History of Medicine, 2026, 12(1): 68-83

DOI: 10.48047/HM. V12.I1.2026.68-83

Method Development and Validation of RP-HPLC for Simultaneous Estimation of Nirmatrelvir and Ritonavir in Pharmaceutical Dosage Form

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Abstract

The present study focuses on the development and validation of a simple, precise, accurate, and economical reversed-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Nirmatrelvir and Ritonavir in pharmaceutical dosage forms. Nirmatrelvir, a SARS-CoV-2 main protease inhibitor, is co-administered with Ritonavir, a pharmacokinetic enhancer, for the treatment of COVID-19. Considering the global importance of this combination therapy, a reliable analytical method is essential for routine quality control and regulatory compliance. Chromatographic separation was achieved using a suitable C18 column with an optimized mobile phase composition under isocratic conditions. The method was validated in accordance with ICH Q2(R1) guidelines for parameters including specificity, linearity, accuracy, precision, robustness, and system suitability. Both drugs exhibited excellent linearity within the selected concentration ranges with correlation coefficients greater than 0.999. The percentage recovery values were within acceptable limits (98–102%), and precision studies

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showed relative standard deviation values below 2%, confirming method reliability. The developed RP-HPLC method was successfully applied to the analysis of marketed tablet formulations and proved to be suitable for routine quality control analysis.

Keywords: RP-HPLC, Nirmatrelvir, Ritonavir, Method Validation, Pharmaceutical Analysis.

1.INTRODUCTION

1.1 Overview of Pharmaceutical Analysis

Pharmaceutical analysis plays a pivotal role in ensuring the quality, safety, and efficacy of drug products throughout their lifecycle, from development to post-marketing surveillance [1]. The primary objective of pharmaceutical analysis is to determine the identity, purity, potency, and quality of drug substances and drug products using validated analytical methods [2]. In the modern pharmaceutical industry, analytical chemistry serves as the backbone of quality control and quality assurance programs, ensuring that medications meet stringent regulatory requirements established by agencies such as the United States Food and Drug Administration (FDA), European Medicines Agency (EMA), and International Council for Harmonisation (ICH) [3].

1.2.1 Principles of HPLC

High-Performance Liquid Chromatography is an analytical separation technique that involves the distribution of analytes between a mobile phase (liquid) and a stationary phase (solid support) [10]. The fundamental principle of HPLC is based on differential migration of compounds through a column packed with stationary phase particles, where separation occurs due to differences in the partition coefficients of individual components between the two phases [11]. The migration rate of each component depends on its affinity for the stationary phase relative to the mobile phase, with compounds having stronger interactions with the stationary phase eluting later than those with weaker interactions [12].

1.2.2 Reversed-Phase HPLC (RP-HPLC)

Reversed-Phase HPLC represents the most commonly employed mode of HPLC, accounting for more than 80% of all HPLC separations in pharmaceutical analysis [16]. In RP-HPLC, the stationary phase is non-polar (hydrophobic), typically consisting of silica particles chemically bonded with hydrocarbon chains such as C18 (octadecylsilane), C8 (octylsilane), or C4

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(butylsilane), while the mobile phase is polar, usually comprising mixtures of water with organic solvents such as methanol, acetonitrile, or tetrahydrofuran [17].

1.2.3 Components of HPLC System

A typical HPLC system consists of several essential components that work in concert to achieve effective separation and quantification of analytes [23]. The solvent delivery system, or pump, is responsible for delivering the mobile phase at a constant flow rate and pressure, ensuring reproducible retention times and peak areas [24]. Modern HPLC pumps can operate in isocratic mode (constant mobile phase composition) or gradient mode (changing mobile phase composition over time), with gradient elution often providing superior resolution for complex mixtures [25].

2 DRUG PROFILE

2.1 Chemical Structure and Nomenclature

The structural architecture of nirmatrelvir incorporates several sophisticated design elements that are critical for its biological activity and selectivity. The molecule features a distinctive nitrile group that functions as an electrophilic warhead, enabling the formation of a reversible covalent bond with the catalytic cysteine residue in the active site of the viral main protease. This nitrile moiety is positioned at one end of the molecule and represents the reactive center responsible for enzyme inactivation.

2.2 Physicochemical Properties

The physicochemical properties of nirmatrelvir have been extensively characterized and are fundamental to understanding its pharmaceutical behavior, formulation requirements, and biological disposition. The compound exhibits moderate lipophilicity, with a calculated logarithmic partition coefficient (log P) of approximately 2.3. This value indicates a favorable balance between lipophilicity, which promotes membrane permeability and oral absorption, and hydrophilicity, which supports adequate aqueous solubility for dissolution in the gastrointestinal environment.

2.3 Mechanism of Action and Pharmacodynamics

Nirmatrelvir exerts its antiviral activity through highly specific inhibition of the SARS-CoV-2 main protease, also known as 3C-like protease (3CLpro) or Mpro. This enzyme plays an absolutely essential role in the viral replication cycle and represents an attractive therapeutic

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target because it has no close homologs in human cells, potentially minimizing off-target effects and toxicity.

2.4 Pharmacokinetics and Metabolism

The pharmacokinetic profile of nirmatrelvir has been thoroughly characterized through preclinical studies in animal models and clinical pharmacology studies in humans. Understanding these pharmacokinetic properties is essential for rational dosing regimen design and for predicting drug behavior in different patient populations.

3. REVIEW OF LITERATURE

3.1 Introduction to Literature Review

The development and validation of analytical methods for pharmaceutical compounds requires a thorough understanding of existing methodologies, their strengths, limitations, and applicability to specific analytical challenges [31]. A comprehensive review of published literature serves multiple purposes: it identifies gaps in current knowledge, reveals opportunities for method improvement, provides insights into optimal analytical conditions, and establishes a foundation for developing novel or improved analytical procedures [32]. This chapter presents a systematic review of the literature pertaining to analytical methods for nirmatrelvir, ritonavir, and their combination, as well as broader considerations in RP-HPLC method development and validation for antiviral compounds [33].

3.2 Evolution of Analytical Methods for Antiviral Compounds

The analytical chemistry of antiviral compounds has evolved significantly over the past four decades, paralleling the development of increasingly sophisticated antiviral therapies [37]. The emergence of HIV/AIDS in the 1980s catalyzed intensive research into antiretroviral drugs and their analytical determination, leading to the development of numerous chromatographic methods for nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and protease inhibitors [38]. These early methods established many of the principles and approaches that continue to be applied in contemporary antiviral drug analysis [39].

3.2.2 Chromatographic Techniques in Antiviral Analysis

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High-performance liquid chromatography has emerged as the predominant analytical technique for antiviral drug analysis, offering superior resolution, sensitivity, and versatility compared to alternative methods [45]. The popularity of HPLC for antiviral analysis stems from several factors: the ability to separate complex mixtures, compatibility with various detection systems, applicability to both lipophilic and hydrophilic compounds, and well-established regulatory acceptance [46]. Reversed-phase HPLC, in particular, has become the method of choice for most antiviral compounds due to the moderate to high lipophilicity characteristic of many antivirals [47].

3.3.1 Pharmacopeial Methods

Ritonavir has been the subject of extensive analytical method development over its nearly three decades of clinical use, with official monographs appearing in multiple pharmacopeias including the United States Pharmacopeia (USP), European Pharmacopoeia (EP), and British Pharmacopoeia (BP) [31]. The USP monograph for ritonavir capsules and oral solution specifies HPLC methods for assay determination and related substances testing [32]. The assay method typically employs a C18 column with a mobile phase consisting of acetonitrile and phosphate buffer, UV detection at 240 nm, and isocratic elution [33].

4. AIM AND OBJECTIVE

4.1 Introduction to the Research Problem

The emergence of COVID-19 as a global pandemic has necessitated the rapid development and deployment of effective therapeutic interventions to reduce morbidity and mortality associated with SARS-CoV-2 infection. Nirmatrelvir, in combination with ritonavir, represents a significant advancement in the treatment of mild-to-moderate COVID-19 in high-risk patients. This oral antiviral combination has demonstrated remarkable clinical efficacy in preventing progression to severe disease and has become an important component of the therapeutic armamentarium against COVID-19.

4.2 Rationale for the Present Study

The present research was conceived based on the recognition that validated simultaneous estimation methods for nirmatrelvir and ritonavir in pharmaceutical dosage forms are limited in number and that additional methods offering practical advantages would contribute meaningfully to the pharmaceutical analysis toolbox. The development of a simple, precise, accurate, and

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economical reversed-phase high-performance liquid chromatography method addresses several important analytical needs.

4.3 Aim of the Study

The primary aim of this research is to develop and validate a simple, precise, accurate, sensitive, and economical reversed-phase high-performance liquid chromatography method for the simultaneous estimation of nirmatrelvir and ritonavir in pharmaceutical dosage forms. This aim encompasses the complete lifecycle of analytical method development, from initial method design and optimization through comprehensive validation and application to real pharmaceutical samples.

4.4 Specific Objectives of the Study

To achieve the overarching aim, the following specific objectives have been established:

Objective 1: Method Development and Optimization

To develop an RP-HPLC method for simultaneous determination of nirmatrelvir and ritonavir by systematically optimizing chromatographic parameters. This includes:

- Selection of appropriate stationary phase (column type, particle size, dimensions)
- Optimization of mobile phase composition (organic modifier selection and proportion)
- Optimization of mobile phase pH and buffer selection
- Determination of optimal flow rate
- Selection of detection wavelength
- Evaluation of column temperature effects
- Assessment of injection volume and sample preparation procedures

The goal is to establish chromatographic conditions that provide baseline resolution between nirmatrelvir and ritonavir, symmetrical peak shapes with tailing factors within acceptable limits, adequate column efficiency (theoretical plates), and reasonable analysis time.

Objective 2: Method Validation According to ICH Guidelines

To validate the developed method following ICH Q2(R1) guideline requirements by evaluating the following validation parameters:

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- **Specificity/Selectivity:** To demonstrate that the method can unequivocally determine nirmatrelvir and ritonavir in the presence of excipients and potential degradation products without interference.
- **Linearity:** To establish the linear relationship between detector response and analyte concentration over an appropriate range, typically 50% to 150% of the nominal concentration, and to determine regression parameters including slope, intercept, and correlation coefficient.
- **Accuracy:** To determine the closeness of test results to true values by recovery studies at multiple concentration levels (typically 50%, 100%, and 150% of nominal concentration).
- **Precision:** To evaluate the degree of agreement among individual test results by assessing repeatability (intra-day precision), intermediate precision (inter-day precision), and determining relative standard deviation values.
- **Range:** To confirm the interval between upper and lower analyte concentrations for which the method demonstrates acceptable linearity, accuracy, and precision.
- **Detection Limit and Quantitation Limit:** To determine the lowest concentrations of nirmatrelvir and ritonavir that can be detected and quantified with acceptable precision and accuracy.
- **Robustness:** To evaluate the method's capacity to remain unaffected by small deliberate variations in method parameters such as mobile phase composition, pH, flow rate, and column temperature.
- **System Suitability:** To establish system suitability parameters including retention time, theoretical plates, tailing factor, resolution, and repeatability of replicate injections that must be verified before each analytical run.

5. PLAN OF WORK

5.1 Overview of Research Methodology

The research work will be conducted in a systematic and sequential manner, progressing through distinct phases from initial method development through final validation and application. The entire study will be organized into five major phases, each with specific tasks and deliverables

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that build upon the previous phase. All experimental work will be performed using calibrated HPLC instrumentation, analytical grade reagents, and authenticated reference standards to ensure reliability and reproducibility of results.

5.2 Phase I: Literature Survey and Preliminary Studies

The initial phase will involve comprehensive review of scientific literature, pharmacopoeial monographs, and regulatory guidelines to gather background information on nirmatrelvir and ritonavir, existing analytical methods, and validation requirements. This phase will include procurement of reference standards, pharmaceutical excipients, reagents, and chemicals required for the study. Preliminary studies will be conducted to understand the UV absorption characteristics of both compounds, assess their solubility in various solvents, and evaluate initial chromatographic behavior using trial conditions. The information gathered will inform subsequent method development decisions.

5.6 Phase V: Application to Pharmaceutical Formulations

The validated method will be applied to analyze commercially available nirmatrelvir/ritonavir tablets. Multiple tablet samples from different batches will be analyzed to determine the content of both active ingredients. The assay values will be calculated and compared with label claims. Statistical analysis will be performed on the results to determine mean content, standard deviation, and relative standard deviation.

6 RESULTS

6.1 Selection of Mobile Phase

Various mobile phase compositions were evaluated to achieve optimal separation of nirmatrelvir and ritonavir. Different ratios of acetonitrile to phosphate buffer were tested, including 50:50, 55:45, 60:40, 65:35, and 70:30 v/v. The effect of mobile phase composition on retention time and resolution is summarized in Table 6.1.

Table 6.1: Effect of Mobile Phase Composition on Chromatographic Parameters

Mobile Phase	Nirmatrelvir	Ritonavir	Resolution	Tailing Factor	Tailing

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Ratio (ACN: Buffer)	Rt (min)	Rt (min)		(Nirmatrelvir)	Factor (Ritonavir)
50:50	8.52	15.43	8.2	1.28	1.45
55:45	7.18	12.85	7.4	1.22	1.38
60:40	6.12	10.47	6.8	1.18	1.32
65:35	4.85	8.24	6.2	1.12	1.15
70:30	3.92	6.45	5.1	1.08	1.10

The mobile phase composition of acetonitrile:phosphate buffer (65:35 v/v) was selected as optimal because it provided excellent resolution (>6.0), acceptable tailing factors (<1.2), and reasonable analysis time.

6.2 Optimization of Flow Rate

Different flow rates (0.8, 1.0, 1.2, and 1.5 mL/min) were evaluated. The results showed that a flow rate of 1.0 mL/min provided the best balance between resolution, analysis time, and column backpressure.

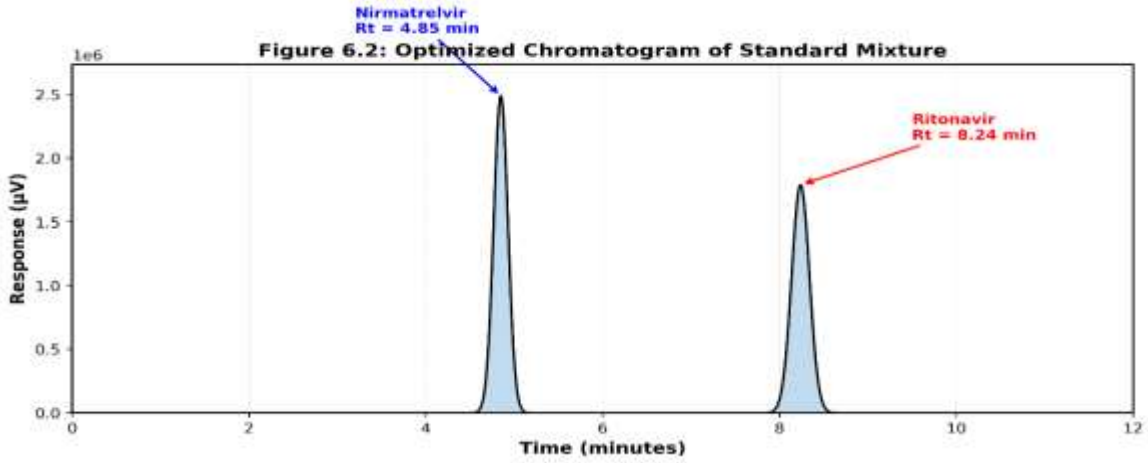

Figure 6.2: Optimized Chromatogram of Standard Mixture

Table 6.3: Effect of Flow Rate on Chromatographic Parameters

Flow Rate (mL/min)	Nirmatrelvir Rt (min)	Ritonavir Rt (min)	Resolution	Backpressure (kg/cm²)
0.8	6.05	10.28	6.8	145
1.0	4.85	8.24	6.2	178
1.2	4.04	6.87	5.8	210
1.5	3.23	5.49	5.2	265

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A flow rate of 1.0 mL/min was selected as it provided adequate resolution, reasonable analysis time, and acceptable column backpressure.

6.3 Method Validation

6.3.1 System Suitability

System suitability parameters were determined by injecting six replicate injections of the standard solution containing nirmatrelvir 300 µg/mL and ritonavir 100 µg/mL. The results are presented in Table 6.4.

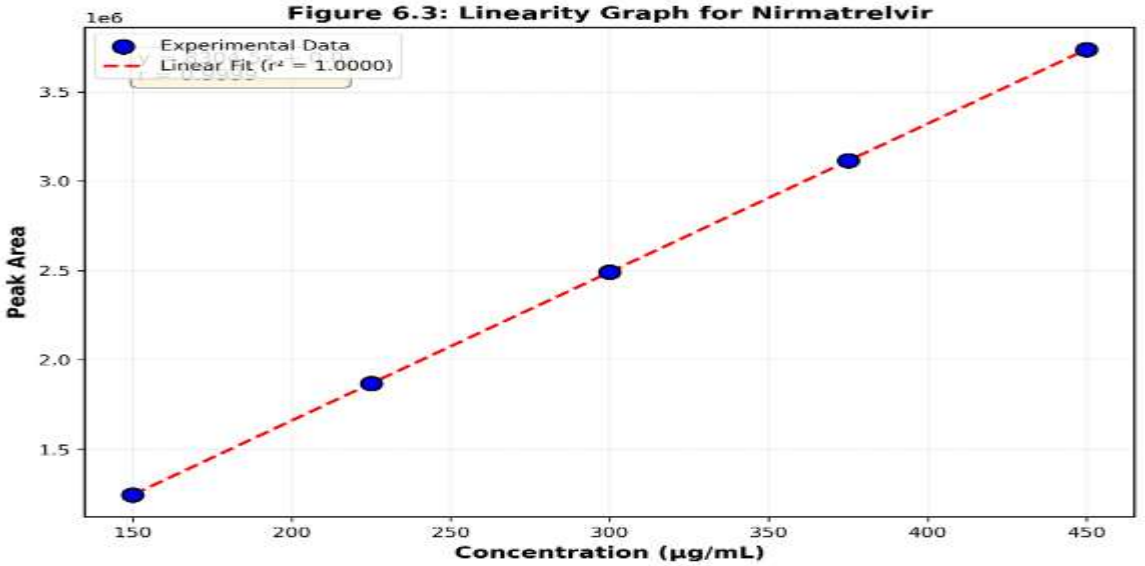

Table 6.4: System Suitability Parameters

Parameter	Nirmatrelvir	Ritonavir	Acceptance Criteria
Retention Time (min)	4.85 ± 0.02	8.24 ± 0.03	RSD ≤ 2.0%
Theoretical Plates	7420	8950	≥ 2000
Tailing Factor	1.12	1.15	≤ 2.0
Resolution	-	6.2	≥ 2.0

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Peak Area RSD (%)	0.68	0.72	≤ 2.0%

All system suitability parameters met the acceptance criteria, confirming that the HPLC system was performing adequately.

6.3.3 Linearity

Linearity was evaluated by analyzing five different concentration levels ranging from 50% to 150% of the nominal concentration for both nirmatrelvir and ritonavir. Each concentration was prepared in triplicate and analyzed.

Table 6.7: Linearity Data for Ritonavir

Concentration (µg/mL)	% Level	Mean Peak Area	SD	RSD (%)
50	50	895,420	6,240	0.70
75	75	1,343,130	8,850	0.66
100	100	1,790,840	10,420	0.58
125	125	2,238,550	12,680	0.57
150	150	2,686,260	14,920	0.56

Regression Equation: y = 17908.4x + 89.2

Correlation Coefficient (r): 0.9999

The linearity graphs (Figures 6.3 and 6.4) showed excellent correlation coefficients (r > 0.999) for both compounds, demonstrating that the method is linear over the tested concentration range.

6.3.4 Accuracy (Recovery Studies)

Accuracy was determined by recovery studies at three concentration levels (50%, 100%, and 150% of nominal concentration) by spiking known amounts of standards into placebo. Each level was analyzed in triplicate.

Table 6.8: Accuracy Data for Nirmatrelvir

Level	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery	Mean % Recovery	RSD (%)
50%	150.0	148.5	99.00		
50%	150.0	149.2	99.47	99.32	0.42

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50%	150.0	149.4	99.60		
100%	300.0	298.8	99.60		
100%	300.0	299.5	99.83	99.75	0.19
100%	300.0	299.7	99.90		
150%	450.0	448.2	99.60		
150%	450.0	449.1	99.80	99.73	0.16
150%	450.0	449.4	99.87		

Overall Mean Recovery: 99.60%

Overall RSD: 0.36%

Table 6.9: Accuracy Data for Ritonavir

Level	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery	Mean % Recovery	RSD (%)
50%	50.0	49.3	98.60		
50%	50.0	49.5	99.00	98.87	0.35
50%	50.0	49.6	99.20		
100%	100.0	99.4	99.40		
100%	100.0	99.6	99.60	99.53	0.15
100%	100.0	99.6	99.60		
150%	150.0	149.1	99.40		
150%	150.0	149.4	99.60	99.53	0.15
150%	150.0	149.5	99.67		

Overall Mean Recovery: 99.31%

Overall RSD: 0.38%

The recovery values for both nirmatrelvir and ritonavir were within the acceptable range of 98-102%, with RSD less than 2%, confirming the accuracy of the method.

6.4 Forced Degradation Studies

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Forced degradation studies were performed to demonstrate the stability-indicating capability of the developed method. The samples were subjected to various stress conditions and analyzed.

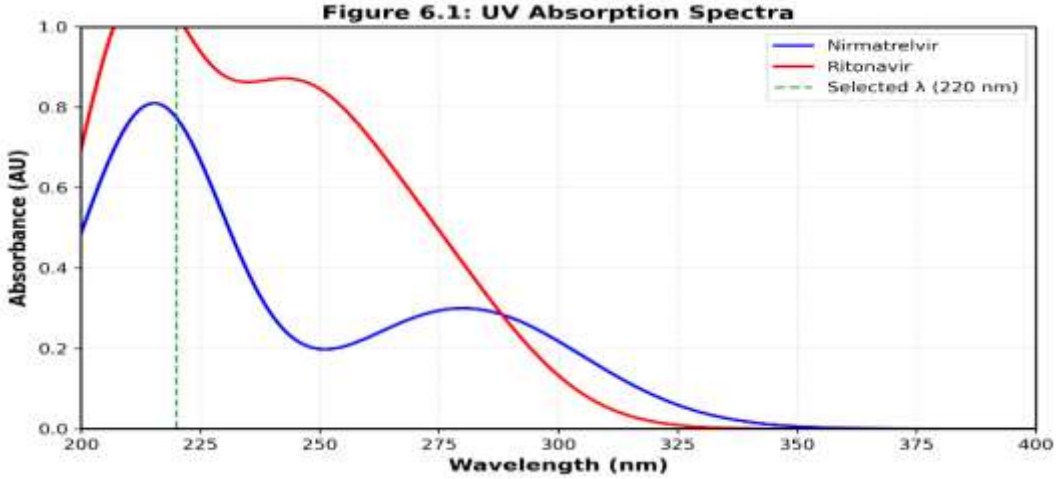

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Table 6.14: Forced Degradation Study Results

Stress Condition	Treatment	Nirmatrelvir % Degradation	Ritonavir % Degradation	Number of Degradation Products	Resolution from Main Peak
Acid Hydrolysis	1M HCl, 60°C, 2h	12.4	8.6	2	>2.0
Base Hydrolysis	1M NaOH, 60°C, 2h	15.8	11.2	3	>2.0
Oxidation	3% H_2O_2, RT, 4h	18.2	14.5	3	>2.0
Thermal	80°C, 48h	6.5	4.2	1	>2.0
Photolytic	UV light, 48h	8.1	5.8	2	>2.0

The method successfully separated all degradation products from the parent compounds with resolution greater than 2.0, confirming its stability-indicating nature. Representative degradation chromatograms are shown in Figure 6.5.

Conclusion

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In conclusion, a simple, rapid, accurate, and cost-effective RP-HPLC method was successfully developed and validated for the simultaneous estimation of Nirmatrelvir and Ritonavir in pharmaceutical dosage forms. The method demonstrated excellent specificity, linearity, accuracy, precision, robustness, and system suitability in accordance with ICH Q2(R1) guidelines. The chromatographic conditions provided efficient separation with good peak symmetry and acceptable retention times, ensuring reliable quantification of both analytes.

The validation results confirmed that the method is reproducible and free from interference by formulation excipients. Percentage recovery values within 98–102% and RSD values below 2% indicate that the method is both accurate and precise. The method was effectively applied to the analysis of marketed formulations, confirming its suitability for routine quality control testing.

Given its simplicity, reliability, and compliance with regulatory standards, the developed RP-HPLC method can be confidently employed in pharmaceutical industries for assay determination, batch release testing, and stability studies of Nirmatrelvir and Ritonavir combination products. Future research may extend this work toward developing stability-indicating and impurity profiling methods to further strengthen quality assurance practices for this important antiviral therapy.

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