"Analytical Method Development And Validation For Simultaneous Estimation Of Antiviral Drug In Bulk And Pharmaceutical Dosage Form By Rp-Hplc "

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ABSTRACT:

Analytical Method Development and validation has been made to develop simple, accurate, precise, and rapid RP-HPLC methods for determination of antiviral drug.The main objective is to develop a dependable and precise RP-HPLC technique to precisely measure the concentration of the antiviral drug in a pharmaceutical product. To develop a method that demonstrates good separation, sensitivity, and selectivity for the antiviral drug in the presence of potential impurities or degradation products. To establish and validate the linearity, precision, accuracy, specificity, and robustness of the developed RP-HPLC method. To determine the limit of detection (LOD) and limit of quantitation (LOQ) for the antiviral drug. The assay method was developed and validated using a Shimadzu Liquid Chromatographic System, comprising an LC-20 AD pump, an SPD-20A UV-Visible detector, and a universal loop injector with a 20µl injection capacity. Separation was conducted on a Thermo fisher Scientific C18 (250mm x 4.6mm, 5µm) with a flow rate of 1.0 ml/min. A 30 µl sample volume was injected, and detection was performed at a wavelength of 221 nm. The mobile phase, consisting of Acetonitrile and Phosphate Buffer in an (70:30 %(v/v) ratio, was employed. The run time was set at 6. Before analyte injection, the column was equilibrated for 15 minutes with the mobile phase.

KEYWORDS: nirmatrelvir ,ritonavir , HPLC, accuracy , stability , LOD, LOQ

INTRODUCTION :

Pharmaceutical analysis is the process of determining the composition, quality, and purity of pharmaceutical products through various scientific techniques. Pharmaceutical analysis also encompasses the detection and quantification of contaminants, degradation products, and potential impurities. This helps in identifying any factors that may compromise the safety or efficacy of the pharmaceutical product. Regulatory authorities, such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), set stringent guidelines for pharmaceutical analysis to ensure that medications meet high standards of quality, safety, and efficacy before reaching the market [2].

Classification of Analytical Method [9,10]

- Non- instrumental Method
- Instrument Method
- Few Analytical Methods

1. Spectroscopic Methods

- UV-visible spectroscopic method
- Infrared spectroscopy
- Mass spectroscopy
- NMR spectroscopy
- Atomic Absorption Spectroscopy

2. Chromatographic Method

- High Performance Liquid chromatography
- Supercritical Fluid chromatography
- High Performance Thin Layer Chromatography
- Gas Chromatography
- LC-MS
- GC-MS

3. Electrochemical Method

- Conductometry
- Voltammetry
- Potentiometry

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography (HPLC) is a powerful analytical technique used for the separation, identification, and quantification of components in a mixture. The fundamental principle of HPLC involves the passage of a liquid sample through a column packed with a stationary phase, typically consisting of silica or other suitable materials. The most used HPLC technique is reverse-phase high-performance Liquid chromatography (RP-HPLC), which excels in Selectivity and sensitivity for a wide range of analytes [17, 18].

Modern HPLC techniques offer real-time compound identification post- separation. HPLC utilizes various detection techniques, including ultraviolet-visible (UV-Vis), mass spectrometry (MS), nuclear magnetic resonance (NMR), and Fourier transform Raman (FTR) spectroscopy, for separating, identifying, and quantifying complex mixtures.

Types of HPLC

High-Performance Liquid Chromatography (HPLC) is a versatile analytical technique used for the separation, identification, and quantification of chemical compounds in a mixture. There are following variants of HPLC, depending upon the phase system (stationary) in the process:

1. Normal Phase HPLC:

In normal phase HPLC, the separation is driven by polarity differences. The stationary phase is polar, typically composed of silica, and the mobile phase is non- polar, commonly using solvents like hexane, chloroform, or diethyl ether. Polar samples are retained on the column, with retention times influenced by their degree of polarity. This method is particularly useful for separating compounds with varying polarities.

2. Reverse Phase HPLC:

In contrast to normal phase HPLC, reverse phase HPLC involves a non-polar or hydrophobic stationary phase and a polar mobile phase. The more non-polar a compound is, the longer it will be retained on the column. This method is widely used for separating and analyzing non-polar and moderately polar compounds, making it suitable for a broad range of applications.

3. Size-Exclusion HPLC:

Size-exclusion HPLC, also known as gel filtration chromatography, focuses on separating molecules based on their size. The column incorporates substrates with precisely controlled pore sizes. Larger molecules elute quickly as they pass

through the larger pores, while smaller molecules are delayed as they navigate through the intricate network of smaller pores.

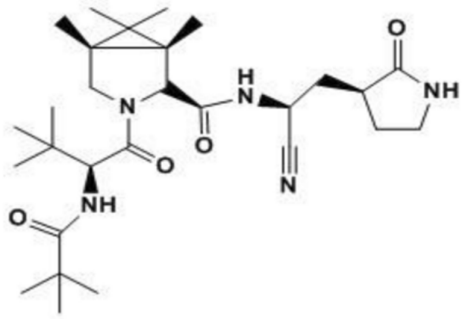
4. Ion-Exchange HPLC:

Ion-exchange HPLC utilizes a stationary phase with an ionically charged surface opposite to the charge of the sample ions. The mobile phase typically consists of an aqueous buffer that controls the pH and ionic strength. The charged analytes interact with the oppositely charged sites on the stationary phase, resulting in their separation based on differences in ion exchange properties.

DRUG PROFILE :

Molecular Structure

NIRMATRELVIR :



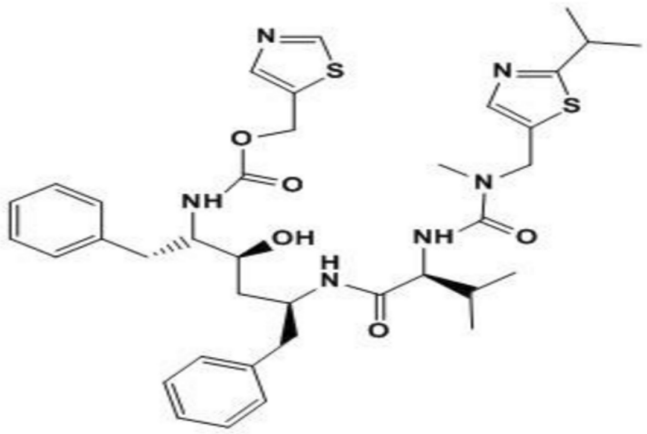
Nirmatrelvir

Molecular Formula	**:**	C23H32F3N5O4
Molecular Weight	**:**	499.5 g/mol
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-trifluoroacetyl) amino] butanoyl]-6,6-dimethyl-3 azabicyclo [3.1.0] hexane-2-carboxamide
Drug Category	**:**	Anti-Viral
Solubility	**:**	Freely soluble in methanol
Half-life	**:**	6.05 hr.
Melting Point	**:**	192.9 °C (379.2 °F)
Storage	**:**	2° - 8°C (35.6° to 46.4°F)
PKa Value	**:**	7.1
Mechanism of Action	**:**	Nirmatrelvir is an antiviral medication used in the treatment of COVID-19. Its mechanism of action involves inhibiting the main protease (Mpro) of the SARS CoV-2 virus. The main protease plays a crucial role in the replication of the virus by cleaving large polyproteins into functional proteins necessary for viral replication. By inhibiting this protease, nirmatrelvir interferes with the viral replication cycle, ultimately reducing the viral load within the body and helping to alleviate symptoms of COVID-19. Nirmatrelvir is often used in combination

with another antiviral medication called ritonavir, which helps to boost its effectiveness by prolonging its presence in the body.

RITONAVIR :

Molecular Structure :



Ritonavir

Molecular Formula :	C37H48N6O5S2
Molecular Weight	720.948 g/mol
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-diphenylhexa n-2-yl]carbamate
Drug Category	Anti-retroviral
Solubility	Freely soluble in methanol , DMSO
Half-life	3 to 5 hr.
Melting Point	126-132°C
Storage	2° - 8°C (35.6° to 46.4°F)
PKa Value	2.8
Mechanism of Action	Ritonavir is a protease inhibitor with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Protease inhibitors block the part of HIV called protease. HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1. Ritonavir binds to the protease active site and inhibits the activity of the enzyme. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs. Modern

protease inhibitors require the use of low-dose ritonavir to boost pharmacokinetic exposure through inhibition of metabolism via the cytochrome P450 3A4 enzyme pathway

MATERIALS AND METHODS :

Active Pharmaceutical Ingredients:

Sr. No	Name	Content	Company
1.	Nirmatrelvir	1 gm.	Zydus Cadila Pvt. Ltd
2.	Ritonavir	1 gm.	Zydus Cadila Pvt. Ltd

Table 1 Active Pharmaceutical Ingredients

Chemicals and Reagents:

Sr. No	Name	Grade	Supplier
1.	Acetonitrile	HPLC	SD fine-chem limited
2.	Water	HPLC	Loba chemie
3.	Methanol	HPLC	Loba chemie
4.	Ortho-phosphoric acid	AR	Loba chemie

Table .2 Chemicals and Reagents

Equipment and Accessories:

Sr. No	Instrument	Brand/Model	Manufacturer/Supplier
1.	HPLC	Shimadzu HPLC	Shimadzu
2.	Software	LC lab Solution	Shimadzu
3.	Column C18	Thermo fisher Scientific	Thermo Scientific C18 (250mm x 4.6mm, 5µm)
4.	Detector PDA	Shimadzu SPD-M20A	Shimadzu
5.	Digital Balance	Aarson	Aarson
6.	pH Meter	Systronics	-
7.	Micropipette(20-200 uL,100-1000uL)	-	Eppendorf
8.	Syring Filter	0.45u	Millipore
9.	Ultra-sonicator	-	PCM

Table 3 Equipment and Accessories

Methods :

Optimized chromatographic condition

The HPLC experiment condition were optimized on the Thermo fisher Scientific C18, (250mm x4.6mm, internal diameter, and 5µm particle size) analytical column**.**

PARAMETERS	CONDITIONS
Column	Thermo fisher Scientific C18(250mm x 4.6mm, 5µm)
Mobile Phase	Acetonitrile: Phosphate Buffer [pH 3.0] (70:30)
Flow Rate	1.0 ml/min
Volume of Injection	10 µL
Detector	PDA detector
Detection Wavelength	221 nm
Run time	6.0 minute

Table 4. Optimized Chromatographic Conditions.

Preparation of standard solution

1.Preparation of Stock Solution 1 of Nirmatrelvir and Ritonavir (1000µg/ml):

Accurately weighed about 10 mg of Nirmatrelvir and 10 mg of Ritonavir of API working standard and transferred to a 100 ml volumetric flask. Add 50 ml of diluent and dissolved properly. Then it was shaken and sonicate for 10 minute and volume was made up to the mark with solvent.

2.Preparation of Stock Solution 2 of Nirmatrelvir and Ritonavir (100µg/ml):

1ml of solution was pipetted out from (Stock solution 1) and transferred to 10 ml volumetric flask and the volume was made up to 10 ml with diluent and sonicated it for 15 min. The concentration of prepared stock solution was 100 µg/ml.

Preparation of analytical solution

a.Preparation of 0.1% OPA Solution:

1ml of Ortho-phosphoric acid (HPLC Grade) was pipetted out and dissolve in 1000 ml of HPLC grade water. And adjust the pH (3.0), finally the solution was filtered by using 0.45 Micron membrane filter, and sonicate it for 10 min.

a) Preparation of Mobile Phase:

Accurately measured 300 ml (30%) of above buffer and 700 ml (70%) of Acetonitrile for HPLC were mixed and degassed in an ultrasonic water bath for 10 min and then filter through 0.45µ filter under vacuum filtration, then this solution is used for HPLC purpose

Method optimization

The assay method was developed and validated using a Shimadzu Liquid Chromatographic System, comprising an LC-20 AD pump, an SPD-20A UV-Visible detector, and a universal loop injector with a 20µl injection capacity. Separation was conducted on a Thermo fisher Scientific C18 (250mm x 4.6mm, 5µm) with a flow rate of 1.0 ml/min. A 30 µl sample volume was injected, and detection was performed at a wavelength of 221 nm. The mobile phase, consisting of Acetonitrile and Phosphate Buffer in an (70:30 %(v/v) ratio, was employed. The run time was set at 6. Before analyte injection, the column was equilibrated for 15 minutes with the mobile phase.

Evaluation of analytical methods (method validation)

1.System Suitability

System suitability is a test to determine the suitability and effectiveness of chromatographic system prior to use. This was done to make sure the chromatographic system was appropriate for the planned purpose. Six replicate injections of standard preparation (30µg/ml) were injected into the liquid chromatographic system and chromatograms were recorded.

1.Specificity

The capacity of an analytical method to quantify an analyte properly in the presence of interferences that would be expected to be present in the sample matrix is known as selectivity.

The mobile phase, standard and sample solution were prepared and inspected in the same way as the analytical method. Any peak in the chromatograms' dilution solution and mobile phase that is near the active substance's retention period shouldn't significantly affect the findings. Nirmatrelvir and Ritonavir has retention time of 2.8 and 3.9 min. the sample or reference solution had a concentration of 30µg/mL.

Acceptances criteria:

There is no interference in the standard peak.

2.Linearity

The linearity of an analytical method is its capability to elicit check consequence which might be at once, or wit the aid of well describe mathematical adjustment, proportional to the concentration of analytes in within a given range.

Linearity is determined by injecting a series of standards of stock solution/diluent using the solvent/mobile phase, at a minimum of five different concentration in the range of 10-50µg/ml. The absorbance was measured at wavelength 221nm.

Linearity of Nirmatrelvir and Ritonavir

Procedure - From the (Standard stock solution 2) of Nirmatrelvir and Ritonavir (**(**100µg/ml), 1ml, 2ml, 3ml, 4ml, 5ml were pipetted out and transferred to separate 10 ml of volumetric flask and the volume was made up to 10 ml with the help of diluent. The linearity of the relationship between peak area and concentration was determined by analyzing five working standards over the concentration range of 10, 20, 30, 40, 50 µg/ml for Nirmatrelvir and 10, 20, 30, 40, 50 µg/ml for Ritonavir.

3.Range

The range of an analytical approach is the range between the upper and lower levels that can be found using the established method with precision, accuracy, and linearity. The linearity test will be conducted within this concentration range.

4.Precision

The degree of agreements between individual test findings obtained when a process is applied repeatedly too many samplings is know as the precision. According to the ICH precision should be performed at three different levels:

5.Repeatability

For repeatability minimum of six determinants were prepared of (30 µg/mL) and of Nirmatrelvir and Ritonavir, respectively. The chromatogram responses were obtained by injecting one by one. The standard deviation & relative standard deviation was calculated for each type of precision.

a.Intra-Day Precision

By collecting three sample of the concentration 20, 30, 40 µg/ml of Nirmatrelvir and Ritonavir on the same day at intervals of two hours for intra- day precision, the reproducibility of the sample application and determination of peak area for the drugs were determined.

B.Inter-Day Precision

By collecting three sample of the concentration of 20, 30, 40 µg/ml of Nirmatrelvir and Ritonavir on the different day for inter-day precision, the reproducibility of the sample application and determination of peak area for the drugs were determined.

6.ACCURACY

The accuracy of an analytical method is the degree of agreement of test results generated by the method to the true value. Accuracy is measured by spiking the sample matrix of interest with a known concentration of analyte standard and analysing the sample using the method being validated.

To demonstrate the accuracy of the titled method, prepared recovery solutions in triplicate in the range of 80%, 100% and 120% of assay concentration and performed the assay as mentioned in test procedure. Calculated percentage recovery of Nirmatrelvir and Ritonavir for all recovery samples. Calculated the mean percentage recovery, relative standard deviation. Also calculated the overall percentage recovery, relative standard deviation.

$$\% \text{ Recovery} = \frac{\text{area of sample x conc.of standard}}{\text{area of standard x conc.of sample}} \text{ x } 100$$

7.LIMIT OF DETECTION (LOD)

Limit of detection is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD was calculated by the standard deviation of the response and the slope.

$$\text{LOD} = \frac{3.3\ x\ \sigma}{s}$$

Where, σ = the Standard Deviation of the Response S = the Slope of the Calibration Curve

LOD are often determined based on single-to-noise ratio, with typical criteria being a single-to-noise ratio is 3:1.

8.Limit Of Quantitation (LOQ)

It is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated by the standard deviation of the response and the slope. The data was obtained from linearity curve and the LOQ was calculated.

$$\text{LOQ} = \frac{10\ x\ \sigma}{s}$$

Where, σ = the standard deviation of the response S = the slope of the calibration curve

LOD are often determined based on single-to-noise ratio, with typical criteria being a single-to-noise ratio is 10:1.

9.Robustness

Robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameter. For HPLC robustness was carried out by changing wavelength, Mobile phase and flow rate. Robustness of a method was done by change in wavelength, Mobile phase or change in flow rate. Injection of 30 µg/mL was prepared from the stock solution and the recorded.

10.Ruggedness

The reproducibility of result under different laboratory condition. Evaluate ruggedness by conducting the method in different laboratory, with different analysis, or using different equipment.

Assay :
Analysis of Marketed Formulation
Brand PAXLOVID

Each tablet contains Nirmatrelvir 150 mg
Ritonavir 100 mg

% Assay determination of Nirmatrelvir and Ritonavir –

Weight and takes 20 Paxlovid tablets. The tablet was crushed to fine powder and amount of powder equivalent to 150 mg of Nirmatrelvir and 100 mg of Ritonavir were weighed and transferred to a 100 ml volumetric flask containing 50ml of diluent. The flask was shaken and sonicate for 10 minute and volume was made up to the mark with diluent. From this solution appropriate dilutions of Nirmatrelvir and Ritonavir were made to get the final concentrations and 20 µl sample was injected into the system to measure peak height, area, retention time. The chromatogram obtained and the area obtained in each chromatogram of five replicate was correlated with regression equation and the amount found is calculate which was within the limit of label claim. The obtained assay values were within the acceptable limit (90-102 %) against the amount claimed in the tablets.

RESULT AND DISCUSSION :

Method Development

Developing a precise and sensitive RP-HPLC method for drug analysis in medications is vital for ensuring consistent quality. RP-HPLC is widely used because it accurately separates and measures components in medicines, guaranteeing their effectiveness and safety.

Selection of Wavelength

The selection of the composition of mobile phase were studied and optimized. Numerous tests were done to optimize the separation of Nirmatrelvir and Ritonavir by adjusting the mobile phase composition. Various organic phase ratios were tried with different lengths of C18 columns. Ultimately, the Thermo Fisher Scientific C18 column (250 X 4.6 mm, 5µm) was chosen for its effective resolution and reasonable run time. Separation was found to be satisfactory with Acetonitrile and phosphate buffer in the ratio of 70:30 %, v/v. UV detection was carried out at 221 nm where both the drugs exhibit maximum absorption. Isocratic mode was chosen as the retention for both the drugs were less than 8 min at a flow rate of 1ml/min. Retention time for Nirmatrelvir and Ritonavir were found to be 2.847 And 3.931 min respectively.

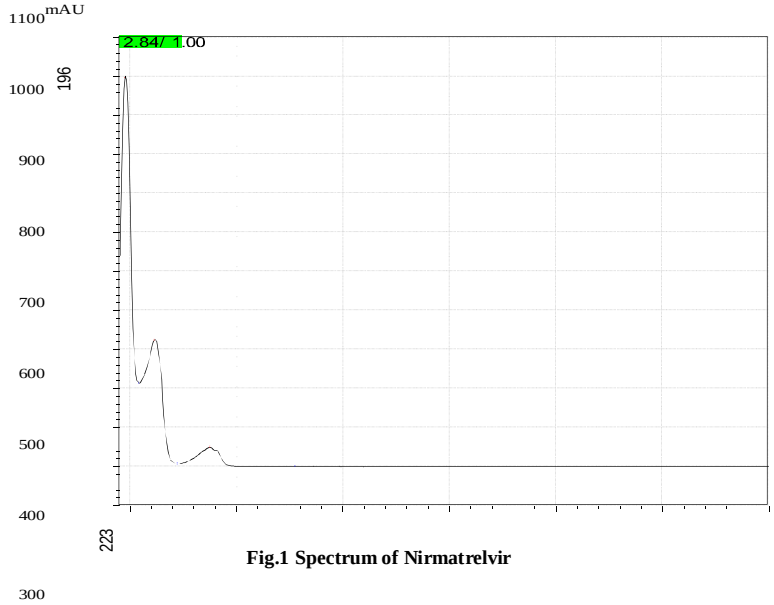


Fig.1 Spectrum of Nirmatrelvir

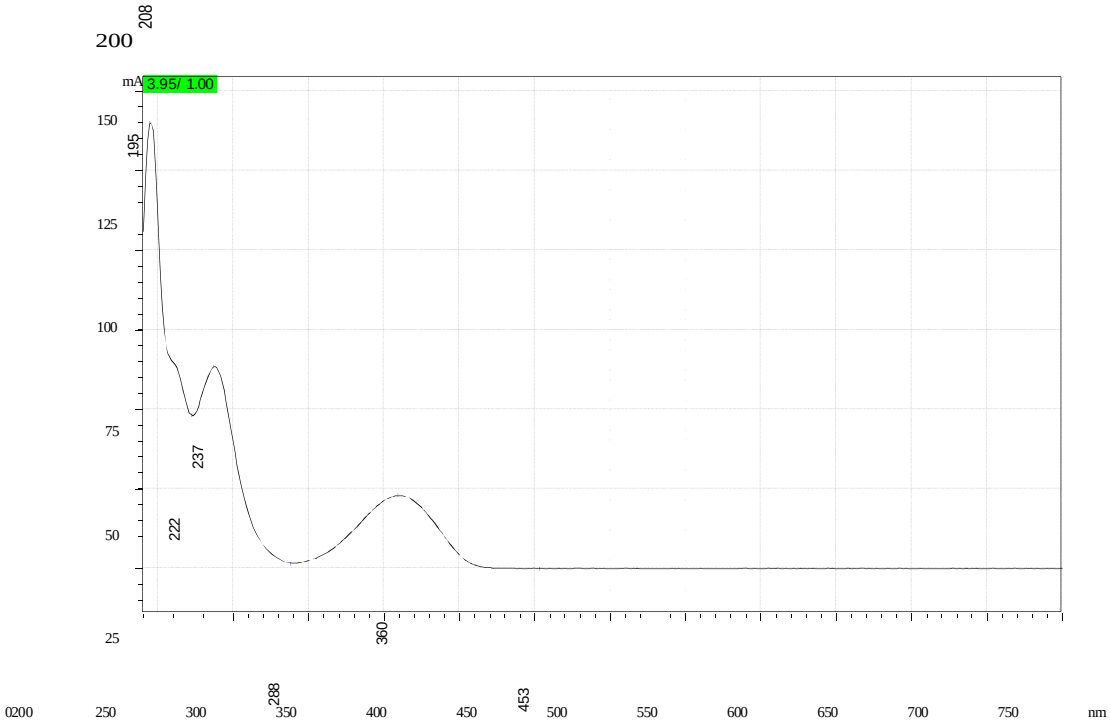


Fig.2 Spectrum of Ritonavir

Analytical Method Validation

System Suitability

Six replicate injections of standard preparation (30µg/mL) were injected into the liquid chromatographic system and chromatograms were recorded.

System Suitability of Nirmatrelvir

Sr. No	Conc. (µg/mL)	Area (µV. sec)	Retention Time	Theoretical Plate	Tailing Factor
1	30	532948	2.847	3936.154	1.534
2	30	533568	2.859	3937.168	1.546
3	30	533941	2.897	3940.182	1.565
4	30	529546	2.921	3955.196	1.578
5	30	534146	2.935	4055.121	1.582
6	30	531445	2.951	4067.138	1.597

Table 5 System Suitability of Nirmatrelvir

System Suitability of Ritonavir

Sr. No	Conc. (µg/mL)	Area (µV. sec)	Retention Time	Theoretical Plate	Tailing Factor
1	30	667095	3.931	4753.853	1.52
2	30	673208	3.949	4753.887	1.539
3	30	674511	3.941	4761.124	1.567
4	30	677426	3.955	4765.254	1.539

5	30	678975	3.963	4774.384	1.548
6	30	685524	3.971	4779.186	1.542

Table 6 System Suitability of Ritonavir

8.Linearity

The method's linearity was assessed by analysing the relationship between the quantity of active compounds and their absorbance's in three solution samples. Linear regression analysis was conducted, and the correlation coefficient (r^2) was calculated based on these results.

The standard stock solution had a concentration of 0.1 mg/ml. A coefficient of determination (r^2) value of 0.999 or higher indicates a strong association. Additionally, the relative standard deviation (RSD %) for each concentration's peak

a.Linearity Curve for the Nirmatrelvir

Sr. No	Concentration (µg/mL)	Area (µV. sec)
1	**10**	185294
2	**20**	354916
3	**30**	532948
4	**40**	722582
5	**50**	909613

Table 7 Linearity of Nirmatrelvir

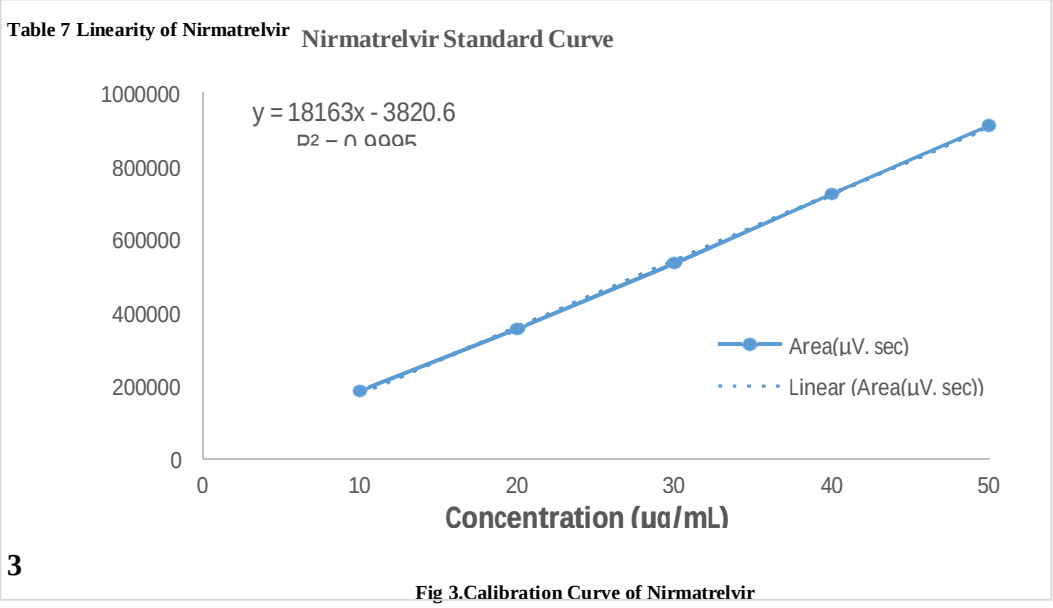


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Fig 3.Calibration Curve of Nirmatrelvir

a.Linearity Curve for the Ritonavir

Sr. No	Conc. (µg/mL)	Area (µV. sec)
1	**10**	227809
2	**20**	447477
3	**30**	677095
4	**40**	892945
5	**50**	1135645

Table 5 Linearity of Ritonavir

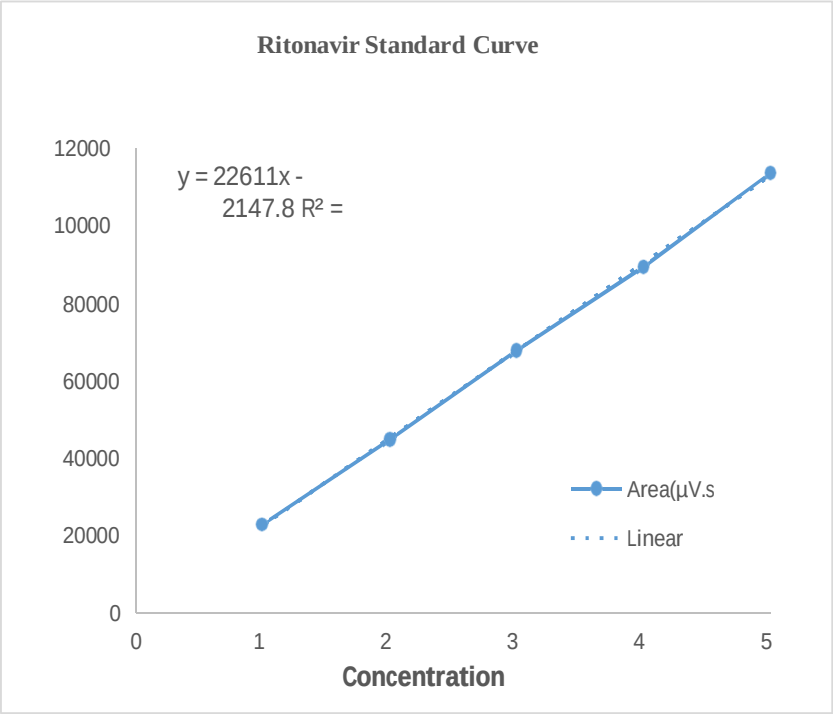

Fig .4 Calibration Curve of Ritonavir

Sr. No	Drug	Rt Time (Min)	Area (mV)	Area (%)	Tailing Factor	T. Plate	Resolution
1	Nirmatrelvir	2.848	19524	45.098	1.521	4016.398	
2	Ritonavir	3.934	237809	54.9082	1.633	4830.235	5.348

Table 6 Linearity of 10 µg/mL of mixture of Nirmatrelvir and Ritonavir

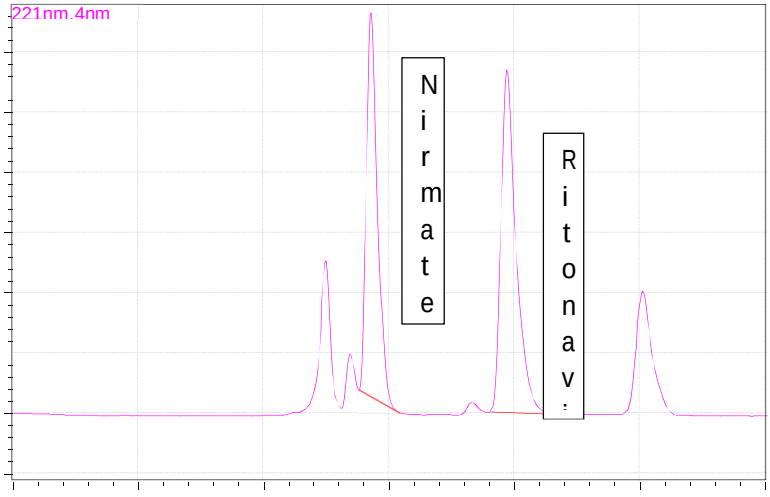

Fig .5 Linearity of 10 µg/mL of mixture of Nirmatrelvir and Ritonavir

9.Range

It appears that the method accuracy, precision and linearity for quantitative analysis were evaluated within the concentration range of 10-50µg/mL for both range Nirmatrelvir and Ritonavir.

10.Precision

Precision is one of the key parameters assessed during method validation. The precision of an analytical method is the degree closeness of agreement between a series of measurements obtained from the multiple sampling of the same sample. Precision include repeatability, inter and intraday precision and reproducibility.

11.Repeatability

For repeatability minimum of 6 determinants were prepared of 30µg/mL conc. of Nirmatrelvir and Ritonavir, respectively. The chromatogram responses were obtained by injecting one by one. The standard deviation & relative standard deviation was calculated for each type of precision.

Sr. No	Peak Area of Nirmatrelvir (µV. sec)	Peak Area of Ritonavir (µV. sec)
1	532948	667095
2	533568	673208
3	533941	674511
4	529546	677426
5	534146	678975
6	531445	685524
MEAN	532599	676123.2
SD	1783.67	6177.56
%RSD	0.334900	0.913673

Table 7 Repeatability of Nirmatrelvir and Ritonavir

Intra-Day Precision

Intra-day precision, also known as within-day precision, evaluates the precision of analytical methods within a single day. It assesses the variation in results obtained from multiple analyses of the same sample within a single day. Intraday precision of conc. 20, 30, 40 µg/mL was prepared and data was obtained for Nirmatrelvir and Ritonavir.

Conc.	20µg/mL	30µg/mL	40µg/mL
Area (µV. sec)	354916	531712	724682
	355001	531820	734758
	360810	540875	734841
MEAN	356909	534802.33	731427
SD	3378.63	5259.361	5841.489
%RSD	0.9466369	0.9834214	0.7986427

Table 8 Intra-Day Precision of Nirmatrelvir

Conc.	20µg/mL	30µg/mL	40µg/mL
Area (µV. sec)	445812	677095	882945
	446924	687157	883075
	448836	687241	873155
MEAN	447190.67	683831	879725
SD	1529.535	5833.698	5690.16
%RSD	0.342032	0.8530906	0.646811

Table 9 Intra-Day Precision of Ritonavir

a. Inter-Day Precision

Inter-day precision, also known as between-day precision, assesses the precision of analytical methods over multiple days. It evaluates the variation in results obtained from multiple analyses of the same sample conducted on different days. Inter-Day precision of conc. 20, 30, 40 µg/mL was prepared and data was obtained for Nirmatrelvir and Ritonavir.

Conc.	20µg/mL (Day 1)	30µg/mL (Day 2)	40µg/mL (Day 3)
Area (µV. sec)	361510	548529	726724
	366411	548615	736823
	366312	544718	736924
MEAN	364744.333	547287.33	733490.333
SD	2801.452	2225.523	5860.034
%RSD	0.7680591	0.4066462	0.7989245

Table 10 Inter-Day Precision of Nirmatrelvir

Conc.	20µg/mL (Day 1)	30µg/mL (Day 2)	40µg/mL (Day 3)
Area (µV. sec)	447482	677101	876460
	447510	687122	876575
	451620	688045	886621
MEAN	448870.667	684089.3	879885.333
SD	2381.03	6069.64	5833.542
%RSD	0.5304498	0.8872590	0.6629888

Table11 Inter-Day Precision of Ritonavir

1. Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. Accuracy may often express in terms of percent recovery of assay of known amount of analyte added. Recovery studies were carried out by addition of standard drug to the sample at 3 different levels of spiking i.e. 80%, 100% and 120% of the actual amount taking into consideration percentage purity of added bulk drug sample.

Sr. No	level of Recovery %	Conc. (µg/mL)	Spiked	Total	Amount Recovery %	% Recovery
1	80%	30	24	54	53.95	99.91
2	80%	30	24	54	54.87	101.61
3	80%	30	24	54	53.85	99.72
4	100%	30	30	60	59.95	99.92
5	100%	30	30	60	59.99	99.98
6	100%	30	30	60	60.89	101.48
7	120%	30	36	66	65.94	99.91
8	120%	30	36	66	66.89	101.35
9	120%	30	36	66	65.85	99.77

Table 12 Accuracy of Nirmatrelvir

Sr. No	level of Recovery %	Conc. (µg/mL)	Spiked	Total	Amount Recovery %	% Recovery
1	80%	30	24	54	53.96	99.93
2	80%	30	24	54	54.91	101.69
3	80%	30	24	54	53.82	99.67
4	100%	30	30	60	59.92	99.87
5	100%	30	30	60	59.98	99.97
6	100%	30	30	60	60.82	101.37
7	120%	30	36	66	65.92	99.88
8	120%	30	36	66	65.96	99.94
9	120%	30	36	66	66.81	101.23

Table 13 Accuracy of Ritonavir

1. LIMIT OF DETECTION (LOD)

The lowest concentration of the analyte that can be consistently detected but not always quantified is known as the limit of detection, or LOD. This guarantees the sensitivity and reliability of the approach by ensuring that singles detected above the LOD can be distinguished from background noise.

Sr. No	Drug	LOD
1	**Nirmatrelvir**	0.317529 µg/mL
2	**Ritonavir**	0.893981 µg/mL

Table 14 LOD of Nirmatrelvir and Ritonavir

1. LIMIT OF QUANTITATION (LOQ)

The lowest concentration of the analyte that can be accurately and precisely measured and quantified is known as the limit of quantification, or LOQ. The lowest concentration standard solutions, when the single-to-noise ratio hits a predetermined level, are analysed to ascertain this.

Sr. No	Drug	LOQ
1	**Nirmatrelvir**	0.962211 µg/mL
2	**Ritonavir**	2.709034 µg/mL

Table 15 LOQ of Nirmatrelvir Ritonavir

2. ROBUSTNESS

Robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameter. For HPLC robustness was carried out by changing wavelength, flow rate and mobile phase composition. The impact of changing in detection wavelength, within $\pm$2 nm, was specifically studied

Sr. No	Wavelength (nm)	Rt. of Nirmatrelvir	Rt. of Ritonavir	Peak Area of Nirmatrelvir	Peak Area of Ritonavir
1	219	2.847	3.931	532942	677080
2	221	2.847	3.931	532948	677095
3	223	2.847	3.931	532951	677098
			MEAN	532947	677091
			SD	4.582576	9.643651
			%RSD	0.000860	0.001424

Table 16 Robustness of Nirmatrelvir and Ritonavir at Wavelength 221±2 nm

Sr. No	Flow rate (ml/min)	Rt. of Nirmatrelvir	Rt. of Ritonavir	Peak Area of Nirmatrelvir	Peak Area of Ritonavir
1	0.1	2.841	3.924	533047	677190
2	1	2.844	3.927	532951	677097
3	1.1	2.847	3.931	532882	676901
			MEAN	532960	677062.667
			SD	82.86736	147.52740
			%RSD	0.015549	0.02179

Table 17 Robustness of Nirmatrelvirs and Ritonavir by changing the Flow rate

Sr. No	Ratio (V/v %)	Rt. of Nirmatrelvir	Rt. of Ritonavir	Peak Area of Nirmatrelvir	Peak Area of Ritonavir
1	65:35	2.843	3.926	532946	677094
2	70:30	2.846	3.929	533052	678096
3	75:25	2.849	3.932	533155	679098
			MEAN	533051.00	678096
			SD	104.5035885	1002
			%RSD	0.01960	0.14777

Table 18 Robustness of Nirmatrelvir and Ritonavir by changing Mobile phase

2.RUGGEDNESS

The ruggedness test of analytical assay method is defined as degree of reproduction of assay results obtained by the successful application of the assay over time and among multiple laboratories and different analyst.

The % recovery at each level and mean recovery should be in between 98.0% to 102%.

3.ASSAY

Analysis of tablet formulation

Brand PAXLOVID

Each tablet contains Nirmatrelvir 150 mg

Ritonavir 100 mg

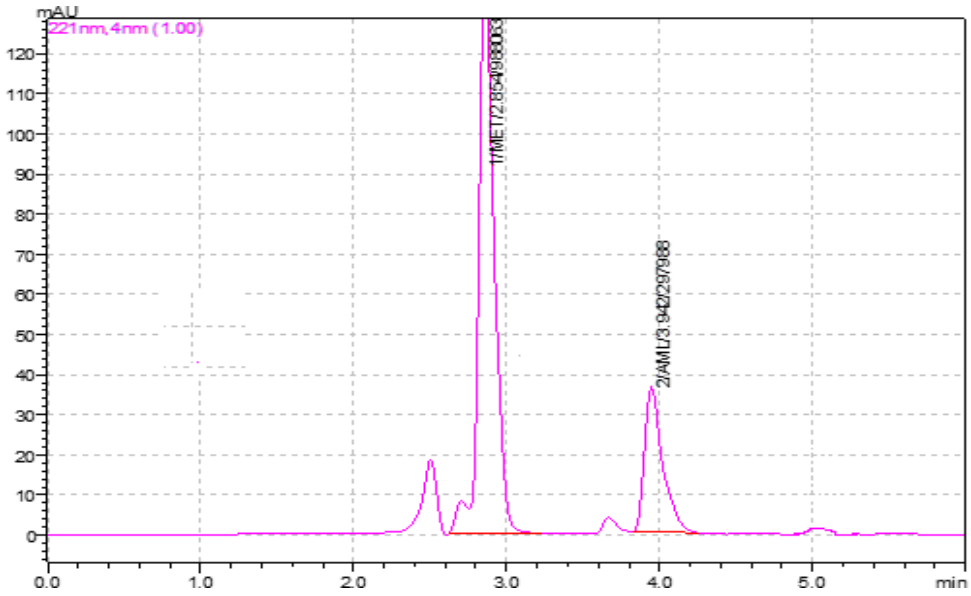

Fig .6Chromatogram of Nirmatrelvir and Ritonavir (30µg/mL) Tablet (Paxlovid)

Sr. No	Drug	Rt Time (Min)	Area (mV)	Area (%)	Tailing Factor	T. Plate	Resolution
1	Nirmatrelvir	2.849	842325	73.7725	1.600	3778.530	
2	Ritonavir	3.939	299462	26.2275	1.845	5056.790	5.356

Table 19 Chromatogram of Nirmatrelvir and Ritonavir (30µg/mL) Tablet (Paxlovid)

Sr. No	Amount Present In (mg)		Amount Found In (mg)		% label claim	
	Nirmatrelvir	Ritonavir	Nirmatrelvir	Ritonavir	Nirmatrelvir	Ritonavir
1	30	20	29.95	19.92	99.83	99.6
2	30	20	29.93	19.91	99.77	99.55
3	30	20	30.21	19.87	100.70	99.35
4	30	20	29.85	20.05	99.50	100.25
5	30	20	29.78	19.91	99.27	99.55

Table 20 Analysis of Nirmatrelvir and Ritonavir Marketed formulation (Paxlovid)

CONCLUSION

In summary and conclusion, the goal of this work was to develop an extremely sensitive, precision, and accurate RP-HPLC method for the analysis of pharmaceutical dosage forms, specifically focusing on Nirmatrelvir and Ritonavir**.**The flow rate was maintained at 1 ml/min, retention time of Nirmatrelvir and Ritonavir was determined to be 2.8 min and 3.9 min. Wavelength selection for simultaneous estimation was based on overlaying UV spectra, resulting in the selection of 221mm.Validation of the developed RP-HPLC method encompassed assessing various parameters such as linearity, precision, accuracy, ruggedness, robustness, LOD, and LOQ. The method displayed excellent linearity, with a regression coefficient (R^2) close to 1. The limit of detection was found to be 0.317529 µg/mL and 0.893981 µg/mL respectively for Nirmatrelvir and Ritonavir. The limit of quantitation was found to 0.962211 µg/mL and 2.709034 µg/mL respectively for Nirmatrelvir and Ritonavir.Precision analysis, indicated by %RSD values of repeatability of Nirmatrelvir is 0.334900 and ritonavir is 0.913673, Intra-day precision of Nirmatrelvir is 0.9466369, 0.9834214, 0.7986477 and Ritonavir is 0.342032, 0.8530906, 0.646811 and Inter-day precision of Nirmatrelvir is 0.7680591, 0.4066462, 0.7989245 and Ritonavir is 0.5304498, 0.8872590, 0.6629888. Accuracy was assessed through recovery studies, with % recovery values of Nirmatrelvir ranging from 99.72% to 101.61% and % recovery values of Ritonavir ranging from 99.67% to 101.69%

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