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Original Research Article

Method development and validation of ritonavir in bulk and pharmaceutical dosage form by using RP-HPLC

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Abstract

Ritonavir, an HIV-1 protease inhibitor, is widely used both as an antiretroviral drug and as a pharmacokinetic enhancer. This study aimed to develop and validate a simple, precise, accurate, robust, and stability-indicating RP-HPLC method for its estimation in bulk and pharmaceutical dosage forms. Chromatographic separation was achieved on a Kromasil C18 column (150 × 4.6 mm, 3.5 µm) using a mobile phase of acetonitrile and 0.2% octane sulfonic acid buffer (20:80 v/v, pH 2.5 adjusted with OPA) at a flow rate of 1.0 mL/min, with detection at 223 nm. Ritonavir eluted at 2.076 min with excellent peak shape (tailing factor 1.12, plate count 9058). The method was linear over 25–150 µg/mL ($R^2 = 0.99988$), accurate (99.9–100.1% recovery), precise (%RSD < 1%), and sensitive (LOD 0.60 µg/mL, LOQ 2.00 µg/mL). Forced degradation confirmed its stability-indicating nature. The method is suitable for routine quality control and stability testing.

Keywords: Ritonavir, RP-HPLC, Method Validation, ICH Q2(R1), Stability-indicating

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1. Introduction

Human immunodeficiency virus (HIV) infection remains a major global health concern, necessitating the continuous development and optimization of antiretroviral therapies.[1,3] Among the therapeutic agents, protease inhibitors (PIs) have played a pivotal role in highly active antiretroviral therapy (HAART) regimens by preventing viral replication and improving patient survival. Ritonavir, a second-generation HIV-1 protease inhibitor, is widely used not only for its antiretroviral activity but also as a pharmacokinetic enhancer. By inhibiting cytochrome P450 3A4 (CYP3A4), Ritonavir increases the plasma concentrations of co-administered protease inhibitors such as Lopinavir, Darunavir, and Atazanavir, thereby enhancing their therapeutic efficacy and reducing dosing frequency.[5-9]

Given Ritonavir's extensive use in fixed-dose combinations and its critical role in HIV management, reliable and sensitive analytical methods are essential for its quality control in both bulk and pharmaceutical dosage forms. Accurate estimation ensures consistent drug content, patient safety, and compliance with pharmacopeial standards. While various analytical techniques such as UV-spectrophotometry, HPTLC, and LC-MS/MS have been reported for determination of Ritonavir, high-performance liquid chromatography (HPLC) remains the most widely employed method owing to its simplicity, reproducibility, and suitability for routine quality control.[9-17]

Despite existing methods, many reported HPLC procedures involve long run times, complex mobile phases, or lack comprehensive validation, limiting their utility for routine analysis. Furthermore, as Ritonavir is susceptible to degradation under stress conditions such as acid, alkali, oxidative, and photolytic environments, it is crucial to develop a method that not only quantifies Ritonavir but also acts as a stability-indicating assay capable of separating the drug from its degradation products.[18-20]

The present study was undertaken with the objective of developing and validating a simple, precise, accurate, and robust reverse-phase HPLC (RP-HPLC) method for

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the estimation of Ritonavir in bulk drug and pharmaceutical dosage form. The method was optimized to achieve sharp peak resolution with a short runtime and was validated in accordance with the International Council for Harmonisation (ICH) Q2(R1) guidelines. Additionally, forced degradation studies were performed to confirm the stability-indicating nature of the method.

2. Materials and Methods

2.1. Chemicals and reagents

Ritonavir reference standard (purity 99.9%) and a marketed formulation (Ritovir) were used for the study. Acetonitrile (HPLC grade), octane sulfonic acid (analytical reagent grade), and ortho-phosphoric acid (analytical reagent grade) were procured from Merck (India). Milli-Q water was used throughout the analysis, and methanol of HPLC grade was employed as the diluent.

2.2. Instrumentation

Chromatographic analysis was performed on a Waters Alliance HPLC system equipped with a photodiode array (PDA) detector. Ancillary instruments included a Shimadzu UV–VIS spectrophotometer for wavelength selection, a Sartorius analytical balance for accurate weighing, an Eutech pH meter for buffer adjustment, and a Unichrome ultrasonicator for degassing and dissolution. Class-A volumetric glassware was used for solution preparation to ensure precision and reproducibility.

2.3. Chromatographic conditions

Several preliminary trials were conducted to optimize the chromatographic parameters, including column type, mobile phase composition, flow rate, and detection wavelength. The finalized method employed isocratic elution on a Kromasil C18 column (150 × 4.6 mm, 3.5 µm particle size). The mobile phase consisted of acetonitrile and 0.2% octane sulfonic acid buffer mixed in a ratio of 20:80 v/v, with the buffer adjusted to pH 2.5 using ortho-phosphoric acid. The flow rate was maintained at 1.0 mL/min, and the detection wavelength was set at 223 nm. The injection volume was 10 µL, with a total runtime of 5 minutes, carried out at ambient temperature (25 °C). Under these optimized conditions, Ritonavir eluted with a sharp and symmetrical peak at a retention time of 2.076 minutes, with a tailing factor of 1.12 and a theoretical plate count exceeding 9000, demonstrating excellent efficiency of the method.

2.4. Preparation of solutions

For the preparation of the standard solution, 10 mg of Ritonavir was accurately weighed, dissolved in 10 mL of methanol, and sonicated until complete dissolution. This solution was further diluted with methanol to obtain a working concentration of 100 µg/mL. For the sample preparation, a quantity of powdered Ritovir tablets equivalent to 17.6 mg Ritonavir was weighed, dissolved in 10 mL of methanol, and sonicated for 30 minutes. The solution was diluted to yield a final concentration of 100 µg/mL and filtered through a 0.45 µm syringe filter before injection into the HPLC system.

2.5. System suitability

System suitability was assessed by evaluating retention time, tailing factor, number of theoretical plates, and reproducibility of peak areas. Acceptance criteria were defined as a tailing factor not more than 2.0, theoretical plates not less than 2000, and relative standard deviation (%RSD) of replicate injections within 2.0%.

2.6. Method validation

The developed method was validated in accordance with the ICH Q2(R1) guidelines for analytical method validation. Specificity was established by analysing blank and placebo chromatograms to ensure no interference at the retention time of Ritonavir. Linearity was assessed over the concentration range of 25–150 µg/mL by preparing six calibration levels, and regression analysis was performed to determine the correlation coefficient. Accuracy was evaluated through recovery studies at three concentration levels (80%, 100%, and 120% of the nominal concentration). Precision was studied at three levels: system precision, assessed using six replicate injections of standard solution; method precision, determined from six independent sample preparations; and intermediate precision, evaluated on different days by different analysts. Robustness was determined by deliberate variations in flow rate (±10%) and mobile phase composition (±2%), while sensitivity was assessed by calculating the limit of detection (LOD) and limit of quantitation (LOQ) based on signal-to-noise ratios of 3 and 10, respectively. Forced degradation studies were carried out to confirm the stability-indicating capability of the method by exposing Ritonavir to various stress conditions, including acidic, alkaline, oxidative, reductive, hydrolytic, photolytic, and thermal environments. Peak purity under each condition was examined using the PDA detector to verify the absence of co-eluting degradation products.

2.7. Forced degradation studies

The stability-indicating property of the method was verified by subjecting Fluorouracil to a range of stress conditions. Acidic hydrolysis was carried out using 1N hydrochloric acid, while alkaline hydrolysis was performed with 1N sodium hydroxide. Oxidative degradation was induced with 10% hydrogen peroxide, and reductive degradation with 10% sodium bisulfite. Hydrolytic degradation was conducted in water, thermal degradation was induced by exposure at 105°C, and photolytic degradation was performed under UV light. Following treatment, samples were neutralized, when necessary, diluted appropriately, and injected into the HPLC system.

The method was able to separate Fluorouracil from its degradation products under all conditions, confirming its stability-indicating capability.

3. Results

3.1. Optimized chromatographic conditions

Several chromatographic trials were conducted with varying mobile phase ratios, buffer systems, and columns. The optimized conditions are summarized in **(Table 1)**. Ritonavir eluted with a sharp, symmetrical peak at a retention time of 2.076 min, demonstrating excellent efficiency and peak shape. The optimized chromatogram is presented in **(Figure 1)**.

Table 1: Optimized Chromatographic Conditions

Parameter	Observation
Instrument Used	Waters Alliance e-2695 HPLC
Injection Volume	10 µL
Mobile Phase	Acetonitrile: Octane Sulfonic acid (20:80, pH 2.5 with OPA)
Column	Kromasil C18 (150 × 4.6 mm, 3.5 µm)
Detection Wavelength	223 nm
Flow Rate	1 mL/min
Runtime	5 min
Temperature	Ambient (25 °C)
Mode of Separation	Isocratic

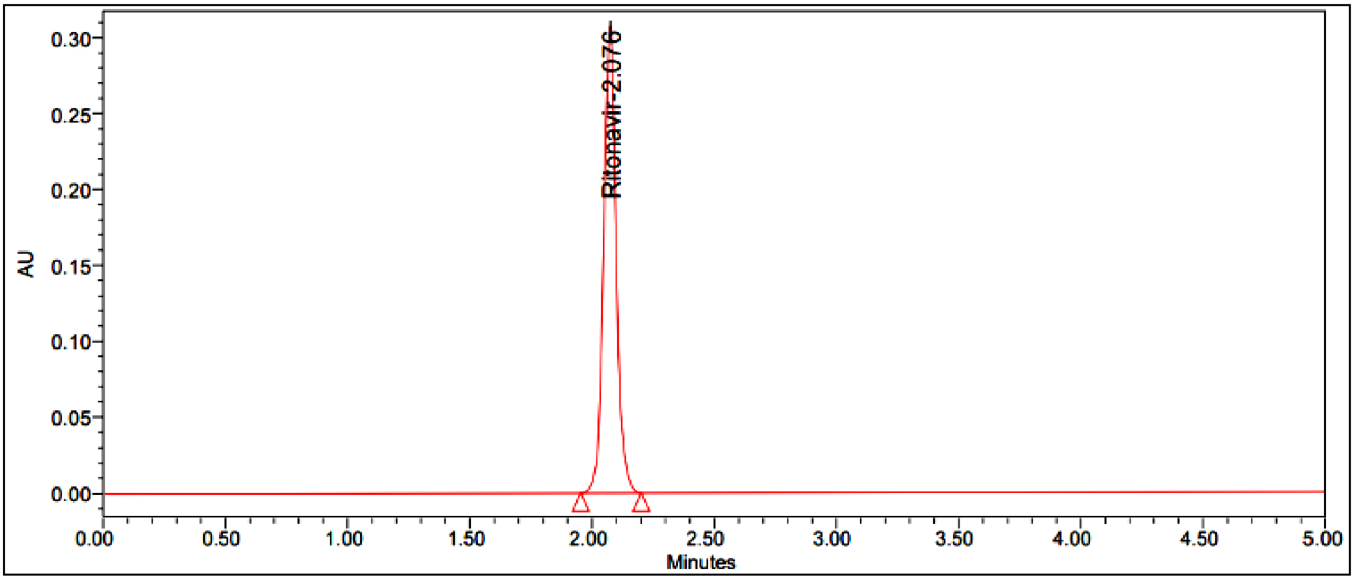

Figure 1: Optimized chromatogram of Ritonavir

3.2. System suitability

System suitability testing confirmed that the method meets ICH Q2(R1) requirements. Ritonavir exhibited a retention time of 2.076 min, theoretical plate count of 9058, tailing factor of 1.12, and %RSD of 0.44 **(Table 2)**. These results confirm excellent column performance, peak symmetry, and reproducibility.

Table 2: System Suitability Parameters for Ritonavir

Parameter	Ritonavir Result	ICH Limit
Retention Time	2.076 min	–
Plate Count	9058	≥ 2000
Tailing Factor	1.12	≤ 2.0
% RSD	0.44	≤ 2.0

3.3. Specificity

No interfering peaks were detected at the retention time of Ritonavir in blank and placebo chromatograms, demonstrating method specificity.

3.4. Precision

The method demonstrated excellent reproducibility across all precision studies **(Table 3)**.

1. System precision: Six replicate injections yielded a mean area of 3,129,318 with %RSD = 0.44.
2. Method precision: Six replicate samples showed %RSD = 0.78.

Intermediate precision: Day-to-day analysis provided %RSD values of 0.63 (Day-1) and 0.58 (Day-2).

Table 3: Precision Results for Ritonavir

S. No	Ritonavir Conc. (µg/mL)	System Precision (Area)	Method Precision (Area)	Intermediate Precision Day-1	Intermediate Precision Day-2
1	100	3131247	3141247	3151024	3125680
2	100	3105269	3126539	3135628	3153147
3	100	3138925	3101171	3106479	3150216
4	100	3145772	3131552	3116880	3145231
5	100	3128541	3105694	3128764	3104986
6	100	3126153	3167905	3157833	3140159
Mean	–	3129318	3129018	3132768	3136570
S.D.	–	13825.52	24469.48	19643.26	18248.54
%RSD	–	0.44	0.78	0.63	0.58

All values were within acceptable ICH limits, confirming precision.

Table 4: Linearity of Ritonavir

Conc. (µg/mL)	Peak Area
25	843320
50	1563981
75	2345947
100	3147218
125	3947819
150	4760234

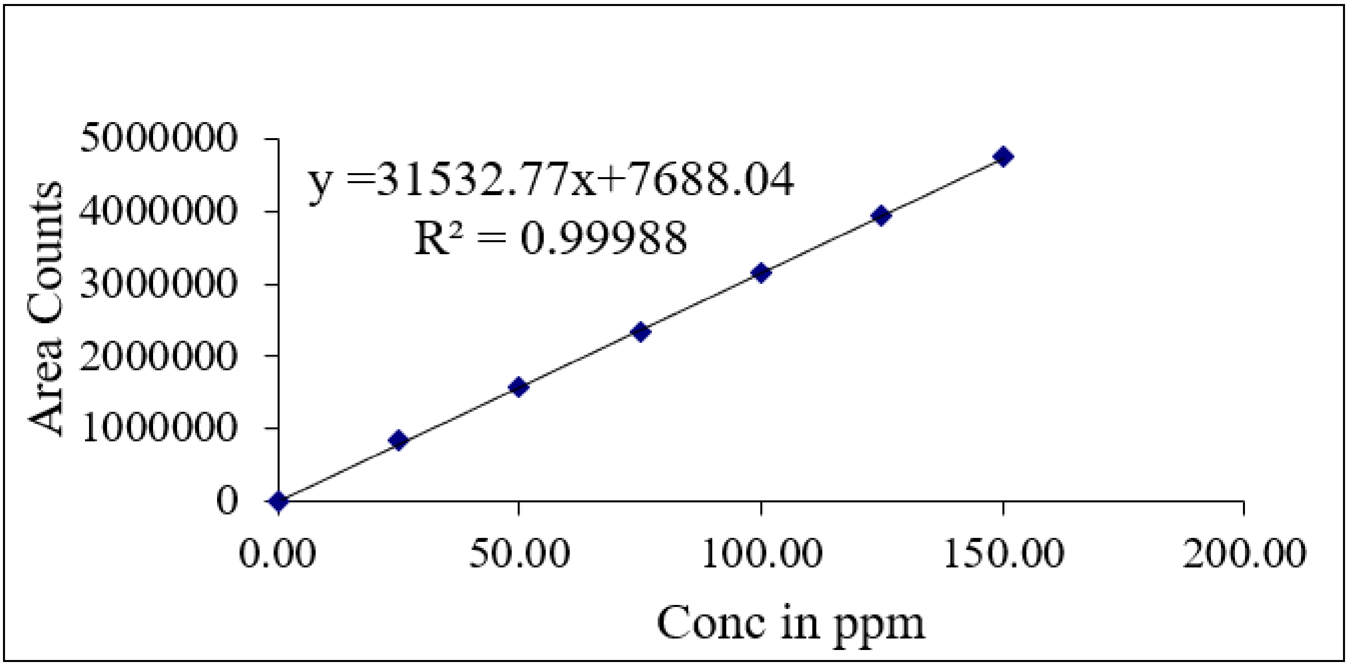

Figure 2: Calibration curve of Ritonavir at 223 nm

3.5. Linearity

Ritonavir exhibited excellent linearity across the concentration range of 25–150 µg/mL. The regression equation was y = 31532.77x + 7688.04, with correlation coefficient $R^2 = 0.99988$ **(Table 4) (Figure 2)**.

3.6. Accuracy

Accuracy was assessed by recovery studies at 80%, 100%, and 120% of target concentration. The mean recovery values ranged from 99.9–100.1%, satisfying the acceptance criteria (98–102%) **(Table 5)**.

Table 5: Accuracy Results for Ritonavir

Concentration Level	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean % Recovery
80%	5612340	18	17.93	99.6	99.9
	5626424	18	17.98	99.9	
	5649701	18	18.05	100.3	
100%	6243178	20	19.95	99.8	100.1
	6280599	20	20.07	100.4	
	6274153	20	20.05	100.3	
120%	6871234	22	21.96	99.8	99.9
	6892385	22	22.03	100.1	
	6864170	22	21.94	99.7	

Table 6: Robustness Results for Ritonavir

Condition	Retention Time (min)	Peak Area	Tailing	Plate Count	%RSD
Flow rate 0.9 mL/min	2.218	3041575	1.18	8925	0.20
Flow rate 1.0 mL/min	2.076	3131247	1.12	9058	0.44
Flow rate 1.1 mL/min	1.945	3270798	1.06	9247	0.10
Organic 18:82	2.384	2927258	1.21	8860	0.40
Organic 20:80	2.072	3105269	1.15	9013	0.44
Organic 22:78	1.822	3405934	1.09	9365	0.27

Table 7: Forced Degradation Results for Ritonavir

Condition	% Degradation	Purity Angle	Purity Threshold	Result
Control	0.0	7.399	11.324	Pass
Acid	4.3	7.384	11.328	Pass
Alkali	2.7	7.383	11.332	Pass
Peroxide	13.3	7.371	11.339	Pass
Reduction	0.9	7.343	11.361	Pass
Thermal	3.6	7.306	11.355	Pass
Photolytic	2.9	7.368	11.314	Pass
Hydrolysis	10.7	7.377	11.321	Pass

3.7. Robustness

Minor deliberate variations in flow rate (±10%) and mobile phase composition (±2%) had negligible effects on retention time, peak area, tailing factor, or plate count. The %RSD remained < 0.5 in all cases, confirming robustness **(Table 6)**.

3.8 Sensitivity

The method demonstrated high sensitivity with LOD = 0.60 µg/mL and LOQ = 2.00 µg/mL, calculated from signal-to-noise ratios of 3 and 10, respectively.

3.9. Forced degradation studies

Ritonavir was subjected to stress conditions including acid, alkali, oxidative, reductive, thermal, hydrolytic, and photolytic degradation. Degradation ranged from 0.9% (reductive) to 13.3% (oxidative). In all cases, the purity threshold exceeded the purity angle, confirming peak purity and establishing the method as stability-indicating **(Table 7)**.

4. Discussion

The present study focused on the development and validation of a robust RP-HPLC method for the estimation of Ritonavir in bulk and pharmaceutical dosage forms. Method development trials demonstrated that the optimized chromatographic conditions—comprising a Kromasil C18 column with acetonitrile and octane sulfonic acid buffer (20:80, pH 2.5)—provided sharp and symmetrical peaks with satisfactory efficiency and reproducibility. The retention time of 2.076 minutes and high theoretical plate count (>9000) ensured a rapid yet reliable separation, meeting the practical requirements for routine analysis.

System suitability testing confirmed the adequacy of the method, with all parameters including tailing factor (1.12), %RSD (0.44), and plate count exceeding the minimum acceptance criteria outlined in ICH Q2(R1). Specificity studies established that no interfering peaks were observed from placebo or blank samples, confirming the selectivity of the method for Ritonavir.

The method displayed excellent precision, with %RSD values below 1% for system, method, and intermediate precision, demonstrating reproducibility across different days and conditions. Linearity was proven over the concentration range of 25–150 µg/mL, with an outstanding correlation coefficient ($R^2 = 0.99988$), which is indicative of the strong proportional relationship between peak area and concentration. Accuracy was validated through recovery studies, yielding mean recovery values between 99.9% and 100.1%, which are well within the acceptable limits of 98–102%, thus confirming the method's reliability in quantifying Ritonavir.

Robustness testing indicated that minor variations in flow rate and mobile phase composition did not significantly impact retention time, peak area, or symmetry, underscoring the ruggedness of the method for routine laboratory use. Furthermore, sensitivity studies revealed a low LOD (0.60 µg/mL) and LOQ (2.00 µg/mL), demonstrating the method's ability to detect and quantify Ritonavir at trace levels, making it suitable even for stability studies.

Forced degradation studies further reinforced the stability-indicating nature of the method. Ritonavir was found to undergo measurable degradation under acidic, alkaline, oxidative, hydrolytic, photolytic, and thermal stress conditions, with oxidative stress producing the highest level of degradation (13.3%). Despite the presence of degradants, peak purity testing confirmed that the Ritonavir peak was spectrally pure in all cases, as the purity threshold consistently exceeded the purity angle. This indicates that the method can reliably distinguish the active drug from its degradation products, fulfilling a critical requirement for stability-indicating assays.

Overall, the results demonstrate that the developed RP-HPLC method meets all analytical performance characteristics required for pharmaceutical quality control.

5. Conclusion

In order to design and validate a simple, fast, and accurate RP-HPLC procedure for the measurement of Ritonavir in pharmaceutical dosage form and bulk, the ICH Q2(R1) recommendations were successfully followed. The method was shown to be linear, accurate, robust, sensitive, precise, and specific. The ability of Ritonavir to be effectively separated from its breakdown products under various stress conditions was confirmed by forced degradation testing. Ritonavir's short duration (5 minutes), strong repeatability, and ability to signal stability make the suggested method perfect for routine quality control, assay determination, and stability testing of the drug in both bulk drugs and final formulations. Regulatory filings and bioanalytical applications that require stability-indicating analytical methods can also use it.

6. Conflict of Interest

None.

7. Source of Funding

None.

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