Development And Validation of Stability-Indicating RP-HPLC Method For Simultaneous Estimation of Ritonavir and Lopinavir in Tablet Dosage Form

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***Abstract*—A simple, accurate, precise, and stability-indicating RP-HPLC method was developed and validated for the simultaneous estimation of Ritonavir and Lopinavir in bulk drug and pharmaceutical tablet dosage forms. Chromatographic separation was performed using an XTerra C18 column with a mobile phase consisting of 0.1% orthophosphoric acid buffer (pH 2.8) and methanol (45:55 v/v) at a flow rate of 1.0 mL/min, with detection at 229 nm. The developed method shows good chromatographic separation with satisfactory retention time, peak symmetry, and system suitability parameters. Forced degradation studies under acidic, alkaline, oxidative, thermal, and photolytic conditions confirmed the stability-indicating capability of the method. Maximum degradation was observed under alkaline conditions. The method was validated according to ICH guidelines for specificity, precision, accuracy, linearity, robustness, LOD, and LOQ, and all parameters were found within acceptable limits. he developed RP-HPLC procedure was successfully utilized for analysis of marketed tablet formulation and was found suitable for routine quality control and stability studies of Ritonavir and Lopinavir.**

***Index Terms*—Ritonavir; Lopinavir; RP-HPLC; Stability-indicating method; Method validation; Forced degradation studies; ICH guidelines; Antiretroviral drugs; Pharmaceutical analysis; Chromatographic method development.**

I. INTRODUCTION

Human Immunodeficiency Virus (HIV) infection continues to remain one of the major global public health challenges despite significant advancements in antiretroviral therapy.[1]The introduction of highly active antiretroviral therapy (HAART) has considerably improved the survival rate and quality of life of HIV-infected patients by effectively suppressing viral replication and reducing disease progression. Among the various classes of antiretroviral agents, protease inhibitors play a crucial role in inhibiting viral maturation and replication by targeting the HIV protease enzyme responsible for cleavage of viral polyproteins into functional proteins. The combined administration of protease inhibitors has become an important therapeutic strategy in the management of HIV infection due to improved antiviral efficacy and reduced development of drug resistance.[2]

Lopinavir and Ritonavir are widely used antiretroviral drugs belonging to the protease inhibitor class. Lopinavir is an effective HIV-1 protease inhibitor that exhibits potent antiviral activity against HIV infection. However, Lopinavir undergoes extensive metabolism by the cytochrome P450 (CYP3A4) enzyme system in the liver, resulting in reduced bioavailability when administered alone.[3] Ritonavir, another HIV protease inhibitor, is commonly co-administered with Lopinavir because of its strong inhibitory effect on CYP3A4-mediated metabolism. Ritonavir acts as a pharmacokinetic enhancer or "booster," thereby increasing plasma concentration, half-life, and therapeutic effectiveness of Lopinavir. Due to this synergistic pharmacokinetic interaction, the fixed-dose combination of Ritonavir and Lopinavir has become an established therapeutic regimen for HIV management and antiviral therapy.[4]

The increasing therapeutic use of combined Ritonavir and Lopinavir formulations necessitates the development of reliable analytical methods for routine quality control, stability assessment, and regulatory compliance. Accurate quantitative estimation of these

drugs in bulk materials and pharmaceutical dosage forms is essential to ensure safety, efficacy, and consistency of pharmaceutical products. Furthermore, during manufacturing, storage,[5] and transportation, pharmaceutical formulations may undergo chemical degradation due to exposure to environmental factors such as heat, light, humidity, oxidation, and hydrolysis. The formation of degradation products can potentially affect therapeutic efficacy and patient safety. Therefore, development of stability-indicating analytical methods capable of separating active pharmaceutical ingredients from their degradation products is critically important in pharmaceutical analysis.[6]

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) is one of the most widely employed analytical techniques for pharmaceutical estimation because of its high sensitivity, selectivity, precision, reproducibility, and suitability for simultaneous multi-component analysis. RP-HPLC methods are extensively used in pharmaceutical industries for assay determination, impurity profiling, dissolution studies, stability testing, and quality control analysis.[7] Stability-indicating RP-HPLC methods are particularly valuable because they allow accurate quantification of active drugs in the presence of degradation products, impurities, and excipients without analytical interference.

A review of literature revealed that several analytical methods including UV spectrophotometry, HPTLC, LC-MS/MS, UPLC, and RP-HPLC methods have been reported for estimation of Ritonavir and Lopinavir either individually or in combination dosage forms.[8] However, many of the previously reported methods possess certain limitations such as longer run time, inadequate sensitivity, insufficient degradation profiling, complex mobile phase composition, higher solvent consumption, or lack of comprehensive validation according to International Council for Harmonisation (ICH) guidelines. Therefore, there remains a need for development of a simple, rapid, economical, accurate, precise, and stability-indicating RP-HPLC method suitable for simultaneous estimation of Ritonavir and Lopinavir in pharmaceutical dosage forms.[9]

Considering these analytical requirements, the present research work was undertaken to develop and validate a stability-indicating RP-HPLC method for simultaneous estimation of Ritonavir and Lopinavir in bulk drug and marketed tablet formulation. The developed method was optimized with respect to chromatographic conditions including mobile phase composition, pH, flow rate, detection wavelength, and column selection to achieve effective separation and satisfactory chromatographic performance. The proposed method was further validated according to ICH Q2(R1) guidelines for parameters such as system suitability, specificity, linearity, precision, accuracy, robustness, limit of detection, and limit of quantitation.[10] In addition, forced degradation studies were carried out under various stress conditions including acidic, alkaline, oxidative, thermal, and photolytic degradation to establish the stability-indicating capability of the method. The developed analytical method is expected to provide a reliable, reproducible, and efficient approach for routine quality control and stability analysis of Ritonavir and Lopinavir pharmaceutical formulations.

II. MATERIALS AND METHODS

Materials

Instruments used:

HPLC – Waters Model NO. E-2695 series Compact System Consisting of XTerra-C18 ODS column

UV spectrophotometer (Shimadzu)

Analytical Balance (Shimadzu)

Sonicator (Toshcon)

Chemicals used:

Methanol HPLC Grade, Buffer, Sodium hydroxide,Ortho Phosphoric acid, Hydrochloric acid, Peroxide

Methods

Characterization of Drugs

Ritonavir and Lopinavir were characterized prior to RP-HPLC method development to confirm their identity, purity, and physicochemical properties. The characterization studies included organoleptic evaluation, melting point determination, solubility studies, FT-IR spectroscopy, and UV–Visible spectroscopic analysis. These preliminary investigations ensured the authenticity and suitability of the drug samples for analytical method development.[11-12]

Organoleptic Evaluation

The organoleptic properties of Ritonavir and

Lopinavir such as color, appearance, texture, and odor were examined visually under normal laboratory conditions. The observed characteristics were matched with standard reference data to verify the identity and purity of the drugs.[13-15]

Determination of Physical Parameters

Melting point determination was performed with a digital melting point apparatus by adopting the capillary tube technique. The powdered samples were gradually heated, and the observed melting ranges were recorded to study purity and thermal behavior.

Solubility studies were performed in different solvents including distilled water, methanol, acetonitrile, ethanol, and phosphate buffer. Excess quantities of the drugs were added separately to each solvent and allowed to equilibrate at room temperature. The solubility behavior was observed visually and categorized qualitatively.[16]

FT-IR Spectroscopy

FT-IR spectroscopy was performed to identify the characteristic functional groups present in Ritonavir and Lopinavir. Drug samples were mixed with potassium bromide and compressed into pellets. The spectra were recorded in the range of 4000–400 cm^{-1} using an FT-IR spectrophotometer, and the obtained peaks were compared with reported reference spectra for confirmation of structural integrity.[17-19]

UV–Visible Spectroscopic Analysis

UV spectroscopic analysis was carried out to determine the absorption characteristics and wavelength of maximum absorbance (λmax) of Ritonavir and Lopinavir. Standard stock solutions of each drug were prepared and scanned in the wavelength range of 200–400 nm using a double-beam UV–Visible spectrophotometer. Ritonavir showed maximum absorbance at 238 nm, while Lopinavir exhibited λmax at 260 nm. These wavelengths were selected for further analytical studies.[20]

Preparation of Calibration Curve

Calibration curves were prepared by serial dilution of standard stock solutions to obtain suitable concentration ranges. The absorbance of each solution was measured at the respective λmax values. Calibration curves were constructed by plotting concentration against absorbance, and regression equations with correlation coefficients were determined to assess linearity.[21-22]

Instruments Used

UV spectroscopic analysis was performed using a double-beam UV–Visible spectrophotometer equipped with matched quartz cells. A calibrated digital analytical balance was used for accurate weighing of samples, and an ultrasonic bath sonicator was employed to ensure complete dissolution of drug samples during solution preparation.[23]

RP-HPLC Method Development

A stability-indicating RP-HPLC method was developed for the simultaneous estimation of Ritonavir and Lopinavir in bulk drugs and tablet dosage forms. The method was optimized by varying chromatographic parameters such as mobile phase composition, pH, flow rate, and detection wavelength to achieve satisfactory separation, peak symmetry, and reproducibility.[24]

Instrumentation

Chromatographic analysis was carried out using a WATERS 2695 HPLC system equipped with a quaternary pump, autosampler, and PDA detector. Separation was achieved using an XTerra C18 column (250 mm × 4.6 mm, 5 µm), and data acquisition was performed using Empower 2 software.[25]

Selection of Detection Wavelength

Standard solutions of Ritonavir and Lopinavir were scanned in the 200–400 nm range using a UV–Visible spectrophotometer. Based on the overlay spectra and adequate absorbance of both analytes, 229 nm was selected as the analytical wavelength for RP-HPLC analysis.[26-27]

Optimization of Chromatographic Conditions

Various combinations of aqueous buffers and organic solvents were investigated during method development. The optimized mobile phase consisted of 0.1% orthophosphoric acid (pH 2.8) and methanol in the ratio of 45:55 v/v, which produced well-resolved and symmetrical peaks.[28-29]

Table 1: Mobile Phase Optimization Trials

Trial No.	Aqueous Phase	Organic Phase	Ratio (v/v)	pH
1	Water	Methanol	60:40	—
2	Water	Acetonitrile	60:40	—
3	0.1% OPA	Methanol	50:50	3.5
4	0.1% OPA	Methanol	45:55	2.8
5	Phosphate Buffer	Acetonitrile	60:40	3.0–4.0

Preparation of Mobile Phase and Diluent

The mobile phase was prepared by combining 0.1% orthophosphoric acid buffer (pH 2.8) with methanol in a 45:55 (v/v) ratio. The prepared solution was filtered through a 0.45 µm membrane filter and subjected to sonication for degassing. A mixture of buffer and methanol in a 50:50 (v/v) ratio was used as the diluent.[30]

Preparation of Standard and Sample Solutions

Standard stock solutions of Ritonavir and Lopinavir were prepared in the diluent to achieve concentrations of 2000 µg/mL and 6000 µg/mL, respectively. Further dilution yielded working concentrations of 60 µg/mL for Ritonavir and 180 µg/mL for Lopinavir.

For sample preparation, powdered tablets equivalent to the required drug content were extracted using diluent, sonicated, filtered, and diluted appropriately to obtain working sample concentrations equivalent to the standard solution.[3]

Chromatographic Procedure

Prior to analysis, the HPLC system was equilibrated with the optimized mobile phase until a stable baseline was achieved. Standard and sample solutions were injected using a 10 µL injection volume. Chromatographic separation was carried out under isocratic conditions at a flow rate of 1.0 mL/min using an XTerra C18 column. Detection was performed at 229 nm and the column temperature was maintained at 30°C. The total run time was set at 10 minutes to achieve complete separation of Ritonavir, Lopinavir, and their degradation products.[32]

Table 2: Final Optimized Chromatographic Conditions

Parameter	Condition
Chromatographic Technique	RP-HPLC
Column	XTerra C18 (250 mm × 4.6 mm, 5 µm)
Mobile Phase	0.1% OPA (pH 2.8): Methanol (45:55 v/v)
Flow Rate	1.0 mL/min
Detection Wavelength	229 nm
Injection Volume	10 µL
Mode of Elution	Isocratic
Column Temperature	30°C
Run Time	10 minutes
Diluent	Buffer: Methanol (50:50 v/v)

System Suitability Testing

System suitability testing was carried out prior to sample analysis to ensure proper performance of the chromatographic system. The working standard solution of Ritonavir and Lopinavir was injected six times under optimized chromatographic conditions. System performance was assessed by evaluating parameters such as retention time, peak area, theoretical plate number, tailing factor, and resolution. System precision and reproducibility were determined by calculating the percentage relative standard deviation (%RSD) of peak areas.[33]

Forced Degradation Studies

Forced degradation studies were performed to confirm the stability-indicating nature of the developed RP-HPLC method. Ritonavir and Lopinavir were exposed to different stress conditions, including acidic, alkaline, oxidative, thermal, and photolytic degradation, in accordance with ICH guidelines. After stress treatment, the samples were neutralized when required, diluted with diluent, filtered through a 0.45 µm membrane filter, and subsequently analyzed under optimized chromatographic conditions.[34-35]

Acidic and Alkaline Hydrolysis

Acid degradation was performed using 0.1 N hydrochloric acid, while alkaline degradation was carried out using 0.1 N sodium hydroxide. After the stress period, the solutions were neutralized, diluted appropriately, filtered, and injected into the HPLC system.

Oxidative Degradation

Oxidative degradation studies were conducted using hydrogen peroxide solution. The stressed samples were diluted with diluent, filtered, and chromatographically analyzed.

Evaluation of Stability-Indicating Capability

The chromatograms obtained from stressed samples were carefully examined to evaluate peak purity, retention behavior, and separation between analyte peaks and degradation products. No interference from degradation products was observed at the retention times of Ritonavir and Lopinavir, confirming the specificity of the developed RP-HPLC method.[36-37]Method Validation

The RP-HPLC method developed for the simultaneous estimation of Ritonavir and Lopinavir was validated in

accordance with ICH Q2(R1) guidelines to establish its suitability for routine pharmaceutical analysis and stability studies.[38-44]

System Suitability

System suitability testing was performed prior to sample analysis to verify the satisfactory performance of the chromatographic system. A mixed working standard solution containing Ritonavir and Lopinavir was injected six times under optimized chromatographic conditions. Parameters such as retention time, peak area, theoretical plate count, tailing factor, and resolution between peaks were evaluated. The percentage relative standard deviation of peak areas was calculated to assess repeatability and system precision.

Specificity

Specificity of the developed method was established by analyzing blank, placebo, standard, and stressed sample solutions individually. The chromatograms were checked for possible interference at the retention times of Ritonavir and Lopinavir.

Precision

Precision studies were carried out to evaluate the repeatability and reproducibility of the method. System precision was assessed using replicate injections of the standard solution, while method precision was evaluated using independently prepared sample solutions. Accuracy

Accuracy of the method was evaluated using recovery studies based on the standard addition technique. Known quantities of Ritonavir and Lopinavir reference standards were added to previously analyzed sample solutions at different concentration levels. Each level was analyzed in three times under optimized chromatographic conditions.

Linearity and Range

The linearity of the method was evaluated by analyzing standard solutions of Ritonavir and Lopinavir across an appropriate concentration range. Calibration curves were constructed by plotting concentration against peak area response. The method demonstrated a direct proportional relationship between concentration and detector response with satisfactory correlation coefficient values, confirming excellent linearity within the selected range.

Stability of Analytical Solution

The stability of analytical solutions was evaluated by storing prepared sample solutions under room temperature and refrigerated conditions. The solutions were analyzed immediately after preparation and after specified storage intervals. The assay results indicated that both Ritonavir and Lopinavir remained stable in solution during the study period without significant changes in analytical response.

Limit of Detection and Limit of Quantitation

The sensitivity of the developed RP-HPLC method was evaluated by determining the limit of detection and limit of quantitation for both analytes. The obtained data confirmed that the method provides adequate sensitivity for the detection and quantification of Ritonavir and Lopinavir at low concentrations.

Robustness

The robustness of the method was evaluated by deliberately varying chromatographic parameters such as flow rate, mobile phase composition, and detection wavelength. The chromatographic performance and assay results remained unaffected by these minor changes, indicating the robustness and reliability of the method under varied analytical conditions.

Ruggedness

Ruggedness studies were carried out by performing the analysis under different laboratory conditions, including different analysts and different days of analysis. Consistent chromatographic performance and assay results confirmed the reproducibility and ruggedness of the developed method.

Analysis of Marketed Formulation

A commercially available tablet formulation containing Lopinavir and Ritonavir was selected to evaluate the applicability of the developed RP-HPLC method for routine pharmaceutical analysis. Lopimune-R® tablets manufactured by Cipla Ltd., India, containing Lopinavir 200 mg and Ritonavir 50 mg per tablet, were used for the study. Twenty tablets were accurately weighed and finely powdered to obtain a homogeneous mixture. An amount of powder equivalent to the labeled drug content was transferred into a volumetric flask and extracted using diluent consisting of buffer and methanol. The mixture was

sonicated to ensure complete extraction of both active pharmaceutical ingredients from the tablet matrix. After removing insoluble excipients and particle matter with a membrane filter, the resultant solution was diluted appropriately to achieve the necessary working concentration.[45-47]

Chromatographic Analysis

The prepared sample solution was analyzed under optimized chromatographic conditions using an XTerra C18 column with a mobile phase consisting of 0.1% orthophosphoric acid buffer and methanol. Prior to sample injection, the chromatographic system was equilibrated until a stable baseline was achieved. Blank and standard solutions were injected to confirm system suitability before analysis of the sample solution. Chromatograms were recorded and peak areas corresponding to Ritonavir and Lopinavir were measured.[48-49]

III. RESULTS AND DISCUSSION

Characterization of Active Pharmaceutical Ingredients

Pre-formulation characterization of Ritonavir and Lopinavir was carried out prior to RP-HPLC method development to confirm their identity, purity, and physicochemical suitability for analysis. The obtained results were found to be in agreement with reported standard values, indicating the suitability of both drugs for further analytical studies.

Organoleptic Evaluation

Both Ritonavir and Lopinavir were observed as fine crystalline powders with uniform texture and characteristic appearance. The samples were odorless and free from visible contamination or discoloration, indicating acceptable physical stability and purity.

Melting Point Determination

Melting point studies showed sharp and narrow melting ranges for both drugs, closely matching reported literature values. The absence of significant variation confirmed the purity and thermal stability of Ritonavir and Lopinavir.

Solubility Studies

Solubility studies revealed that both drugs were practically insoluble in water but showed good solubility in organic solvents such as methanol and acetonitrile. Moderate solubility was observed in ethanol and phosphate buffer. These findings supported the selection of suitable solvents and mobile phase components during RP-HPLC method development.

Overall, the characterization studies confirmed the authenticity, purity, and physicochemical suitability of Ritonavir and Lopinavir for development of a reliable and reproducible stability-indicating RP-HPLC analytical method.

FTIR Spectral Analysis

FTIR spectroscopy was performed to confirm the structural identity and functional group integrity of Ritonavir and Lopinavir prior to RP-HPLC method development. The spectra were recorded over the range of 4000–400 cm^{-1} using the KBr pellet technique. The observed absorption bands were compared with reported standard reference spectra for confirmation of molecular structure and purity.

FTIR Spectrum of Ritonavir

The FTIR spectrum of Ritonavir showed characteristic absorption peaks corresponding to various functional groups present in the drug molecule. Peaks observed in the region of 2924.09 cm^{-1} and 2855.72 cm^{-1} confirmed aliphatic C–H stretching vibrations. A strong absorption band at 1730.15 cm^{-1} indicated carbonyl stretching of amide or ester groups. Aromatic ring stretching vibrations were observed around 1618.28 cm^{-1} and 1502.55 cm^{-1}. The bands in the region of 1330.88–1018.40 cm^{-1} confirmed C–N and C–O stretching vibrations associated with amide and ester functionalities. Additional peaks in the fingerprint region represented aromatic bending and skeletal vibrations. The obtained spectrum was found to be in close agreement with standard reported spectra, confirming the identity, purity, and structural integrity of Ritonavir.

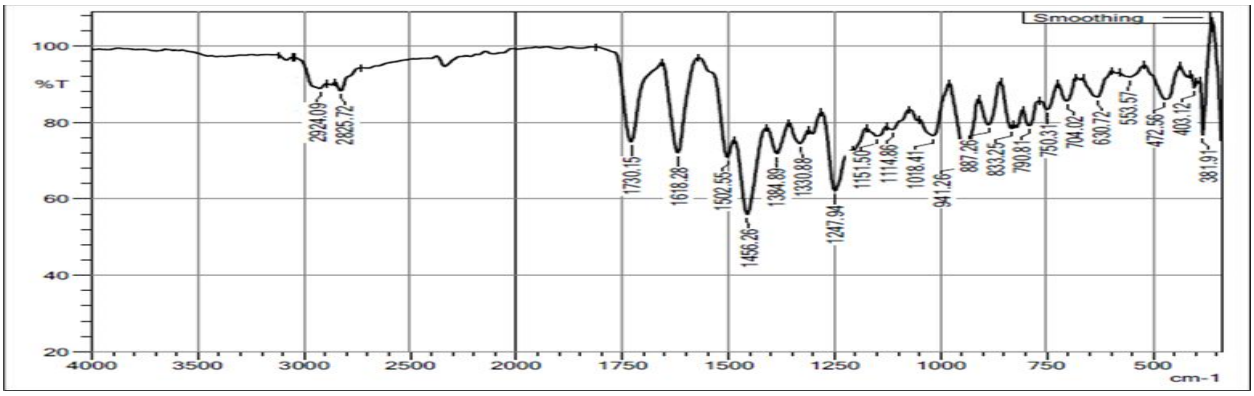

Figure1: FTIR Spectra of Ritonavir pure

FTIR Spectrum of Lopinavir

The FTIR spectrum of Lopinavir exhibited characteristic absorption bands confirming the presence of important functional groups within the molecule. A broad peak at 3323.35 cm^{-1} indicated N–H stretching vibrations of the amide group, while peaks at 2922.16 cm^{-1} and 2856.58 cm^{-1} represented aliphatic C–H stretching vibrations. Strong absorption bands at 1735.93 cm^{-1} and 1680.21 cm^{-1} confirmed ester and amide carbonyl stretching respectively. Peaks corresponding to amide linkages and aromatic functionalities were also observed in the expected regions. The fingerprint region showed characteristic molecular vibrations confirming the structural framework of the drug. The obtained FTIR spectrum closely matched reported standard spectra, thereby confirming the authenticity and purity of Lopinavir.

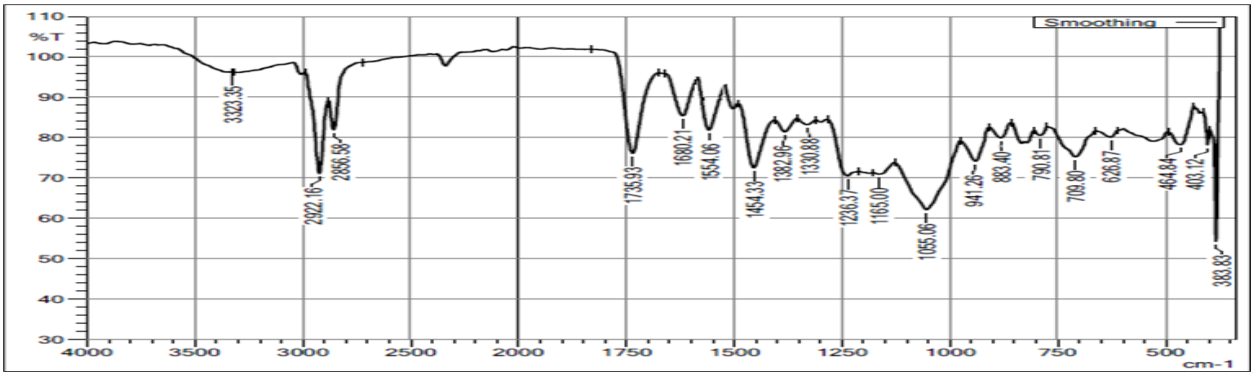

Figure2: FTIR Spectrum of Lopinavir pure

UV Spectroscopic Analysis

Determination of λ max

UV spectroscopic analysis was carried out to determine the wavelength of maximum absorbance (λmax) of Ritonavir and Lopinavir for subsequent analytical studies. Standard solutions of both drugs were scanned individually in the wavelength range of 200–400 nm using a UV–Visible spectrophotometer. Ritonavir exhibited maximum absorbance at 238 nm, whereas Lopinavir showed maximum absorbance at 260 nm.

Development of Standard Calibration Curve

Calibration curves for Ritonavir and Lopinavir were constructed by plotting concentration against absorbance values obtained at their respective λmax wavelengths.

Ritonavir exhibited excellent linearity with a regression equation of $y = 0.0098x + 0.0012$ and a correlation coefficient (R^2) value of 0.998. Similarly, Lopinavir showed linear behavior with a regression equation of $y = 0.0112x + 0.0040$ and an R^2 value of 0.9991.

The high correlation coefficient values confirmed compliance with Beer–Lambert's law and demonstrated the suitability of the developed UV spectroscopic method for quantitative estimation of

Ritonavir and Lopinavir in bulk and pharmaceutical dosage forms.

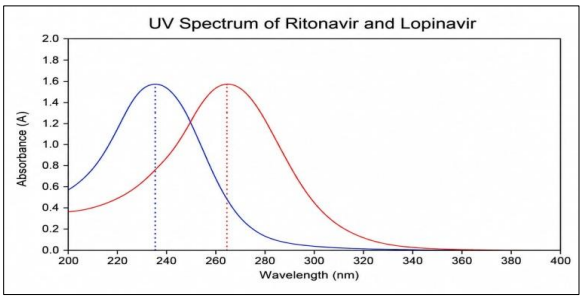

Figure 3: UV Spectrum showing λ max of Ritonavir at 238 nm and Lopinavirat 260 nm

RP-HPLC Method Development

The developed stability-indicating RP-HPLC method enabled the simultaneous determination of Ritonavir and Lopinavir in bulk drug and tablet dosage forms Different chromatographic conditions were optimized by varying mobile phase composition, pH, and organic modifiers to obtain sharp peaks, acceptable retention time, and good resolution.

Initial trials using water with methanol or acetonitrile produced poor peak symmetry and inadequate separation. Improved chromatographic performance was achieved using acidic buffer systems. The optimized chromatographic conditions consist of 0.1% orthophosphoric acid buffer (pH 2.8) and methanol in the ratio of 45:55 v/v using an XTerra C18 column (250 mm × 4.6 mm, 5 µm). The developed method produced symmetrical and well-resolved peaks with satisfactory theoretical plate count and minimal tailing.

Chromatographic analysis was performed with a WATERS 2695 HPLC system equipped with PDA detector and Empower 2 software. The developed method demonstrated good reproducibility, shorter run time, and suitability for routine quality control analysis.

Selection of Detection Wavelength

UV spectral analysis showed that Ritonavir and Lopinavir exhibited maximum absorbance at 238 nm and 260 nm respectively. However, 229 nm was selected as the common analytical wavelength for simultaneous estimation because both drugs showed sufficient absorbance at this wavelength with enhanced sensitivity and minimal mobile phase interference.

Overall, the optimized RP-HPLC method was found to be easy, accurate, precise, robust, economical, and stability-indicating for simultaneous estimation of Ritonavir and Lopinavir.

Forced Degradation Studies of Ritonavir and Lopinavir

Forced degradation studies were carried out under acidic, alkaline, oxidative, thermal, and photolytic stress conditions according to ICH guidelines to establish the stability-indicating capability of the developed RP-HPLC method. The chromatograms obtained under stress conditions demonstrated effective separation of degradation products from the principal analyte peaks without interference. Peak purity analysis confirmed that both Ritonavir and Lopinavir peaks remained spectrally pure under all stress conditions. Among the different stress conditions studied, alkaline hydrolysis produced maximum degradation, whereas thermal degradation showed the least degradation for both drugs.

Forced degradation studies confirmed the stability-indicating capability of the developed RP-HPLC method. Ritonavir and Lopinavir showed degradation under acidic, alkaline, oxidative, thermal, and photolytic stress conditions, with degradation products well separated from the main analyte peaks. Maximum degradation was observed under alkaline conditions, where Ritonavir and Lopinavir showed 10.85% and 11.18% degradation respectively, while minimum degradation was observed under thermal stress. The absence of interfering peaks confirmed the specificity and selectivity of the method. Overall, the developed RP-HPLC method was found to be accurate, specific, and suitable for stability analysis and routine quality control of Ritonavir and Lopinavir formulations.

Table 3: Forced Degradation Study Parameters of Ritonavir

Sr. No.	Degradation Condition	Peak Area	% Recovered	% Degraded
1	Acidic Degradation	1845624	92.54	7.46
2	Alkaline Degradation	1778142	89.15	10.85
3	Oxidative Degradation	1912035	95.87	4.13
4	Thermal Degradation	1941848	97.36	2.64
5	Photolytic (UV) Degradation	1864577	93.49	6.51

Table 4: Forced Degradation Study Parameters of Lopinavir

Sr. No.	Degradation Condition	Peak Area	% Recovered	% Degraded
1	Acidic Degradation	1063548	93.28	6.72
2	Alkaline Degradation	1012746	88.82	11.18
3	Oxidative Degradation	1094835	96.03	3.97
4	Thermal Degradation	1112054	97.54	2.46
5	Photolytic (UV) Degradation	1074421	94.23	5.77

Method Development by RP-HPLC

An RP-HPLC method was successfully developed for simultaneous estimation of Ritonavir and Lopinavir by optimizing mobile phase composition, pH, and chromatographic conditions. Initial trials showed poor resolution and peak tailing, whereas acidic mobile phase conditions significantly improved peak symmetry and separation.

The optimized conditions consisted of 0.1% orthophosphoric acid buffer (pH 2.8) and methanol (45:55 v/v) on an XTerra C18 column, which produced sharp, well-resolved peaks with satisfactory retention time and system suitability parameters.

Table 5: Various Trials and Optimization of Chromatographic Conditions

Trial No.	HPLC System	Chromatographic Conditions	Observations	Remarks
1	WATERS 2695 HPLC with PDA detector	Mobile Phase: Water: Methanol (70:30) Column: XTerra C18 (250 × 4.6 mm, 5 µm) Flow rate: 1 mL/min Wavelength: 229 nm	Broad peaks with poor resolution	Rejected
2	WATERS 2695 HPLC with PDA detector	Mobile Phase: Water: Acetonitrile (70:30) Column: XTerra C18 (250 × 4.6 mm, 5 µm) Flow rate: 1 mL/min Wavelength: 229 nm	Peak tailing observed	Rejected
3	WATERS 2695 HPLC with PDA detector	Mobile Phase: Phosphate Buffer (pH 4.5): Methanol (50:50) Column: XTerra C18 (250 × 4.6 mm, 5 µm) Flow rate: 1 mL/min Wavelength: 229 nm	Peaks separated but poor peak symmetry	Rejected
4	WATERS 2695 HPLC with PDA detector	Mobile Phase: 0.1% OPA: Methanol (50:50) Column: XTerra C18 (250 × 4.6 mm, 5 µm) Flow rate: 1 mL/min Wavelength: 229 nm	Improved peak shape and resolution	Accepted for further optimization
5	WATERS 2695 HPLC with PDA detector	Mobile Phase: 0.1% OPA (pH 2.8): Methanol (45:55) Column: XTerra C18 (250 × 4.6 mm, 5 µm) Flow rate: 1 mL/min Wavelength: 229 nm	Sharp peaks with excellent resolution and acceptable retention time	Optimized

Blank Chromatogram

The blank chromatogram obtained using diluent showed no interfering peaks at the retention times of Ritonavir and Lopinavir.

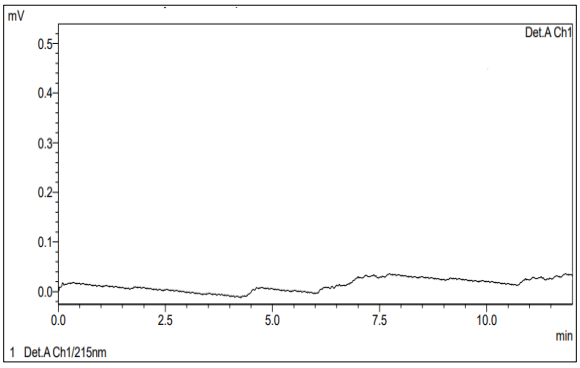

Figure 4: Blank Chromatogram

Optimized Chromatographic Conditions

Final optimization was achieved using 0.1% orthophosphoric acid buffer (pH 2.8) and methanol in the ratio of 45:55 v/v at a flow rate of 1 mL/min. Under these chromatographic The developed stability-indicating RP-HPLC method enabled the simultaneous determination of Ritonavir and Lopinavir in bulk drug and tablet dosage formsThe developed stability-indicating RP-HPLC method enabled the simultaneous determination of Ritonavir and Lopinavir in bulk drug and tablet dosage formsThe developed stability-indicating RP-HPLC method enabled the simultaneous determination of Ritonavir and Lopinavir in bulk drug and tablet dosage formsThe developed stability-indicating RP-HPLC method enabled the simultaneous determination of Ritonavir

and Lopinavir in bulk drug and tablet dosage formsThe developed stability-indicating RP-HPLC method enabled the simultaneous determination of Ritonavir and Lopinavir in bulk drug and tablet dosage formsThe developed stability-indicating RP-HPLC method enabled the simultaneous determination of Ritonavir and Lopinavir in bulk drug and tablet dosage formsconditions, both Ritonavir and Lopinavir exhibited sharp, symmetrical, and well-resolved peaks with acceptable retention time and baseline stability.

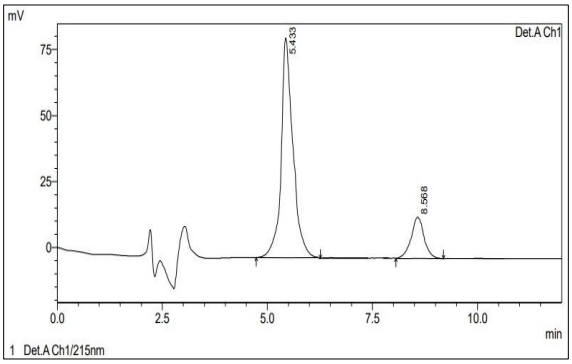

Figure 5: Optimized HPLC Chromatogram of Ritonavir and Lopinavir

System Suitability and Specificity

The developed RP-HPLC method showed satisfactory system suitability parameters for both Ritonavir and Lopinavir. Retention time, peak area, tailing factor, and theoretical plate count were found within acceptable limits, indicating good chromatographic performance and reproducibility. The retention times of Ritonavir and Lopinavir were observed at approximately 5.4 min and 8.6 min, respectively. Specificity studies confirmed the absence of interference from blank and excipient peaks at the retention times of both analytes, demonstrating the specificity of the developed method.

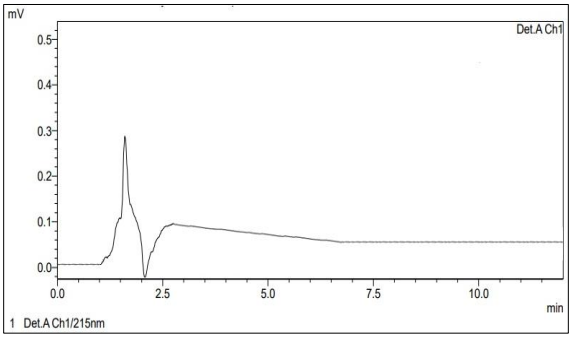

Figure 6: Blank Chromatogram

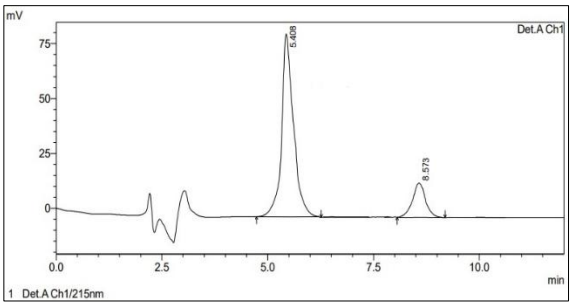

Figure 6: System SuitabilityChromatogram of Standard Ritonavir and Lopinavir 5

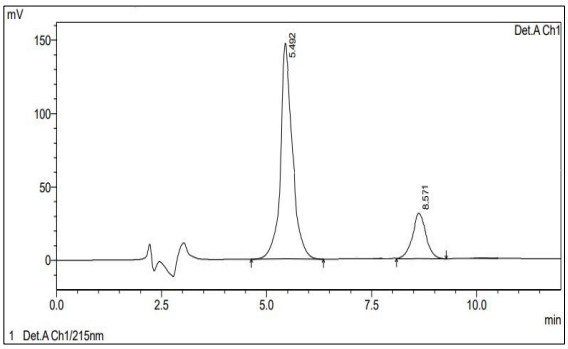

Figure 7: Standard Chromatogram of Mixture of Ritonavir and Lopinavir

Precision

System precision and method precision studies showed %RSD values below 2% for both Ritonavir and Lopinavir, confirming excellent repeatability and precision of the method. Intraday and inter-day precision studies at different concentration levels also produced acceptable %RSD values within the specified limits, indicating good intermediate precision and reproducibility.

Table 6: System Precision Data of Ritonavir and Lopinavir

Sr.No.	Peakareasof RTV	Peakareasof LPV
1	3781654	1046827
2	3786584	1045762
3	3695201	1086384
4	3756484	1073268
5	3786879	1056891
6	3687549	1048967
Mean	3749058.50	1059683.16
SD (±)	46130.69	16608.40
RSD (%)	1.230	1.567
Acceptance criteria	% RSDshouldnotbemorethan2	

Accuracy

Accuracy of the method was evaluated by recovery studies using the standard addition method at 80%, 100%, and 120% levels. The mean percentage recovery was found to be approximately 100.25% for Ritonavir and 99.80% for Lopinavir, confirming the accuracy and reliability of the developed method.

Table 7: Recovery study for Ritonavir and Lopinavir

Ritonavir							
Level	Set	Amount added(µg/ml)	Amount found(µg/ml)	%Recovery	Mean	SD	%RSD
80%	1	100	99.94	100.06	100.45	0.769	0.765
	2	100	100.05	99.95			
	3	100	98.68	101.33			
100%	1	200	199.96	100.02	100.30	0.391	0.390
	2	200	199.73	100.13			
	3	200	198.52	100.74			
120%	1	300	299.84	100.05	100.01	0.058	0.058
	2	300	300.17	99.94			
	3	300	299.88	100.04			
Lopinavir							
Level	Set	Amount added(µg/ml)	Amount found(µg/ml)	%Recovery	Mean	SD	%RSD
80%	1	10	9.93	99.36	99.46	1.017	1.023
	2	10	9.84	98.49			
	3	10	10.05	100.52			
100%	1	20	19.99	99.95	100.09	0.152	0.152
	2	20	20.01	100.07			
	3	20	20.05	100.25			
120%	1	30	29.95	99.86	99.96	0.100	0.100
	2	30	30.01	100.06			
	3	30	29.98	99.95			

Linearity and Range

The method exhibited good linearity over the concentration range of 100–500 µg/mL for Ritonavir and 10–50 µg/mL for Lopinavir. The calibration curves showed excellent correlation coefficients, indicating a direct proportional relationship between concentration and peak area.

Table 8: Linearity and Range for Ritonavir and Lopinavir

oncentrationi nµg/mlfor RTV	agePeakArea*	oncentrationin µg/mlfor LPV	age PeakArea*
100	1986815	10	515479
200	3785275	20	1058223
300	5806467	30	1499394
400	7882647	40	2057241
500	9996578	50	2541361
Slope	19912	Slope	50781
CC	68340	CC	9097.4

Stability, Sensitivity, Robustness, and Ruggedness

Solution stability studies confirmed that both drugs remained stable for 24 hours under refrigerated and room temperature conditions. The developed method demonstrated adequate sensitivity with low LOD and LOQ values for both analytes. Robustness studies performed by varying flow rate and chromatographic conditions showed no significant changes in chromatographic performance, as %RSD values remained below 2%. Ruggedness studies under different environmental conditions also confirmed the reproducibility and reliability of the method.

Table 9: Data of Ruggedness for Ritonavir and Lopinavir

RTV				
Change in Parameters	Area of Standard	Mean	SD	%RSD
25°C	1987364	1974169	15649.50	0.792
	1978263			
	1956879			

37°C	3786389	3788685	2569.21	0.067
	3791460			
	3788206			
60 °C	5808637	5841052	65425.70	1.120
	5798163			
	5916357			
LPV				
Change in Parameters	Area of Standard	Mean	SD	%RSD
25°C	515624	516660	4377.37	0.847
	521462			
	512893			
37°C	1059351	1058790	959.582	0.090
	1057682			
	1059337			
60 °C	1495226	145547	1871.72	0.125
	1493856			
	1497558			

Analysis of Marketed Formulation

The developed RP-HPLC method was successfully applied for simultaneous estimation of Ritonavir and Lopinavir in marketed tablet formulation. Well-resolved and symmetrical peaks were obtained without interference from excipients, confirming the specificity of the method. The retention times of Ritonavir and Lopinavir were found to be 5.485 min and 8.534 min, respectively. The assay values were 97.31% for Ritonavir and 98.43% for Lopinavir, which were within acceptable pharmacopeial limits. The tailing factor and theoretical plate count values indicated satisfactory chromatographic performance and column efficiency. Overall, the developed method was found to be accurate, precise, reproducible, and suitable for routine quality control analysis of combined Ritonavir and Lopinavir tablet dosage forms.

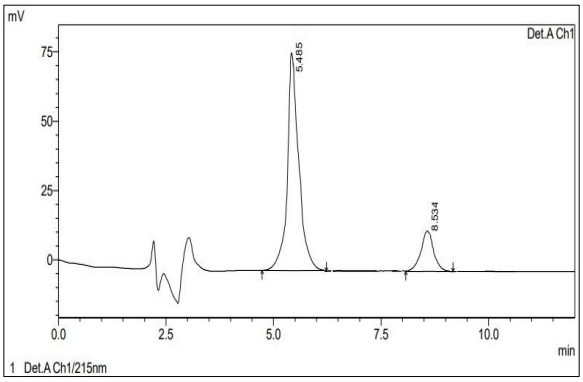

Figure 8: Chromatogram of Marketed Formulation

Table 10: Evaluation Parameters of Marketed Formulation

Sr. No.	Name	Retention Time (min)	Area (µV*sec)	Tailing Factor	Theoretical Plate Count	% Assay
1	Ritonavir	5.485	1933369	1.121	245.203	97.31
2	Lopinavir	8.534	507386	1.284	2367.764	98.43

IV. CONCLUSION

The present study successfully developed and validated a simple, accurate, precise, robust, and stability-indicating RP-HPLC method for simultaneous estimation of Ritonavir and Lopinavir in bulk drug and pharmaceutical tablet dosage forms. The optimized chromatographic conditions provided efficient separation with satisfactory peak symmetry, retention time, and reproducibility. Forced degradation studies confirmed the stability-indicating capability of the method by effectively separating degradation products formed under acidic, alkaline, oxidative, thermal, and photolytic stress conditions. Validation studies performed according to ICH guidelines demonstrated acceptable specificity, precision, accuracy, linearity, robustness, ruggedness, sensitivity, and reproducibility of the developed method. The developed RP-HPLC method was successfully applied to marketed tablet formulation analysis and produced assay values within acceptable pharmacopeial limits. Therefore, the proposed method can be effectively employed for routine quality control analysis, stability studies, and pharmaceutical analysis of Ritonavir and Lopinavir in combined dosage forms.

V. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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