RESEARCH PAPER

Method Development and Validation Study of Nirmatrelvir using a Stability-Indicating HPLC method along with Comprehensive Impurity Characterization and Toxicity Profiling

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ABSTRACT
A sensitive, stability-indicating and precise high-performance liquid chromatography (HPLC) method was established for the quantitative determination of Nirmatrelvir (NTR) and its associated impurity in bulk drug and tablet formulations. The analytical method was developed using a simple mobile phase of water and acetonitrile (20:80, v/v) under isocratic elution at 1 mL/min and chromatographic separation was achieved on a Phenomenex C18 column (250 × 4.6 mm, 5 µm). The effectiveness of the method is established using impurity-spiked samples. The validation was done in compliance with ICH Q2(R1) guidelines. The method demonstrated excellent specificity, linearity (1.64–29.94 µg/mL; R^2 = 0.9998), accuracy (94–101%), and precision (%RSD < 2%). As per ICH Q3A/B regulatory guidelines, we used caronic anhydride which is a reactive cyclic anhydride for impurity characterization. Moderate systemic toxicity (LD_{50} ≈ 500 mg/kg) was seen in the in-silico toxicity assessment using PROTOX 3.0 platform. Also, the invitro MTT cytotoxicity assay was done on PBMC cells to study the cytotoxic effect of impurity and established safety profile of the drug. The method development and validation of Nirmatrelvir is confirmed to be a practical, eco-friendly and cost-effective evaluation for routine quality control of the drug. Effective separation of products confirms that a stability indicating method which is accurate, specific and reliable has been developed. Estimation of risks associated with the impurity and its cytotoxicity through computational and invitro methods provides a framework for evaluation of formulations with Nirmatrelvir in the future.

Keywords: Nirmatrelvir, Caronic Anhydride, HPLC, Impurity Profiling, Method Validation, In-silico Toxicity, MTT Assay
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INTRODUCTION

Developing a valuable and stable analytical method is the foundation to ensure safety and efficacy of the drug while maintaining compliance with regulatory guidelines (1). Nirmatrelvir which is used as the first line therapy for COVID 19 infection, is a direct SARS-CoV-2 3CL protease inhibitor. It is given in combination with Ritonavir, as it enhances the pharmacokinetic exposure of Nirmatrelvir. The quantification, degradation and impurity testing of the active pharmaceutical ingredient (API) becomes very essential as it plays a crucial role in treating infection. (2,3,4). Caronic Anhydride is a degradation impurity which can be potentially toxic due to its increased chemical reactivity (5,6).The chemical nomenclature of Nirmatrelvir is "(1R,2S,5S)-N - ((1S)-1 - Cyano – 2 - ((3S) - 2- oxopyrrolidin – 3 - yl) ethyl) – 3 - ((2S) - 3, 3 – dimethyl -2-(2,2,2-trifluoroacetamido) butanoyl)-6,6-dimethyl-3- azabicyclo (3.1.0) hexane-2 carboxamide, a structurally complex, effective oral antiretroviral drug (7,8). Impurity profiling for Caronic Anhydride was evaluated under stress conditions to identify the stability of the drug and potential degradation pathways (9) International regulatory guidelines, especially ICH Q14 for method development and ICH Q2(R2) for method validation, emphasize the need for validated stability-indicating methods. In addition, ICH Q3A/B establishes reporting, identification, and qualification thresholds for impurities, focusing on potentially reactive moieties such as cyclic anhydrides (10,11). HPLC is an

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extensively used analytical method in medical, biological and chemical fields for its non-destructive nature. It can also be used for thermally liable compounds unlike Gas Chromatography (GC). It offers multiple techniques to choose from in terms of selecting a stationary phase and type of detectors, making it a highly viable option for method development (12) There are several studies conducted on method development, validation of the drug and degradation of Nirmatrelvir, along with Ritonavir in some studies. (13,14,15,16) Although there are previous studies, our study aims to establish a multifaceted approach on the complete chemical profile of the drug which includes a simple cost-effective precise method development, validation compared across three formulations: API, bulk tablet formulation and impurity. Along with these a simultaneous impurity risk assessment is done using computational in-silico toxicity and invitro cytotoxicity which reduces dependency on animal studies. Recent analytical research has also shifted toward greener, economical, and rapid chromatographic techniques, with HPLC remaining the most widely adopted tool for routine quality control.

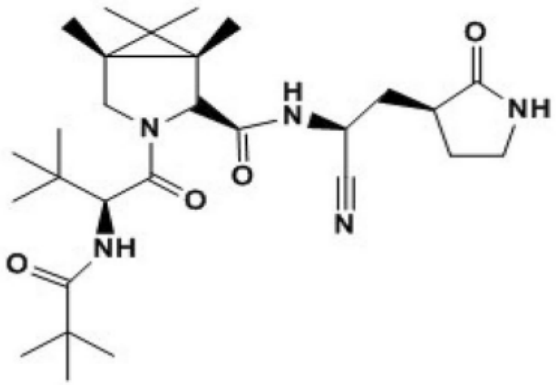


Fig 1: Structures of Nirmatrelvir (13)

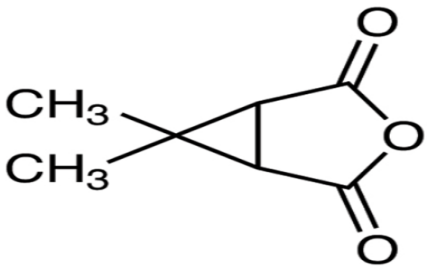


Fig 2: Structure of Caronic anhydride

Impurity Profiling and Cytotoxicity of Caronic Anhydride in Nirmatrelvir

Caronic Anhydride - Impurities in Nirmatrelvir, can form during synthesis, formulation, or degradation, which is a risk to safety and efficacy. ICH Q3A (for drug substances)/B (for drug products) guidelines quotes this cyclic anhydride impurity as highly reactive, which can potentially cause irritation or hypersensitivity via nucleophilic attacks on membranes/proteins. Therefore, monitoring the impurity profile is crucial for process control and stability of the formulations. (9)

Cytotoxicity Evaluation

Cytotoxicity assays evaluate the extent of cellular injury that helps in deciding the therapeutic window and cellular selectivity, which is vital for antivirals like Nirmatrelvir to omit the host cells. (17) The main cytotoxicity study types include acute (single high dose), sub-chronic (repeated short-term), chronic, genotoxicity (DNA damage), reproductive/ developmental studies and carcinogenicity studies. (18) They comply with the ICH/OECD guidelines, supporting safe dosing. (19,20)

MTT Assay

The MTT assay was preferred for screening Nirmatrelvir (API), tablets, and Caronic Anhydride impurity, for its sensitivity, reproducibility, and simplicity. Metabolically active cells reduce yellow MTT $3-(4{,}5-dimethylthiazol-2-yl)-2{,}5-diphenyltetrazoliumbromid$ to purple formazan through mitochondrial dehydrogenases at an absorbance level of 570 nm that correlates with viability.(21)

- **Mechanism:** Metabolically active cells form intracellular formazan crystals, which is solubilised in DMSO and checked for the absorbance in microplate reader.
- **Advantages:** MTT assays are rapid, quantitative, sensitive, versatile, non-radioactive and safe.
- **Limitations:** Due to mitochondrial reliance, assay results are susceptible to reducing agent interference.

MATERIALS AND METHODS

Method development usually requires the choice of columns, mobile phase, detectors, and method of quantization etc. The analytical method was developed systematically optimizing the key chromatographic parameters to achieve peak symmetry, good resolution and flow rates. Multiple C18 columns were assessed with different flow rates ranging from 0.8 to 1.2 mL/min. Detector wavelengths were evaluated using a PDA scanner across 190–800 nm. Different proportions of water and acetonitrile were also tested to achieve sharper peaks and reduced retention times. The imperative for establishing a reliable analytical method arises because existing methods are either expensive, time-consuming or not accurate and precise. [22]

Materials and Reagents

- Nirmatrelvir API (97.5% purity) and Caronic Anhydride impurity standards were obtained from AA Scientifics, Hyderabad, India.
- HPLC-grade water and acetonitrile were purchased from SD Fine Chemicals and Loba Chemie, respectively. All chemicals were of analytical grade.
- Marketed Nirmatrelvir tablets (150 mg) were procured from an authorized pharmacy.

Instrumentation

A Waters 2690/5 HPLC system equipped with a PDA detector (190–800 nm) was used. Sample injections were performed using a 20 µL loop. Data was processed using Empower SPs software.

Methods

***Chromatographic Optimization*:** The mobile phase was passed through a 0.45 µm membrane and degassed before use. To achieve optimal resolution, the following conditions were finalized:

- Column: Phenomenex C18 (250 × 4.6 mm; 5 µm)
- Mobile phase: Water: Acetonitrile (20:80, v/v)
- Flow rate: 1.0 mL/min
- Elution: Isocratic
- Detection: PDA, optimized wavelength
- Injection volume: 10 µL
- Temperature: Ambient

Preparation of HPLC Solutions and standards:

Mobile Phase: HPLC- grade water was mixed with HPLC-grade Acetonitrile in 20:80 ratio (20ml water and 80 ml Acetonitrile). It passed through 0.45 µm membrane filter under vacuum filtration and degassed in an ultrasonic bath for 10 minutes.

Reference Stock Solution: For this solution 10 mg of Nirmatrelvir was accurately weighed and dissolved in the diluent (Acetonitrile) using sonication. Final volume was adjusted to obtain a concentration of 998 ug/ml.

Test Sample Solution: To prepare the test sample solution, commercially available (Nirmatrelvir) tablets were purchased and used. 10 mg of Nirmatrelvir was accurately weighed and diluent (Acetonitrile) was added to a 10ml dry volumetric flask. It is then passed through 0.45 µ injection filter and then subjected to sonication for 15 min to dissolve the drug.

Calibration Standards: Eight levels of calibration standards were used, all prepared individually by diluting the reference stock solution (998 ug/ml) with acetonitrile in 10ml volumetric flasks to obtain the final concentrations as1.64, 4.44, 7.92, 10.56, 15.09, 16.77, 23.95, and 29.94 µg/mL. These series of working solutions were present in concentrations ranging from low to high levels which are used for specific purposes in method validation like evaluating the linearity, constructing a calibration curve and to define the methods working range. Also, an additional 3 quality control (QC) solutions were prepared independently from the same reference stock solution. By diluting the appropriate amounts of the stock solution with acetonitrile in 10ml volumetric flasks the final concentrations obtained were 31.94 µg/mL for high-quality control (HQC), 16.61 µg/mL for medium-quality control (MQC), and 1.83 µg/mL low-quality control (LQC). These solutions are used during method validation to evaluate accuracy and precision. All solutions were mixed thoroughly and used immediately or stored under appropriate conditions to ensure stability throughout the analysis.

HPLC Validation:

The developed HPLC method was validated as per ICH guidelines to find out the reliable method for quantitative analysis of Nirmatrelvir. To confirm whether the developed method produce consistent, accurate, reproducible results within a defined range. The four particular characteristics tested were selectivity, linearity, accuracy, and precision.

Selectivity of the developed method is measured by injecting the blank and lower limit of quantification (LLOQ) into the chromatographic system and calculate the peak area and retention time. Inject diluent and evaluate the interference at RT of API. Inject each (Blank and LLOQ) into chromatographic system and measure the peak area and retention time.

Linearity of the method is established by injecting multiple concentrations of the standard solutions in view of producing results that are directly proportional to the concentration of analytes in samples within a specific range. Linearity of the method was evaluated by plotting a calibration curve with concentration on X-axis versus

peak area on Y-axis and calculating correlation coefficient to confirm.

Accuracy indicates how adjacent are the test results from the developed method to the true value. Three quality control levels (HQC, MQC, and LQC) were evaluated, each at a specified limit. Percentage recoveries were measured for all three concentrations to demonstrate the trueness of the method.

Precision was evaluated by achieving closely agreeing results with repeated analysis of homogeneous samples under the same conditions.

In-Silico Toxicity Assay

Computerized tools like PROTOX 3.0 is used to evaluate the hepatotoxicity, mutagenicity, and carcinogenicity without having to take an animal testing. The toxicity predictions for Caronic Anhydride are classified by LD50: >5000 mg/kg (non-toxic), 300–5000 mg/kg (toxic when swallowed), or ≤300 mg/kg (poses high risk/death). The use of such software's is not only cost-effective, but also these are methods that guides early safety profiling.

Cytotoxicity Assay

PBMC (Peripheral Blood Mononuclear Cells) cells were cultured in liquid medium (RPMI) + 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin maintained at 37°C in the presence of 5% CO_2.

First, the cultured PBMC cells were isolated and collected in a 15 ml tube. Then, the cells were plated in RPMI medium that contains 10 % FBS and 1% antibiotic solution for 24-48 hrs at a density of 1×10^5 cells/ml cells/well (200 µL) in a 96-well tissue culture plate at a temperature of 37°C. In the wells that were previously washed with sterile PBS, the samples were treated with 5 different concentrations, in a serum-free RPMI medium. Each of the samples was replicated three times, and the cells were incubated in a 5% CO2 humidified incubator for 24 hrs at 37°C. Once after the incubation, MTT (10 µL of 5 mg/ml) was added to each sample in the well and the cells were again incubated for another 2 to 4 hrs till the purple precipitates were clearly visible under an inverted microscope. Then the medium along with MTT (220 µL) was removed from the wells and washed with 1X PBS (200 µl). Finally, DMSO (100 µL) was added to dissolve the formazan crystals that are formed in the cells, and the plate was shaken for 5 minutes. The absorbance for each well was measured using a microplate reader (Thermo Fisher Scientific, USA) at 570 nm. The percentage cell viability and IC50 values were calculated using Graph Pad Prism 6.0 (USA). (23,24)

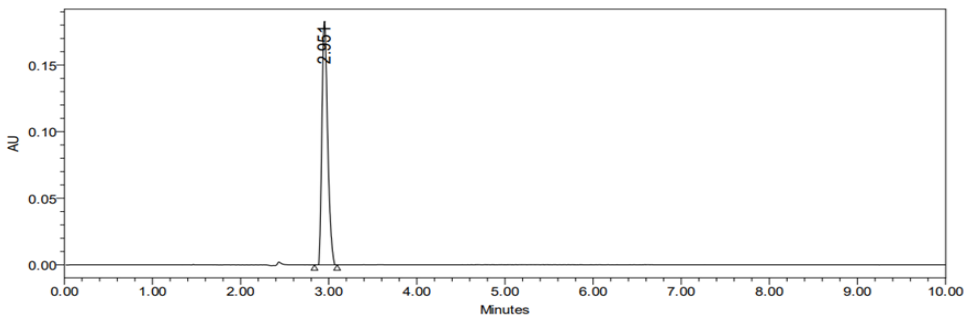
Formula: Cell viability % = Test OD/Control OD X 100

RESULTS

The results of chromatographic separation of Nirmatrelvir showed well resolved peaks and a consistent recovery time at 2.9 minutes in all three formulations.

Table 1: Summary results of Nirmatrelvir

NIRMATRELVIR (API)				**NIRMATRELVIR (Tablet)**			**NIRMATRELVIR Impurity (Caronic Anhydride)**		
S. No	**Sample ID/ Injection**	**RT**	**Area**	**Sample ID/ Injection**	**RT**	**Area**	**Sample ID/ Injection**	**RT**	**Area**
1	Nirmatrelvir API Inj- 01	2.9	825041	Nirmatrelvir Tablet Inj-01	2.9	228181	Imp Caronic Anhydride Inj-01	2.9	156124
2	Nirmatrelvir API Inj-02	2.9	920101	Nirmatrelvir Tablet Inj- 02	2.9	227897	Imp Caronic Anhydride -Inj 02	2.9	155115
3	Nirmatrelvir API Inj-03	2.9	924010	Nirmatrelvir Tablet Inj-03	2.9	228865	Imp Caronic Anhydride- Inj-03	2.9	156113
	Average	**3**	**889717.3**	**Average**	**3**	**228314.3**	**Average**	**2.9**	**155784**



	STD DEV	**0**	**56045.4**	**STD DEV**	**0**	**497.6**	**STD DEV**	**0**	**579.4**
	%RSD	**0**	**6.3**	**%RSD**	**0**	**0.2**	**%RSD**	**0.1**	**0.4**

Fig 3: HPLC Chromatogram of Nirmatrelvir API (100ug/ ml

Fig 4: HPLC Chromatogram of Nirmatrelvir Tablet (100ug/ ml)

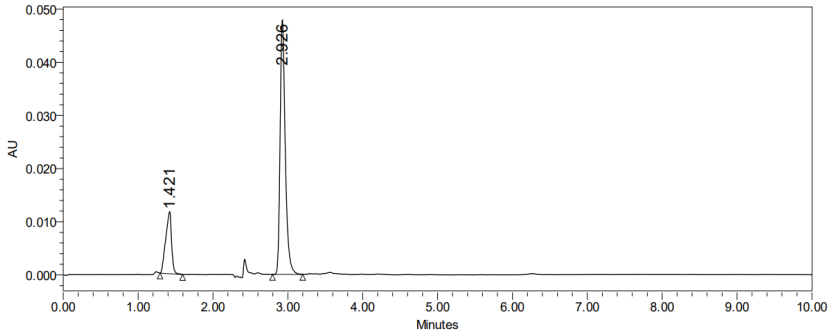
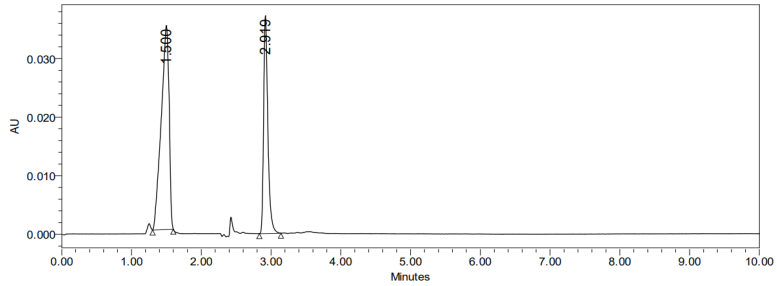


Fig 5: HPLC Chromatogram of Nirmatrelvir Impurity (Caronic Anhydride) (100ug/ ml)



Selectivity:

***Observation*:** In this method, % Interference of Analyte less than 20% for Nirmatrelvir was seen during retention times. It was, therefore, asserted that this approach was unique.

Table 2: Results of Selectivity – Nirmatrelvir

S No	**Sample ID**	**Analyte RT**	**Analyte Area**
1	BLK-01	1.3	254
2	BLK-02	1.3	652
3	BLK-03	1.3	325
4	BLK-04	1.3	254
5	BLK-05	1.3	554
6	BLK-06	1.3	412
7	LLOQ-01	3.31	20045
8	LLOQ-02	3.31	21548
9	LLOQ-03	3.31	22548
	Average		**21380.3**
	Standard Deviation		**1259.9**
	%RSD		**5.9**

Table 3: Results of Selectivity _ Interferences – Nirmatrelvir

Sample ID	**% Interference of Analyte- Nirmatrelvir**
BLK-01	1.19
BLK-02	3.05

BLK-03	1.52
BLK-04	1.19
BLK-05	2.59
BLK-06	1.93

Linearity:

***Observation*:** The correlation coefficient was found to be 0.9998, the slope was found to be 12102 and intercept was found to be 2894.6 for Nirmatrelvir.

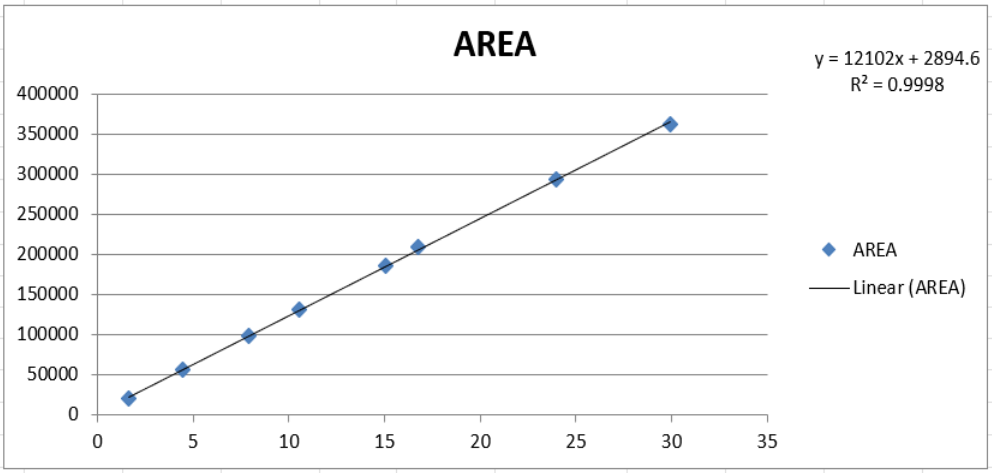


Fig 6: Calibration plots of Nirmatrelvir

Table 4: Results of Linearity – Nirmatrelvir

S.NO	SOLUTION	CONC	AREA
1	SS01	1.64	20769
2	SS02	4.44	56222
3	SS03	7.92	98829
4	SS04	10.56	131284
5	SS05	15.09	185982
6	SS06	16.77	208837
7	SS07	23.95	293903
8	SS08	29.94	362356
	Average		**169772.8**
	Standard Deviation		**117044.4**
	%RSD		**68.9**
	Correlation Coefficient		**0.9998**

Accuracy

***Observation*:** Nirmatrelvir showed mean recoveries were found to be 98.03%. The limit for mean % recovery is 92-99% which is within the limit. Hence it can be said that the proposed method was accurate.

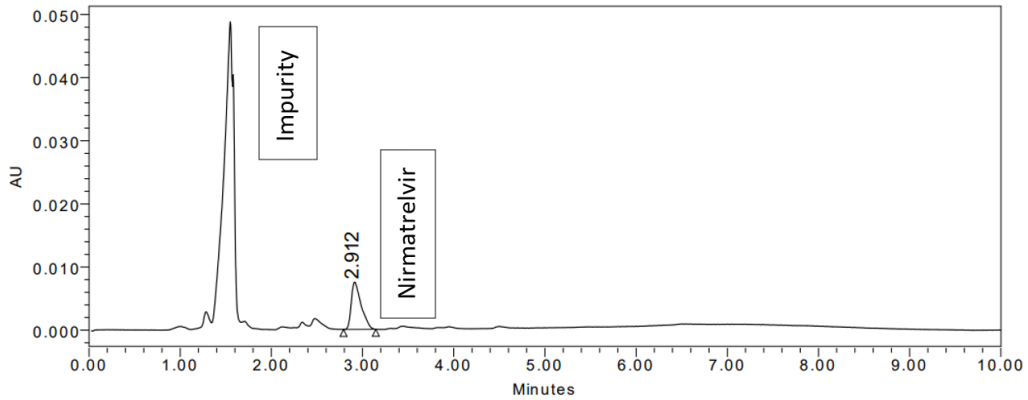


Fig 7: Chromatograms of Accuracy- Nirmatrelvir

Table 5: Accuracy Batch – Nirmatrelvir

S No	Sample	Concentration (ng/ml)	Analyte Area	Calculated Concentration	Accuracy
1	SS01	1.64	20769	1.48	90.2
2	SS02	4.44	56222	4.41	99.3
3	SS03	7.92	98829	7.93	100.1
4	SS04	10.56	131284	10.61	100.5
5	SS05	15.09	185982	15.13	100.3
6	SS06	16.77	208837	17.02	101.5
7	SS07	23.95	293903	24.04	100.4
8	SS08	29.94	362356	29.70	99.2

Table 6: Results of Accuracy

S No	Sample	Concentration (ng/ml)	Analyte Area	Calculated Concentration	Accuracy
1	LQC-01	1.83	24789	1.81	99.0
2	LQC-02	1.83	24650	1.80	98.4
3	LQC-03	1.83	24658	1.80	98.4
4	LQC-04	1.83	24752	1.81	98.8
5	LQC-05	1.83	24683	1.80	98.5
6	LQC-06	1.83	24726	1.81	98.7
7	MQC-01	16.61	195672	15.93	95.9
8	MQC-02	16.61	194526	15.83	95.3
9	MQC-03	16.61	195566	16.64	100.2
10	MQC-04	16.61	192682	15.68	94.4
11	MQC-05	16.61	197852	16.11	97.0
12	MQC-06	16.61	196256	15.98	96.2
13	HQC-01	31.94	385623	32.32	101.2
14	HQC-02	31.94	386253	31.67	99.2
15	HQC-03	31.94	384582	31.53	98.7

16	HQC-04	31.94	379852	31.59	98.9
17	HQC-05	31.94	379552	31.12	97.4
18	HQC-06	31.94	385625	31.62	99.0

Table 7: Results of Accuracy: Concentration Vs Analyte

S No	Sample	Nirmatrelvir % Recovery
1	LQC	98.6
2	MQC	96.5
3	HQC	99.0
Mean		98.03

Precision:

***Observation*:** Since the obtained mean RSD (%) is within the accepted limit (≤2%), there was no significant difference found. So, this method was found to be precise at the selected wavelength.

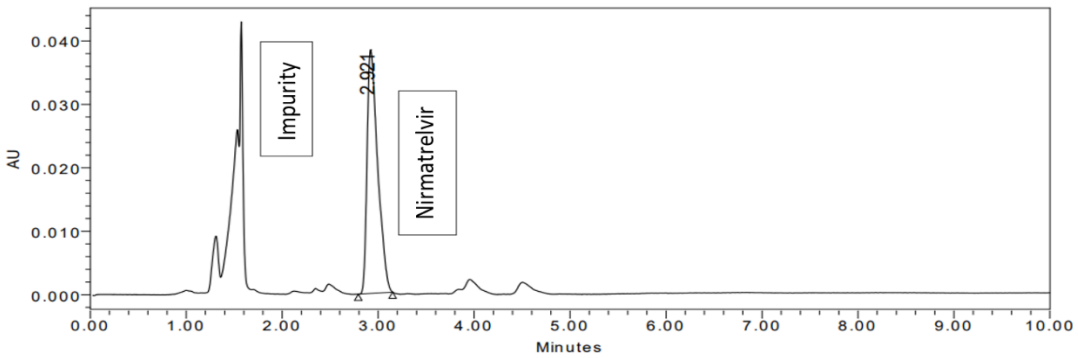


Fig 8: Chromatograms of Precision- Nirmatrelvir

Table 8: Results of Precision- Nirmatrelvir

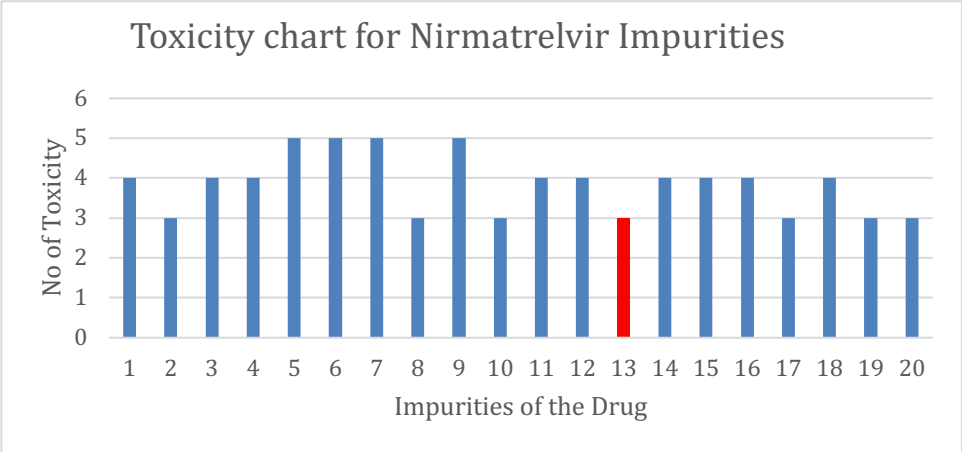
Injection	Area or Nirmatrelvir
Injection-1	385623
Injection-2	386253
Injection-3	384582
Injection-4	379852
Injection-5	379552
Injection-6	385625
Average	383581.2
Standard Deviation	3053.8
%RSD	0.80

Impurity Profiling

In-silico Toxicity The Protox 3.0 results for LD50

Table 9: Impurity in In-silico Toxicity

Impurity	LD50 (mg/kg)	Predicted Organ Toxicity	Regulatory Concern
Caronic Anhydride	500	Respiratory Toxicity, Neurotoxicity, Echo toxicity	ICH Q3A/B



Caronic Anhydride- Nirmatrelvir Impurities

Fig 9: Toxicology Chart for NTR Impurity: Caronic Anhydride

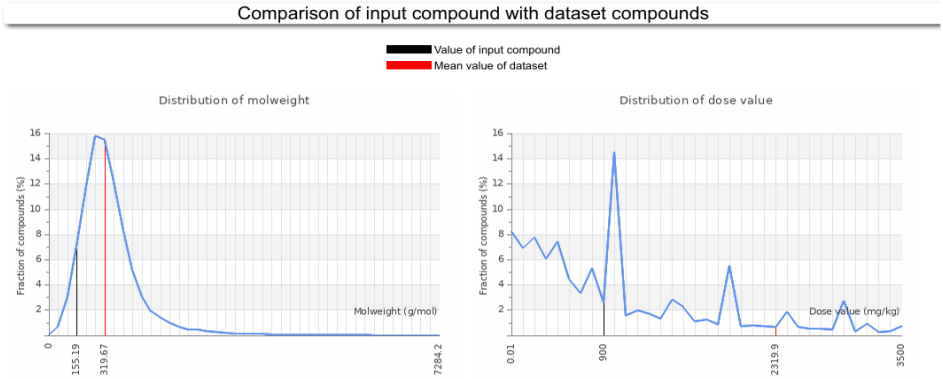


Fig 10: NTR Impurity- Caronic Anhydride

MTT Assay

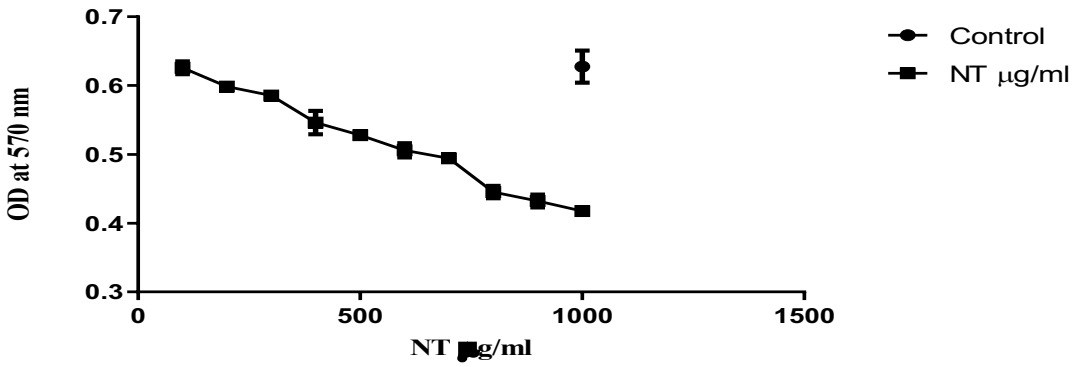


Fig11: Graph of sample Nirmatrelvir (NT)

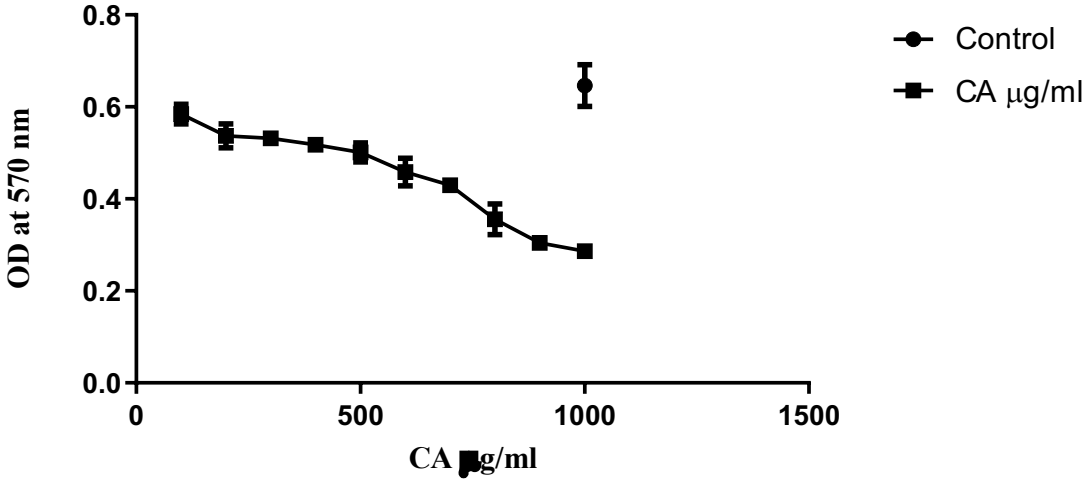


Fig 12: Graph of sample Caronic Anhydride (CA)

Table 10: Cell Viability of same sample Nirmatrelvir (NT)

S. No.	Tested sample concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean value (%)
1	Control	100	100	100	100
2	1000 µg/ml	68.9256	67.84	63.1902	66.651935
3	900 µg/ml	71.7355	67.52	67.638	68.964525
4	800 µg/ml	72.2314	72.8	68.0982	71.043188
5	700 µg/ml	81.8182	78.24	76.5337	78.863975
6	600 µg/ml	81.8182	81.12	79.1411	80.693095
7	500 µg/ml	87.1074	83.68	81.9018	84.22976
8	400 µg/ml	89.0909	85.44	86.8098	87.113575
9	300 µg/ml	96.3636	93.76	90.184	93.435895
10	200 µg/ml	97.686	96.32	92.4847	95.496871
11	100 µg/ml	101.818	100.32	97.3926	99.843607

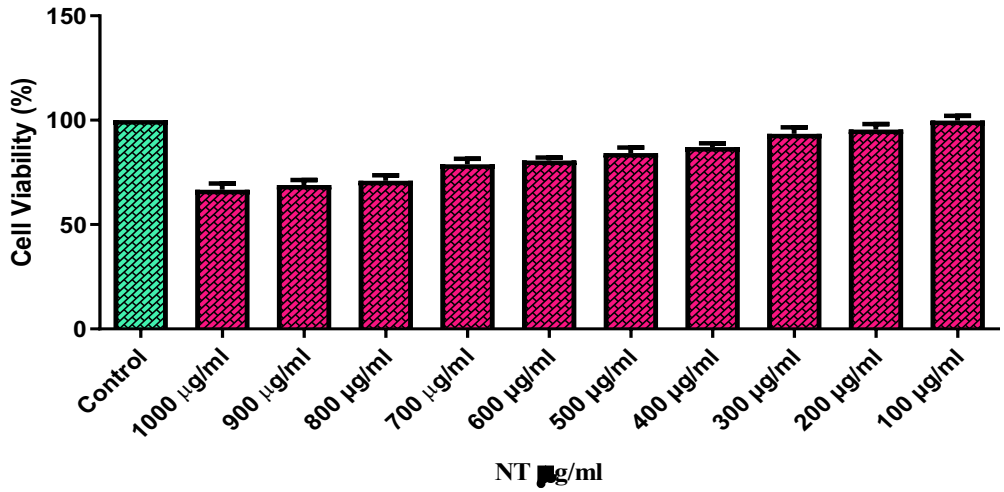


Fig 13: Results of Cell Viability for Nirmatrelvir

Table 11: Cell Viability of same sample Caronic Anhydride (CA)

S. No.	Tested sample concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
1	Control	100	100	100	100
2	1000 µg/ml	47.308	41.404	44.745	44.486
3	900 µg/ml	48.613	44.556	48.567	47.245
4	800 µg/ml	52.529	55.731	56.688	54.982
5	700 µg/ml	70.147	62.178	67.675	66.667
6	600 µg/ml	74.878	61.318	77.707	71.301
7	500 µg/ml	80.750	69.198	83.439	77.796
8	400 µg/ml	84.339	74.355	82.325	80.340
9	300 µg/ml	86.460	76.074	85.191	82.575
10	200 µg/ml	82.708	78.653	88.535	83.299
11	100 µg/ml	91.517	84.384	96.178	90.693

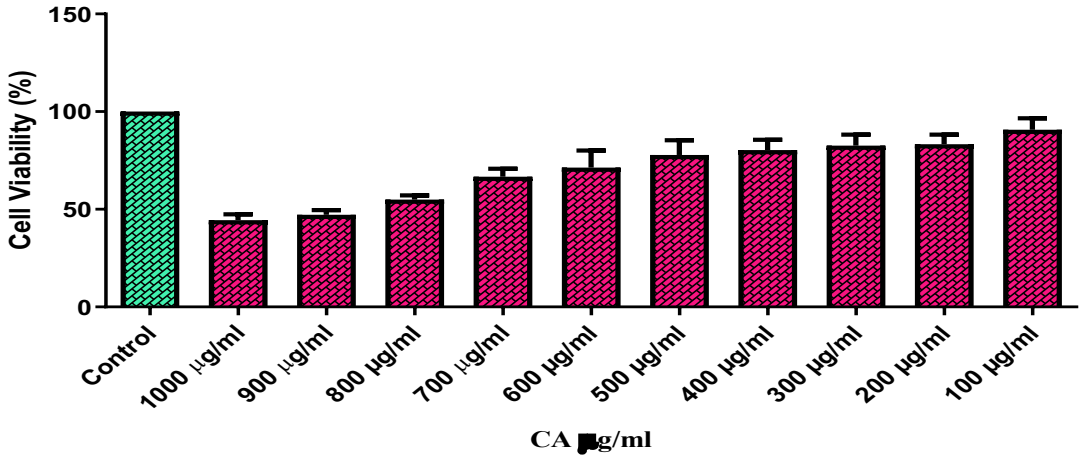


Fig 14: Results of Cell Viability for Caronic Anhydride

Table 12: OD Values of sample at 570 nm (NT and CA)

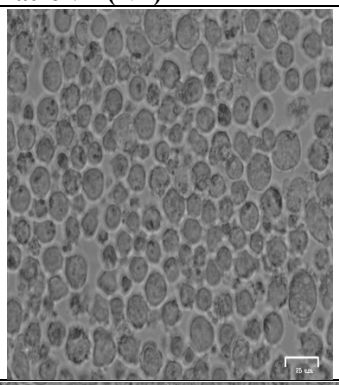
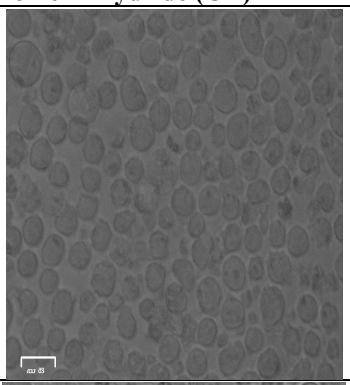
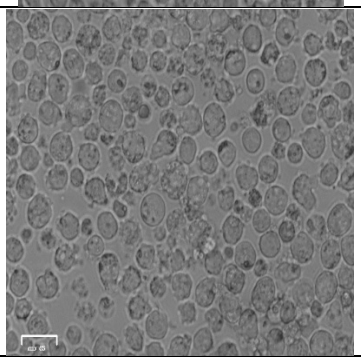
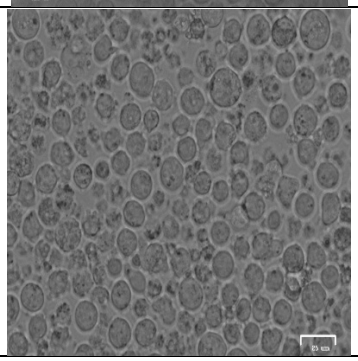
S. No.	Tested sample concentration (µg/ml)	OD value at 570 nm (in triplicates) NT			OD value at 570 nm (in triplicates) CA		
1	Control	0.605	0.625	0.652	0.613	0.698	0.628
2	1000 µg/ml	0.417	0.424	0.412	0.29	0.289	0.281
3	900 µg/ml	0.434	0.422	0.441	0.298	0.311	0.305
4	800 µg/ml	0.437	0.455	0.444	0.322	0.389	0.356
5	700 µg/ml	0.495	0.489	0.499	0.43	0.434	0.425
6	600 µg/ml	0.495	0.507	0.516	0.459	0.428	0.488
7	500 µg/ml	0.527	0.523	0.534	0.495	0.483	0.524

8	400 µg/ml	0.539	0.534	0.566	0.517	0.519	0.517
9	300 µg/ml	0.583	0.586	0.588	0.53	0.531	0.535
10	200 µg/ml	0.591	0.602	0.603	0.507	0.549	0.556
11	100 µg/ml	0.616	0.627	0.635	0.561	0.589	0.604

Table 13: IC50 Value of tested sample (NT and CA)

Log(inhibitor) vs Normalized Response	Nirmatrelvir (NT)	CA (Caronic Anhydride)
Best-fit values		
LogIC50	2.629	2.785
IC50 value of test sample (µg/m)	425.9	610.2
95% CI (profile likelihood)		
LogIC50	2.504 to 2.753	2.752 to 2.815
IC50	318.9 to 566.1	564.3 to 653.3
Degrees of Freedom	29	28
R squared	0.6844	0.8974
Sum of Squares	10381	3442
Sy.x	18.92	11.09
# of X values	30	30
# Y values Analyzed	30	30

In Vitro Cytotoxicity Assay

Concentration (µg/ml)	Nirmatrelvir (NT)	Caronic Anhydride (CA)
Control		
100		

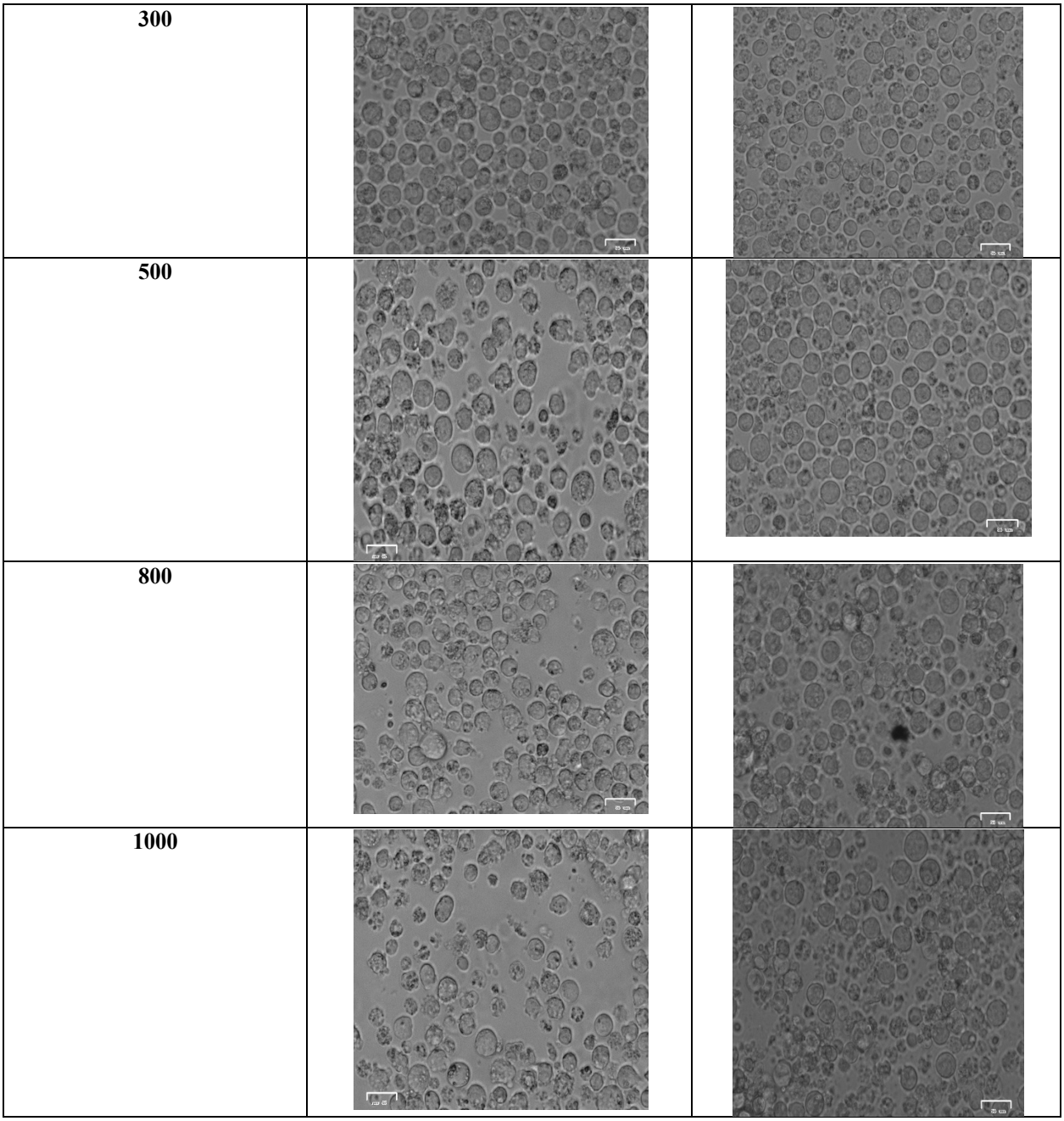


Fig 15: Images of control cells and cells treated with Nirmatrelvir (NT) and Caronic Anhydride (CA) at different concentrations in the in-vitro cytotoxicity assay.

DISCUSSION

The developed HPLC method showed strong selectivity, as evidenced by the minimal interfering peaks at the retention time of Nirmatrelvir and its impurity. The calibration curve showed excellent linearity across the tested range - 1.64–29.94 µg/mL, yielding a correlation coefficient of 0.9998, that proves a high degree of proportionality between concentration and detector response. Accuracy results further supported the reliability of the study, which was within the acceptable limits stated by the ICH guidelines, with mean percentage recovery recorded at 98.03%.

Impurity profiling recognised Caronic Anhydride as the main primary impurity requiring regulatory and safety concerns. As a result of its reactive cyclic anhydride structure, there is a possible risk of mucosal irritation and hypersensitivity reactions for the identified impurity. Eventually, Caronic Anhydride caters under the control of ICH Q3A/B thresholds, reinforcing the need for a robust analytical system capable of detecting and quantifying it with precision.

The In-silico toxicity predictions of this study, classified Caronic Anhydride as, possessing moderate acute oral toxicity, with an estimated LD_{50} of ≃500 mg/kg. The computer-based model has also showed potential

hepatotoxicity and immunotoxicity. These findings in the study highlights the importance of drug safety that should be maintained throughout the shelf life of the drug, by following the LD_{50} limits within the regulatory guidelines.

Cytotoxicity is often interpreted based on IC_{50} criteria, where IC_{50} values ≥100 µg/mL denote that its non-toxic; between 10–60 µg/mL means moderate toxicity; and if the value is ≤10 µg/mL that indicates high toxicity. The MTT assay results from our study showed that Nirmatrelvir exhibits low cytotoxicity, falling in line with its expected pharmacological safety profile. In contrary, Caronic Anhydride showed a noticeably high IC_{50} value, that indicats lower cytotoxic potential in comparison with the active pharmaceutical ingredient. Although the cytotoxic nature of the product is relatively less in in-vitro studies, the impurity's reactive chemical nature and in-silico toxicity reports warns careful monitoring procedures that has to be done during formulation and stability studies.

Collectively, the chromatographic performance, impurity assessment, and toxicity findings confirms that the developed method is very reliable and suitable for routine quality control that provides a comprehensive approach in evaluating both the analytical and biological safety aspects of Nirmatrelvir and its related impurities.

CONCLUSION

The HPLC method developed in this study for the simultaneous estimation of Nirmatrelvir and its associated impurity, Caronic Anhydride, was proved to be reliable, rapid, and also cost-effective. Its simplicity, robustness and optimized conditions make it ideal for routine analysis in pharmaceutical quality control laboratories, enabling decreased retention times and overall run times, supporting high-throughput analysis for both bulk materials and marketed formulations.

The study also highlights the regulatory and toxicological significance of Caronic Anhydride with its identification as a reactive process-related impurity, combined with its outcomes of in-silico toxicity predictions, reinforces the need for vigilant monitoring during manufacturing and stability testing. These findings contribute to a comprehensive impurity control strategy in par with current ICH expectations for COVID-19 antiviral agents.

Most importantly, cytotoxicity evaluation provided valuable insight into the biological safety of the final formulation which when present within specified limits, the impurity is unlikely to cause significant cytotoxic effects, thereby supporting the overall safety profile of the product.

In summary, the combined analytical, toxicological, and cytotoxicity assessments presented in this work gives a solid foundation to ensure the quality, efficacy, and safety of Nirmatrelvir containing pharmaceutical products.

AUTHORS CONTRIBUTION:

The Authors Confirm their Contribution to the paper as follows: Study Conception and design were contributed by SJ; data collection and contributed by BR; analysis and interpretation of results were contributed by KN, TP and JN: Manuscript drafted by EM, SD. All the authors reviewed the results and approved the final version of the manuscript.

CONSENT FOR PUBLICATIONS

Not Applicable

CONFLICT OF INTEREST

The Author declares no conflict of interest, financial or otherwise

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LIST OF ABBREVIATIONS:

HPLC- High Performance light Chromatography
NTR- Nirmatrelvir
C18- Octadecyl carbon Chain
%RSD- Relative Standard Deviation
COVID-19- Coronavirus Disease 2019
ICH- International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
SARS-CoV-2- Severe acute respiratory syndrome coronavirus 2
3 CL Protease- 3-chymotrypsin-like protease
GC- Gas Chromatography
PDA- Photodiode Array detector
API- Active Pharmaceutical Ingredient
HQC-High Quality Control
MQC- Medium Quality Control
LQC- Low Quality Control
RT- Retention Time
LLOQ- Lowest Limit of Quantification
LD- Lethal Dose
CA- Caronic Anhydride
OECD- Organization for Economic Co-operation and Development.
MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO- Dimethyl sulfoxide
PBMC- Peripheral Blood Mononuclear Cells
RPMI- Phenol red-free media
OD- Optical Density

IC50- Half-maximal inhibitory concentration