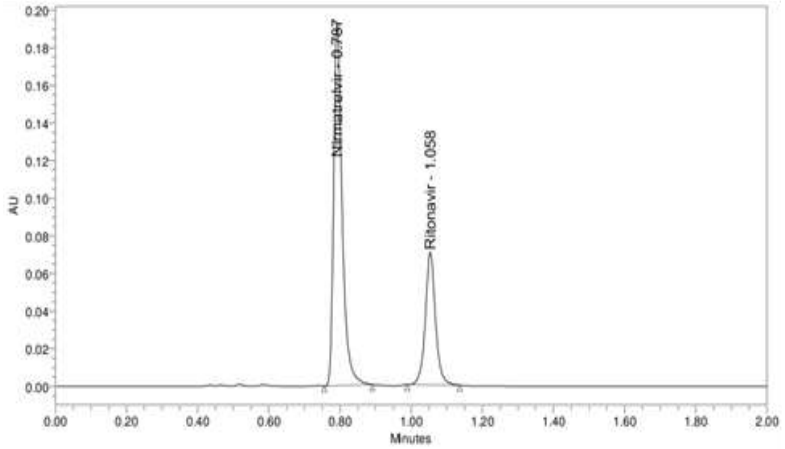

Figure 3. Optimized Chromatogram

4.2 Linearity:

The detector respond was found to be linear in the concentration range of Nirmatrelvir and Ritonavir peak areas are measured. The calibration curves of Nirmatrelvir and Ritonavir are shown in figures 4 - 6 respectively and calibration data is Table 3

Table 3. Calibration data of Nirmatrelvir and Ritonavir

% level of concentration	Nirmatrelvir		Ritonavir	
	conc. (µg/ml)	Response	Conc. (µg/ml)	Response
0	0	0	0	0
25	15	1712922	5	568417
50	30	3480126	10	1144464
75	45	5217596	15	1705012
100	60	6846454	20	2256606
125	75	8574878	25	2821428
150	90	10372303	30	3405412
(y=mx+b)	y = 114686x + 13045		y = 112957x + 6810.8	
Slope(m)	114686		112957	
Intercept(b)	13045		6810.8	
R2	0.999		0.999	

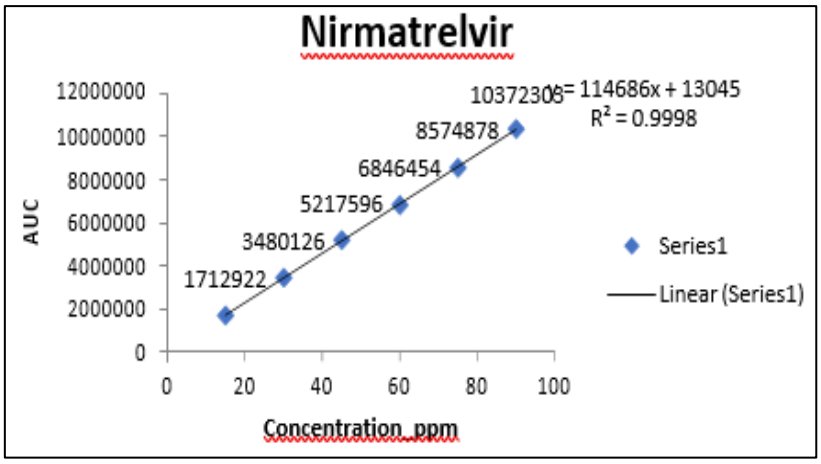

Figure 4. Calibration data of Nirmatrelvir

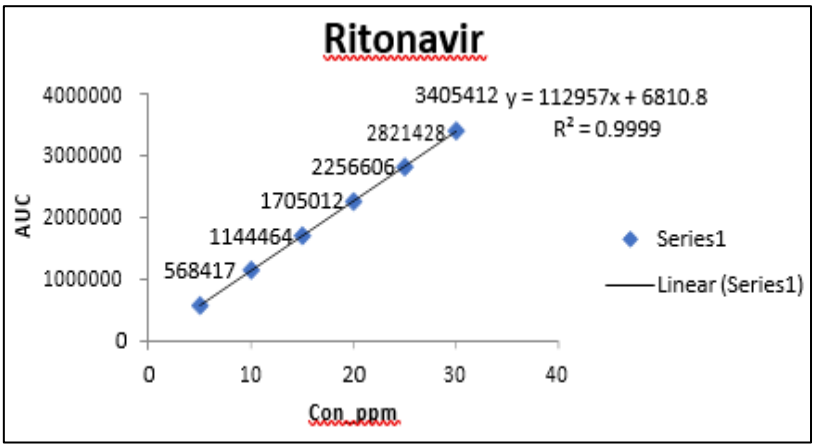

Figure 5. Calibration data of Ritonavir

4.3 Precision:

From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 1.2% and 0.5% respectively for Nirmatrelvir and Ritonavir. As the limit of Precision was less than "2" the system precision was passed in this method.

Table 4. System Precision of Nirmatrelvir and Ritonavir

Nirmatrelvir	Ritonavir
6816686	2263535
6827575	2274625
6836573	2263527
6835577	2263475
6806464	2284635
6746566	2257364
6811574	2267860
33868.6	9940.2
0.5	0.4

Table 5. Intraday precision and inter day Precision

Intraday Precision		Inter day Precision	
Nirmatrelvir	**Ritonavir**	**Nirmatrelvir**	**Ritonavir**
6816574	2273534	6834757	2293524
6836466	2253646	6802464	2263525
6845757	2274635	6843264	2276645
6805757	2263635	6893644	2253643
6836658	2287353	6833685	2285655
6804646	2263635	6823456	2254365
6824310	2269406	6838545	2271226
17612.2	11674.5	30416.0	16658.5
0.3	0.5	0.4	0.7

4.4 Accuracy

Three concentrations 50%, 100%, 150%, were injected in a triplicate manner and amount Recovered and % Recovery were displayed in Table 6

Table 6. Accuracy of Nirmatrelvir and Ritonavir

	Nirmatrelvir				Ritonavir			
% Level	**Peak Area**	**Amount Spiked (µg/ml)**	**Amount recovered (µg/ml)**	**% Recovery**	**Peak Area**	**Amount Spiked (µg/ml)**	**Amount recovered (µg/ml)**	**% Recovery**
50%	10355565	30	30.2	100.6	1475217	10	10.06	100.64
	10345654	30	30.1	100.3	1475498	10	9.96	99.57
	10364678	30	30.3	100.9	1476524	10	10.09	100.91
100%	13767866	60	59.9	99.9	1968502	20	20.20	100.99
	13747456	60	59.8	99.6	1958226	20	20.11	100.55
	13784645	60	60.1	100.1	1969182	20	20.17	100.87
150%	17259463	90	90.4	100.4	2456565	30	29.94	99.79
	17264646	90	90.4	100.5	2459456	30	29.84	99.46
	17245547	90	90.3	100.3	2447656	30	30.09	100.29

4.5 Limit of Detection and Limit of Quantification

LOD value for Nirmatrelvir was found to be 0.33 and Ritonavir was 0.03 respectively. LOQ value for Nirmatrelvir and Ritonavir were found to be 1.02 and 0.08 respectively. These low LOD and LOQ values indicate that the proposed RP-UPLC method is sensitive.

4.6 Robustness

Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines

Table 7. Robustness data of Nirmatrelvir

Parameter	Optimized condition	Used condition	Peak area	Retention Time	Plate Count	Tailing factor
Flow rate (±0.1ml/min)	1ml/min	0.27ml/min	6806363	0.922	4527	1.23
		0.3ml/min	6816686	0.789	4585	1.26
		0.33ml/min	6836255	0.718	4506	1.26

Column temp. (±50c)	300c	270c	6806475	0.920	4538	1.24
		300c	6827575	0.790	4536	1.23
		330c	6826446	0.718	4658	1.25
Mobile phase Composition (5%v/v)	40:60	70:30	6903363	0.736	4648	1.23
		65:35	6836573	0.791	4585	1.24
		60:40	6806363	0.853	4579	1.27

Table 8. Robustness data of Ritonavir

Parameter	Optimized condition	Used condition	Peak area	Retention Time	Plate Count	Tailing factor
Flow rate (±0.1ml/min)	1ml/min	0.27ml/min	2267565	1.230	6848	1.18
		0.3ml/min	2263535	1.049	6804	1.19
		0.33ml/min	2200905	0.960	6824	1.19
Column temp. (±50c)	300c	270c	2265474	1.236	6906	1.17
		300c	2274625	1.051	6836	1.19
		330c	2257464	0.951	6837	1.20
Mobile phase Composition (5%v/v)	40:60	70:30	2294635	0.954	6826	1.20
		65:35	2263527	1.051	6854	1.18
		60:40	2255462	1.190	6795	1.19

4.7 Forced Degradation Studies

Degradation of both Nirmatrelvir and Ritonavir shown in table 9 and Chromatogram in Figure 6 – 11

Table 9. Degradation data of Nirmatrelvir and Ritonavir

	Nirmatrelvir			Ritonavir		
FD condition	Area	% recoved	% Degradation	Area	% recoveed	% Degradation
Acid	6446353	94.54	5.46	2144577	94.47	5.53
Alkali	6483535	95.09	4.91	2188566	96.41	3.59
Oxidation	6794336	99.65	0.35	2266874	99.86	0.14
Thermal	6784776	99.51	0.49	2201563	96.98	3.02
Photolytic	6736355	98.80	1.20	2206353	97.19	2.81
Neutral	6736464	98.80	1.20	2217353	97.68	2.32

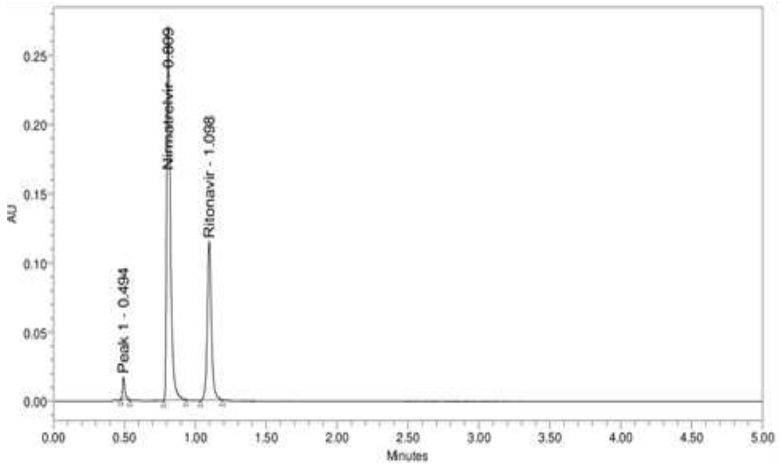

Figure 6. Acid degradation chromatogram

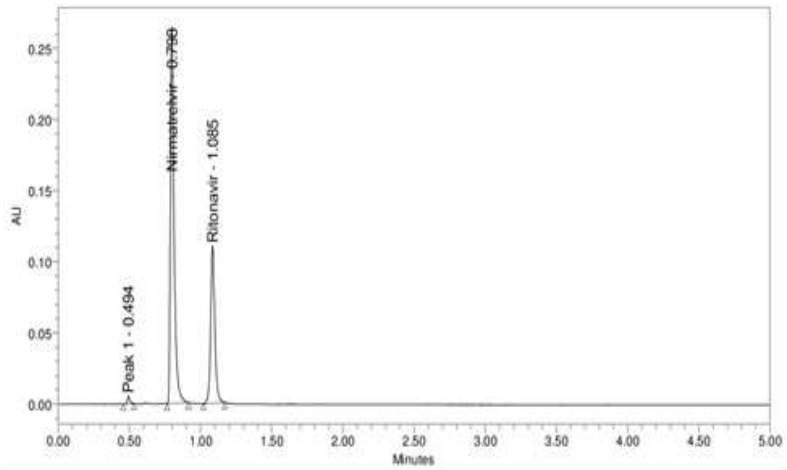

Figure 7. Base degradation chromatogram

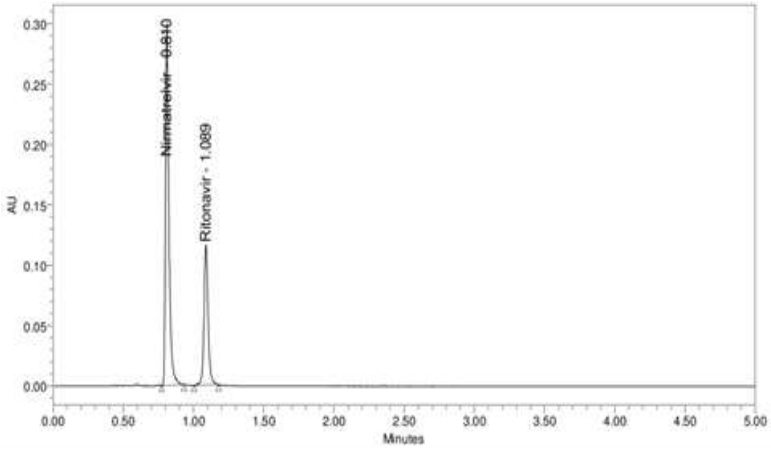

Figure 8. Peroxide degradation chromatogram

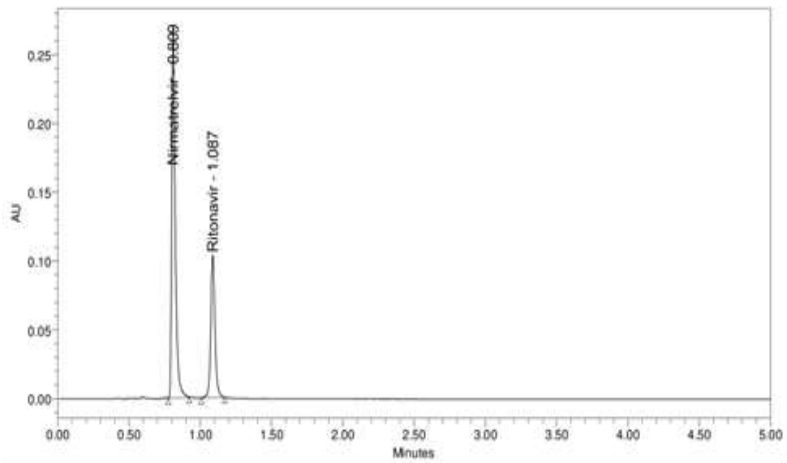

Figure 9. Thermal degradation chromatogram

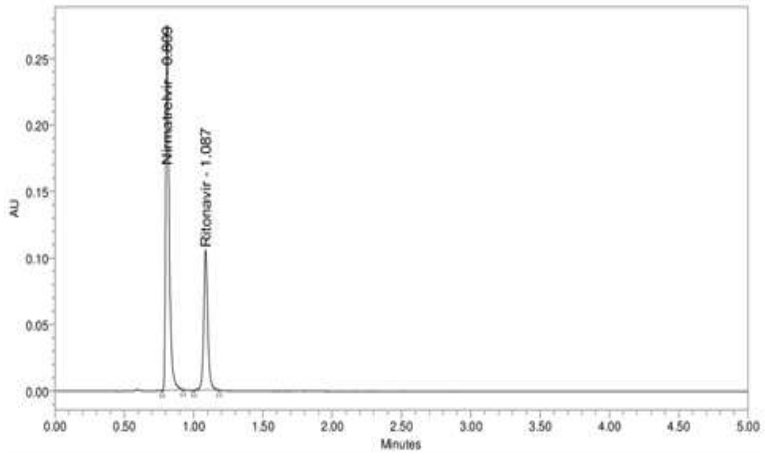

Figure 10. Uv degradation chromatogram

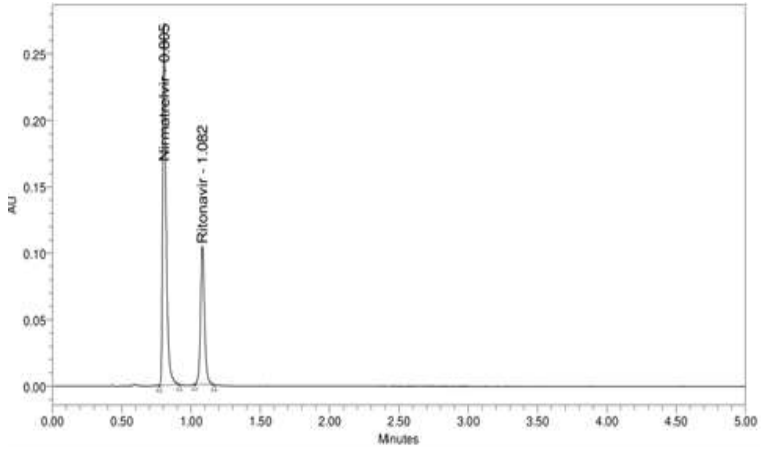

Figure 11. Water degradation chromatogram

DISCUSSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Ritonavir and Nirmatrelvir in Tablet dosage form. Retention time of Nirmatrelvir and Ritonavir were found to be 0.787 min and 1.058 min. %RSD of the Nirmatrelvir and Ritonavir were and found to be 0.5 and 0.4 respectively. %Recovery was obtained as 100.29% and 100.34% for Nirmatrelvir and Ritonavir. LOD, LOQ values were obtained from regression equations of Nirmatrelvir and Ritonavir were 0.33ppm, 0.03ppm and 1.02ppm, 0.08ppm respectively. Regression equation of Nirmatrelvir is y = 114686x + 13045, and of Ritonavir is y = 112957x + 6810.8, Retention times are 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.

CONCLUSION

The developed UPLC method for the estimation of selected drugs is simple, rapid, accurate, precise, robust and economical. The mobile phase and solvents are simple to prepare and economical, reliable, sensitive and less time consuming. The sample recoveries were in good agreement with their respective label claims and they suggested non-interference of formulation recipients in the estimation and can be used in laboratories for the routine analysis of selected drugs. Since the system validation parameters of UPLC method used for estimation of selected drugs in pure and have shown satisfactory, accurate and reproducible results (without any interference of recipients) as well, it is deduced that the simple and short proposed methods be most useful for analysis purpose. The present work concluded that stability indicating assay method by RP-UPLC was simple, accurate, precise, and specific

and has no interference with the placebo and degradation products. Hence these can be used for routine analysis of Nirmatrelvir and Ritonavir.

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