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Original Article

A NEW ROBUST ANALYTICAL METHOD DEVELOPMENT, VALIDATION, AND STRESS DEGRADATION STUDIES FOR ESTIMATING RITONAVIR BY UV-SPECTROSCOPY AND HPLC METHODS

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ABSTRACT

Objective: To specify ritonavir and its commercial dosage form, the current study set out to design and validate concise, precise, and efficient high-performance liquid chromatography and spectrophotometric methods. The developed spectroscopy and chromatographic methods are reliable, precise, accurate, and specific for estimating ritonavir.

Methods: The method is superior to previously described methods due to its shorter retention duration, use of an affordable and easily accessible mobile phase, UV detection, and improved peak resolution. The maximum absorbance was determined by analysing multiple concentration ranges of ritonavir at 10-60 µg/ml using the UV-spectrophotometric method. The chromatographic separation was performed with a mobile phase composed of acetonitrile and 0.1% formic acid (1:1 v/v) pumped at a 1.0 ml/min flow rate on a phenyl (150 x 4.6 mm, 3.5 µm) column.

Results: Obeyed Beer-Lambert law over the 10-60 µg/ml and 25-150 µg/ml concentration range of ritonavir for the UV-spectrophotometric and HPLC methods, respectively. The absorbance at 273 nm was selected as the maximum absorbance throughout the UV-spectroscopic study. The detection and quantification limits for UV-spectroscopic are 0.89 and 2.93 µg/ml, whereas for the HPLC method are 0.78 and 2.57 µg/ml, respectively. In the accuracy and precision validation studies, the amount of recovery and percentage of RSD was excellent with acceptance limits as per International Conference on Harmonization (ICH) guidelines.

Conclusion: The suggested method has been approved following standards established by the ICH. The developed methods can be employed to analyse ritonavir API and pharmaceutical dosage forms and provide better specificity, excellent separation, and specified analyte and degradation substances.

Keywords: Ritonavir, Spectrophotometry, HPLC, ICH guidelines, Method validation, Degradation study

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INTRODUCTION

In 2008, around 33.4 million HIV-positive individuals worldwide, nearly 2 million of whom passed away. By lowering HIV-related morbidity and death, highly active antiretroviral therapy (HAART) has renewed hope for those with HIV/AIDS [1]. Ritonavir is (5S, 8S, 10S,11S)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl) -4-thiazolyl]-3, 6-dioxo-8, 11-bis (phenylmethyl)-2, 4, 7, 12-tetraazatridecan-13-oic acid 5-thiazolyl methyl ester (fig. 1) [2]. Between 1995 and 1997, the United States Food and Drug Administration (FDA) approved the protease inhibitor ritonavir, transforming HIV therapy. Ritonavir has an HIV-1 impedance profile [3]. It is seldom employed for its antiviral activity but is a booster for other protease inhibitors [4]. Ritonavir can inhibit severe acute respiratory syndrome (SARS) or novel coronavirus [5], and it can also inducer of several metabolizing enzymes like CYP1A4, glucuronosyl transferase, CYP2C9, and CYP2C19; the magnitude of drug interactions is difficult to predict, particularly for drugs that are metabolized by multiple enzymes or have low intrinsic clearance by CYP3A [6]. Ritonavir is strongly protein-bound, with a half-life of 3-5 h. Due to the CYP3A4 isoenzyme system's auto-induction, it will reduce its metabolism [7]. Contrarily, the side effects cause headaches, diarrhoea, weariness, heartburn, and changes in food tastes [8].

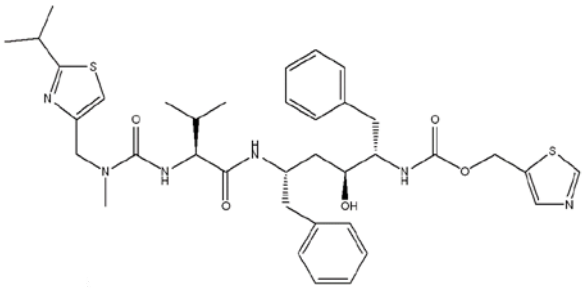


Fig. 1: Structure of ritonavir

We asserted through the literature review that is using various analytical methods, ritonavir was evaluated. Ritonavir has been evaluated using various techniques, including bio-analytical, high-performance liquid chromatography, ultra-high-performance liquid, high-performance thin-layer chromatography, ultraviolet–visible spectroscopy, capillary electrophoresis, liquid-chromatography–mass spectrometry, and LC-ESI-MS, both alone and in combination with other drugs in pharmaceutical formulations [9-19]. However, a few streamlined UV-spectrophotometric and HPLC techniques employed for regular analysis of ritonavir alone were published with erroneous justification; therefore, these assembled data might not be helpful for future research. In this regard, our main aim is to develop

robust, rapid, sensitive, selective, linear, and precise types of UV-spectrophotometric and HPLC methods for determining ritonavir and performing stability-indicating stress degradation studies. The method was validated as per the United States Pharmacopeia [20] and ICH guidelines [21]. The proposed analytical method developed by the appropriate selection of less toxic solvents is an enormous challenge because better separation and quantification analysis is much more important. Linearity, accuracy, precision, specificity, the limit of detection (LOD), and the limit of quantification (LOQ) are performed and used to ascertain the drug content of ritonavir in various pharmaceutical products following ICH Q2(R1) criteria.

MATERIALS AND METHODS

Apparatus

Shimadzu 1800 UV spectrophotometer was used for this analysis, with 1 cm matched quartz cells for all measurements and UV probe 4.2 series software. Water alliance High-performance liquid chromatography system with phenyl (150 x 4.6 mm, 3.5 µm) column and PDA detector was used for chromatographic separation. For data analysis and integration, empower software was used. The investigation employed a digital analytical balance (Mettler Toledo, India), an ultrasonic sonicator (UCA 701, Unichrome, India), and validated borosilicate glass pipettes, volumetric flasks, beakers, and an adjustment of pH was made using a consort Helico LI-120 pH meter.

Chemicals and reagents

Mylan Laboratories Ltd., Hyderabad, India provided pure samples of ritonavir (99.9%). In comparison, the reagents and chemicals utilized were analytical HPLC grade based. Acetonitrile and methanol were purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Membrane filters (0.45 µm) purchased from Phenomenex (USA) were. The commercial dosage of the ritonavir tablets (ritomune-100) has been obtained from the local market. All the chemicals used were analytical reagent grade, and the solvents were HPLC grade. Ideal Chemicals Pvt. Ltd, India, provided analytical grade formic acid, acetonitrile of liquid chromatography (LC) grade, sodium hydroxide, hydrogen peroxide, and hydrochloric acid. Analytical balance (AX 200; Shimadzu Analytical Pvt. Ltd., India), and pH meter were used during the study.

Development of UV-spectrophotometric and HPLC methods to identify ritonavir

Employed UV-spectroscopic method (Method A)

UV-Vis spectroscopic scanning is an easy-to-use, non-intrusive analytical method with the potential to support formulation development mechanistically. UV scanning over the previous five years has shown potential in various drug substances and delivery methods. It is also qualitatively and quantitatively useful in some more specialized research. This technique is frequently used in many other industries as a kinetic and monitoring study to ensure authenticity analysis, quality monitoring, and purity [22]. The simplest way to run various analyses is using the principle of UV-Spectroscopy. A blank solution for the mobile phase was maintained. From 200 to 400 nm, samples were recorded. Following the linearity analysis, the λ_{max} was confirmed to be 273 nm. Fig. 2 represents the UV spectrum of ritonavir in mentioned conditions.

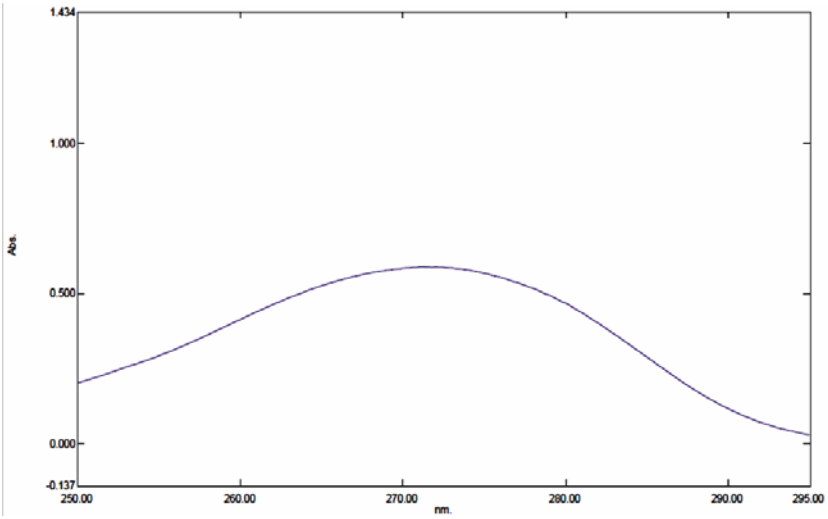


Fig. 2: UV spectrum of ritonavir

Employed high-performance liquid chromatography method (Method B)

Chromatography is the backbone of separation science and is universally used in all research laboratories and pharmaceutical industries. To evaluate drug products, high-performance liquid chromatography is a crucial analytical tool. The different pharmaceuticals and drug-related degradants that can develop in storage or production, as well as any drugs and drug-related impurities that may be added during synthesis should be able to be separated, detected, and quantified using HPLC procedures. An HPLC method's performance qualities and limitations are established through validation, and the influences that might affect these characteristics and the degree to which they might vary are also identified [23, 24].

Optimization of chromatographic conditions by HPLC method

In the normal phase, HPLC is used to determine and separate most classes of chemical compounds by using non-polar solvents for the mobile phase and polar components for the stationary phase. Simultaneously, chromatographic conditions were optimized to establish a routine analysis of ritonavir, with excellent technique reproducibility and analytical throughput. Numerous columns were used for this analysis, like the Inertsil ODS, C_{18}, C_8, Kromasil nonpolar, and cyano columns. The method was utilized by Waters Alliance HPLC with Phenyl (150 x 4.6 mm, 3.5 µm) column at a flow rate of 1 ml/min at 2.34 min retention time with a mobile phase composition of acetonitrile and 0.1% formic acid (1:1 v/v). Further optimization attempts included manipulating the composition of the mobile phases and flow rate, but no significant changes were observed. The injection volume was controlled at 20 µl, and the column temperature was set ambient at 25 °C. Empower 2 software version was applied for data acquisition and analysis. Fig. 3 represents the optimized chromatogram of ritonavir in the mentioned conditions.

Preparation of mobile phase for methods A and B

One milliliter of HPLC-grade formic acid was diluted with 100 milliliters of distilled water to create 0.1% formic acid. 100 ml of the 0.1% formic acid solution was combined with 100 ml of HPLC grade acetonitrile to make a 1:1 v/v, a combination of acetonitrile and 0.1% formic acid.

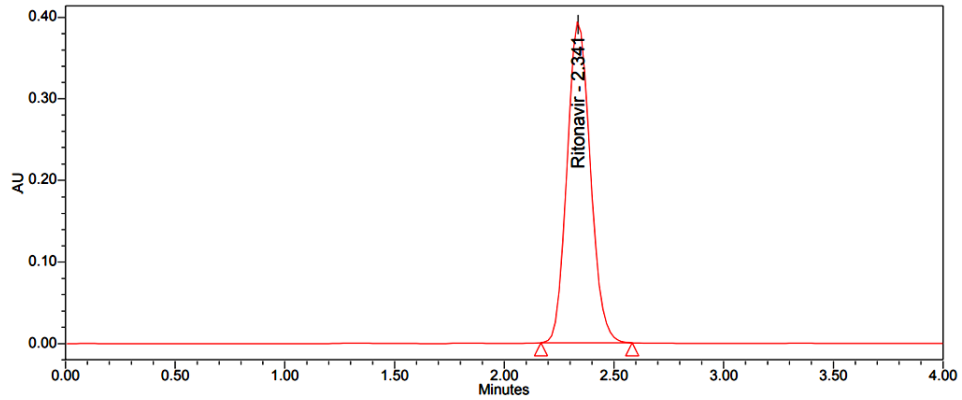


Fig. 3: HPLC chromatogram of ritonavir

Sample preparations for methods A and B

Preparation of solution for standard drug

10 mg of ritonavir working standard was transferred into a 10 ml clean, dry volumetric flask, use a scale, added mobile phase, then sonicated the mixture to thoroughly dissolve it and bring the volume up to the desired level. Samples were prepared for procedures A and B by dilution with the mobile phase made from the working standard according to the requirement. All solutions were stored at 15 °C until analysis and were found stable for at least 10 d.

Preparation of solution for commercial formulation

Ten commercial tablets (ritomune-100) were carefully weighed for the assay analysis, and the average weight was established. The tablets were uniformly crushed to create a fine powder. A quantity of 25 mg of ritonavir powder was added to a volumetric flask with a 25 ml capacity. It was sonicated for 15 min with enough acetonitrile to dissolve the medication. The volume was then adjusted with the mobile phase to the desired level. The Whatman filter paper was used to filter the resulting solution. The sample solutions for procedures A and B were created by diluting the filtration solution with the mobile phase. The percentage estimation of the drug was then computed using the assay formula.

Method validation of ritonavir by HPLC and UV-spectroscopic method

A crucial task in the pharmaceutical sector is method validation. Validation data are used to verify that the analytical method utilised for a particular test is appropriate for its goals. These findings show the analytical approach's effectiveness, reliability, and consistency. Following ICH Q2 (R1) recommendations, the analytical methodology was carried out to validate analytical methods for system suitability, linearity, detection limit, quantification, accuracy, precision, and robustness for both methods A and B.

Stress degradation studies by HPLC and UV-spectroscopic method

In the pharmaceutical industry, forced degradation studies offer a method for analysing the stability of drug samples. The chemical stability of the molecule has an impact on the safety and effectiveness of drug products. Information on molecule stability offers the information needed to choose the best formulation, container, storage environment, and shelf life. These data are crucial for regulatory documentation and play a significant part. Stability studies of novel drug compounds must be conducted before filling out the registration dossier. As per International Conference on Harmonization (ICH) guidelines (Q1A), stability studies must be performed to propose new drug substances and/or products' shelf life. To assess the suggested method's stability indicating characteristics and specificity, stress degradation studies by HPLC and UV-spectroscopic method of ritonavir were also carried out, followed by ICH recommendations [25, 26] and Mondal *et al.*, (2016) [27]. All solutions used in stress studies were prepared at an initial concentration of 1 mg/ml of ritonavir and further diluted in the mobile phase to give a final concentration of 60 µg/ml for method A and 100 µg/ml for method B and filtered the solutions before injection. Acid degradation was conducted in 0.5M hydrochloric acid; alkaline degradation was conducted using 0.5N sodium hydroxide, and solutions for oxidative stress studies were prepared using 10 % hydrogen peroxide refluxed for 90 min at 60 °C. The drug solution was heated in a thermostat for thermal stress degradation testing, and the sample solution was cooled and used. Photostability was exposed to UV light for 6 h under a UV chamber (365 nm) and analysed.

RESULTS

Method validation

The developed spectroscopy and chromatography methods for estimating ritonavir are accurate, precise, robust, and specific. The method is better than previously reported methods because of its less retention time, economical and readily available mobile phase used, UV detection, and better resolution of peaks. The run time is relatively short, enabling rapid quantification of many samples in routine and quality-control analysis of the ritonavir formulation. All these factors make this method suitable for quantifying ritonavir in bulk drugs and pharmaceutical dosage forms without interference. The results were based on the International Conference on Harmonization guidelines.

Linearity

An analytical technique called linearity provides test results directly proportional to the analyte in the test sample. In Linearity studies, calibration curves were graphed in a concentration range of 10-60 µg/ml for method A and 25-150 µg/ml for method B. As a result, the linear regression equation of method A is y = 0.0265x+0.0584 with a correlation coefficient of 0.9992 (fig. 4 and 5), and method B is y = 29577x+37774 with a correlation coefficient of 0.9985 (fig. 6 and 7).

Precision

When RSD in precision studies was less than 2%, the suggested procedure had acceptable reproducibility. The performance of intraday and interday precision and the percent RSD for the response of six replicate measurements in both methods A and B were within the acceptable ranges. Results from the intraday and interday precision studies are summarized in tables 1 and 2.

Accuracy

The percentage of recovery values in the accuracy studies demonstrates that the proposed method is accurate and that interference response exists. Adding an adequate amount of ritonavir standard stock solution to the sample solution evaluated accuracy at three different concentration levels (50%, 100%, and 150%). Three replicate measurements are performed for methods A and B, showing that the percent recovery was within the allowed ranges (tables 3 and 4).

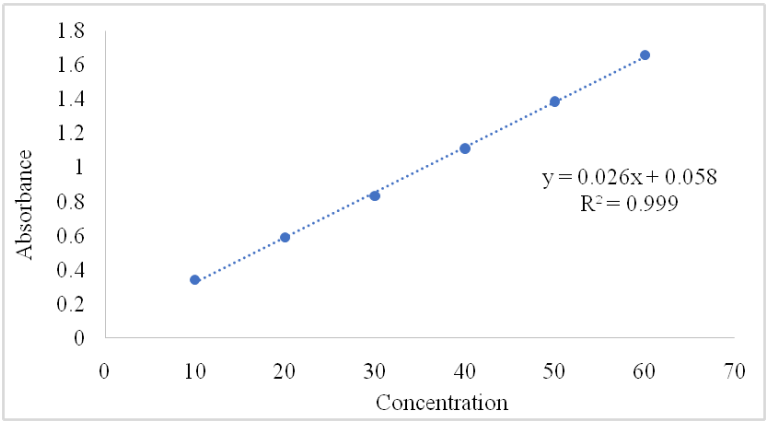


Fig. 4: Calibration curve of ritonavir for method A (UV-spectroscopic method)

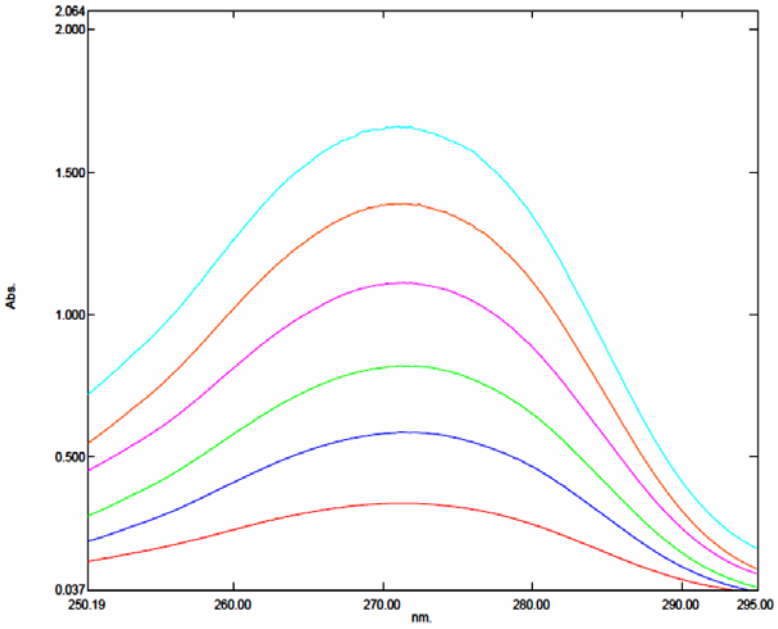


Fig. 5: Overlay spectrum of ritonavir for method A (UV-spectroscopic method)

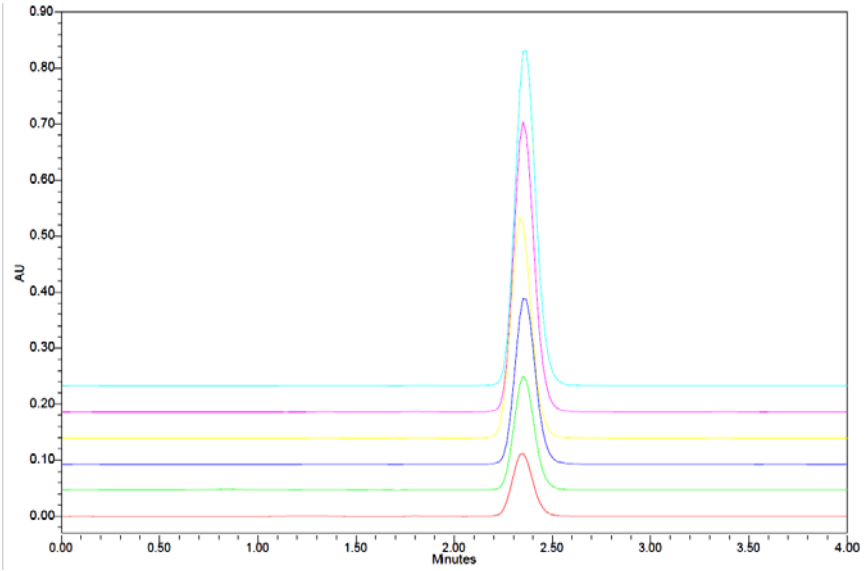


Fig. 6: Overlay chromatogram of ritonavir for method B (HPLC method)

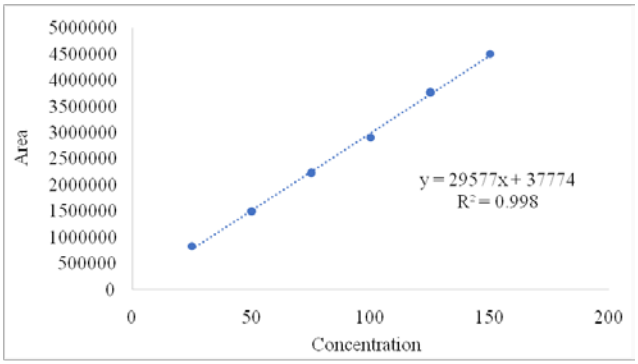


Fig. 7: Calibration curve of ritonavir for method B (HPLC method)

Table 1: Intraday precision for methods A and B of ritonavir

S. No.	Conc. (µg/ml)	Method A	Method B	%RSD	
		Absorbance	Area	Method	
				A	B
1	50	1.388	1498563	0.058%	0.041%
2	50	1.387	1497895		
3	50	1.386	1498336		
4	50	1.387	1498523		
5	50	1.388	1499125		
6	50	1.388	1499652		

Method A (UV-spectroscopic method), Method B (HPLC method).

Table 2: Intraday precision for methods A and B of ritonavir

S. No.	Conc. (µg/ml)	Method A	Method B	%RSD	
		Absorbance	Area	Method	
				A	B
1	50	1.375	1489568	0.47%	0.91%
2	50	1.372	1474586		
3	50	1.385	1458963		
4	50	1.389	1489562		
5	50	1.385	1496325		
6	50	1.379	1485693		

Method A (UV-spectroscopic method), Method B (HPLC method).

Table 3: Ritonavir accuracy observations for method A (UV-spectroscopic method)

Conc. (µg/ml)	Amount of drug added (µg/ml)		Amount recovered (µg/ml)	% Recovery
	Pure	Formulation		
15	10	5	14.96	0.23 %
15	10	5	14.89	
15	10	5	14.92	
30	10	20	29.94	0.22 %
30	10	20	29.85	
30	10	20	29.81	
45	10	35	44.98	0.16 %
45	10	35	44.86	
45	10	35	44.99	

Table 4: Ritonavir accuracy observations for method B (HPLC method)

Conc. (µg/ml)	Amount of drug added (µg/ml)		Amount recovered (µg/ml)	% Recovery
	Pure	Formulation		
37.5	20	17.5	37.32	0.27 %
37.5	20	17.5	37.24	
37.5	20	17.5	37.12	
75	20	55	74.98	0.28 %
75	20	55	74.56	
75	20	55	74.78	
112.5	20	92.5	112.36	0.08 %
112.5	20	92.5	112.51	
112.5	20	92.5	112.53	

Table 5: The ritonavir robustness data for several approach techniques using UV and HPLC techniques

Method	Condition	%RSD
A	Wavelength at 275 nm	0.56
	Wavelength at 273 nm	0.38
B	Flow rate of 0.8 ml/min	0.23
	Flow rate of 1.2 ml/min	0.25
	Mobile phase of acetonitrile and 0.1% formic acid 40:60 (v/v)	0.51
	Mobile phase of acetonitrile and 0.1% formic acid 60:40 (v/v)	1.12
	Temperature at 27 °C	0.21
	Temperature at 23 °C	0.58

*Mean of six observations (n=6). Method A (UV-spectroscopic method), and Method B (HPLC method).

Robustness

To execute the robustness assessment by altering the wavelength (±2 nm) in method A and the flow rate (±0.1 ml/min), mobile phase ratio, and temperature (±3 °C) in method B. All the parameters were passed with no notable changes. The percent RSD was within the acceptable range (table 5).

Limits of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ parameters were determined from the regression equation of ritonavir; LOD = 3.3 σ/S, LOQ = 10 σ/S, where the standard deviation of the response (σ) and S is the slope of the corresponding calibration curve. In the LOD analysis, the detection limits for methods A and B were 0.89, and 0.78 µg/ml, while the LOQ was 2.93, and 2.57 µg/ml, respectively. Table 6 displays the relevant LOD and LOQ values for ritonavir.

Analysis of marketed formulations

The commercially available ritomune-100 mg formulations of ritonavir assay were carried out, and the purity percentage was assessed by methods A and B. Neither substantial variation was found during the percentage purity analysis. The interpretation findings for the marketed tablets of ritonavir are depicted in table 7.

Table 6: Employing UV and HPLC techniques, the sensitivity assessments (LOD and LOQ) of ritonavir

Method	LOD (µg/ml)	LOQ (µg/ml)
Method A	0.89	2.93
Method B	0.78	2.57

*Mean of three observations (n=3). Method A (UV-spectroscopic method), Method B (HPLC method).

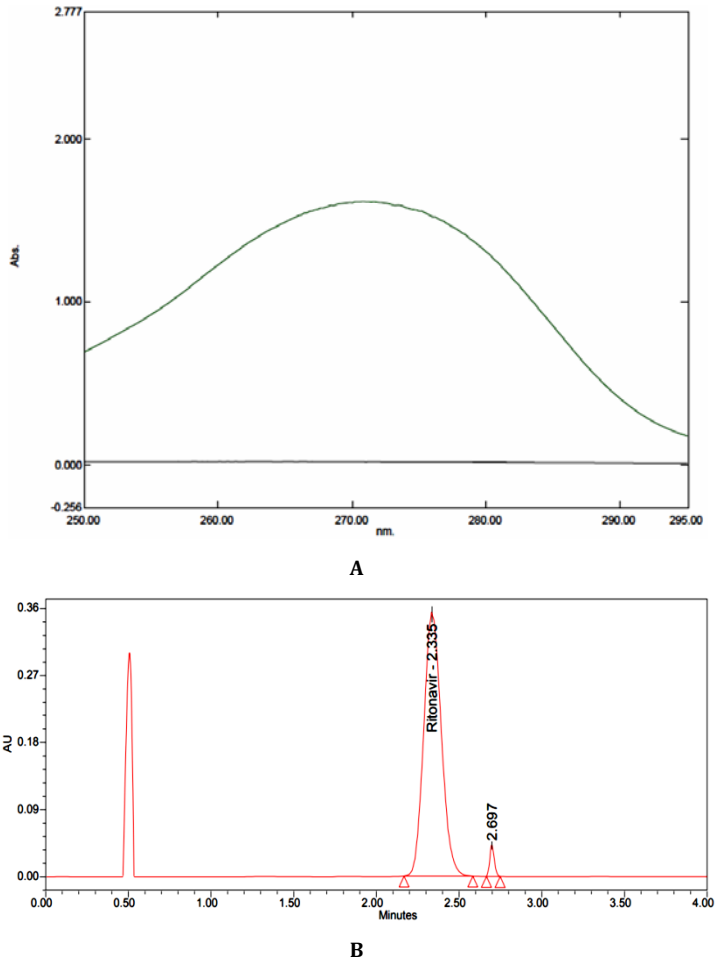


Fig. 8: The oxidative stress degradation studies spectrum and chromatogram for methods A and B

Table 7: Assay data for the commercially available ritonavir formulations (ritomune-100 mg) using UV and HPLC techniques

Drug and label claim	Amount estimated (mg/tab)		Purity (% w/w)±SD, (%RSD)	
	Method		Method	
	A	B	A	B
Ritomune (100 mg)	99±0.69	99±0.75	99.58±0.78 (0.31%)	99.68±0.65 (0.24%)

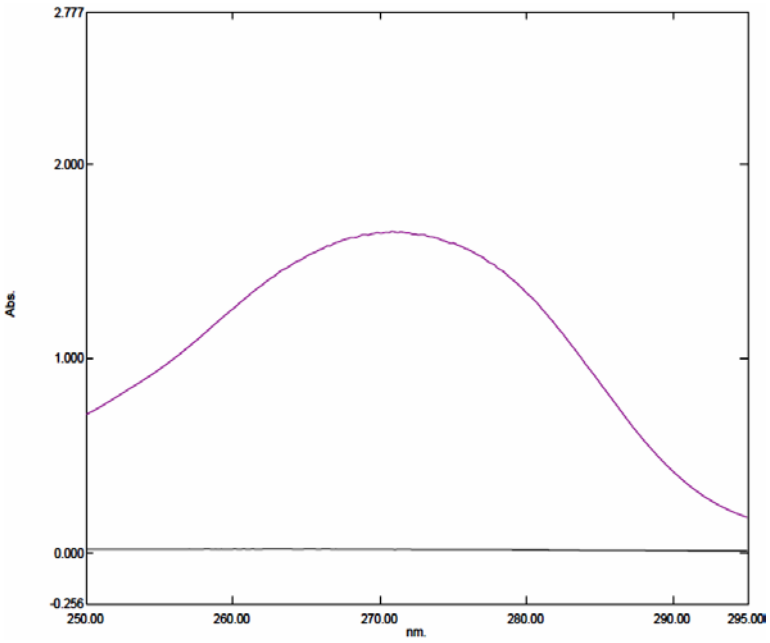
Data are expressed as mean±SD, n=3, Method A (UV-spectroscopic method), Method B (HPLC method).

Stress degradation studies

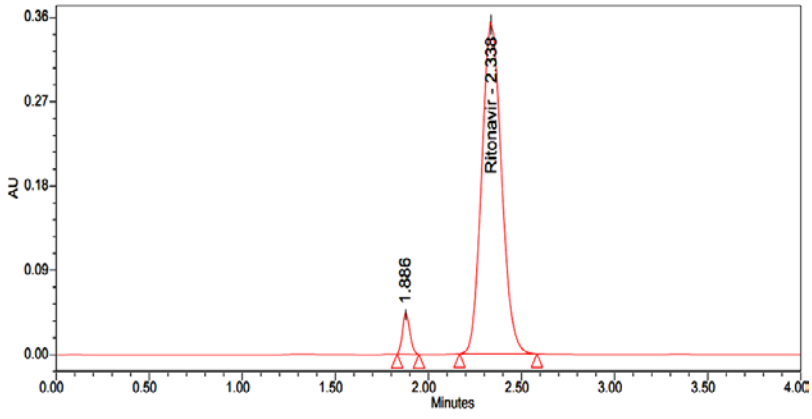
Studies on stress degradation were carried out under various stressful conditions by UV-Spectroscopic and HPLC methods, but no significant degradation was observed.

The highest degradation percentage was observed in oxidation stress tribunals, where the UV-spectroscopic method observed 3.17 % degradation, respectively. However, the HPLC method showed 14.11 % degradation at 2.335 min of retention time (fig. 8).

Studies on acid stress degradation indicated that the UV-spectroscopic method exhibited 1.56 % degradation. In contrast, the HPLC method showed a degradation percentage of 12.02 % at 2.338 min of retention time (fig. 9).



A

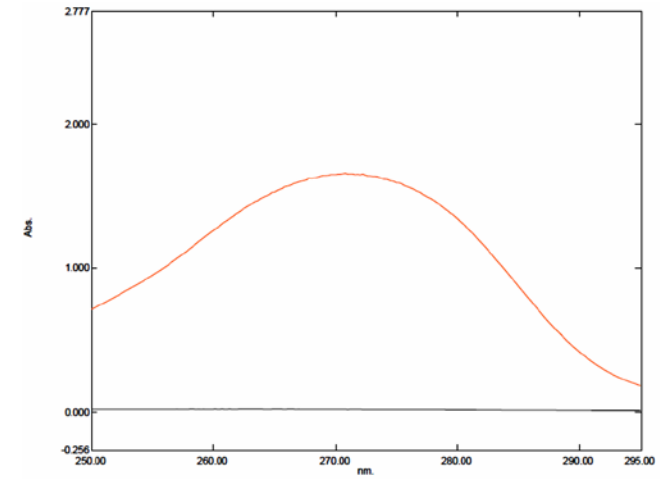


B

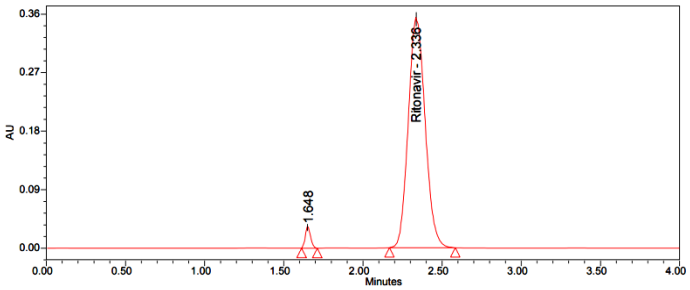
Fig. 9: The acid stress degradation studies spectrum and chromatogram for methods A and B

In investigations on alkali stress degradation, it was revealed that the UV-spectroscopic method exhibited degradation rates of 1.01 %, respectively. In contrast, the HPLC method had a degradation rate of 7.90 % at 2.336 min of retention time (fig. 10).

Less percentage of degradation was observed in thermal stress degradation studies, with the UV-spectroscopic method finding 0.17 % and the HPLC method finding 0.51 % at a 2.337 min retention time (fig. 11).

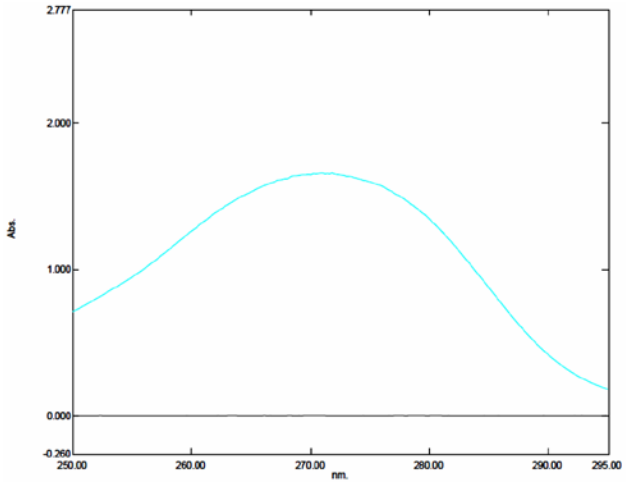


A

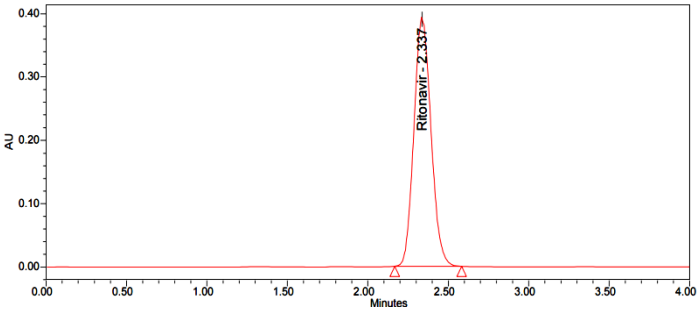


B

Fig. 10: The alkali stress degradation studies spectrum and chromatogram for methods A and B



A



B

Fig. 11: The thermal stress degradation studies spectrum and chromatogram for methods A and B

Regarding photolytic stress degradation, the UV-spectroscopic method showed a degradation percentage of 0.05 %; however, in HPLC method showed a degradation percentage of 0.003 % at a 2.333 min retention time (fig. 12).

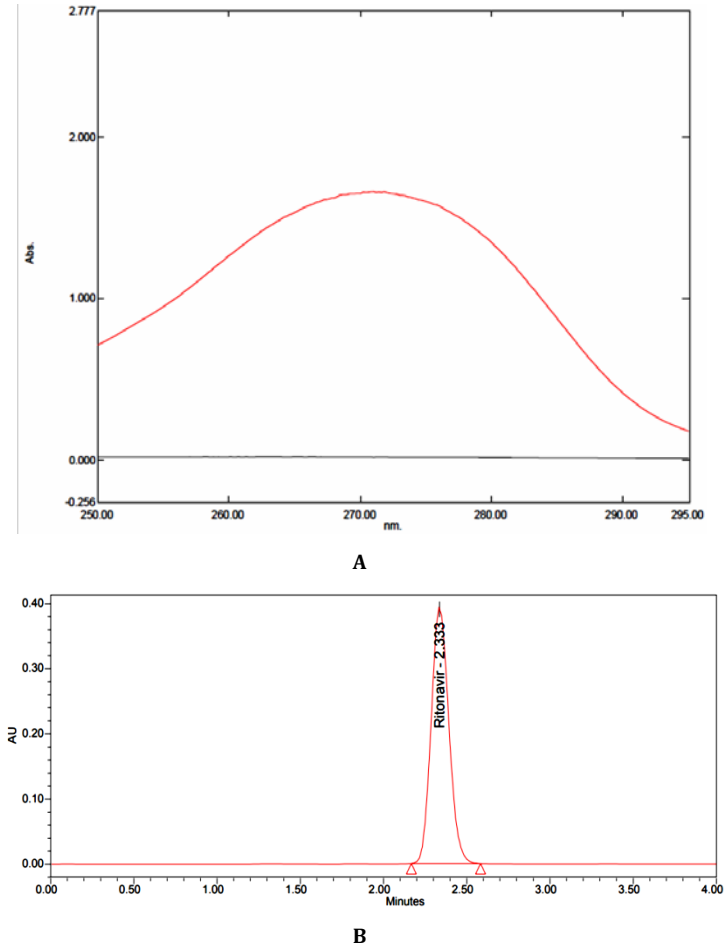


Fig. 12: The photolytic stress degradation studies spectrum and chromatogram for methods A and B

The desired outcomes of tests on stress degradation are listed in table 8, and the validation parameters for the UV-Spectrophotometric and HPLC methods are summarised in table 9, respectively.

Table 8: Overview of ritonavir UV-spectrophotometric and HPLC validation parameters

Parameters	Method A	Method B
λmax	273 nm	273 nm
Linearity (µg/ml)	10-60 µg/ml	25-150 µg/ml
Regression coefficient	0.9992	0.9985
Regression equation (y=mx+c)	y = 0.0265x+0.0584	y = 29577x+37774
Intra-day precision (% RSD)	0.058%	0.041%
Inter-day precision (% RSD)	0.47%	0.91%
Robustness (% RSD)	0.38-0.56	0.21-1.12
LOD (µg/ml)	0.89	0.78
LOQ (µg/ml)	2.93	2.57

Method A (UV-spectroscopic method), Method B (HPLC method).

Table 9: The desired outcome of ritonavir stress degradation studies employing UV-spectroscopy and HPLC

Degradation Condition	Method A	Method B	% Degradation	
	Absorbance	Area	Method	
			A	B
Oxidation	1.616	2495874	3.17 %	14.11 %
Acid	1.643	2556528	1.56 %	12.02 %
Alkali	1.652	2676358	1.01 %	7.90 %
Thermal	1.666	2891137	0.17 %	0.51%
Photolytic	1.668	2906006	0.05 %	0.003 %

Method A (UV-spectroscopic method), Method B (HPLC method)

DISCUSSION

The findings of the current investigation indicate that efficient separation and selectivity were achieved in a shorter runtime using the developed and validated method. Using a gradient technique, the chromatogram was monitored with the mobile phase flow rate. The system suitability parameters were evaluated [28]. The designed and validated approach was linear, accurate, precise, and robust against the wide concentration of ritonavir, which might help qualitative and quantitative validation. The significant objective of this study was to pinpoint the spectroscopic and chromatographic techniques that were reliable enough to produce an appropriate separation of the components with a good spectrum for the UV-spectroscopic method and chromatogram within a reasonable run time for the high-performance liquid chromatographic method [29-31]. The target analytical profile was created to identify critical method attributes influencing critical quality attributes, and a systematic risk analysis was conducted. The most important quality variables were specificity, resolution, separation factor, and retention time [32, 33]. Mobile phase characteristics were discovered to be the most crucial for the specified analysis based on risk priority number. Therefore, three parameters were selected as crucial technique features: the acetonitrile ratio, the formic acid ratio, and the flow rate in the mobile phase. This experimental effort discovered the most significant absorption peak at 273 nm after obtaining the UV spectrum. The mobile phase of acetonitrile and 0.1% formic acid (1:1 v/v) at a flow rate of 1 ml/min characterizes the recognized point by combining the unique crucial method features. Elutes were analysed using a PDA detector at a detection wavelength of 273 nm. The design space presents the operable method region where the changes will not affect the quality of the analysis. International Council validated the proposed method for Harmonization (ICH) guidelines, Validation of Analytical Procedures: Text and Methodology Q2 (R1). According to studies on stress degradation, UV-spectroscopic and HPLC analysis of ritonavir solutions demonstrated no indications of insignificance degradation [34].

CONCLUSION

The current research proposes an accurate, efficient, and specific UV-spectroscopic and HPLC approach for routine ritonavir analysis. It may be used to identify related substances or contaminants during storage conditions and estimate the analyte of interest without interferences. Using a waters alliance, HPLC with phenyl (150 x 4.6 mm, 3.5 µm) column and composition of acetonitrile and 0.1% formic acid (1:1 v/v) as a mobile phase in this study resulted in superior analyte elution with high resolution, increased plate count, increased capacity factor, and reduced tailing. The reported development methods were validated as per ICH Q2 (R1) guidelines. UV-spectroscopic and HPLC methods can analyse the ritonavir analyte in bulk and dosage form.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTERESTS

For this work, the authors report that they have no conflicts of interest.

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