RESEARCH PAPER

Development and Validation of a QbD-Based Stability-Indicating HPLC Method with LC–MS Characterization of Nirmatrelvir in Bulk Drug and Pharmaceutical Formulation

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Abstract

Purpose:

The purpose of this study was to develop and validate a quality by design (QbD)–based stability-indicating HPLC method for the quantitative determination of nirmatrelvir in bulk drug and pharmaceutical formulations, along with LC–MS characterization of its degradation products.

Methods:

Method development was carried out using a QbD approach supported by central composite design to optimize critical chromatographic parameters. Forced degradation studies were performed under acidic, alkaline, oxidative, thermal, and photolytic conditions in accordance with ICH guidelines. Chromatographic separation was achieved on a NucleoSil C8 column (250 × 4.6 mm, 5 µm) using an isocratic mobile phase of methanol and 0.1% formic acid (70:30, v/v) at a flow rate of 1.2 mL/min, with UV detection at 215 nm. The method was validated for linearity, accuracy, precision, specificity, robustness, and sensitivity. Degradation products were characterized using LC–MS.

Results:

Nirmatrelvir showed significant degradation under acidic and alkaline conditions, forming three major degradation products (DP1, DP2, and DP3), while minimal degradation was observed under oxidative, thermal, and photolytic stress. The retention time of nirmatrelvir was 4.037 min. The method demonstrated excellent linearity over the concentration range of 4–24 µg/mL ($R^2 = 0.999$), with satisfactory accuracy (recoveries close to 100%), precision (%RSD < 2), robustness, and specificity. The limits of detection and quantification were 0.646 µg/mL and 1.957 µg/mL, respectively. LC–MS analysis enabled structural characterization of the degradation products and proposal of probable degradation pathways.

Conclusion:

A robust, precise, and stability-indicating QbD-based HPLC method was successfully developed and validated for nirmatrelvir. The method effectively separates the drug from its degradation products and is suitable for routine quality control and stability studies of nirmatrelvir in bulk drug and pharmaceutical formulations.

Keywords: Nirmatrelvir; Quality by Design; Stability-indicating HPLC; Forced degradation; LC–MS; Method validation

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1.Introduction

Stability-indicating analytical methods are defined as validated quantitative procedures capable of detecting changes over time in the chemical, physical, or microbiological properties of a drug substance or drug product, while accurately measuring the active pharmaceutical ingredient and its degradation products without interference, as outlined in the draft 1998 guidelines[1]. Such methods are essential to ensure drug quality, safety, and efficacy throughout the product lifecycle.

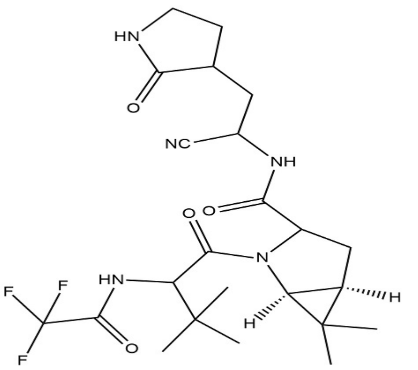

Fig.1. Structure of Nirmatrelvir

Nirmatrelvir, an antiviral agent used in the treatment of COVID-19, has the molecular formula $C_{23}H_{32}F_3N_5O_4$. It is a colorless to pale powder and is reported to be freely soluble in 1-butanol (Figure 1)[2,3]. Like many pharmaceutical compounds, nirmatrelvir is susceptible to degradation upon exposure to various environmental and chemical stress conditions, which may lead to a loss of 5–20% of drug content over time. Conventional stability testing approaches often rely on trial-and-error experimentation, which is laborious, time-consuming, and cost-intensive.

A systematic and science-based approach, such as Quality by Design (QbD), offers a more efficient alternative by minimizing experimental uncertainty through risk assessment and structured method optimization [4,5]. QbD enables the identification of critical method parameters and ensures robust analytical performance with predictable quality outcomes. However, a review of the available literature reveals only a limited number of QbD-based stability-indicating methods reported for nirmatrelvir, with insufficient disclosure of quantitative degradation data and the absence of LC–MS-based degradation product characterization [6–11].

In this context, the present study focuses on the development and validation of a QbD-based stability-indicating HPLC method for nirmatrelvir in accordance with ICH guidelines. The method aims to quantitatively assess the drug under various forced degradation conditions, evaluate degradation pathways, and characterize the formed degradation products using LC–MS. The proposed risk-based analytical approach is designed to provide a robust, reliable, and reproducible method suitable for routine quality control and stability assessment of nirmatrelvir in bulk drug and pharmaceutical formulations.

2. Material and Methods

2.1 Chemicals and reagents

Methanol (Analytical Reagent Grade), HPLC grade water. Every chemical and reagent was acquired from S. D. Fine Chemicals Ltd., Mumbai.

2.2 Instruments

Borwin Chromatography Model PU 2080 Plus used Software (1.50) for HPLC. The sample injection port has a 20µl loop, along with a NucleoSil C8 column (250 mm × 4.6 mm, 5 µm), JASCO UV-2075 UVVIS detector, JASCO V-730 UV-Visible double beam spectrophotometer, Shimadzu ATX-224 electronic weighing scale, and ELGA UHQ II water purification Conductivities below 0.05µS/cm were used. The Shimadzu LCMS-2050

performed LC-MS. A positive-mode ESI ion source has a 0–2000 m/z mass range. Desolation temperature was 450 degrees Celsius, nebulizer gas flow was 2 liters per minute, drying gas flow was 5 liters per minute, capillary voltage was 3 kilovolts, and Qarray voltage was 20/120 volts A 2 × 50 mm, 2.2 micron Shin-pack XR-ODS column was used at 40 °C. Mobile Phases A and B were water with 0.1% ammonium acetate and methanol. The flow was 0.5 mL/min.

2.3 Preparation of Standard Stock Solution

For standard Nirmatrelvir stock solution, dissolve 10 mg in 10 mL methanol to achieve 1000 µg/mL concentration. To make a 100 µg/mL solution, dilute 1 mL of the stock solution with 10 mL of mobile phase. Dilutions were made using methanol.

3. Results and Discussion

3.1. Selection of Critical Chromatographic Parameters Using Design-Expert® Software

Table.1 The optimal chromatographic settings by design expert software

Level of Variable	Factor	
	Mobile Phase Composition % (Methanol: 0.1 % Glacial Acetic Acid)	**Flow Rate(ml/min**
Low Level(-1	60:40	0.8
Mediun Level(0	70:30	1.0
High Lev (1)	80:20	1.2

summarizes the chromatographic variables and their respective levels selected for method optimization using Design-Expert® software. Mobile phase composition and flow rate were identified as critical method parameters and evaluated at three levels to study their impact on chromatographic performance.

3.1 Selection of Detection Wavelength

Subsequent using methanol and the standard stock solution, dilutions were produced examined over the 200 to 400 nm wavelength range, hence obtaining the spectrum. Nirmatrelvir had significant absorption at 215 nm in contrast to 264 nm. Thus, 215 nm was designated as the wavelength for detection and quantification in further study. (Figure 2)

Table.2 Critical material attributes (CMAs)

Ru	Factor-1 % Metha ol	Facto -2 Flow Rate	Respon e-1 Retenti n Time	Respon e-2 Tailing Factor	Respons e-3 Theoreti cal Plat
1	60	0.8	5.067	1.345	6178.952
2	60	1	5.034	1.155	6470.536
3	60	1.2	4.941	1.130	6564.652
4	70	0.8	4.113	1.033	7588.914
5	70	1	4.008	1.021	7737.820
6	70	1.2	3.901	1.052	7834.741
7	80	0.8	2.707	1.206	5634.229
8	80	1	2.622	1.166	5704.964
9	80	1.2	2.521	1.128	5713.795

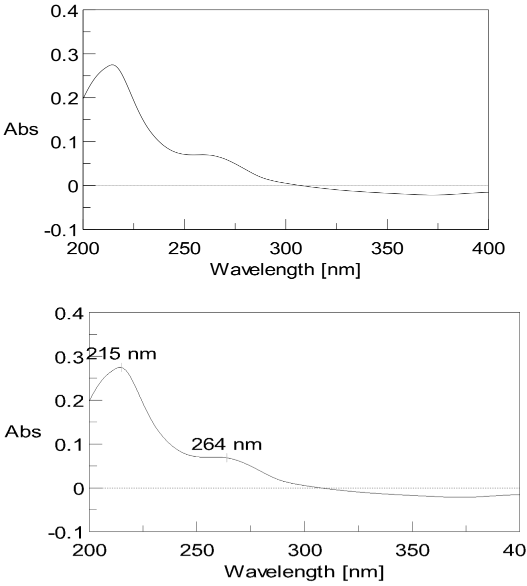

Fig.2.UV Spectra of Nirmatrelvir (10 µg/ml)

3.2 QbD approach to mobile phase and chromatographic conditions

Chromatographic assays used a 10 µg/mL Nirmatrelvir working solution. The process was designed and optimized using Quality by Design. We assessed methodological issues using Central Composite Design (CCD). We analysed retention duration, tailing factor, and theoretical plates in various circumstances. This gave Nirmatrelvir unique chromatographic settings. The Design Expert tool determined chromatographic parameters. (Table 1) Optimising HPLC parameters relied on retention duration, tailing factor, and theoretical plates. These were crucial material characteristics (CMAs) for the product profile. Tables 2, 3, 4, 5, 6 and Figure 3

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Table. 3 Constraints

Name	Goal	Low r Limi	Upp r Limi	Low r Weig ht	Upp r Weig ht	Import ance
A: % Metha ol	is target = 70	60	80	1	1	3
B: Flow Rate	is in range	0.8	1.2	1	1	3
Retent on Time	is in range	3.5	4.5	1	1	3
Tailing Factor	is in range	1	1.1	1	1	3
Theore ical Plate	Maxi ize	5634 23	7834. 74	1	1	3

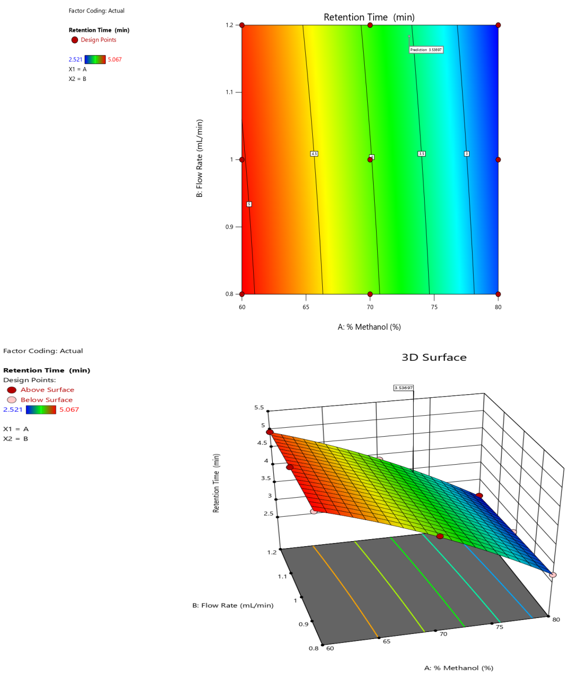


Fig.3.2D contour plot and 3D surface plot of Nirmatrelvir by using central composite design for the effect of the combination of factors on the Retention Time

Table. 4 Solutions found

Nun ber	% Meth anol	Flo w Rat e	Reter tion Time	Tail ng Fact or	Theor etical Plate	Desira bility	
1	**70.05 7**	**1.1 84**	**4.037**	**1.01 7**	**7532.6 38**	**1**	**Selec ted**
2	70.03 2	1.1 82	4.038	1.01 9	7531.9 39	0.997	

Table.5 System suitability parameters for Nirmatrelvir

Drug	**Concentr ation (µg/ml)**	**RT (Mir)**	**Area**	**Plate**	**Asymm etry**
Nirmatı elvir	8	3.87 6 ± 0.17 3	741766 564	7245. 58	1.08

Summary of Chromatographic parameters selected

Table.6 Summary of Chromatographic parameters

Sr. No.	**Parameter**	**Conditions used for Analysis**
1	Column	NucleoSil C8 Column (250 mm × 4.6 mm, 5 µm)
2.	Mobile phase	Methanol: 0.1 % Formic Acid (70:30 v/v)
3.	Flow rate	1.2 ml/min
4.	Detection Wavelength	215 nm
5.	Sample injector	20 µl loop
6.	Column temperature	Ambient

3.3 Validation

3.3.1 Linearity

Mobile phase dilutions of Nirmatrelvir (100 g/ml) stock solution yielded six concentrations. Each focus received 6 copies. A linear correlation was seen between peak area and concentration at 4-24 µg/ml. The results are in Table 7. Plotting peak area versus concentrations produced Figures 4 and 5's calibration curve.

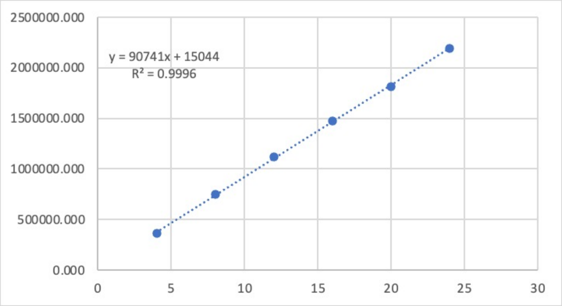


Fig.4.Calibration Curve of Nirmatrelvir

Table.7 Linearity study of Nirmatrelvir

Repli cates	**Concentrations of Nirmatrelvir**					
	4 µg/m	**8 µg/m**	**12 µg/ml**	**16 µg/ml**	**20 µg/ml**	**24 µg/ml**
	Peak Area					
1	373127.265	741766.564	1118252.050	1478782.375	1776096.196	2203127.493
2	361029.375	772769.375	1138786.455	1434437.977	1855806.638	2129463.574
3	357626.465	759964.261	1115168.455	1517558.595	1800905.953	2263994.775
4	353763.375	724326.953	1096699.420	1490014.400	1786360.632	2196239.325
5	362264.595	740217.695	1145246.460	1451358.069	1852323.802	2165888.032
6	361386.620	749705.880	1117226.595	1480195.612	1804792.052	2198205.989
Mean	361532.949	748125.121	1121896.573	1475391.171	1812714.212	2192819.865
Std. Dev.	6494.080	16848.233	17582.609	29261.810	33655.498	44614.261
%RSD	1.796	2.252	1.567	1.983	1.857	2.035

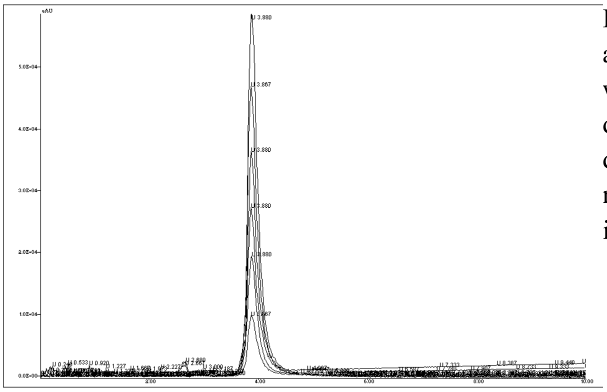
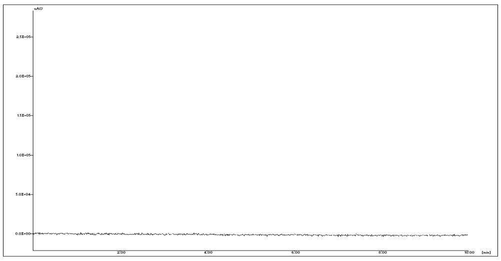


Fig.5.Overlay Chromatogram of linearity of Nirmatrelvir



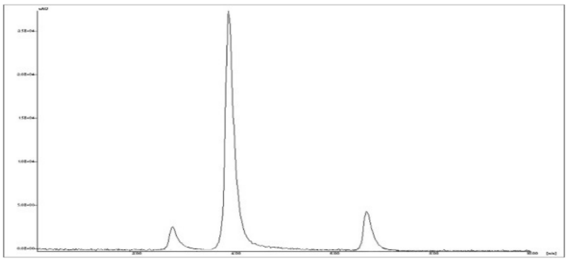
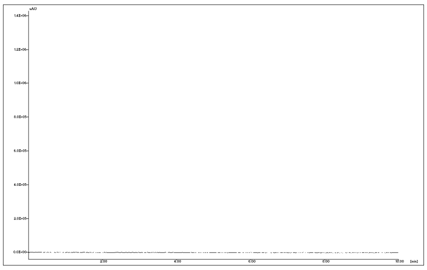


Fig.6.Acid Blank Injection
Fig.7.Chromatogram of acid degradation



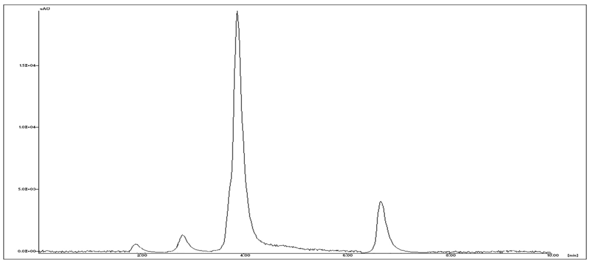


Fig.8. Alkali Blank Injection
Fig.9.Chromatogram of alkaline hydrolysis

3.3.2 Range

Nirmatrelvir = 4-24 µg/ml

3.3.3 Precision

Intra- and inter-day variance tests verified procedure accuracy. In intraday trials, nirmatrelvir (8, 12, 16 µg/ml) was tested for one day and the percentage relative standard deviation (RSD) was determined. Three replicates of different doses over three days were used to compute the relative standard deviation (RSD) %. Tables 8 and 9 show intraday/interday fluctuations.

Table.8 Intra-day precision study Nirmatrelvir

Theo. Conc (mcg/ml)	Area	Practical Conc	% Assay	Avg*	SD	RSD
8	740311.650	7.993	99.909			
8	744316.930	8.037	100.461	100.037	0.376	0.376
8	739096.650	7.979	99.742			
12	1098752.410	11.943	99.524			
12	1108517.850	12.050	100.421	99.944	0.451	0.451
12	1102696.637	11.986	99.886			
16	1466780.580	15.999	99.992			
16	1479277.674	16.136	100.853	100.365	0.442	0.440
16	1470547.575	16.040	100.251			

*Average of three determinations

3.3.4.LOD and LOQ

The formula yields LOD and LOQ. -

$$LOD = \frac{3.3\ \sigma}{S} \qquad LOQ = \frac{10\,\sigma}{S}$$

σ=Y-intercept's standard deviation;
S =slope.

3.3.5 Specificity

Peak purity profiling tests evaluated the method's specificity. The highest purity values surpassed 998, indicating the lack of interference from degradation products, contaminants, or matrix constituents. (Table. 11)

Table. 11 The peak purity value

Drug	Purity tail	Purity front
Nirmatrelvir	998.94	999.12

3.3.6 Assay

Weigh 20 Tablets (Nirmatrelvir 150 mg- Primovir) and smash them to powder in motor and pestle. To get a 1000 µg/ml concentration, measure 10 mg of Nirmatrelvir tablet powder and dissolve in 10 ml of methanol. The mobile phase diluted one 1 ml of the standard stock solution to 10 ml, yielding a 100 µg/ml Nirmatrelvir solution. A 10 µg/ml solution was achieved by dilution in the mobile phase. There were 6 operations. Sample solutions and areas were recorded. Concentration and recovery were calculated using the linear equation. Table 12 shows results.

Table. 12 Results of formulation analysis (Assay)

Sr. No	Area	Concentration (µg/ml)	% Recovery
01	739303.248	7.982	99.770
02	741230.008	8.003	100.036
03	742700.865	8.019	100.238
04	742529.816	8.017	100.215
05	739436.352	7.983	99.788
06	738568.875	7.974	99.669
Mean	740628.194	7.996	99.953
S. D	1771.501	0.020	0.244
% RSD	0.239	0.244	0.244

3.3.7 Accuracy

Recoveries were performed by adding a reference medicine to the Sample at 50%, 100%, and 150% to validate the procedure. Selected sample concentration was 4 g/ml. Solution chromatograms were obtained by sending them in triplicate under constant chromatographic conditions. Nirmatrelvir concentrations were determined using a linearity equation. Table 13 presents the findings.

Table.13 Recovery study of Nirmatrelvir

Level	Sample Conc. (µg/ml)	Std Conc. (µg/ml)	Area	Amount Recovered	% Recc ery	% Recovery (Mean ±%RSD)
50 %	8	4	1108629	12.052	100.41	100.282 ± 0.355
			1102840	11.988	99.89	
			1109565	12.062	100.57	
100 %	8	8	1472247	16.059	100.38	100.317 ± 0.481
			1478110	16.124	100.72	
			1464153	15.970	99.81	
150 %	8	12	1830518	20.007	100.06	99.962 ± 0.262
			1823876	19.934	99.67	
			1833106	20.036	100.19	

3.3.8 Robustness

The method's robustness was proven by mobile phase composition, detection wavelength (1 nm), and flow rate (0.05 ml/min). The method's resilience showed that moderate operational parameter changes did not alter key peaks after targeted analytical parameter tweaks, proving its strength. Table 14.

Table.14 Robustness study

	FLOW RATE (± 0.05 ml/min)		
	0.95	1	1.05
	749492.407	756198.955	737910.429
	737348.594	741139.266	758133.713
	749469.493	743070.811	742438.306
Avg	745436.831	746803.011	746160.816
STD DEV	7004.628	8194.238	10613.111
% RSD	0.940	1.097	1.422
	MP COMPOSITION (± 2 ml Composition)		
	68:32	70:30	72:28
	742907.805	738091.42	748229.326
	741042.749	724326.953	739657.002

	743362.662	745300.0198	742885.044
Avg	742437.739	735906.131	743590.457
STD DEV	1229.317	10655.937	4329.479
% RSD	0.166	1.448	0.582
	DETECTION WAVELENGTH (± 1 nm)		
	214	215	216
1	754686.560	747250.625	746610.480
2	751139.704	752644.977	754279.515
3	740283.089	744373.847	772769.375
Avg	748703.118	748089.816	757886.457
STD DEV	7504.512	4198.938	13447.285
% RSD	1.002	0.561	1.774

Summary of validation study

Table 15 presents an overview of the validation parameters.

Table.15 Summary of validation study

Sr. No.	**Validation Parameter**	**Nirmatrelvir**
1.	Linearity	Y=16804x+22843 R² = 0.999
2.	Range	2-12 µg/ml
3.	Assay (Mean ± % RSD)	100.137 ± 0.422
4.	Precision	(% RSD)
	A) Intraday precision	0.379 – 0.542
	B) Interday precision	0.128-0.841
5.	Accuracy	% Recovery (Mean ± % RSD)
	50 %	99.930 ± 0.576
	100 %	100.157 ± 0.417
	150 %	100.271 ± 0.597
6.	LOD	0.166 µg/ml
7.	LOQ	0.503 µg.ml
8.	Specificity	Specific
9.	Robustness	Robust

Summary of stress degradation study of Nirmatrelvir is given in Table

Table.16 Summary of stress degradation study of Nirmatrelvir

Sr. No.	**Stress Degradation Condition**	**% Recovery**	**% Degradation**	**R.T. o degraded products**
1	Acid (1 N HCl, Kept fo 24 Hr)	87.53	12.47	D1- RT 2.845 min D2 – RT 6.678 min
2	Alkali (1 N NaOH, Kept for 24 Hr)	81.65	18.65	D1- RT 2.836 min D2 – RT 6.669 min D3 – RT 1.912 min
3	H_2O_2, 30% (Kept for 24 Hr)	98.46	1.54	--
4	Dry Heat (100^0C for 6 hr)	97.94	2.06	--
5	Photo stability [UV 200 watt hrs/square meter Florescence, 1.2 million Lux. Hrs]	99.12	0.88	--

3.4 Stress degradation studies of bulk drug

Acidic and alkaline hydrolysis, oxidation, heat, and photolysis were used to investigate stress deterioration. Every experiment had Nirmatrelvir RS and blank samples. The pharmaceutical solution emphasized the blank similarly. In solids, photolytic degradation and dry heat were done.

3.4.1 Acidic hydrolysis

1 ml methanolic 1 N HCl and 1 ml Nirmatrelvir working standard solution (1000 µg/ml) were incubated in darkness for 24 hours. The solution was neutralized with 1 N NaOH, adjusted to 10 ml, and diluted with 2 ml of mobile phase to obtain 20 µg/ml before injection under optimum chromatographic conditions. (Fig. 6,7)

Acid Degradation:

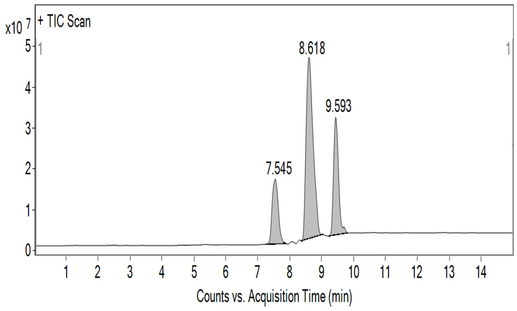
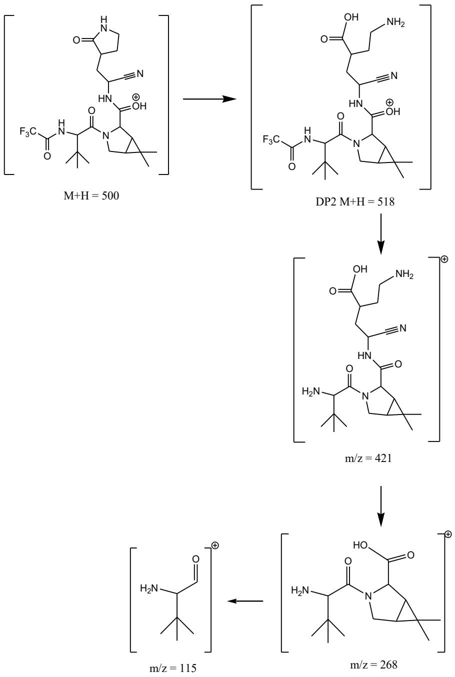


Fig.10.LC-MS Chromatograph of Nirmatrelvir subjected to Acidic stress (Degradant peak D1 –

7.545 min; Degradant peak D2 – 9.593 min; / Nirmatrelvir Peak at RT – 8.618 min)

3.4.2 Alkaline hydrolysis

Working standard solution of Nirmatrelvir (1000 µg/ml) was combined with 1 ml of 1 N aqueous sodium hydroxide and kept in darkness for 24 hours. Next, neutralize with 1 N HCl and adjust to 10 ml. Dilute 2 ml with mobile phase to 20 µg/ml and inject under ideal chromatographic conditions. (Figure 8, 9)



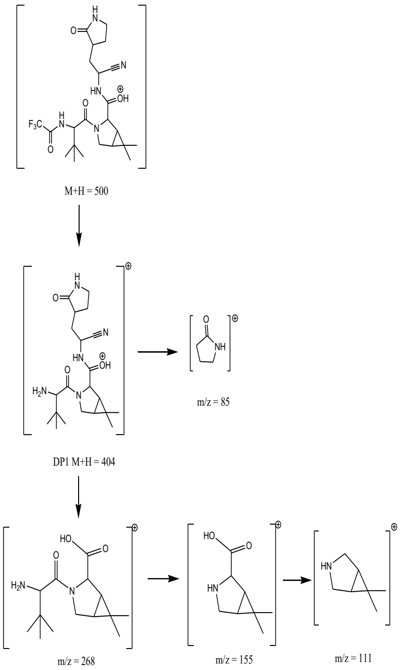


Fig.11. Probable degradation pathway for Degradant DP2 of Nirmatrelvir under Acid/Alkali stress sample

Fig.12.Probable degradation pathway for Degradant DP1 of Nirmatrelvir under Acid/Alkali stress sample

Alkali Degradation:

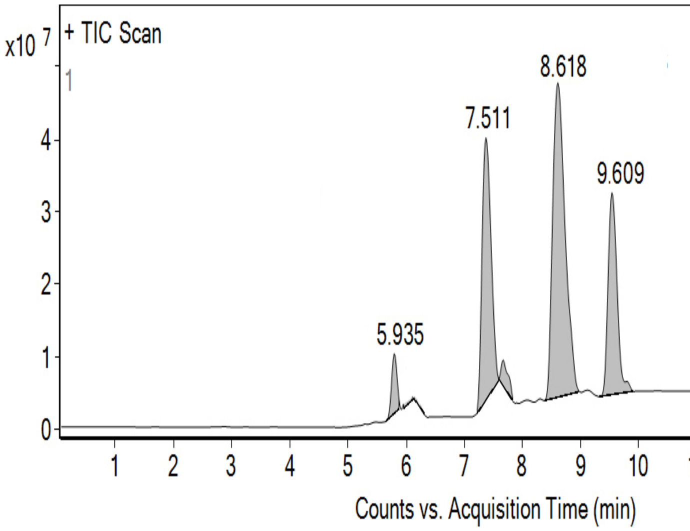
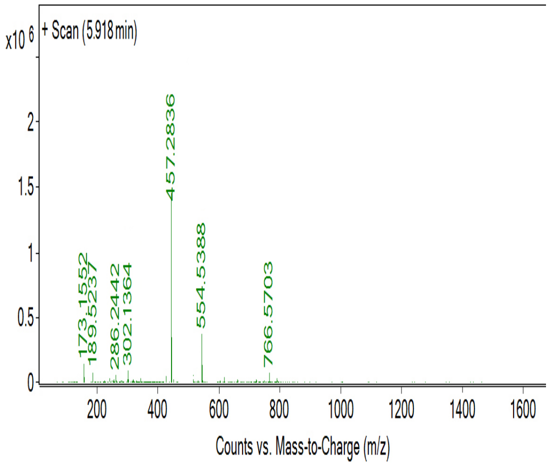


Fig r LC-MS Chromatograph of Nirmatrelvir subjected to Alkali stress (Degradant peak D1 – 7.511 min; Degradant peak D2 – 9.609 min; Degradant peak D3 – 5.935 min / Nirmatrelvir Peak at RT – 8.618 min)



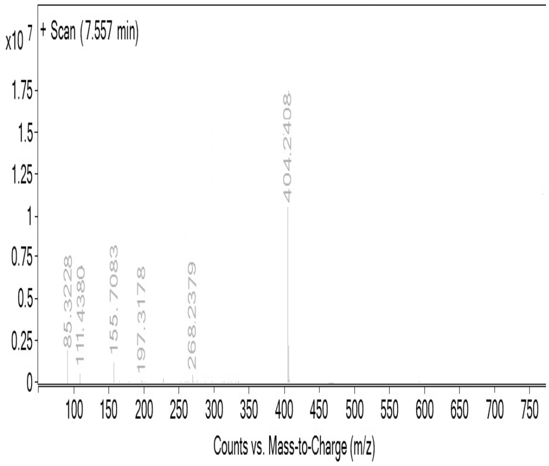


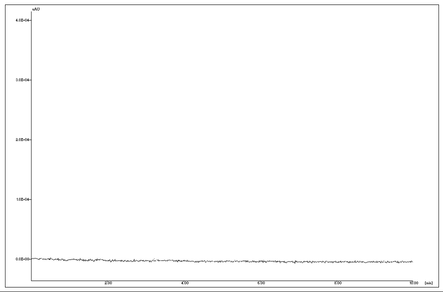
Fig.13.MS Spectrum of DP2 of Nirmatrelvir Acid/Alkali stress sample

Fig.14.MS Spectrum of DP3 of Nirmatrelvir under Alkali stress sample

3.4.3 Oxidative Degradation

Mix one milliliter of 30% H2O2 with one milliliter of Nirmatrelvir (1000 □u00b5g/ml) working standard solution. Darkly incubate 24 hours. To get a final volume of 10 ml (20 µg/ml), dilute 2 ml with mobile phase and inject under optimal chromatographic conditions.

(Figures 10, 11)



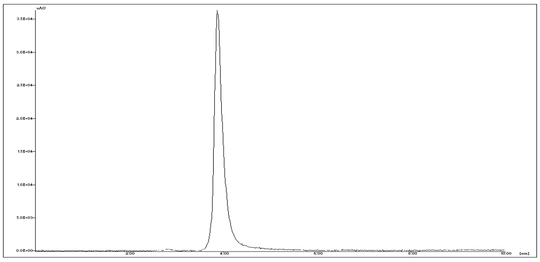


Fig.15. Blank H_2O_2 injection

Fig.16.Chromatogram of oxidation

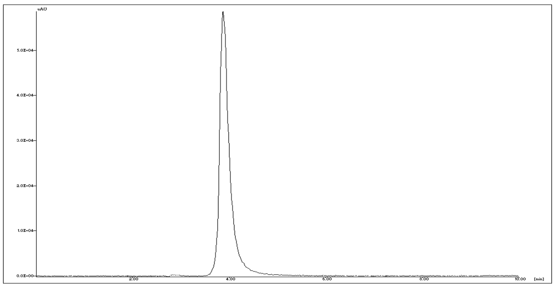


Fig.17**.**Chromatogram of Nirmatrelvir (20 µg/ml) after exposing to dry heat

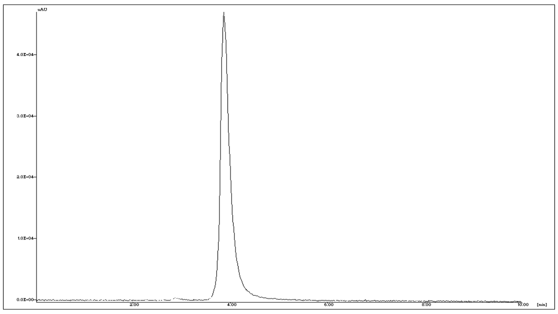


Fig.18**.**Chromatogram of Nirmatrelvir (20 µg/ml) after photo degradation

3.4.4 Degradation under dry heat

Dry heat testing medicines in a 1000°C oven took six hours. This chemical was isolated and processed to 100 g/ml using conventional solution preparation. For best chromatographic conditions, dilute a two-milliliter solution aliquot with mobile phase to ten milliliters (20 µg/ml) and inject. Figure 12

3.4.5 Photo-degradation studies.

The medication was photolytically tested using 200 watt-hours per square meter UV light and 1.2 million Lux fluorescent light. The material was processed to

100 µg/ml using standard solution preparation following exposure. Dilute two milliliters of the solution with mobile phase to ten (20 µg/ml) and inject under optimal chromatographic conditions. (Figure 13)

3.5 LC-MS study

A study was conducted on both the conventional medication and the forced degradation samples of the drug under acidic and alkaline conditions, during which deterioration was noted in the creation of a stability-indicating test technique.

HPLC using a Shin-pack XR-ODS (2 X 50 mm, 2.2 micron) analytical column successfully separated two degradation products under acidic conditions and three degradation products under alkaline conditions. Exact mass measurements to four decimal places using LC–ESI/MS, it was discovered and characterized. (Figure 14, 15, 16)

Standard Nirmatrelvir

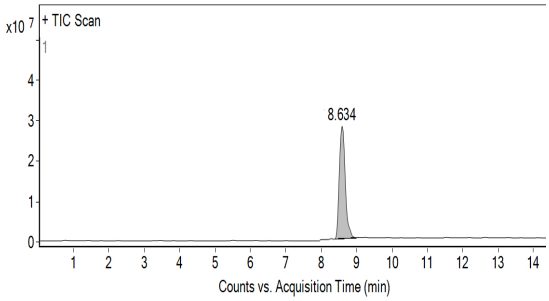


Fig.19.LC-MS Chromatograph of Nirmatrelvir standard (RT – 8.634 min)

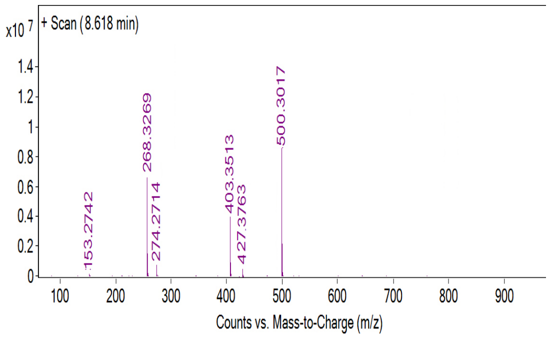


Fig.20. LC-MS Spectrum of Nirmatrelvir standard (Retention Time – 8.618 min, M+H peak- m/z 500)

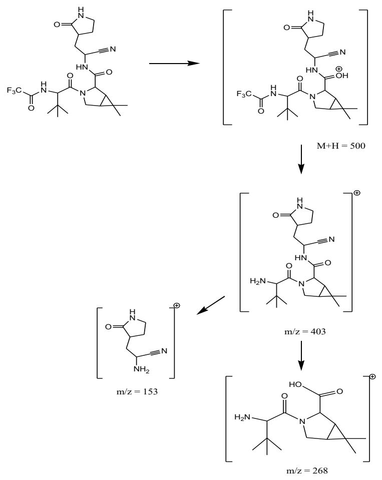


Fig.21.Probable degradation pathway (fragmentation pattern) of Standard Nirmatrelvir Molecule

4. Conclusion

Using Quality by Design (QbD), a robust and reliable HPLC technique was designed and validated to quantify nirmatrelvir and its degradation products. This methodical approach discovered and refined important procedure parameters for high precision, accuracy, and specificity. The approach separated the intact medication from breakdown products under diverse stress conditions, including acidic and alkaline hydrolysis, showing stability. Degradation produced three main products, DP1, DP2, and DP3, which were successfully resolved from the parent molecule. The NucleoSil C8 column and isocratic elution of Methanol and 0.1% Formic Acid in a 70:30 (v/v) ratio were optimized for chromatography. The best wavelength for detecting nirmatrelvir and its contaminants was 215 nm. Main compound retention duration was 4.037 minutes, allowing effective separation in a short run time. The approach was suitable for regular quality control and stability investigations of nirmatrelvir-containing pharmaceutical formulations and had high analyte resolution. The proposed method was suitable for stability research and quality monitoring due to its linearity, accuracy, sensitivity, and robustness. LC-MS also characterized degradation products structurally and proposed degradation routes. This validated technique tracks nirmatrelvir stability in bulk medicine and pharmaceutical formulations using a consistent analytical equipment to verify product effectiveness and safety throughout shelf life.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the research work presented in this report.

Consent for Publications

All authors have read and approved the final version of this manuscript for publication.

Availability of Data and Material

All data generated or analyzed during this study are included in the manuscript and are available within the document.

Authors' Contributions

Ms. **Surekha Shegar** designed and conducted the research work, performed data analysis, and prepared the manuscript.

Dr. Minal Ghante[2]supervised the research design, guided the experimental work, and reviewed and approved the final version of the manuscript.

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