"Method development and method validation for estimation of Nirmatelvir and Ritonovir by using RP-HPLC method"

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ABSTRACT

A simple, Accurate, precise method was developed for the simultaneous estimation of the Nirmatrelvir and Ritonavir in pharmaceutical dosage form. Chromatogram was run through Std Agilent C18 150 x 4.6 mm, 5□. Mobile phase containing Buffer 0.01N KH2po4 (2.2Ph): Acetonitrile taken in the ratio 60:40 was pumped through column at a flow rate of 0.8ml/min. Buffer used in this method was 0.01N Kh2po4. Temperature was maintained at 30°C. Optimized wavelength selected was 265 nm. The retention time of Nirmatrelvir and Ritonavir were found to be 2.241 min and 2.816 min. %RSD of the Nirmatrelvir and Ritonavir were and found to be 0.4 and 0.6 respectively. %Recovery was obtained as 99.15% and 99.77% for Nirmatrelvir and Ritonavir respectively. LOD, LOQ values obtained from regression equations of Nirmatrelvir and Ritonavir were 0.21, 0.16 and 0.65, 0.48 respectively. The regression equation of Nirmatrelvir is y = 16802x + 995.5, and y = 16802x + 995.5 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. 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.

Key Words: Nirmatrelvir, Ritonavir, RP-HPLC, Chromatogram

1.INTRODUCTION

The quality of a drug plays an important role in ensuring the safety and efficacy of the drugs. Quality assurance and control of pharmaceutical and chemical formulations is essential for ensuring the availability of safe and effective drug formulations to consumers. Hence Analysis of pure drug substances and their pharmaceutical dosage forms occupies a pivotal role in assessing the suitability to use in patients. The quality of the analytical data depends on the quality of the methods employed in generation of the data (1). Hence, development of rugged and robust analytical methods is very important for statutory certification of drugs and their formulations with the regulatory authorities. The quality and safety of a drug is generally assured by monitoring and controlling the assay and impurities effectively. While assay determines the potency of the drug and impurities will determine the safety aspect of the drug. Assay of pharmaceutical products plays an important role in efficacy of the drug in patients. The wide variety of challenges is encountered while developing the methods for different drugs depending on its nature and properties. This along with the importance of achieving the selectivity, speed, cost, simplicity, sensitivity, reproducibility and accuracy of results gives an opportunity for researchers to come out with solution to address the challenges in getting the new methods of analysis to be adopted by the pharmaceutical industry and chemical laboratories. Different physico-chemical methods (1) are used to study the physical phenomenon that occurs because of chemical reactions. Among the physico-chemical methods, the most important are optical (refractometry, polarimetry, emission and fluorescence methods of analysis), photometry (photocolorimetry and spectrophotometry covering UV- Visible, IR Spectroscopy and nepheloturbidimetry) and chromatographic (column, paper, thin layer, gas liquid and high-performance liquid chromatography) methods. Methods such as nuclear magnetic resonance (NMR) and para magnetic resonance (PMR) are becoming more and more popular. The combination of mass spectroscopy (MS) with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures which are based on complex formation; acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have also been used in

pharmaceutical analysis. The number of new drugs is constantly growing. This requires new methods for controlling their quality. Modern pharmaceutical analysis must need the following requirements.

1. The analysis should take minimal time.
2. The accuracy of the analysis should meet the demands of Pharmacopoeia.
3. The analysis should be economical.
4. The selected method should be precise and selective.

2.MATERIALS AND METHODS

Materials:

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

Instruments:

- Electronics Balance-Denver
- p^H meter -BVK enterprises, India
- Ultrasonicator-BVK enterprises
- Waters HPLC System series with Binary pumps, Photo Diode array detector and manual sampler integrated with empower software
- Lab india UV double beam spectrophotometer with UV win5 software was used for measuring absorbances of Nirmatrelvir and Ritonavir solutions.

Methods:

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

Preparation of buffer:

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 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).

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).

Validation:

System suitability parameters:

The system suitability parameters were determined by preparing standard solutions of Nirmatrelvir (15) and Ritonavir (10ppm) 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.

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%.

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)

25% Standard solution: 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (3.75µg/ml of Nirmatrelvir and 2.5µg/ml of Ritonavir)

50% Standard solution: 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (7.5µg/ml of Nirmatrelvir and 5µg/ml of Ritonavir)

75% Standard solution: 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (11.25µg/ml of Nirmatrelvir and 7.5µg/ml of Ritonavir)

100% Standard solution: 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (15µg/ml of Nirmatrelvir and 10µg/ml of Ritonavir)

125% Standard solution: 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (18.75µg/ml of Nirmatrelvir and 12.5 µg/ml of Ritonavir)

150% Standard solution: 1.5ml each from two standard stock solutions was pipettede out and made up to 10ml (22.5µg/ml of Nirmatrelvir and 15µg/ml of Ritonavir)

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)

Preparation of 50% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Acceptance Criteria:

The % Recovery for each level should be between 98.0 to 102

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.

Robustness conditions like Flow minus (0.7ml/min), Flow plus (0.9ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much effected and all the parameters were passed. %RSD was within the limit.

LOD sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of Nirmatrelvir, Ritonavir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

LOQ sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each of Nirmatrelvir, Ritonavir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

Degradation studies:

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 60^0c. For HPLC study, the resultant solution was diluted to obtain 15µg/ml& 10µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:

To 1 ml of stock ssolution Nirmatrelvir and Ritonavir, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 15µg/ml& 10µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:

To 1 ml of stock solution Nirmatrelvir and Ritonavir, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 15µg/ml& 10µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:

The standard drug solution was placed in oven at 105°C for 1 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 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/m^2 in photo stability chamber For HPLC study, the resultant solution

was diluted to obtain 15µg/ml& 10µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxing the drug in water for 1hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to 15µg/ml& 10µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

3.RESULTS AND DISCUSSION

Optimized wavelength selected was 215nm.

Method development: Method development was done by changing various, mobile phase ratios, buffers etc.

Trial 1:

Chromatographic conditions:

Mobile phase	**:**	Acetonitrile : Water (60:40 v/v)
Flow rate	**:**	**1** ml/min
Column	**:**	Agilent C18 (4.6 x 150mm, 5µm)
Detector wavelength	**:**	215nm
Column temperature	**:**	30°C
Injection volume	**:**	10.0□L
Run time	**:**	10.0 min
Diluent	**:**	Water and Acetonitrile in the ratio 50:50 v/v
Results	:	In this trail only one peak was eluted, so, further trial is carried out.

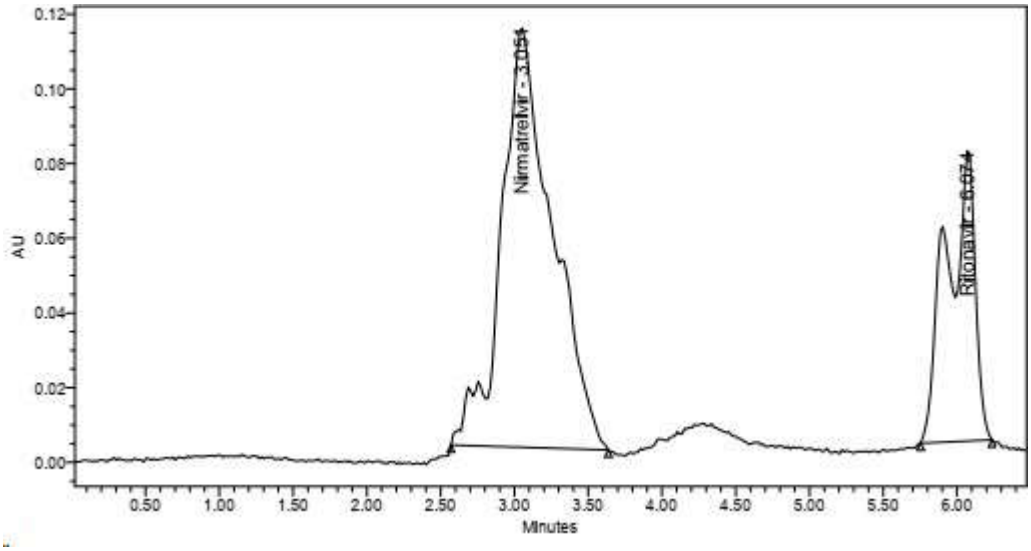


Fig 3.1.Trial chromatogram 1

Trial 2 :

Chromatographic conditions:

Mobile phase	**:**	Water**:** Methanol (50:50)
Flow rate	**:**	**1** ml/min
Column	**:**	Agilent C18 (4.6 x 250mm, 5µm)
Detector wave length	**:**	215nm
Column temperature	**:**	30°C
Injection volume	**:**	10□L
Run time	**:**	5 min
Diluent	**:**	Water and Acetonitrile in the ratio (50:50)

Results : Both peaks were eluted but both peaks were eluted with broad shape and peak splitting is observed. so, Further trial is carried out.

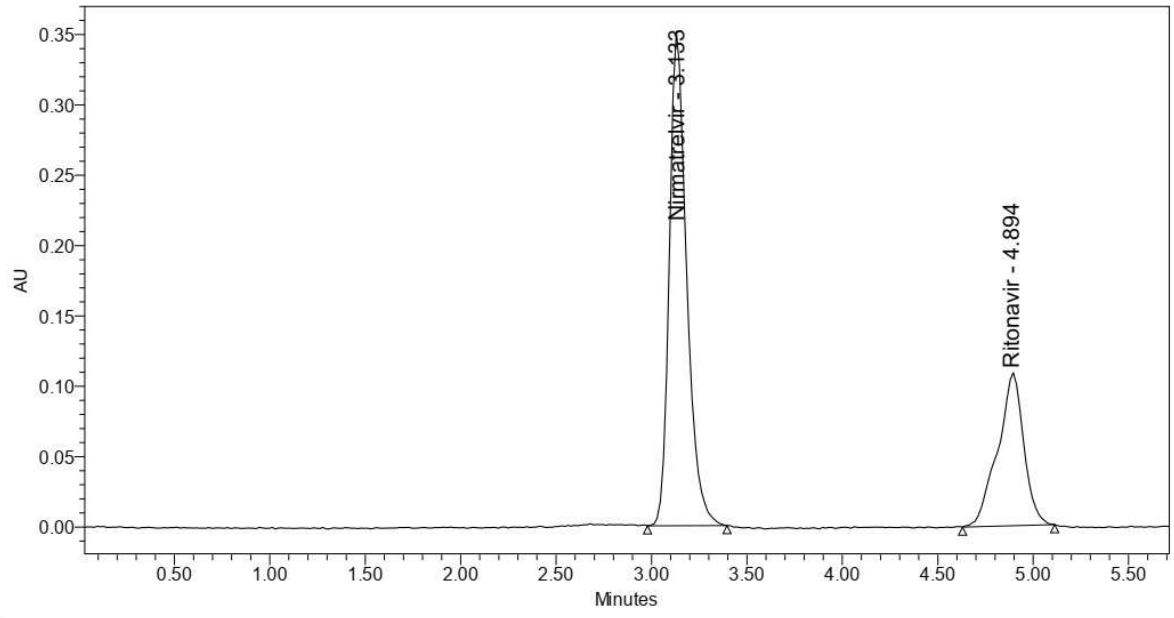


Fig 3.2 Trial chromatogram 2

Trial 3:

Chromatographic conditions:

Mobile phase	**:**	0.1%OPA**:** Methanol (50:50)
Flow rate	**:**	**1** ml/min
Column	**:**	Agilent C18 (4.6 x 250mm, 5µm)
Detector wave length	**:**	215nm
Column temperature	**:**	30°C
Injection volume	**:**	10□L
Run time	**:**	5 min
Diluent	**:**	Water and Acetonitrile in the ratio (50:50)

Results : Both peaks were eluted but Ritonavir peak shape is not good so, Further trial is carried out.

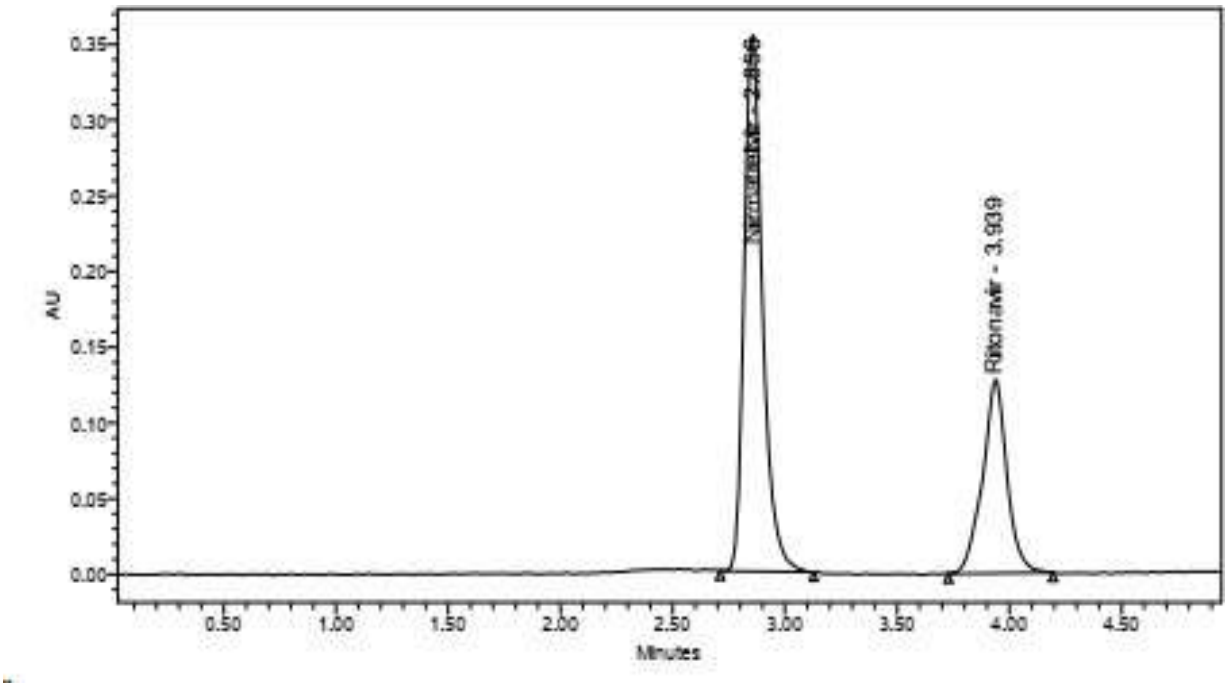


Fig 3.3 Trial chromatogram 3

Trial 4:

Chromatographic conditions:

Mobile phase : 0.1% OPA:Acetonitrile(50:50)

Flow rate : **1** ml/min

Column : Agilent C18 (4.6 x 150mm, 5µm)

Detector wave length : 215nm

Column temperature : 30°C

Injection volume : 10□L

Run time : **5** min

Diluent : Water and Acetonitrile in the ratio 50:50

Results : Nirmatrelvir and Ritonavir both peaks are eluted but Ritonavir peak retention time more than literature review. So, further trails are carried out.

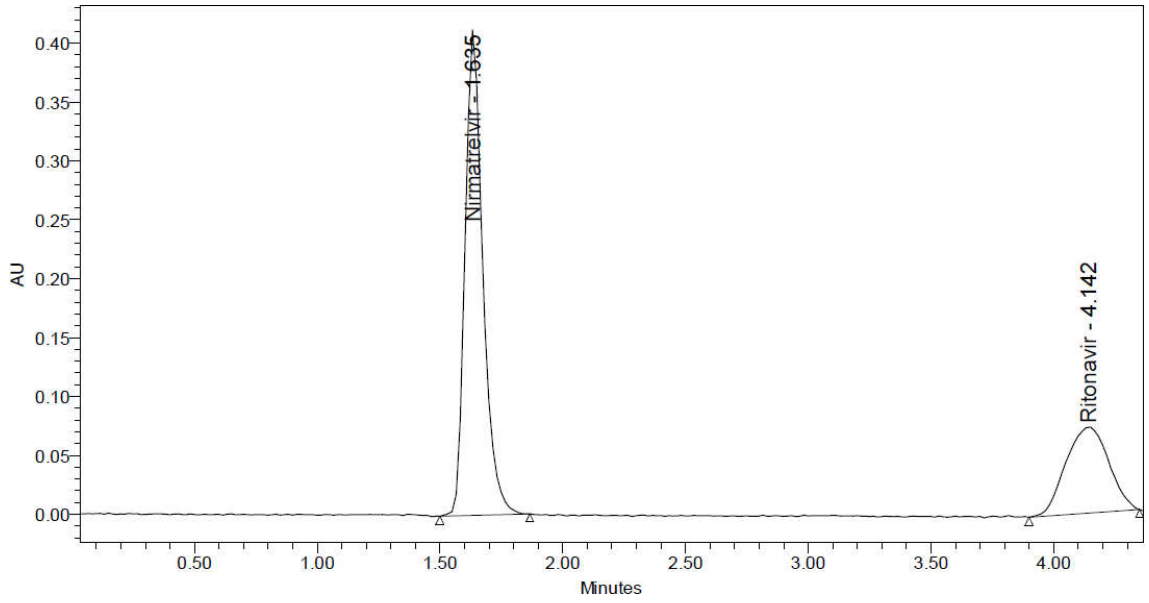


Fig 3.4 Trial chromatogram 4

Optimized method:

Chromatographic conditions:

Mobile phase	**:**	0.01N Kh2po4:Acetonitrile(50:50)
Flow rate	**:**	1 ml/min
Column	**:**	Agilent C8 (4.6 x 150mm, 5µm)
Detector wave length	**:**	215nm
Column temperature	**:**	30°C
Injection volume	**:**	10□L
Run time	**:**	**10**min
Diluent	**:**	Water and Acetonitrile in the ratio 50:50
Results	**:**	Both peaks have good resolution, tailing Factor, theoretical plate count and resolution.

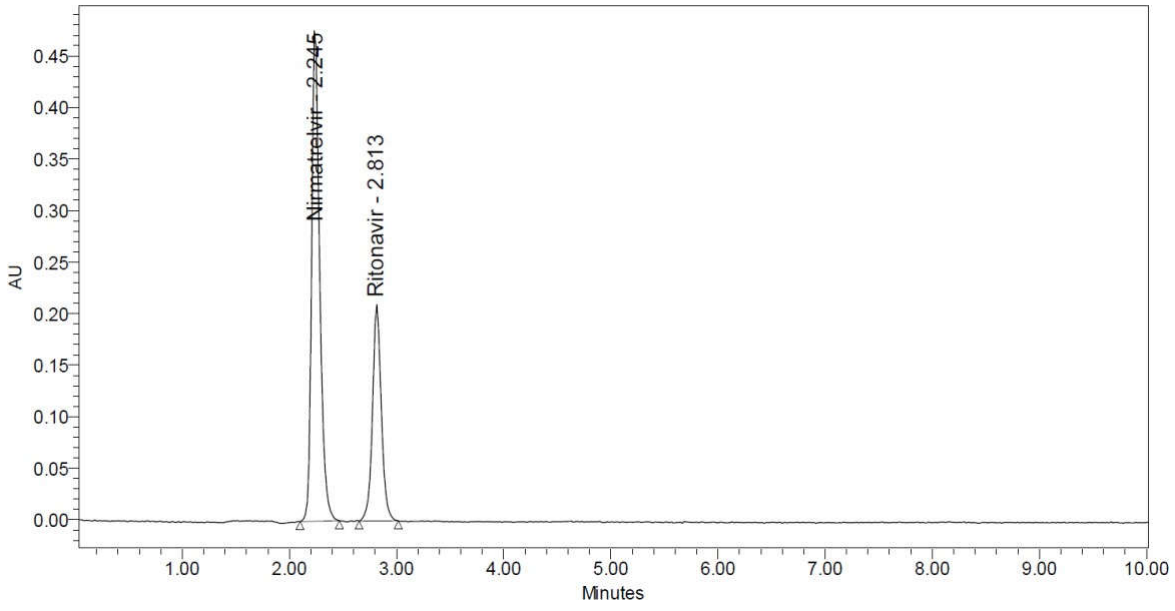


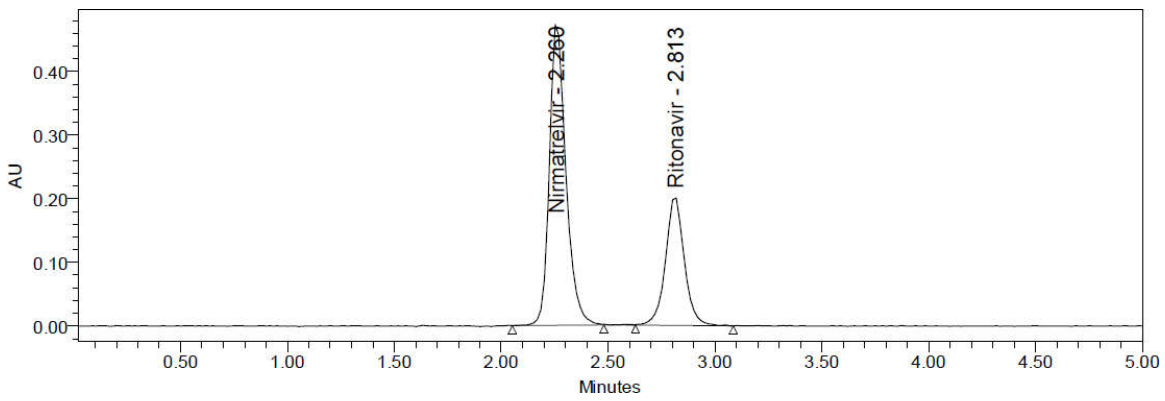
Fig 3.5 Optimized Chromatogram

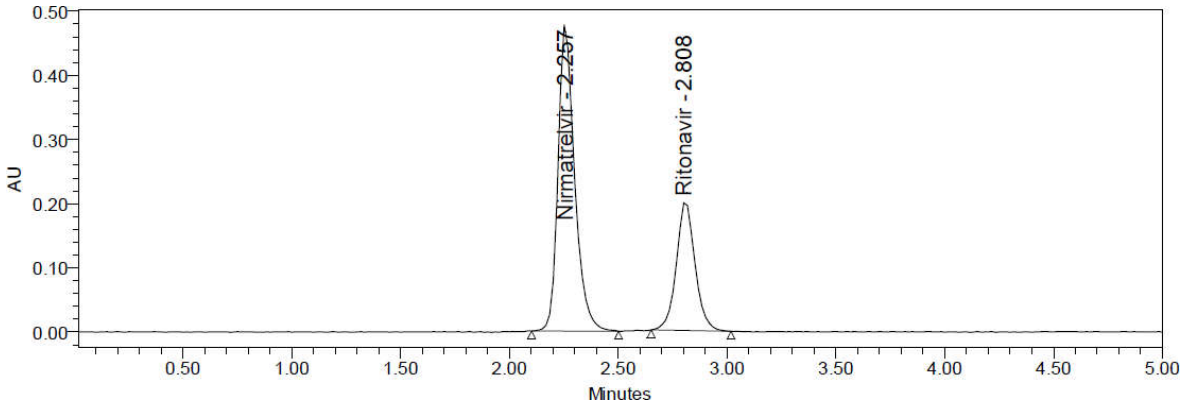
Observation: Nirmatrelvir and Ritonavir were eluted at 2.241 min and 2.816 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

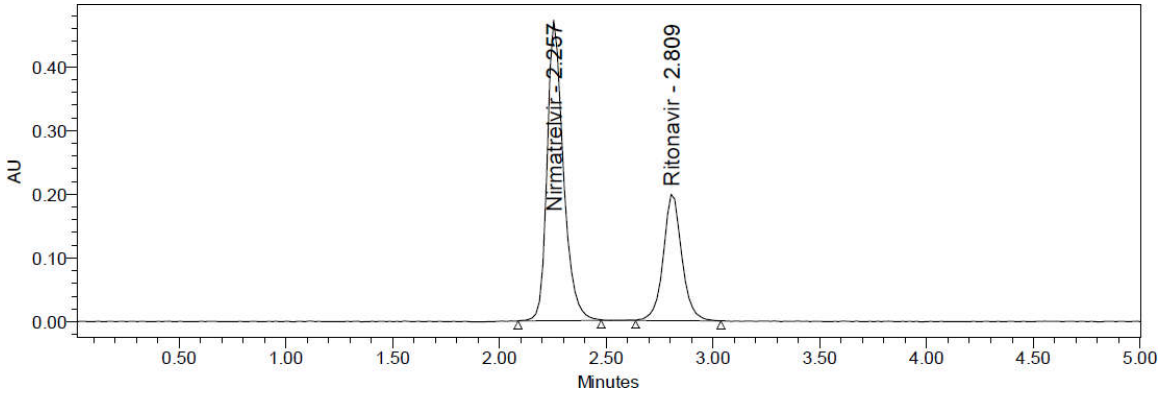
System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines

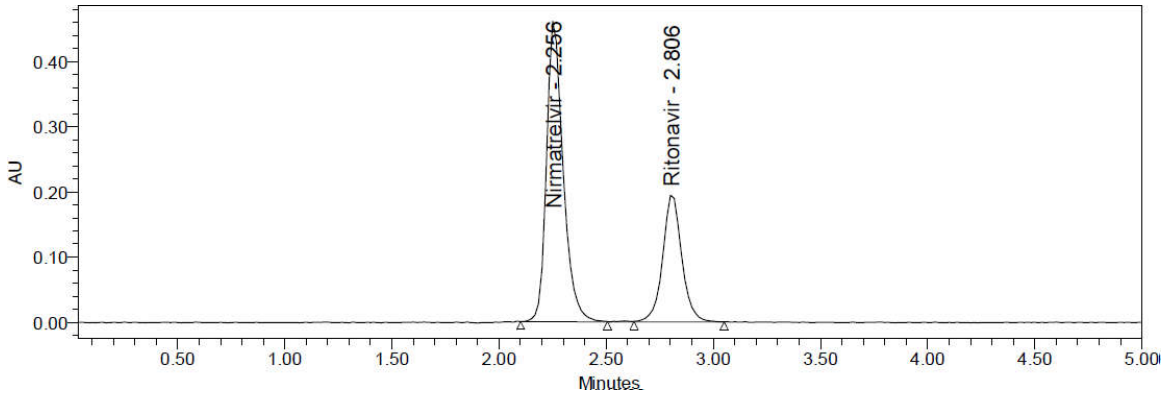
Table: 3.1 System suitability parameters for Nirmatrelvir and Ritonavir

S no	Nirmatrelvir			Ritonavir			
Inj	RT(min)	USP Plate Count	Tailing	RT (min)	USP Plate Count	Tailing	Resoluton
1	2.256	4131	1.29	2.806	5264	1.04	3.5
2	2.257	4355	1.29	2.808	5484	1.07	3.6
3	2.257	4284	1.27	2.809	5575	1.06	3.7
4	2.258	4410	1.28	2.812	5241	1.03	3.6
5	2.260	4330	1.27	2.813	5242	1.05	3.7
6	2.262	4285	1.25	2.813	5339	1.03	3.6




Nirmatrelvir - 2.257
Ritonavir - 2.808
AU
Minutes


Nirmatrelvir - 2.257
Ritonavir - 2.809
AU
Minutes


Nirmatrelvir - 2.256
Ritonavir - 2.806
AU
Minutes

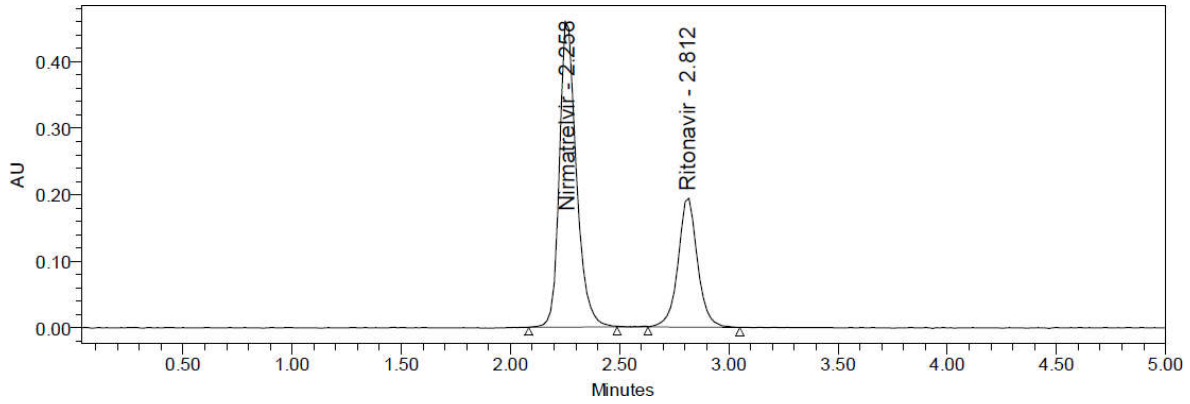


Fig 3.6 System suitability Chromatogram

Discussion: According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Validation:

Specificity:

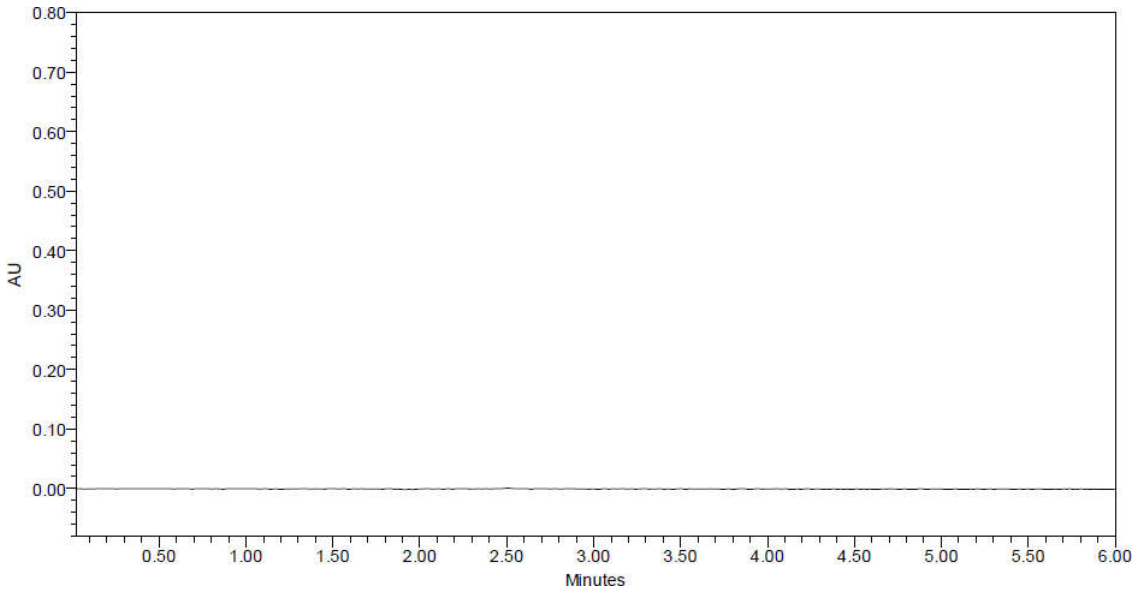


Figure No. 3.07. Chromatogram of blank.

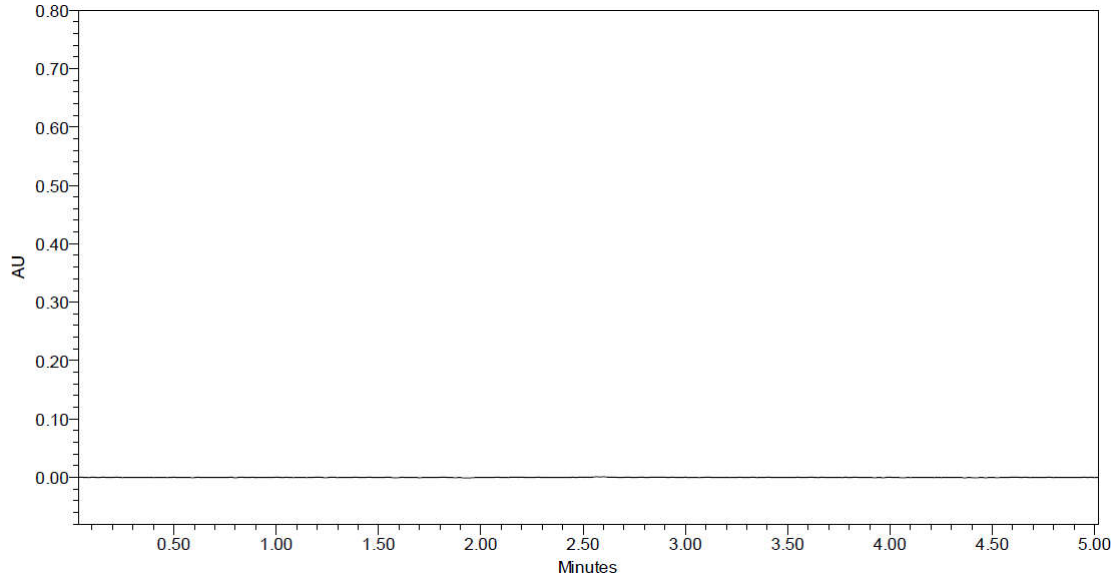


Figure No. 3.8 Chromatogram of placebo

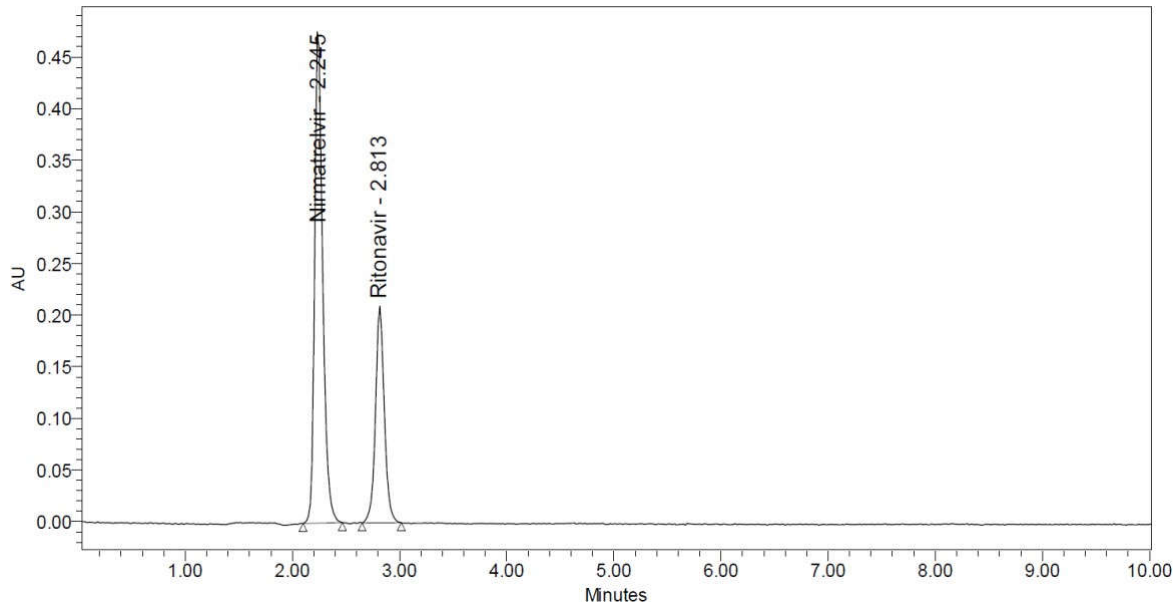


Fig 3.09 Typical Chromatogram

Discussion: Retention times of Nirmatrelvir and Ritonavir were 2.258 min and 2.812 min respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Linearity:

Table 3.2 Linearity table for Nirmatrelvir and Ritonavir.

Nirmatrelvir		Ritonavir	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
3.75	63530	2.5	30323
7.5	130051	5	59595
11.25	186426	7.5	87814
15	252346	10	122107
18.75	318423	12.5	147962
22.5	378385	15	175627

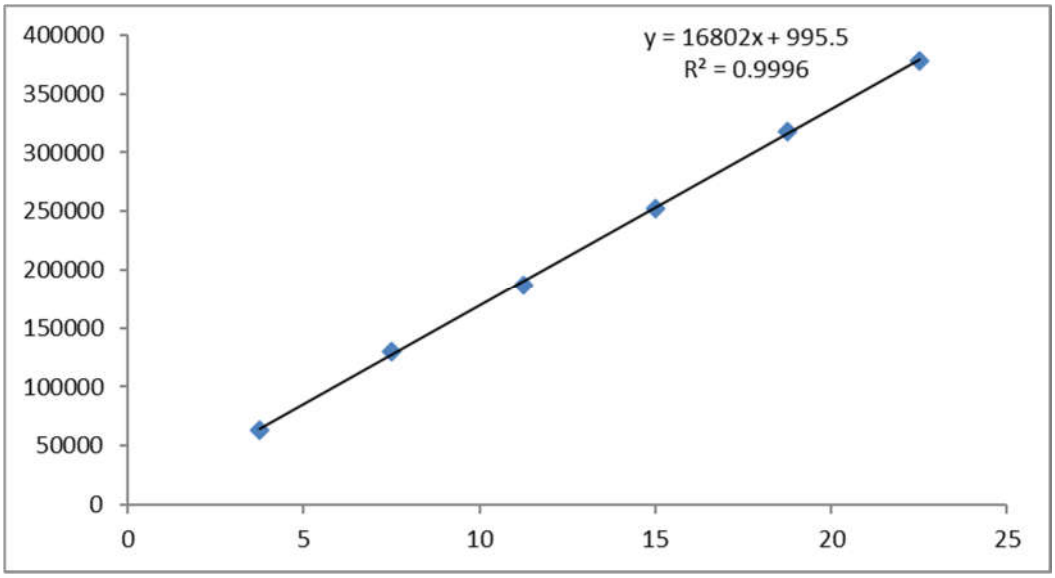


Fig No. 3.10 Calibration curve of Nirmatrelvir

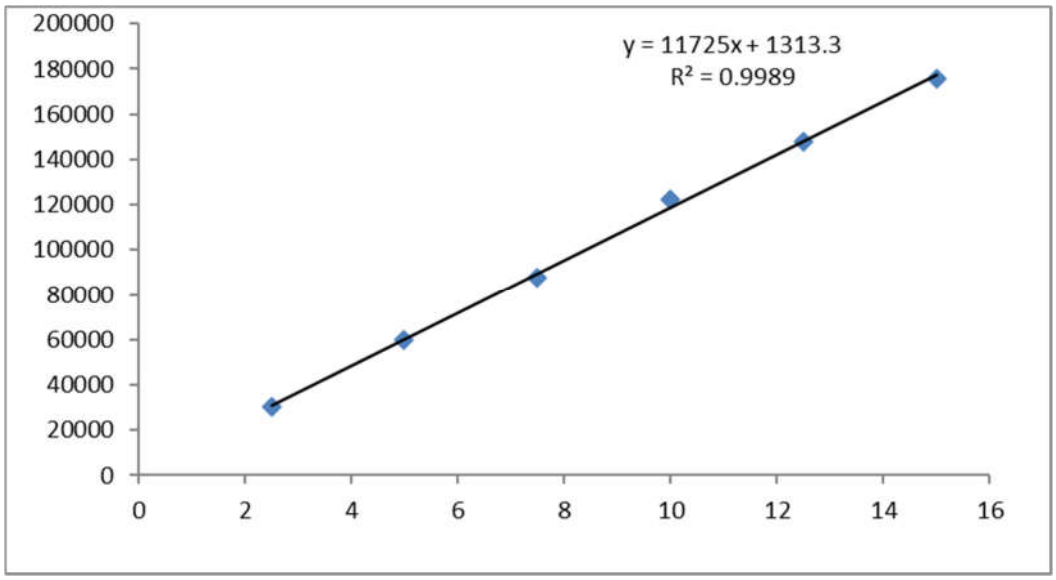
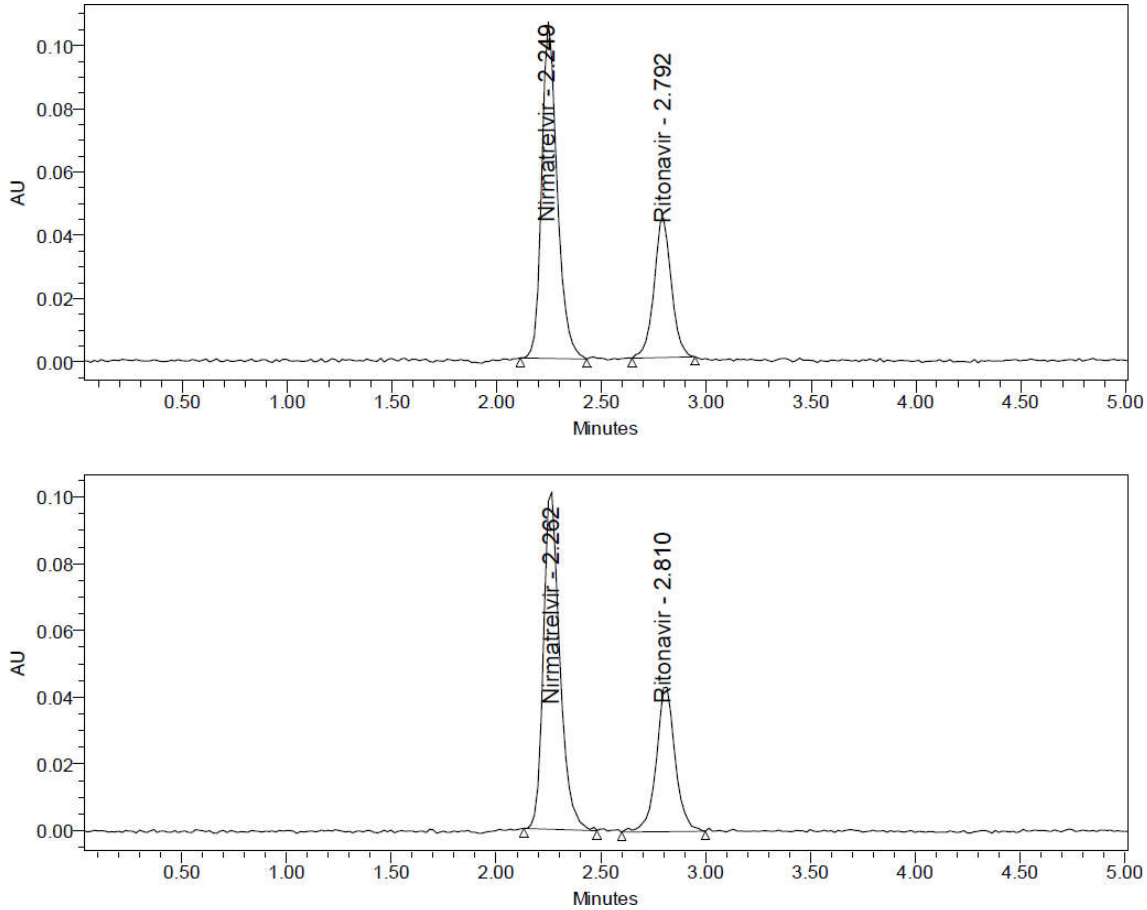
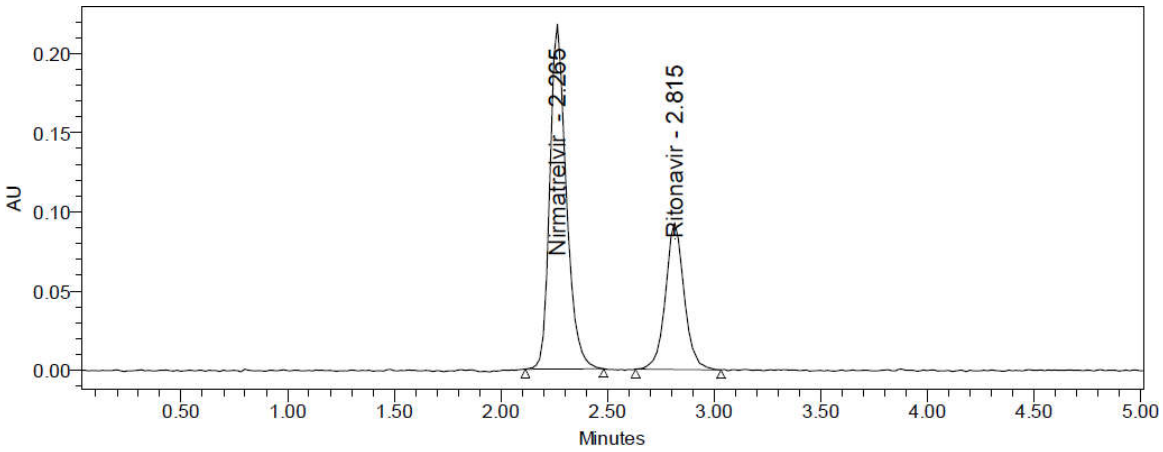


Fig No. 3.11 Calibration curve of Ritonavir



Discussion: Six linear concentrations of Nirmatrelvir (3.75-22.5µg/ml) and Ritonavir (2.5-15µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Nirmatrelvir was y = 16802x + 995.5 and of Ritonavir was y = 11725x + 1313.3. Correlation coefficient obtained was 0.999.

Fig. No. 3.12 Linearity 25% Chromatogram of Nirmatrelvir and Ritonavir



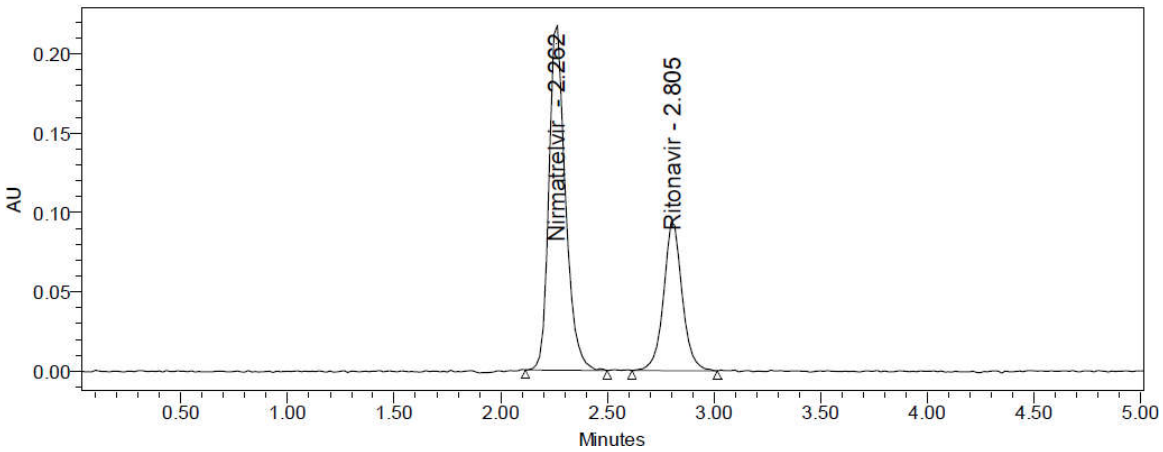


Fig No. 3.13 Linearity 50% Chromatogram of Nirmatrelvir and Ritonavir

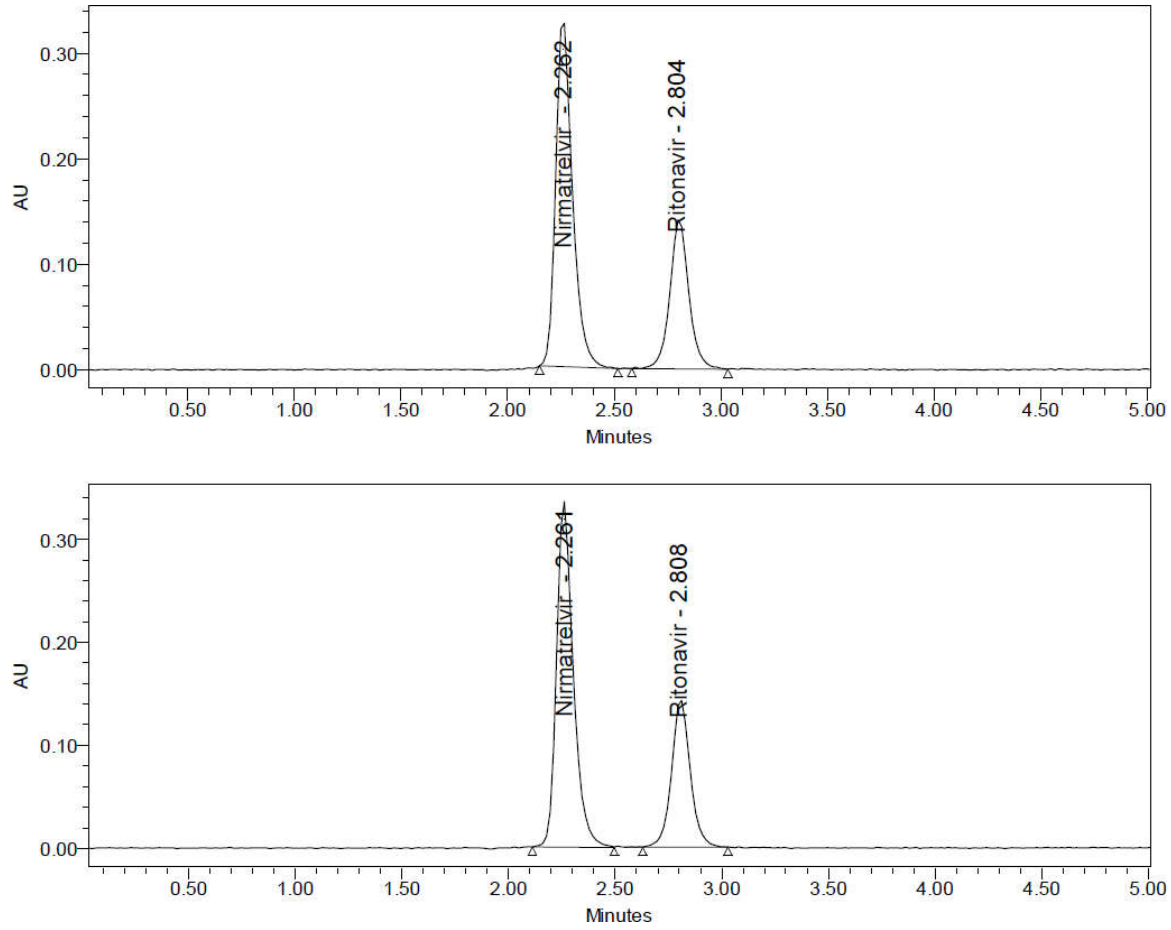


Fig No. 3.14 Linearity 75% Chromatogram of Nirmatrelvir and Ritonavir

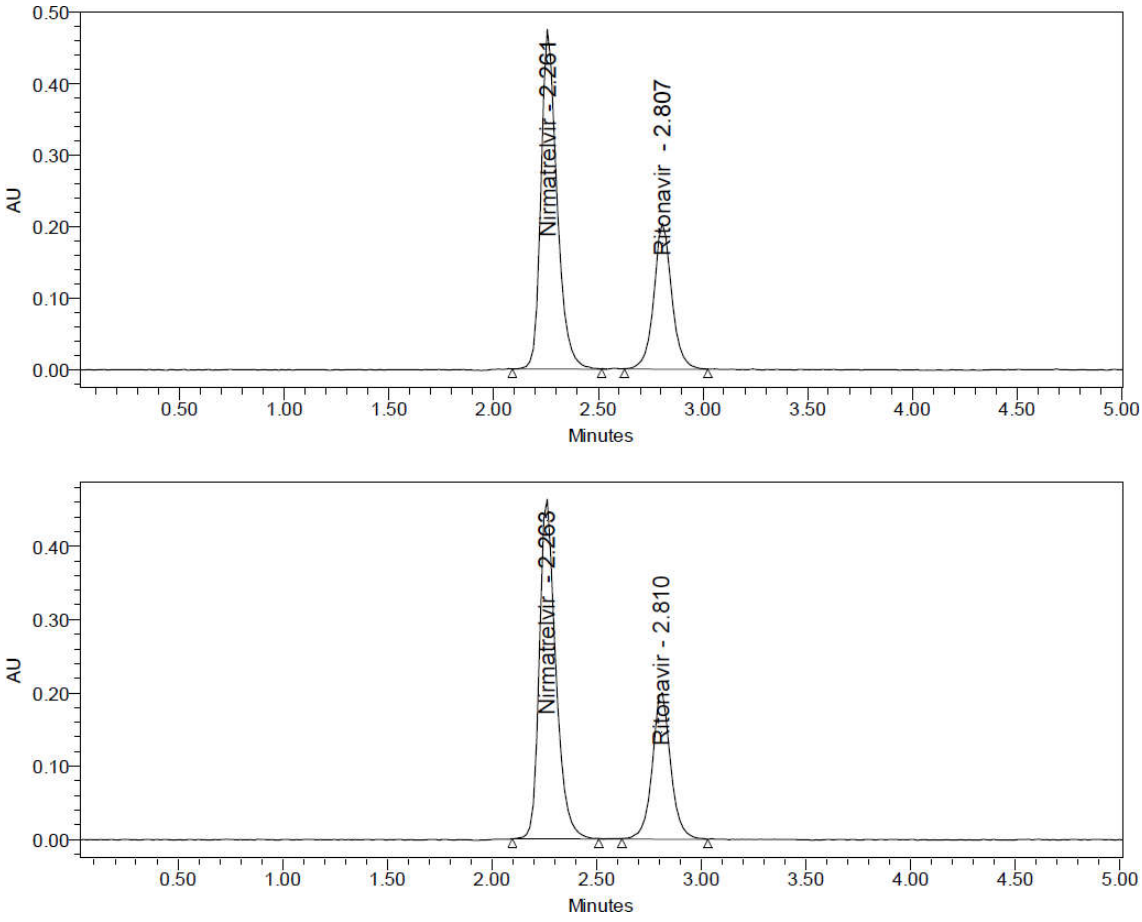


Fig No. 3.15 Linearity 100% Chromatogram of Nirmatrelvir and Ritonavir

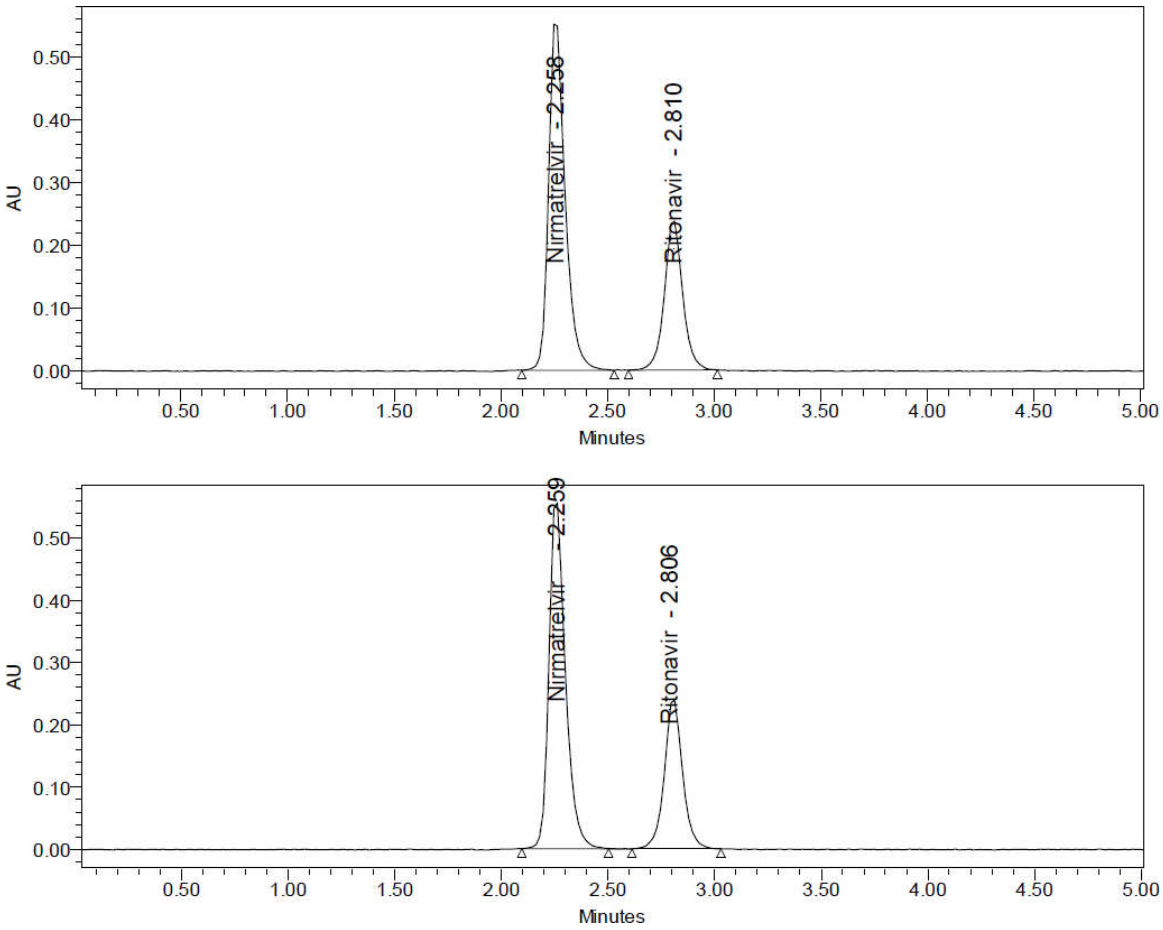


Fig No. 3.16 Linearity 125% Chromatogram of Nirmatrelvir and Ritonavir

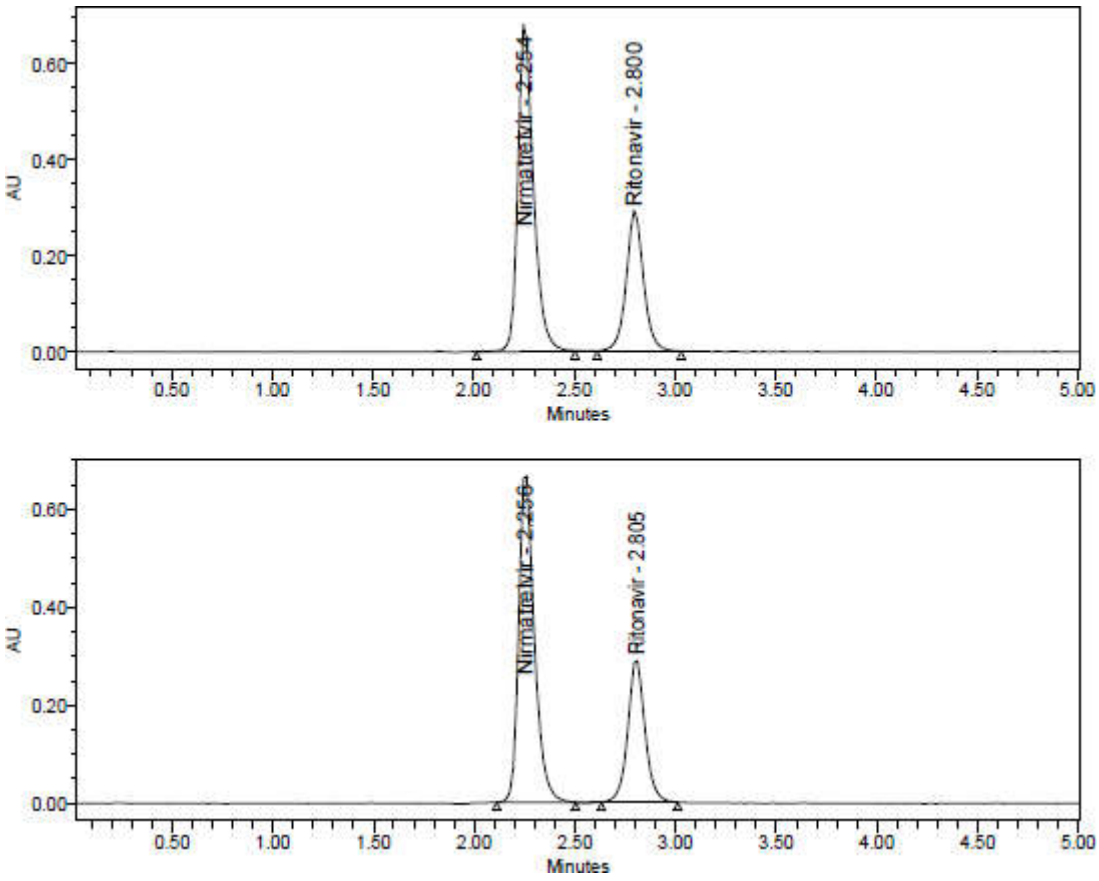


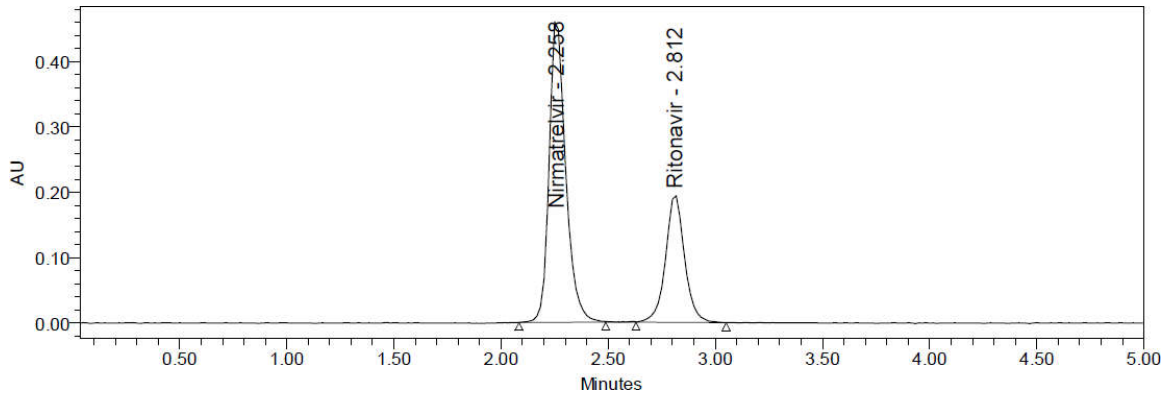
Fig No. 3.17 Linearity 150% Chromatogram of Nirmatrelvir and Ritonavir

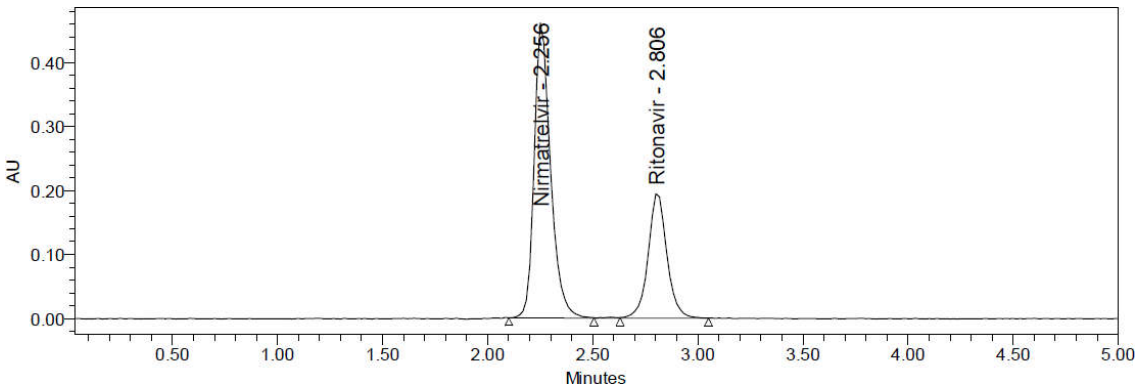
Precision:

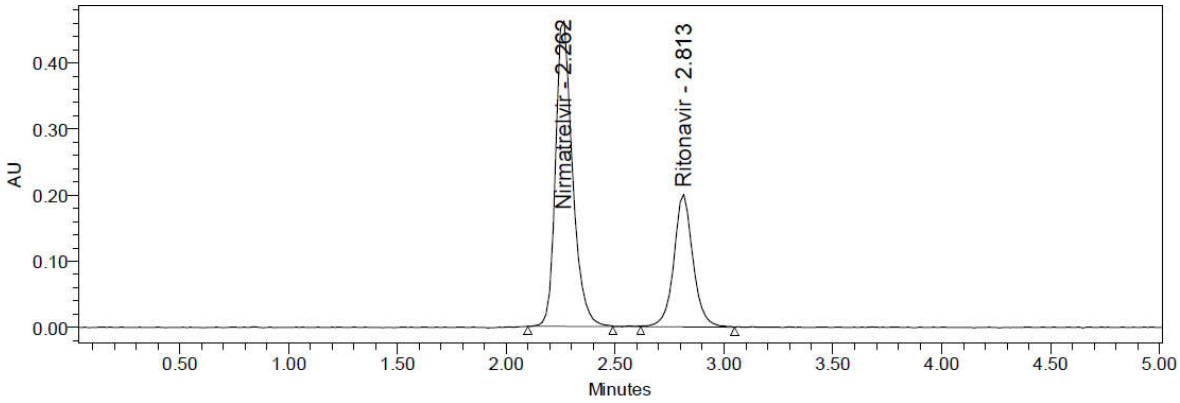
System Precision:

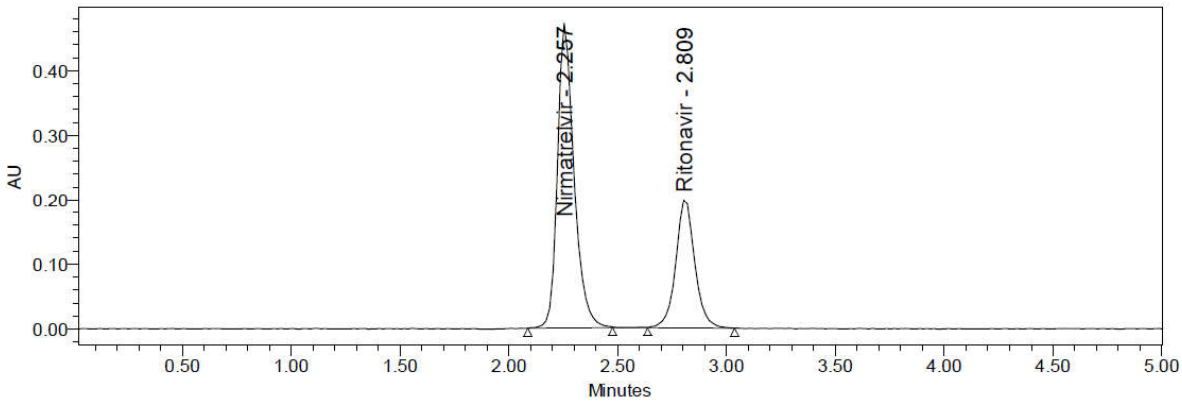
Table 3.3 System precision table of Nirmatrelvir and Ritonavir

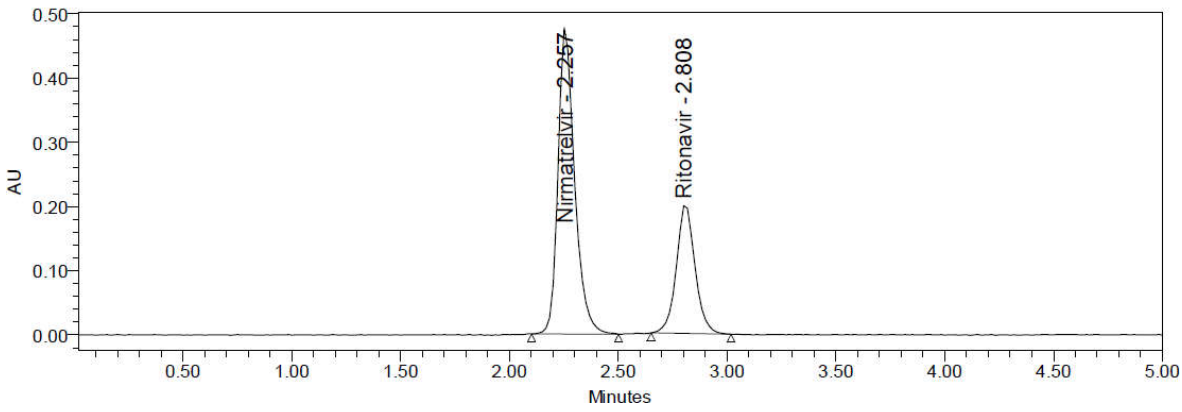
S. No	Area of Nirmatrelvir	Area of Ritonavir
1.	252676	119169
2.	252661	119165
3.	252638	118338
4.	252495	119901
5.	254357	118480
6.	254772	118065
Mean	253267	118853
S.D	1016.0	681.3
%RSD	0.4	0.6




Nirmatrelvir - 2.256
Ritonavir - 2.806
AU
Minutes


Nirmatrelvir - 2.262
Ritonavir - 2.813
AU
Minutes


Nirmatrelvir - 2.257
Ritonavir - 2.809
AU
Minutes


Nirmatrelvir - 2.257
Ritonavir - 2.808
AU
Minutes

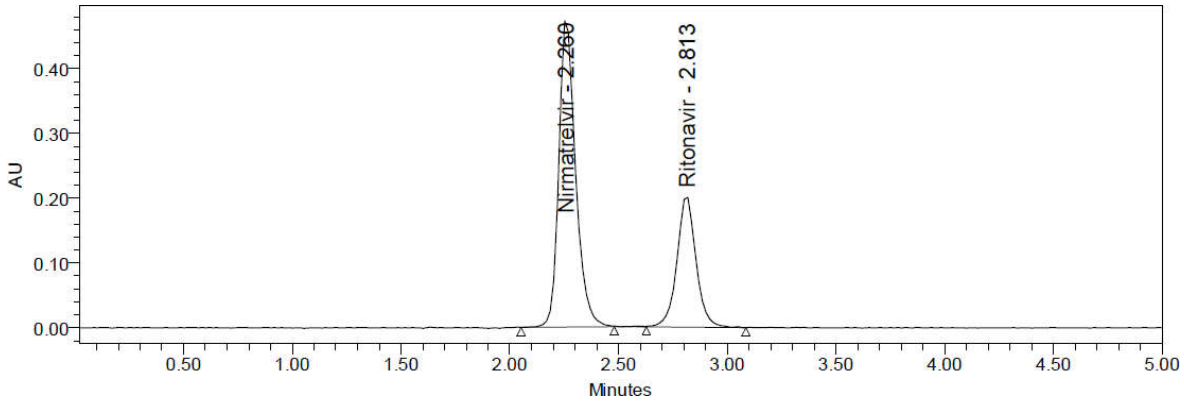


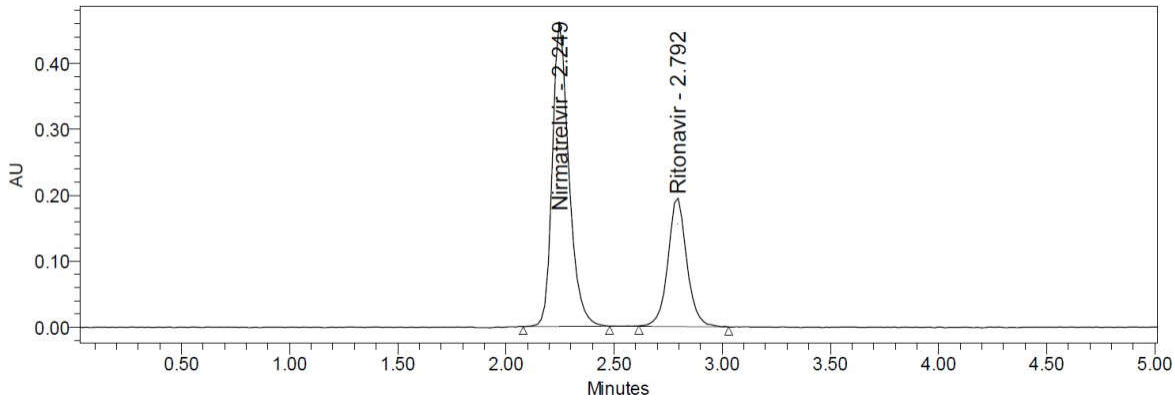
Fig 3.18 System precision chromatogram

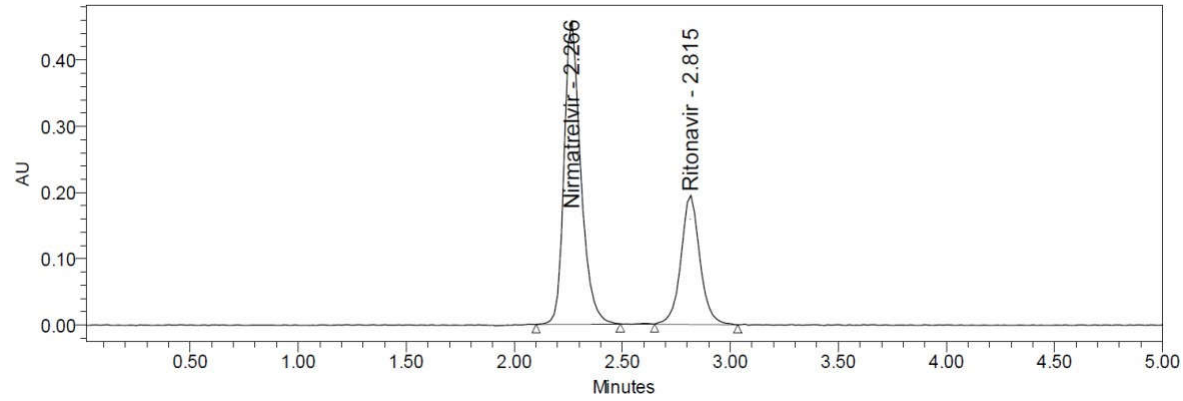
Discussion: From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.4% and 0.6% respectively for Nirmatrelvir and Ritonavir. As the limit of Precision was less than "2" the system precision was passed in this method.

Repeatability:

Table 3.4 Repeatability table of Nirmatrelvir and Ritonavir

S. No	Area of Nirmatrelvir	Area of Ritonavir
1.	251414	118516
2.	252509	118393
3.	252274	118673
4.	256473	118782
5.	253323	118796
6.	252751	118832
Mean	253124	118665
S.D	1756.1	176.1
%RSD	0.7	0.1


Nirmatrelvir - 2.249
Ritonavir - 2.792
AU
Minutes
0.00
0.10
0.20
0.30
0.40
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00


Nirmatrelvir - 2.266
Ritonavir - 2.815
AU
Minutes
0.00
0.10
0.20
0.30
0.40
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00

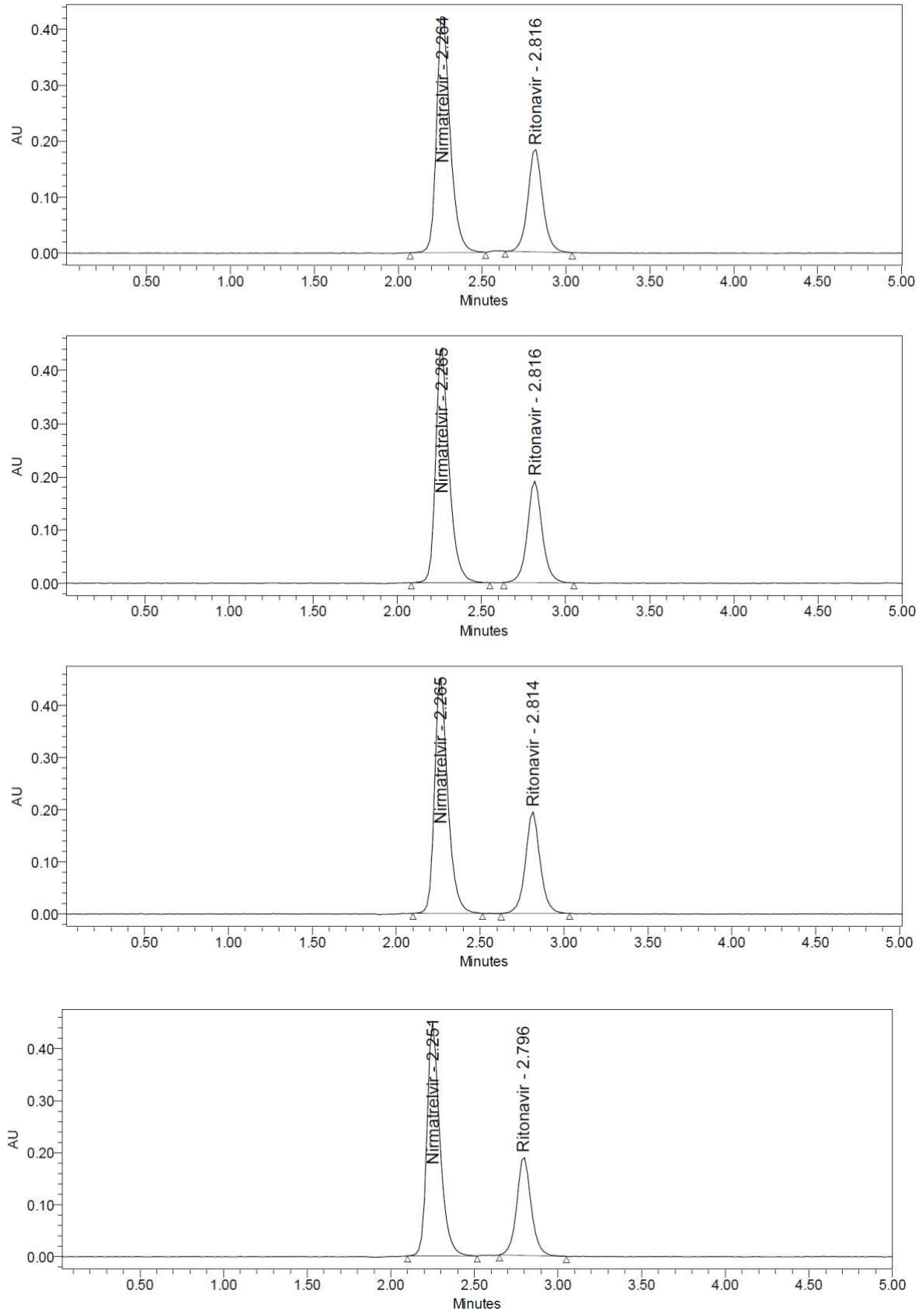


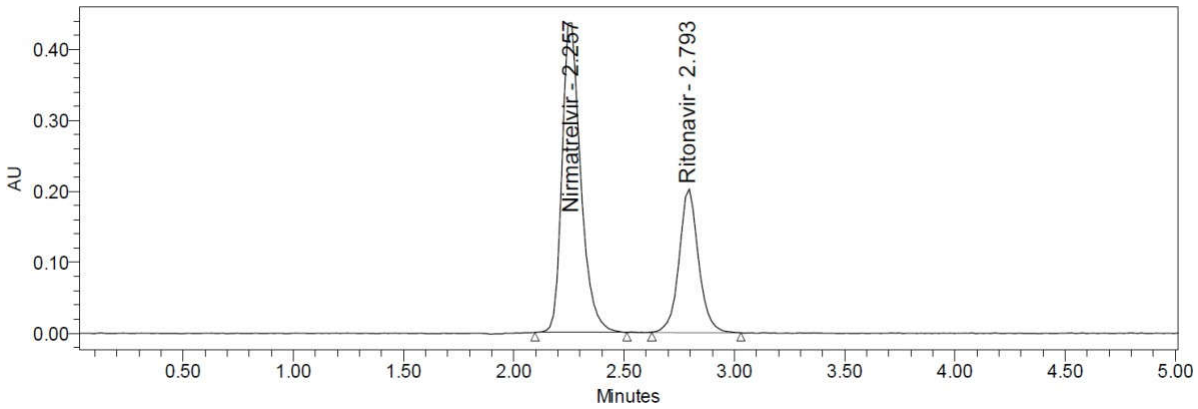
Fig No. 3.19 Repeatability chromatogram

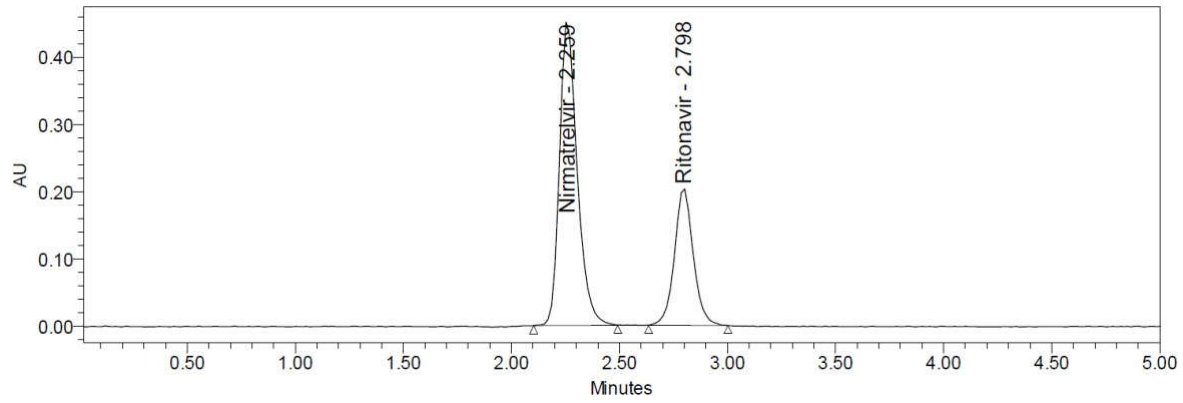
Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.7% and 0.1% respectively for Nirmatrelvir and Ritonavir. As the limit of Precision was less than "2" the system precision was passed in this method.

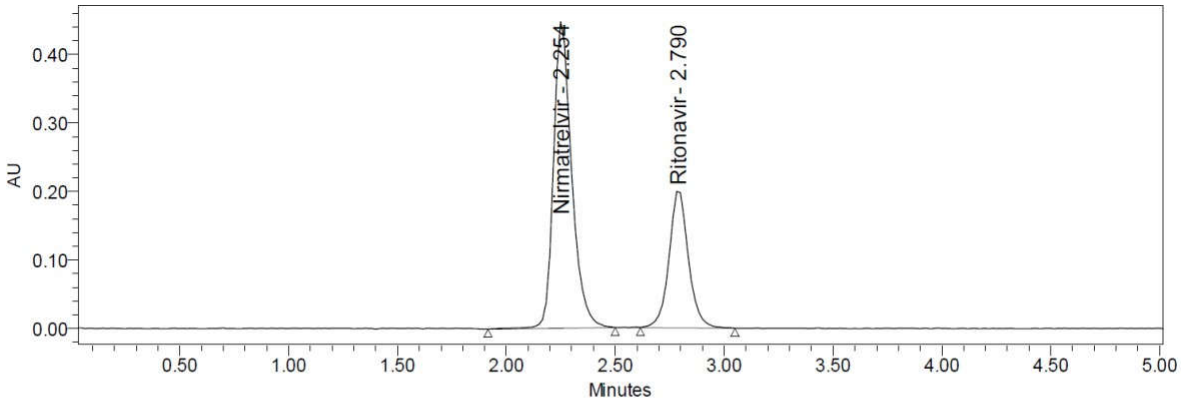
Intermediate precision (Day_ Day Precision):

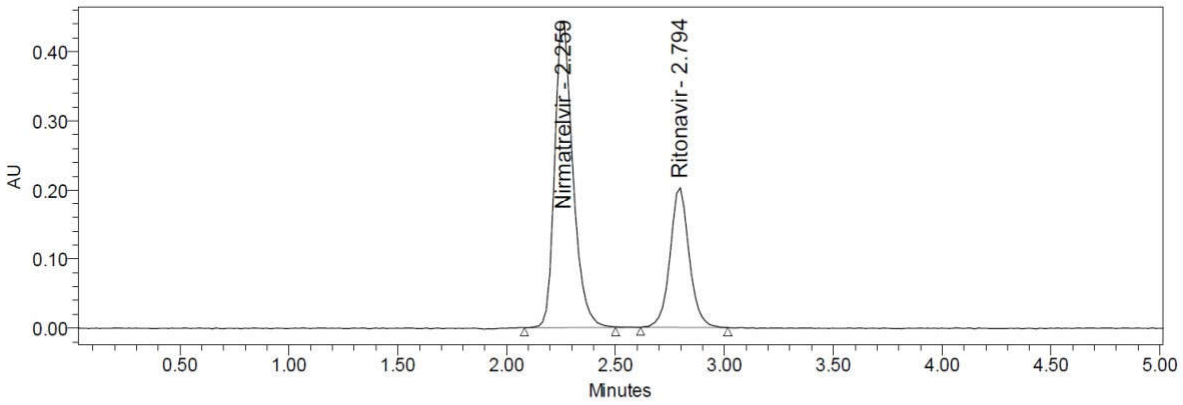
Table 3.5 Intermediate precision table of Nirmatrelvir and Ritonavir

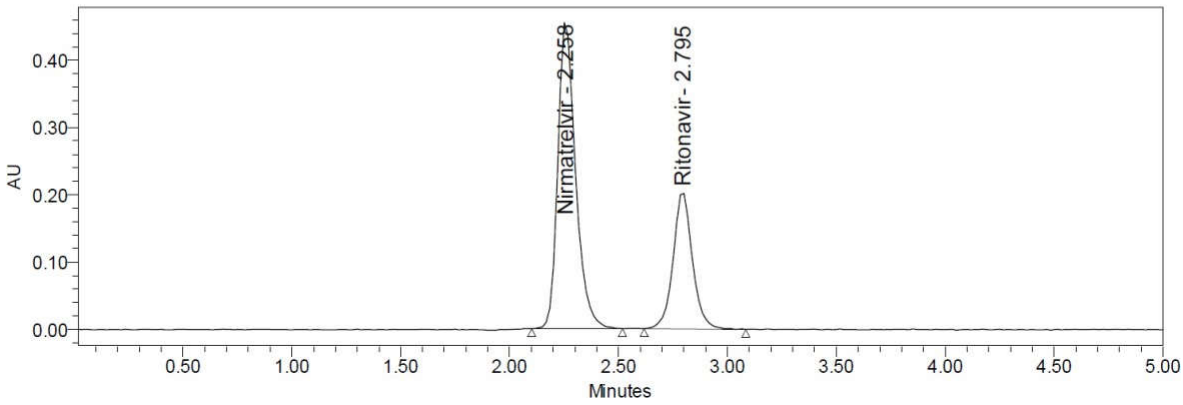
S. No	Area of Nirmatrelvir	Area of Ritonavir
1.	248551	110678
2.	249426	110488
3.	246627	110077
4.	246743	110417
5.	243234	111001
6.	248538	110191
Mean	247187	110475
S.D	2228.7	334.8
%RSD	0.9	0.3




Nirmatrelvir - 2.259
Ritonavir - 2.798
AU
Minutes


Nirmatrelvir - 2.254
Ritonavir - 2.790
AU
Minutes


Nirmatrelvir - 2.259
Ritonavir - 2.794
AU
Minutes


Nirmatrelvir - 2.258
Ritonavir - 2.795
AU
Minutes

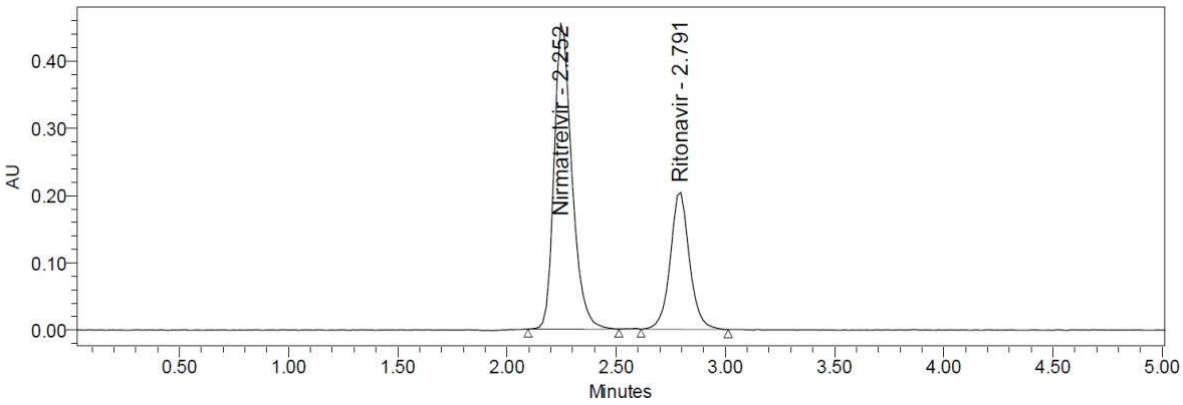


Fig: 3.20 Inter Day precision Chromatogram

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.9% and 0.3% respectively for Nirmatrelvir and Ritonavir. As the limit of Precision was less than “2” the system precision was passed in this method.

Accuracy:

Table 3.6 Accuracy table of Nirmatrelvir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	7.5	7.38	98.35	99.15%
	7.5	7.45	99.39	
	7.5	7.47	99.55	
100%	15	14.85	98.99	
	15	14.91	99.42	
	15	14.71	98.06	
150%	22.5	22.40	99.55	
	22.5	22.44	99.75	
	22.5	22.35	99.33	

Table 3.7 Accuracy table of Ritonavir

% Level	**Amount Spiked (µg/mL)**	**Amount recovered (µg/mL)**	**% Recovery**	Mean %Recovery
50%	5	5.0	99.6	99.77%
	5	5.0	99.6	
	5	5.0	99.8	
100%	10	9.9	99.5	
	10	10.0	99.9	
	10	10.0	100.1	
150%	15	15.0	100.0	
	15	14.9	99.4	
	15	15.0	100.1	

Discussion: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.15% and 99.77% for Nirmatrelvir and Ritonavir respectively.

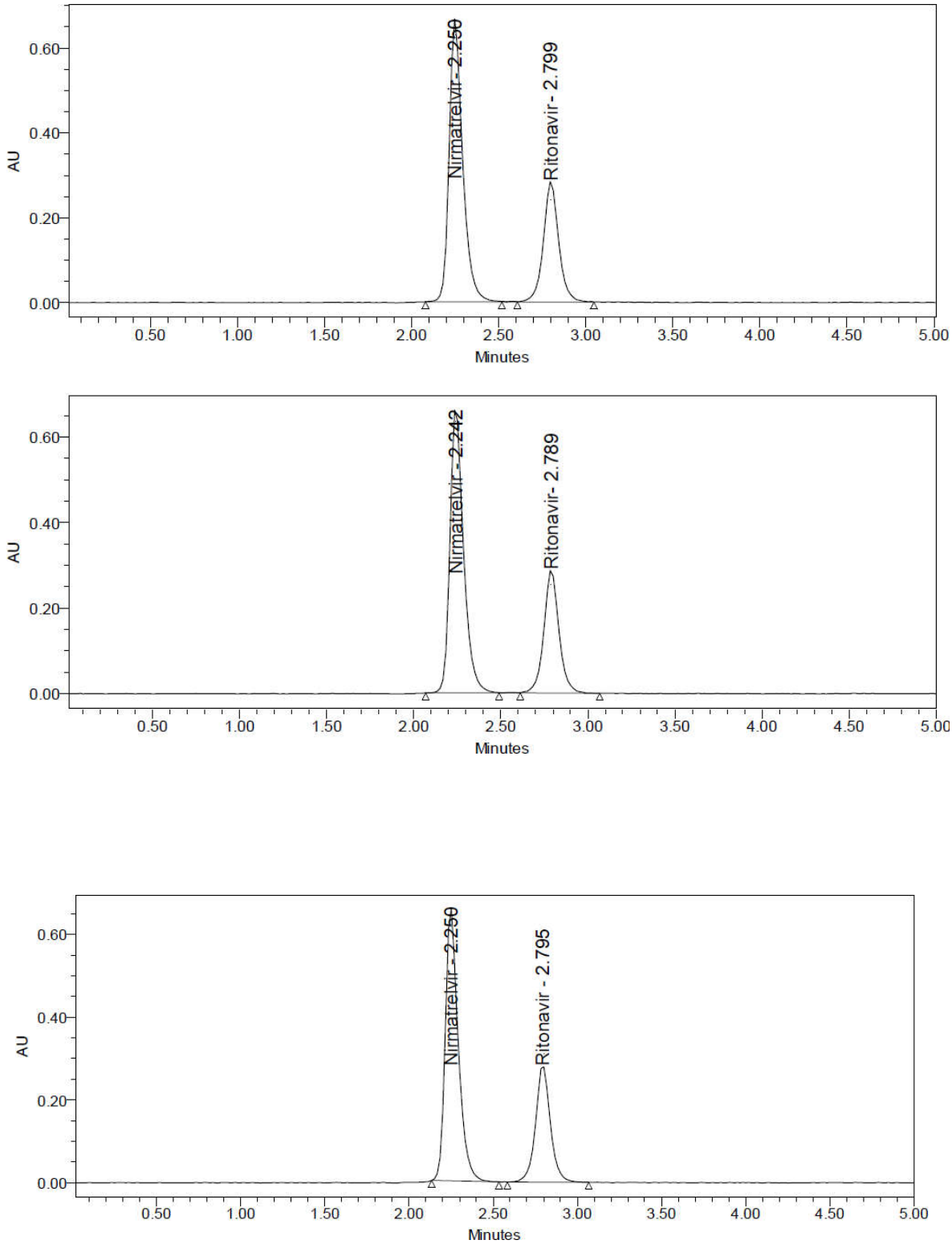


Fig No. 3.21 Accuracy 50% Chromatogram of Nirmatrelvir and Ritonavir

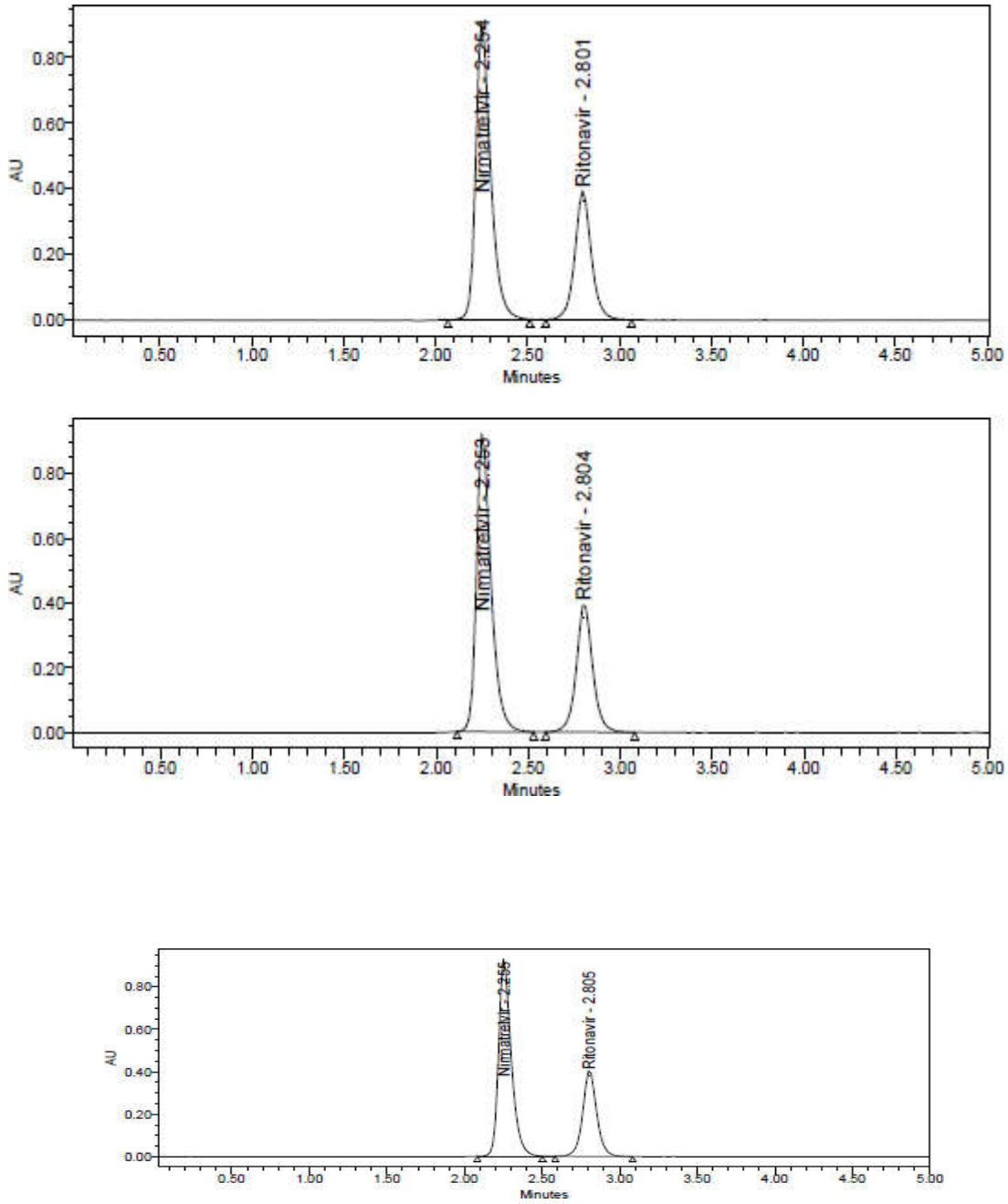


Fig No. 3.22 Accuracy 100% Chromatogram of Nirmatrelvir and Ritonavir

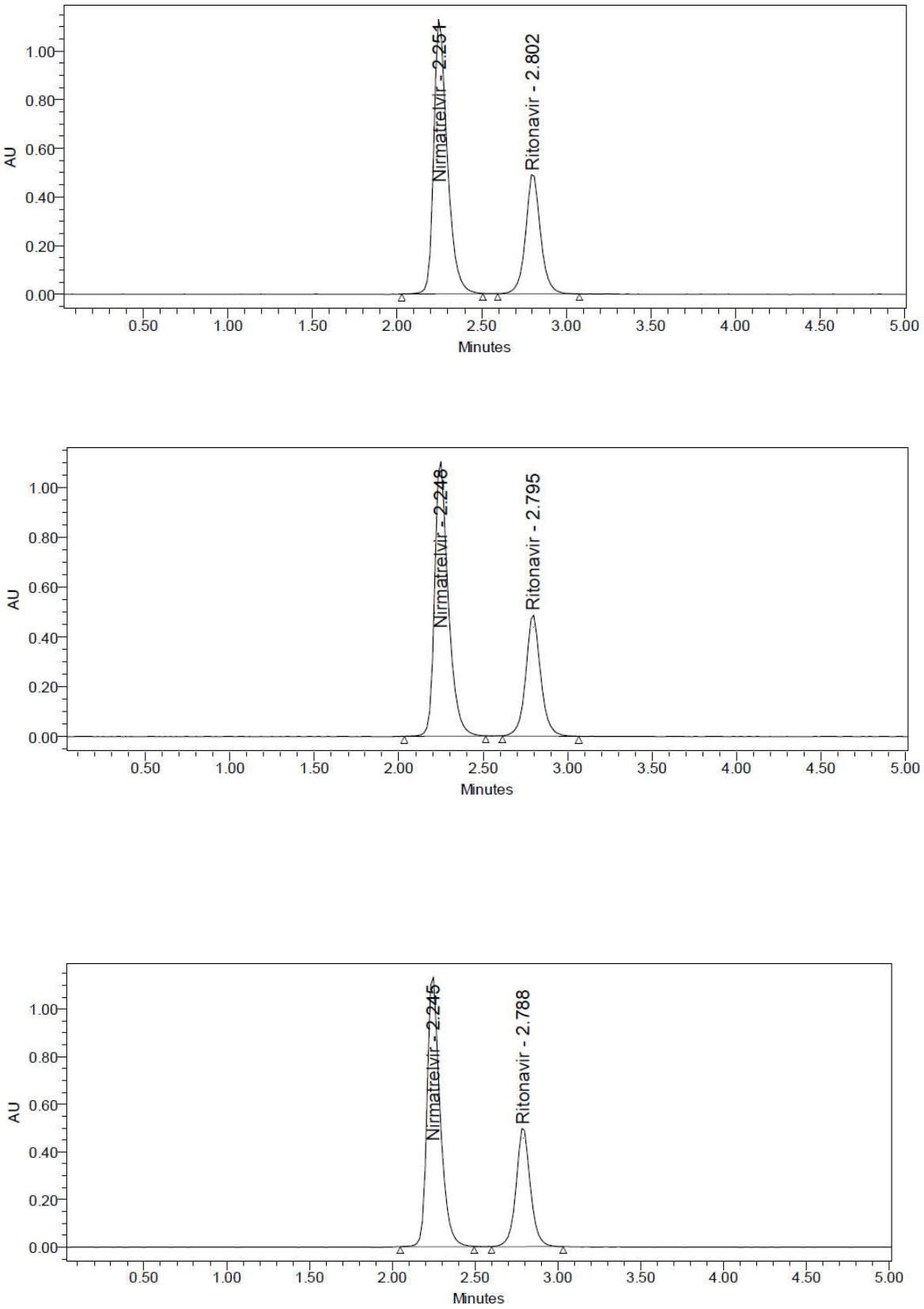


Fig No. 3.23 Accuracy 150% Chromatogram of Nirmatrelvir and Ritonavir

Sensitivity:

Table 3.8 Sensitivity table of Nirmatrelvir and Ritonavir

Molecule	LOD	LOQ
Nirmatrelvir	0.21	0.65
Ritonavir	0.16	0.48

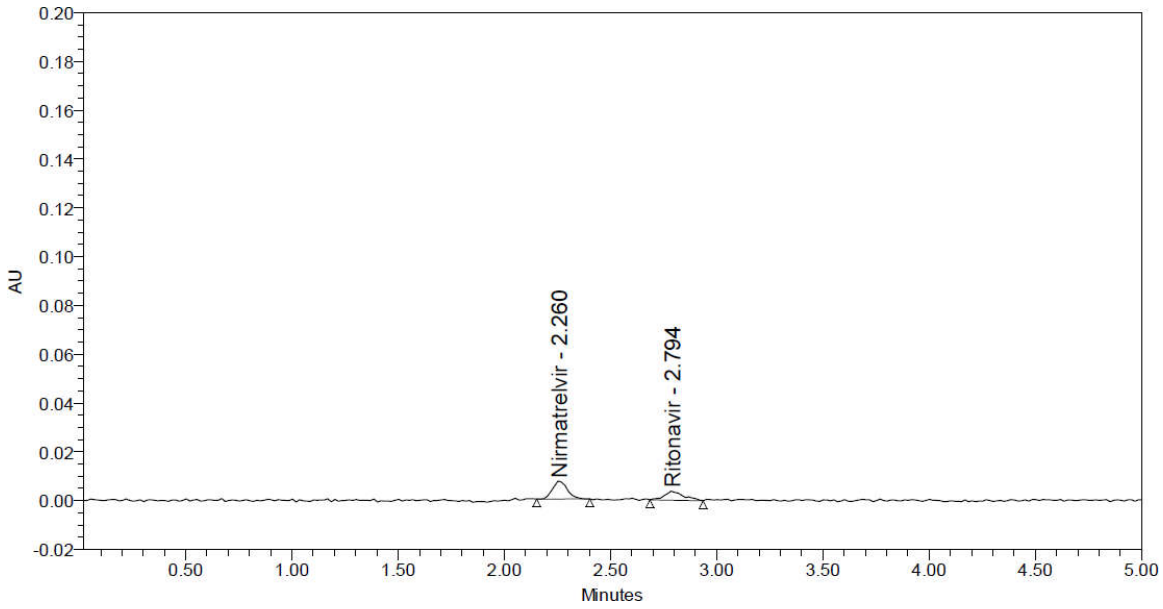


Fig. No. 3.24 LOD Chromatogram of Standard

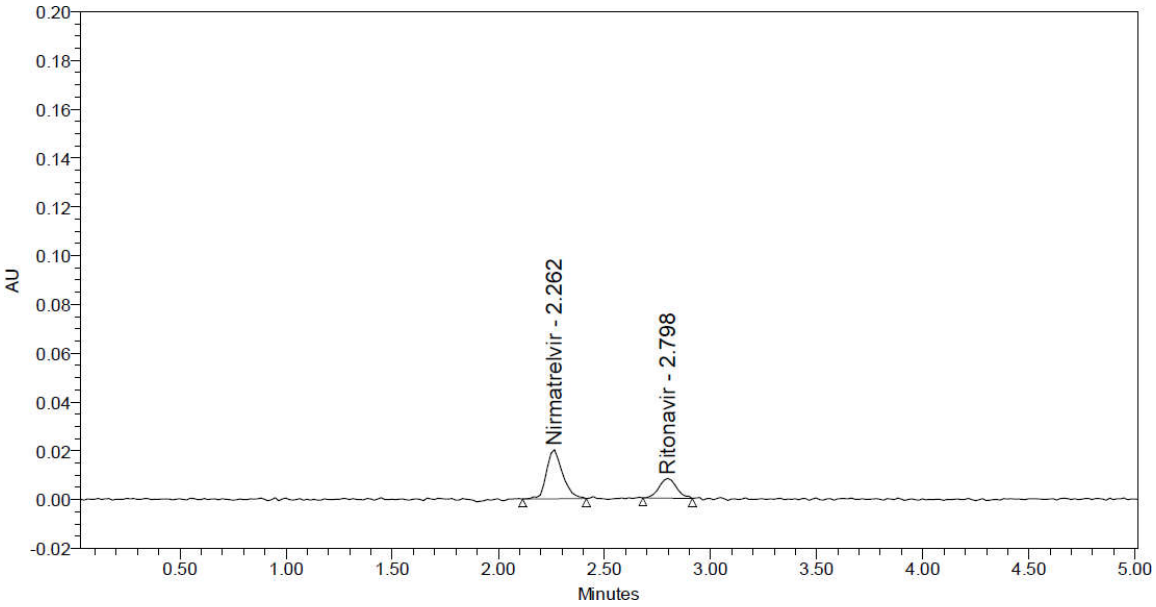


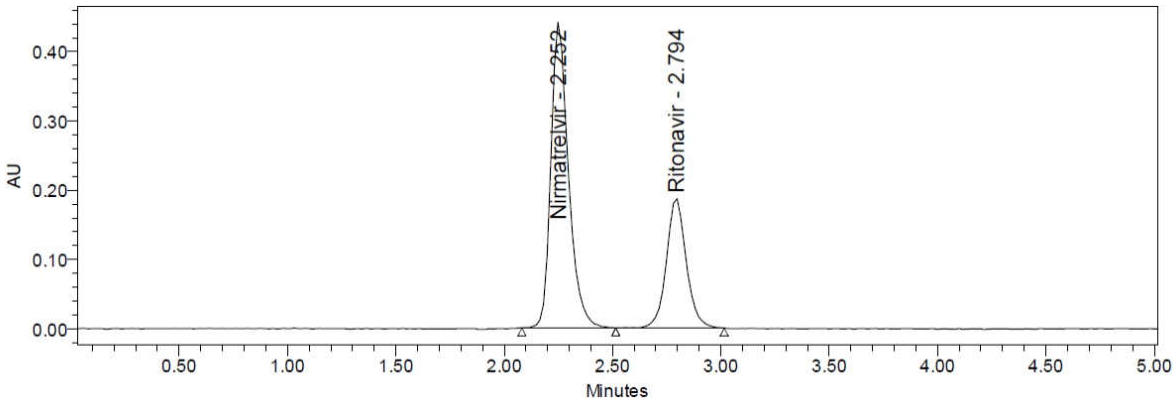
Fig. No. 3.25 LOQ Chromatogram of of Standard

Robustness:

Table 3.9 Robustness data for Nirmatrelvir and Ritonavir.

S.no	Condition	%RSD of Nirmatrelvir	%RSD of Ritonavir
1	Flow rate (-) 0.7ml/min	0.2	0.2
2	Flow rate (+) 0.9ml/min	0.4	0.5
3	Mobile phase (-) 55B:45A	0.4	0.5
4	Mobile phase (+) 65B:35A	0.2	0.1
5	Temperature (-) 25°C	0.1	0.3
6	Temperature (+) 35°C	0.3	0.7

Discussion: Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus (45B:55A), mobile phase plus (55B:45A), temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.



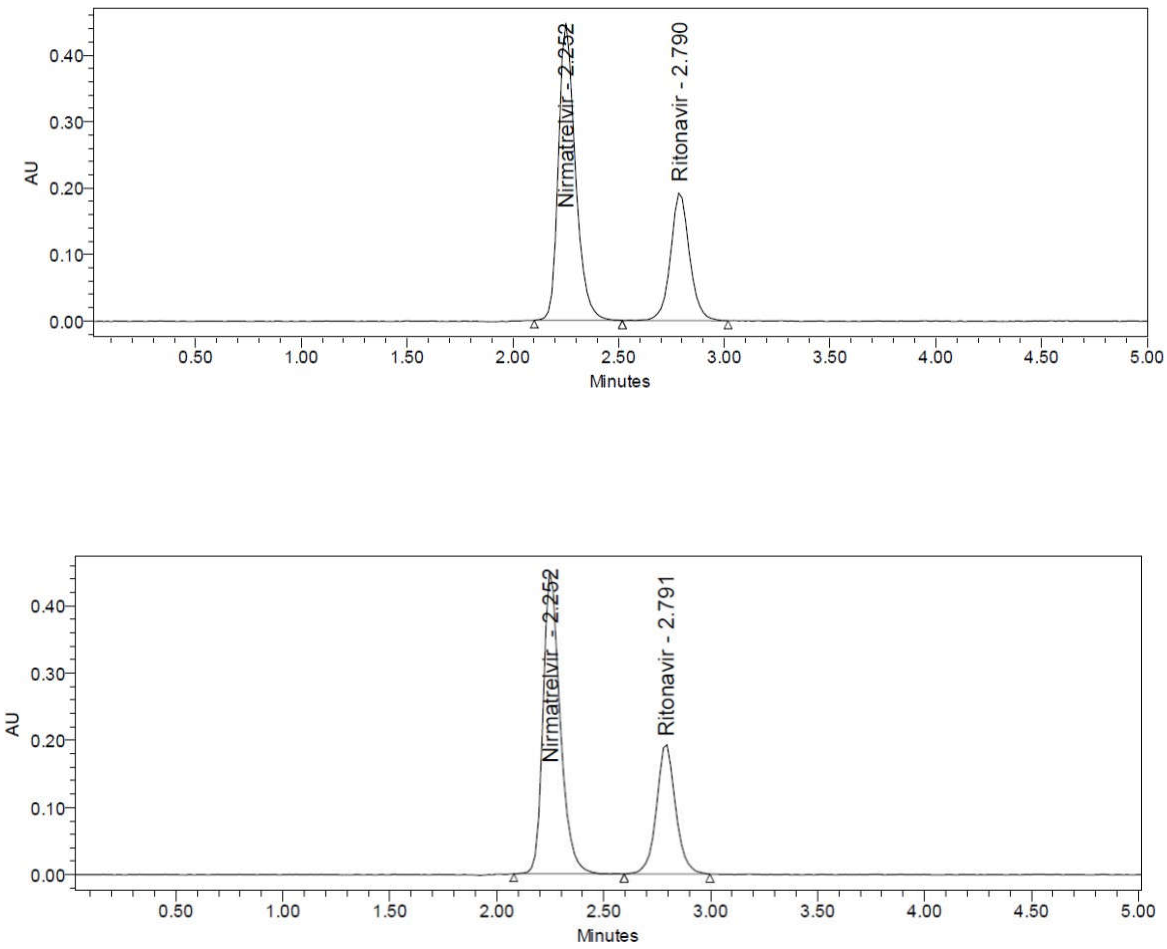
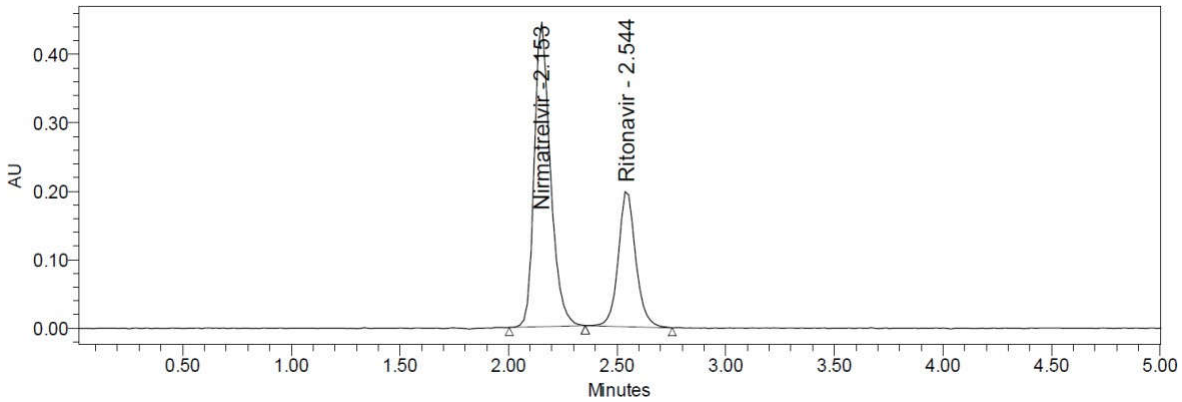


Fig No. 3.26 Flow minus Chromatogram of Nirmatrelvir and Ritonavir



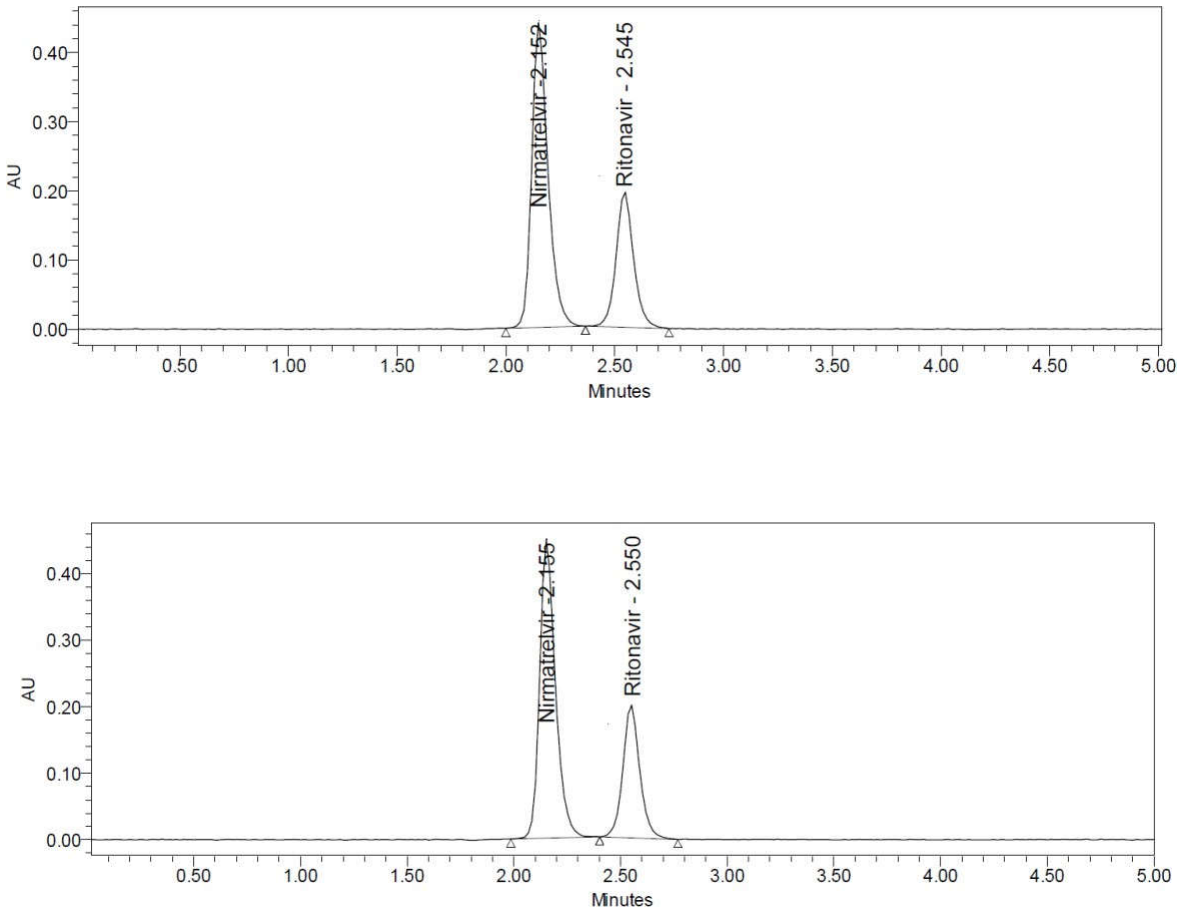
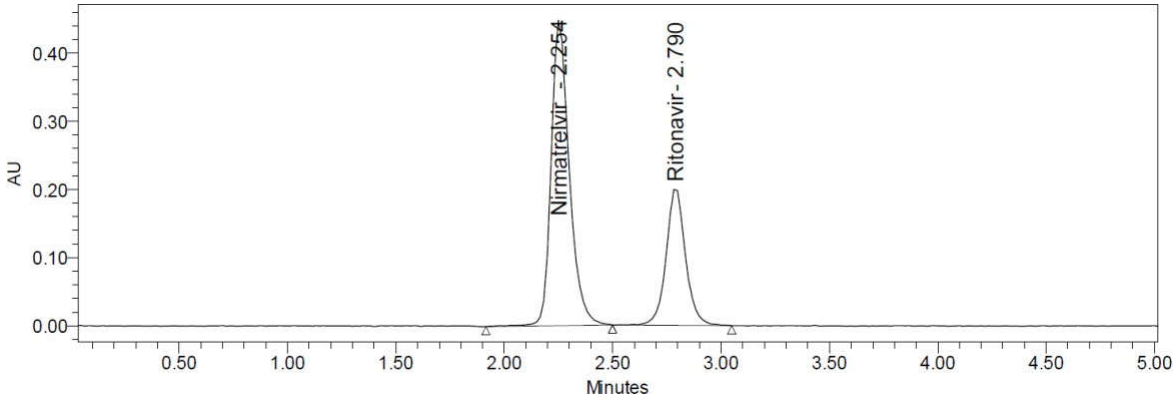


Fig No. 3.27 Flow plus Chromatogram of Nirmatrelvir and Ritonavir.



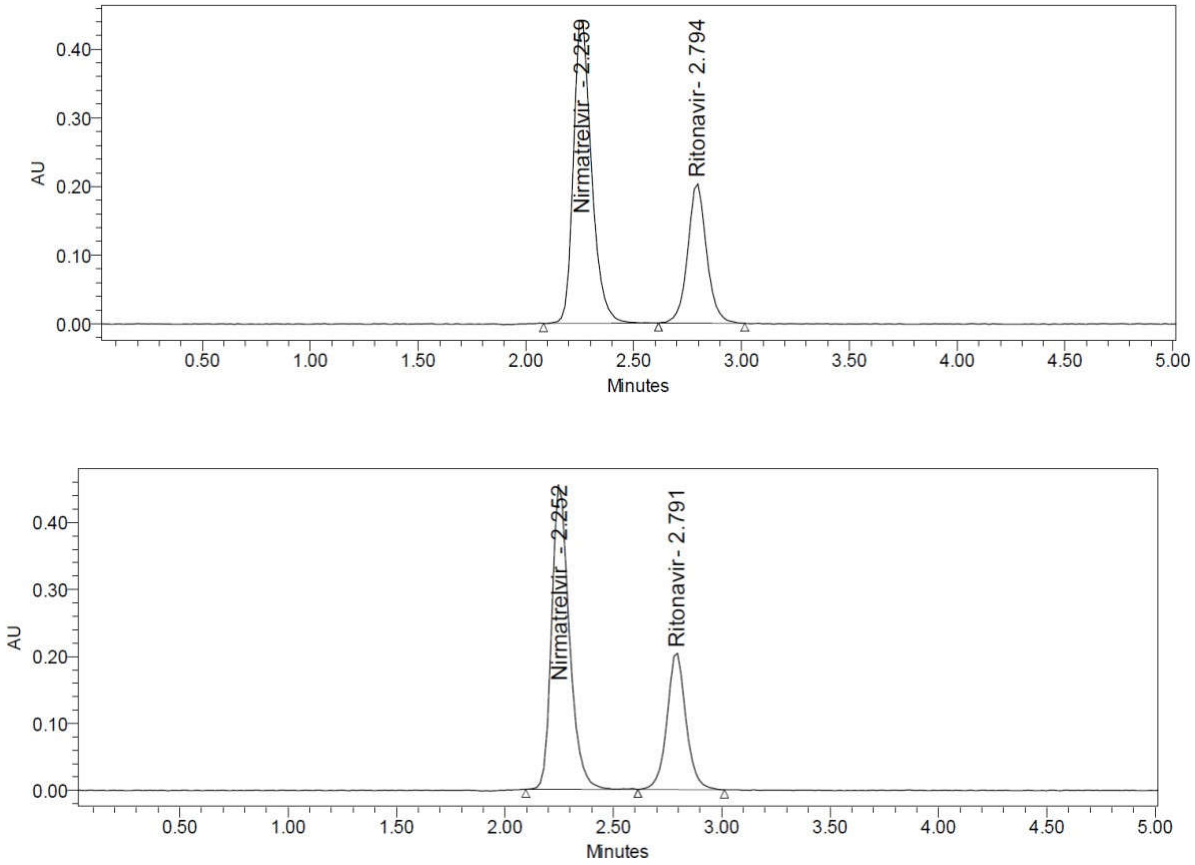
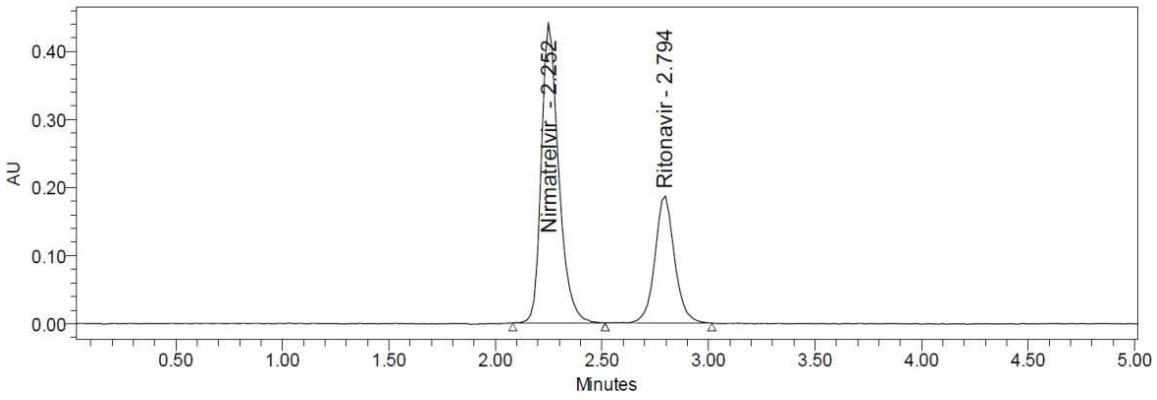


Fig No. 3.28 Mobile phase minus Chromatogram of Nirmatrelvir and Ritonavir.



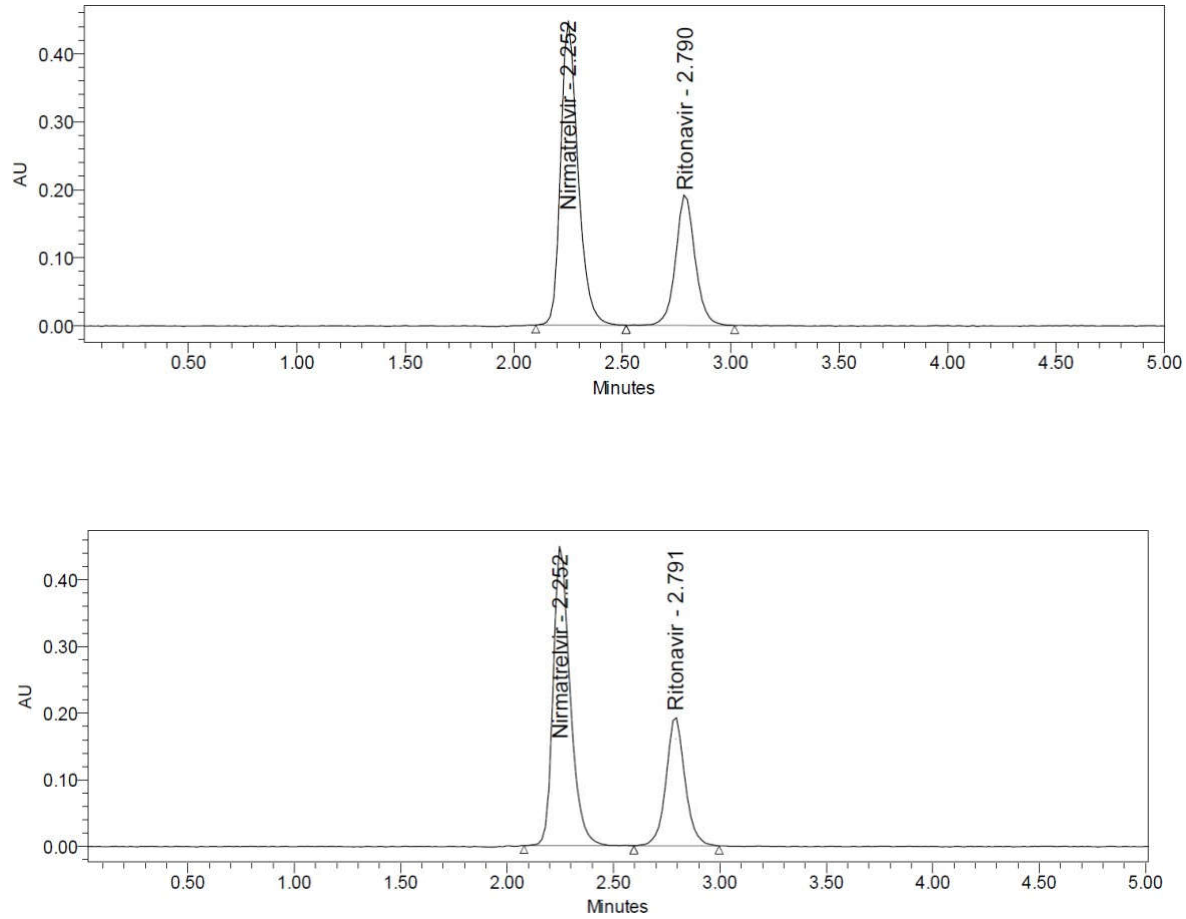
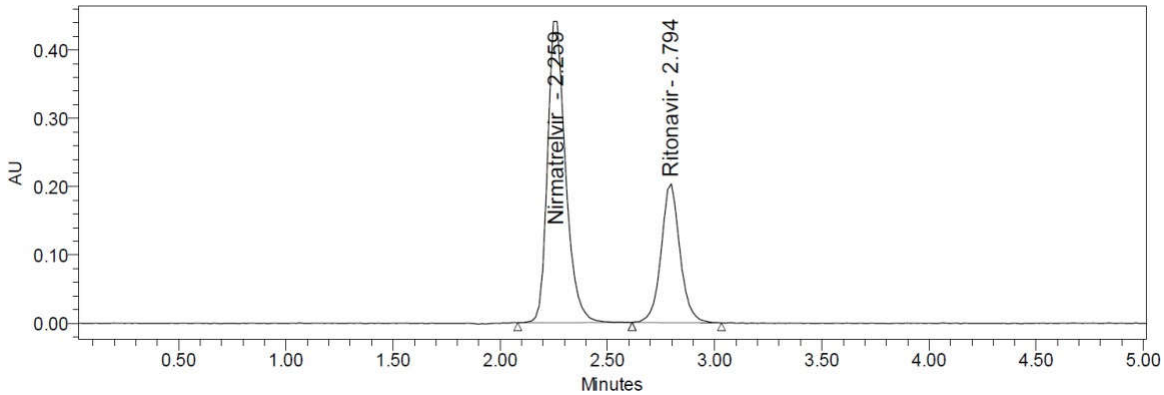
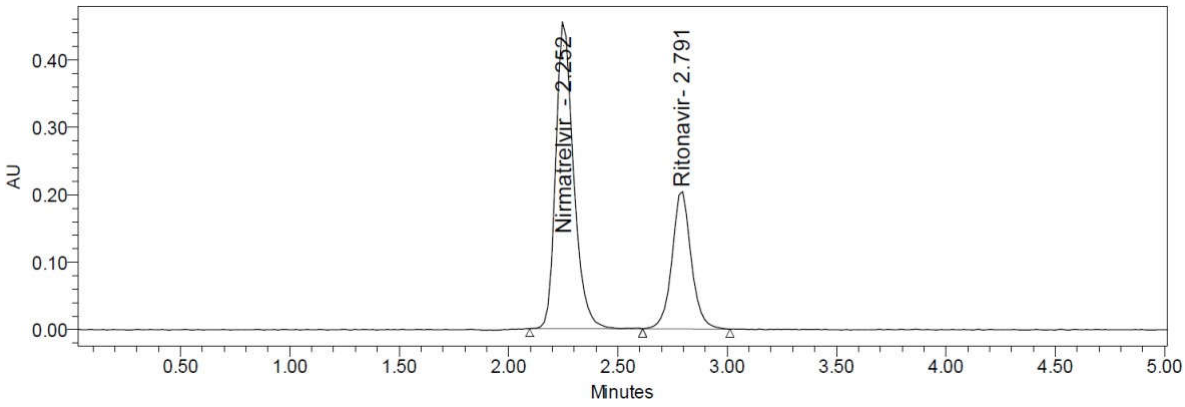


Fig No. 3.29 Mobile phase Plus Chromatogram of Nirmatrelvir and Ritonavir.





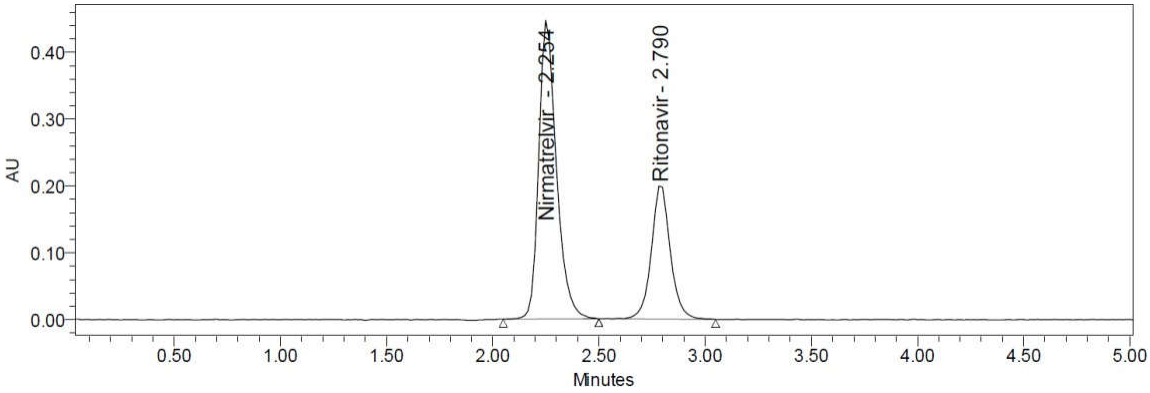


Fig No. 3.30 Temperature minus Chromatogram of Nirmatrelvir and Ritonavir

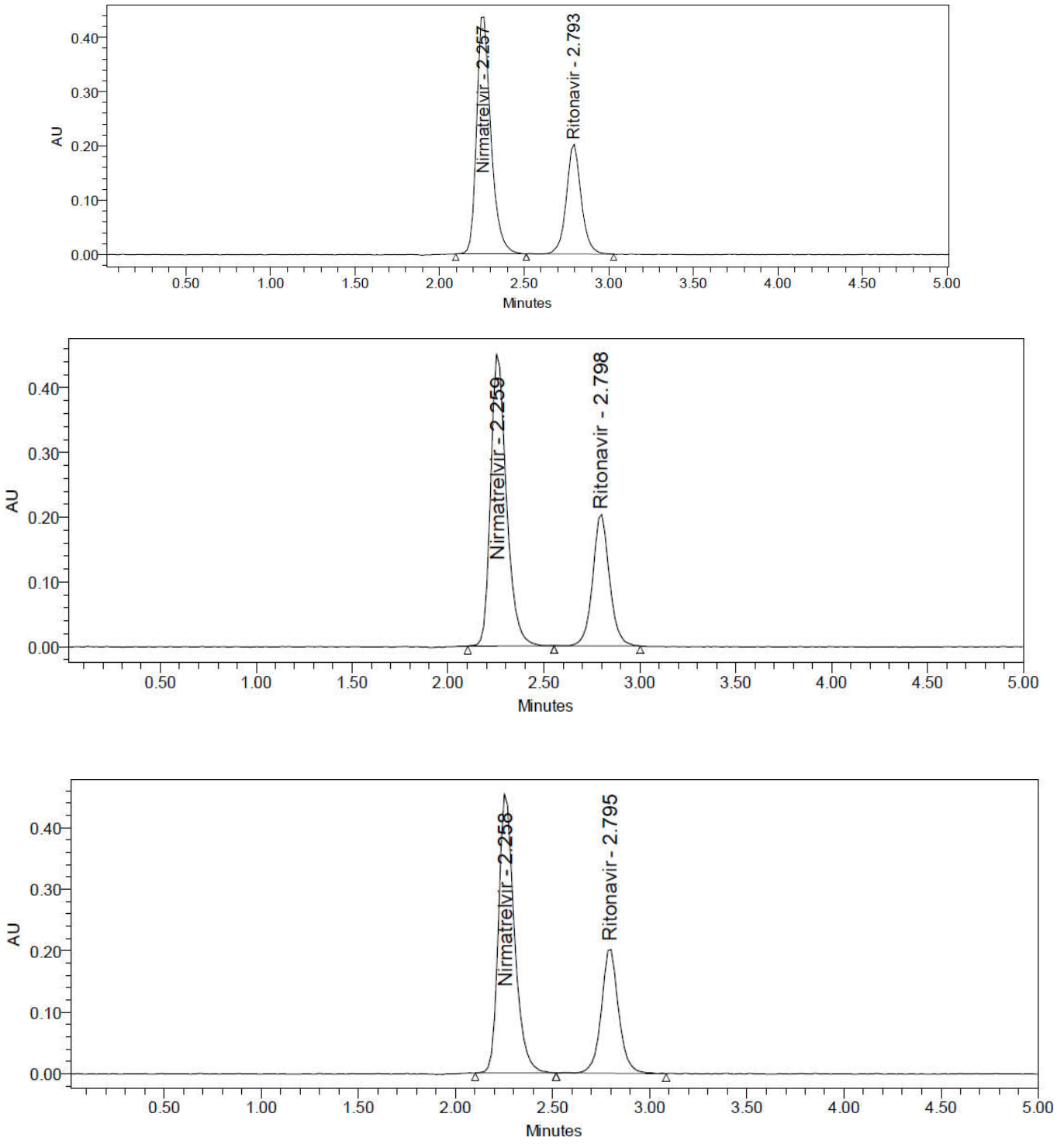


Fig No. 3.30 Temperature plus Chromatogram of Nirmatrelvir and Ritonavir

Assay: bearing the label claim Nirmatrelvir 150mg, Ritonavir 100mg. Assay was performed with the above formulation. Average % Assay for Nirmatrelvir and Ritonavir obtained was 99.84% and 99.74% respectively

Table 3.10Assay Data of Nirmatrelvir

S.no	Standard Area	Sample area	% Assay
1	252676	251414	99.17
2	252661	252509	99.60
3	252638	252274	99.51
4	252495	256473	101.16
5	254357	253323	99.92
6	254772	252751	99.70
Avg	253267	253124	99.84
Stdev	1016.0	1756.1	0.69
%RSD	0.4	0.7	0.7

.

Table 3.11 Assay Data of Ritonavir

S.no	Standard Area	Sample area	% Assay
1	119169	118516	99.62
2	119165	118393	99.51
3	118338	118673	99.75
4	119901	118782	99.84
5	118480	118796	99.85
6	118065	118832	99.88
Avg	118853	118665	99.74
Stdev	681.3	176.1	0.15
%RSD	0.6	0.1	0.1

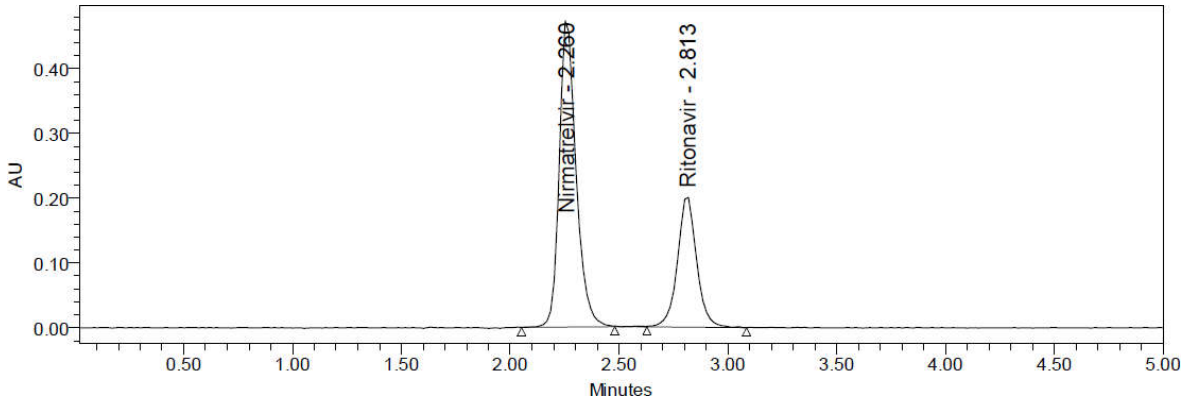


Fig 3.31 Chromatogram of working standard solution

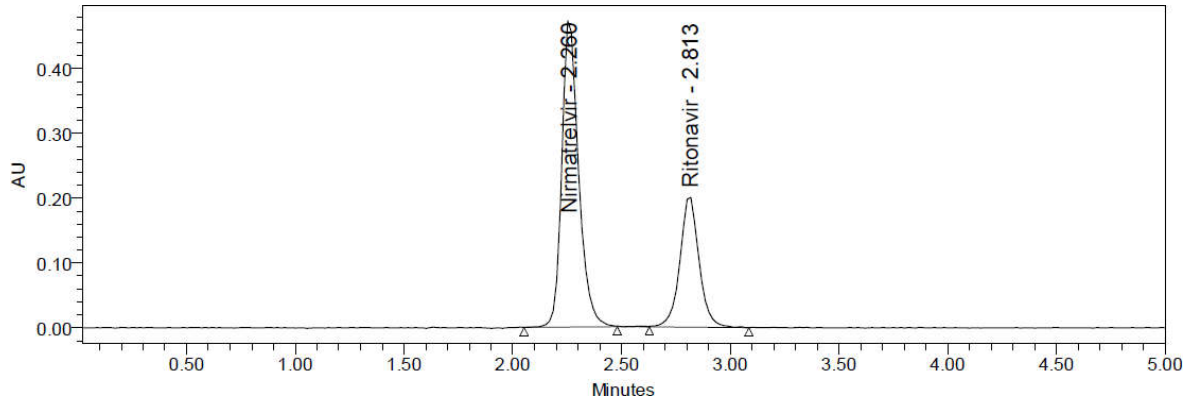


Fig No. 3.32 Chromatogram of working sample solution

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 analysis.

Table 3.12.degradation data

Type of degradation	**Nirmatrelvir**			**Ritonavir**		
	AREA	%RECOVERED	% DEGRADED	AREA	%RECOVERED	% DEGRADED
Acid	240493	94.86	5.14	112076	94.20	5.80
Base	242104	95.50	4.50	113067	95.04	4.96
Peroxide	243912	96.21	3.79	115019	96.68	3.32
Thermal	246873	97.38	2.62	116020	97.52	2.48
Uv	249523	98.42	1.58	117346	98.63	1.37
Water	251418	99.17	0.83	118126	99.29	0.71

Degradation chromatograms

Acid degradation chromatogram

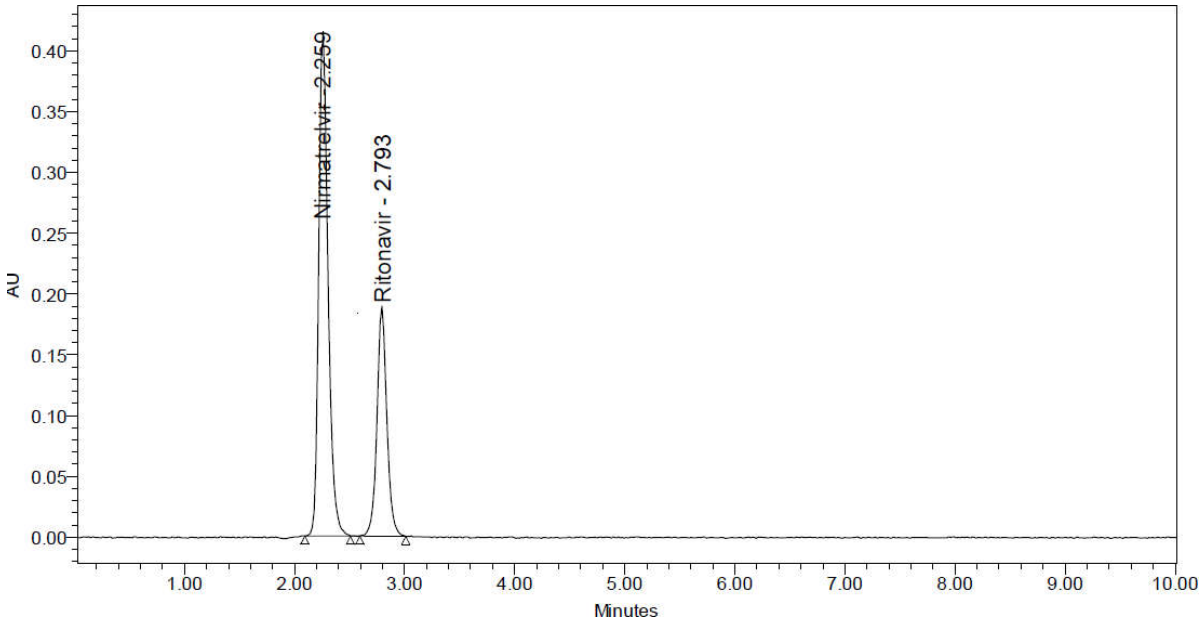


Fig.3.33 acid

Base degradation chromatogram

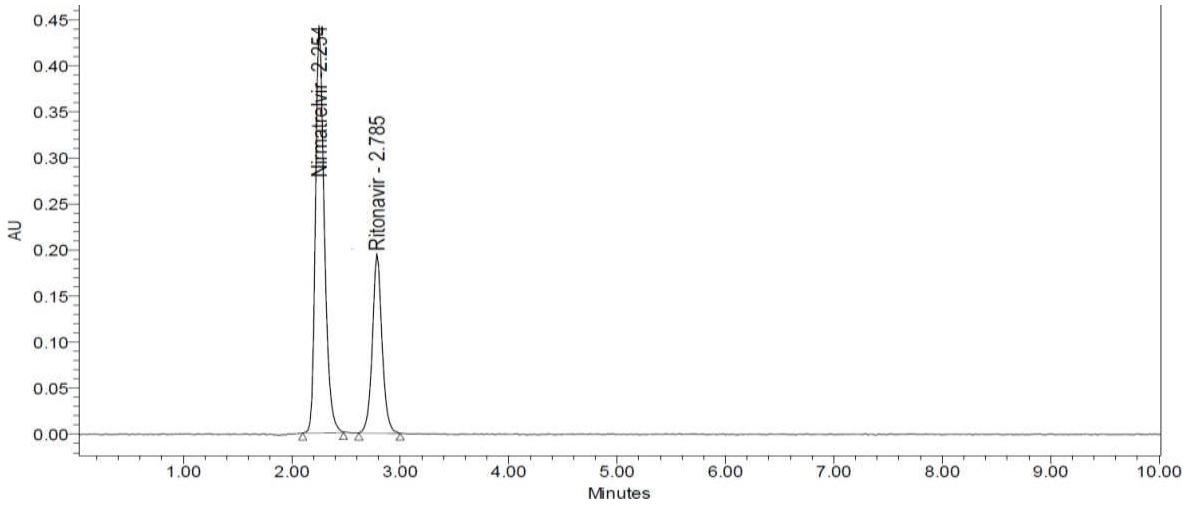


Fig.3.34 base

Peroxide degradation chromatogram

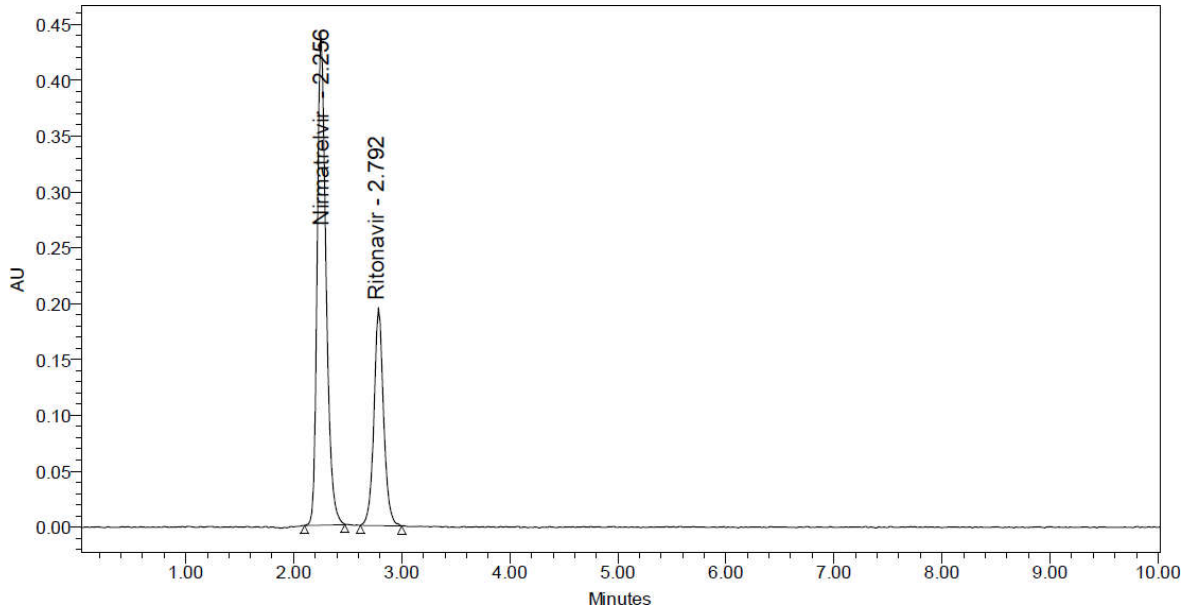


Fig.3.35 peroxide

Thermal degradation chromatogram

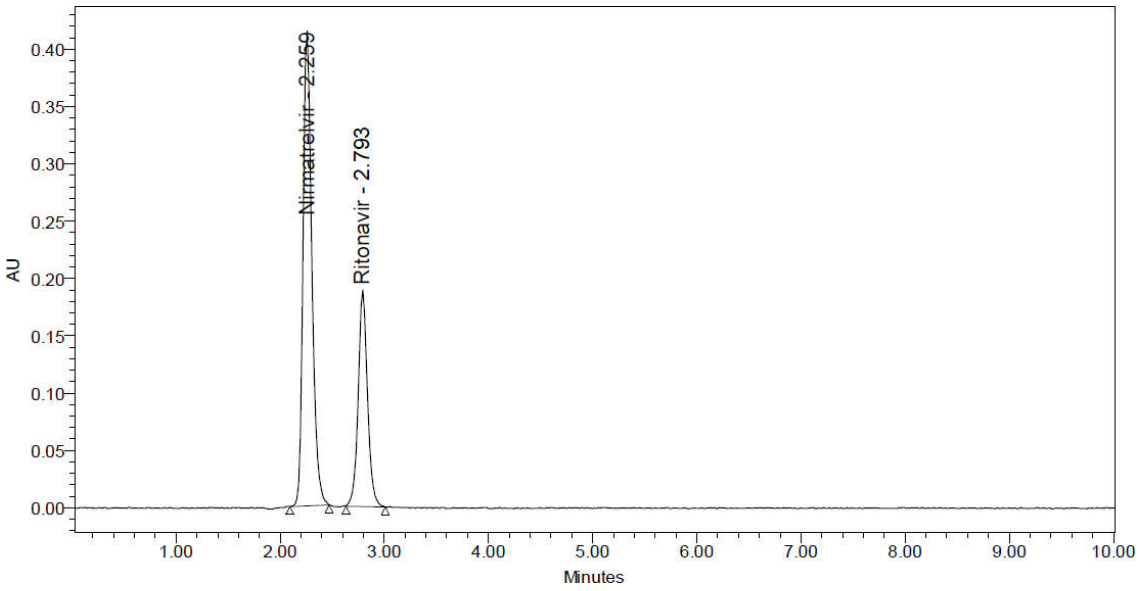


Fig.3.36 thermal

Uv degradation chromatogram

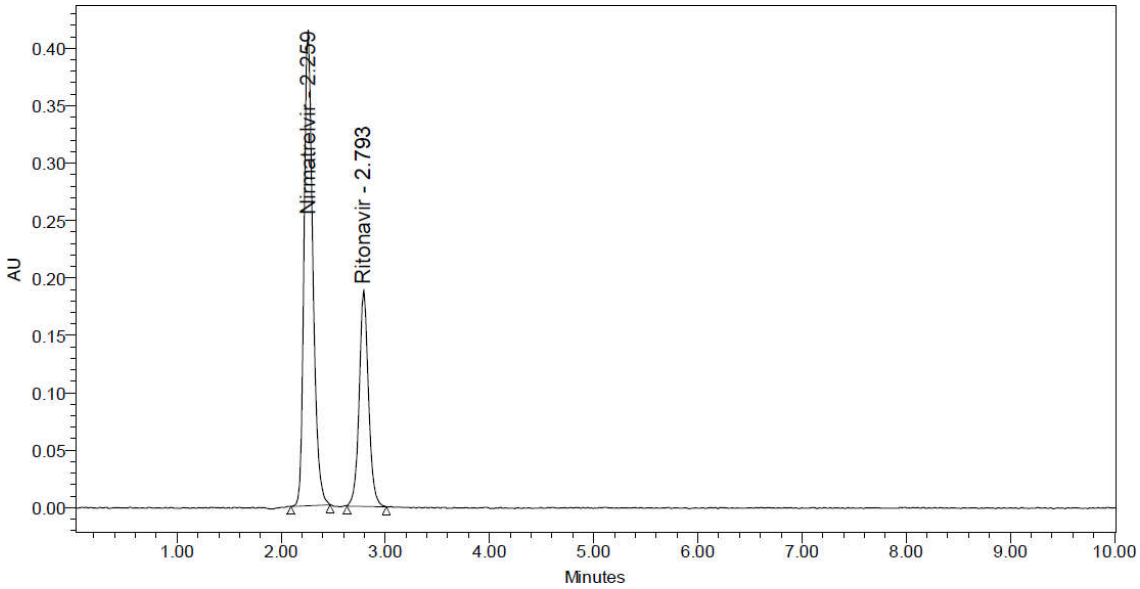


Fig.3.37 uv

Water degradation chromatogram

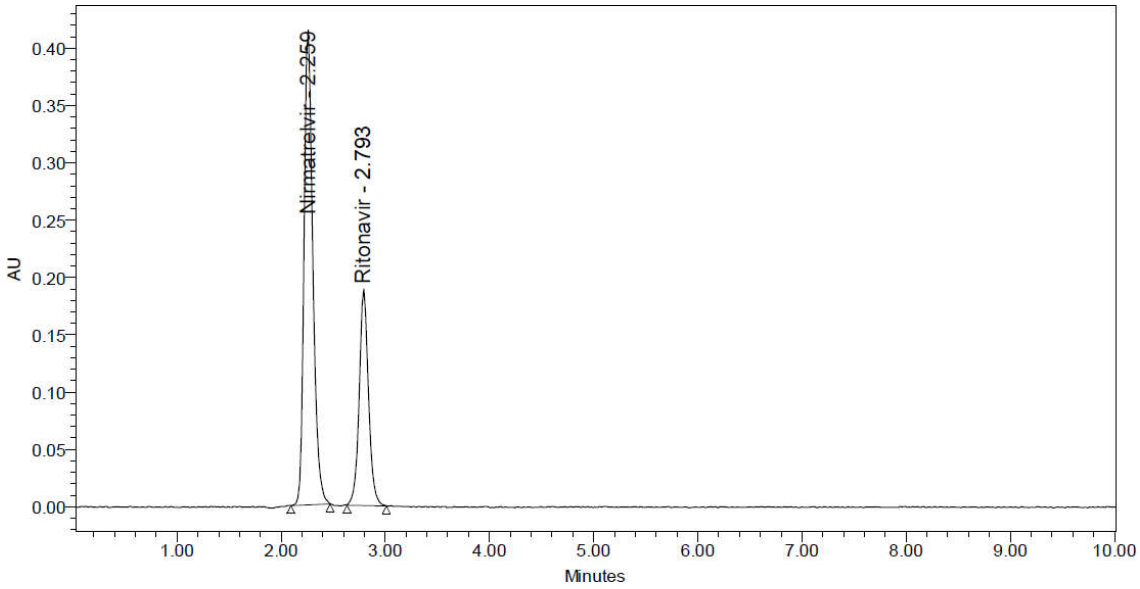


Fig.3.38 water

4.SUMMARY AND CONCLUSION

4.1 Summary Table

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)		16802	16802	
Intercept(c)		995.5	995.5	
Regression equation (Y=mx+c)		y = 16802x + 995.5	y = 16802x + 995.5	
Assay (% mean assay)		99.84%	99.74%	90-110%
Specificity		Specific	Specific	No interference of any peak
System precision %RSD		0.4	0.6	NMT 2.0%
Method precision %RSD		0.7	0.1	NMT 2.0%
Accuracy %recovery		99.15%	99.77%	98-102%
LOD		0.21	0.16	NMT 3
LOQ		0.65	0.48	NMT 10
Robustness	**FM**	0.2	0.2	%RSD NMT 2.0
	FP	0.4	0.5	
	MM	0.4	0.5	
	MP	0.2	0.1	
	TM	0.1	0.3	
	TP	0.3	0.7	

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 2.241 min and 2.816 min. %RSD of the Nirmatrelvir and Ritonavir were and found to be 0.4 and 0.6 respectively. %Recovery was obtained as 99.15% and 99.77% for Nirmatrelvir and Ritonavir respectively. LOD, LOQ values obtained from regression equations of Nirmatrelvir and Ritonavir were 0.21, 0.16 and 0.65, 0.48 respectively. Regression equation of Nirmatrelvir is y = 16802x + 995.5, and y = 16802x + 995.5 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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