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Method Development And Validation For The Simultaneous Determination Of Lopinavir And Ritonavir By Using Rp-Hplc

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Abstract: A simple, rapid, precise, and sensitive RP-HPLC method was developed and validated for the simultaneous quantification of Lopinavir and Ritonavir in pharmaceutical dosage forms. Separation was achieved on a Waters Symmetry Shield RP-18 (150 × 4.6 mm, 3.5 µm) column using a mobile phase of Methanol: Heptane Sulfonic acid buffer (pH 2.5, adjusted with OPA) in a 10:90 v/v ratio, at a flow rate of 1.0 mL/min. Detection was performed at 236 nm using a PDA detector. The method exhibited theoretical plates >2000 and tailing factors <2 for both drugs, with %RSD <2.0. Validation as per ICH guidelines confirmed the method's accuracy, precision, robustness, and suitability for routine quantitative and stability studies of Lopinavir and Ritonavir.

Index Terms **-** HPLC, Lopinavir and Ritonavir.

I. INTRODUCTION

Analytical chemistry plays a vital role in the pharmaceutical industry by ensuring the safety, quality, and efficacy of drug formulations. Among the various analytical tools, High-Performance Liquid Chromatography (HPLC) has become a method of choice for the accurate and reliable quantification of active pharmaceutical ingredients (APIs) and impurities. Reverse-Phase HPLC (RP-HPLC), in particular, is widely used because of its high resolution, reproducibility, and suitability for separating compounds with varying polarities in a single run [1].

Lopinavir and Ritonavir are antiretroviral drugs belonging to the class of protease inhibitors, widely used in combination therapy for the treatment of Human Immunodeficiency Virus (HIV-1) infection. Lopinavir acts by inhibiting the HIV-1 protease enzyme, preventing the cleavage of viral polyproteins into functional proteins required for viral replication. Ritonavir, on the other hand, functions both as a protease inhibitor and as a pharmacokinetic enhancer by inhibiting cytochrome P450 3A4 (CYP3A4), thereby reducing the metabolic degradation of lopinavir and increasing its plasma concentration. This combination, marketed as *Kaletra*, has proven highly effective as part of highly active antiretroviral therapy (HAART). Ensuring the quality and stability of these fixed-dose combinations is essential for maintaining therapeutic efficacy. Because both drugs are co-formulated, simultaneous estimation becomes necessary for routine quality control, dosage accuracy, and stability studies [2-5]. Conventional analytical techniques such as UV spectrophotometry and TLC lack the sensitivity and selectivity needed for complex formulations, whereas RP-HPLC provides superior separation, accuracy, and precision. Several RP-HPLC methods have been reported for the simultaneous estimation of Lopinavir and Ritonavir using different stationary phases, mobile phase compositions, and detection wavelengths. Most studies achieved good resolution but required long run times or complex mobile phases. Therefore, there is a continuous need to develop a simple, rapid, and economical method that can produce accurate and reproducible results under routine laboratory conditions. Method development in RP-HPLC involves the systematic selection and optimization of chromatographic parameters such as stationary phase, mobile phase composition, pH, flow rate, and detection wavelength to achieve efficient separation and

peak symmetry. Validation of the developed method according to International Council for Harmonisation (ICH) guidelines is crucial to confirm its reliability for analytical use. Validation parameters such as accuracy, precision, specificity, linearity, robustness, limit of detection (LOD), and limit of quantitation (LOQ) ensure that the method performs consistently and accurately within the desired range. In the present study, a simple, sensitive, and stability-indicating RP-HPLC method was developed and validated for the simultaneous quantification of Lopinavir and Ritonavir in bulk and tablet dosage forms. Chromatographic separation was achieved using a Waters Symmetry Shield RP-18 column (150 × 4.6 mm, 3.5 µm) with a mobile phase consisting of Methanol and Heptane Sulfonic acid buffer (pH 2.5, adjusted with O-phosphoric acid) in the ratio of 10:90 v/v [6-9]. The flow rate was maintained at 1 mL/min, and detection was carried out at 236 nm using a photodiode array detector under ambient temperature. The method produced sharp, well-resolved peaks with theoretical plate counts exceeding 2000 and tailing factors less than 2 for both analytes [10].

The method was validated as per ICH Q2(R1) guidelines for parameters including system suitability, linearity, precision, accuracy, robustness, and sensitivity. The %RSD values were found to be less than 2%, indicating excellent precision. The low LOD and LOQ values confirmed the method's sensitivity, while deliberate variations in chromatographic conditions demonstrated its robustness. Forced degradation studies under acidic, basic, oxidative, photolytic, and thermal conditions further confirmed the stability-indicating nature of the developed method, as degradation peaks were well separated from those of the parent compounds. Thus, the developed RP-HPLC method provides a reliable, efficient, and economical approach for the routine analysis and stability evaluation of Lopinavir and Ritonavir in bulk and pharmaceutical formulations. The simplicity and reproducibility of this method make it highly suitable for quality control laboratories and regulatory compliance testing. By ensuring accurate quantification of both drugs in combined dosage forms, the proposed method supports consistent therapeutic efficacy and enhances pharmaceutical quality assurance in antiretroviral therapy [11-13].

II. MATERIALS AND METHODS

Instruments and Equipment

All chromatographic analyses were performed on a Waters Alliance e2695 HPLC system equipped with a quaternary pump, auto sampler, column oven, and a photodiode array (PDA) detector. Data acquisition and processing were performed using Empower 3 software. The separation was achieved using a Waters Symmetry Shield RP-18 column (150 × 4.6 mm, 3.5 µm). Other instruments used included a digital pH meter, ultrasonic bath (PCI Analytics), and a vacuum filtration assembly fitted with 0.45 µm nylon filters.

Table 1: List of Instruments used in the Study

S. No.	Instrument	Model/Make
1	HPLC System	Waters Alliance e2695
2	Detector	Photodiode Array (PDA)
3	Column	Waters Symmetry Shield RP-18 (150 × 4.6 mm, 3.5 µm)
4	Sonicator	PCI Ultrasonic Cleaner
5	pH Meter	Elico Digital pH Meter
6	Filtration Unit	Millipore Vacuum Filter Assembly
7	Software	Empower 3 HPLC Software

Table 2: List of Chemicals and Reagents

S. No.	Chemical/Reagent	Grade/Source
1	Lopinavir	CMS Laboratories, Hyderabad
2	Ritonavir	CMS Laboratories, Hyderabad
3	Methanol	HPLC Grade, Merck Pvt. Ltd.
4	Heptane Sulfonic Acid	Analytical Grade, Merck
5	Orthophosphoric Acid	AR Grade, Merck Pvt. Ltd.
6	Milli-Q Water	In-house purification system

The chromatographic method was optimized after several trials to achieve the best separation of Lopinavir and Ritonavir. The analysis was performed using a Waters Alliance e2695 HPLC system equipped with a PDA detector and a Waters Symmetry Shield RP-18 column (150 × 4.6 mm, 3.5 µm). The mobile phase consisted of Methanol and Heptane Sulfonic acid (pH 2.5, adjusted with OPA) in a 10:90 ratio, filtered through a 0.45 µm membrane filter. The flow rate was maintained at 1 mL/min, the detection wavelength was 236 nm, the injection volume was 10 µL, and the total run time was 6 minutes. Acetonitrile was used as the diluent.

For the preparation of the standard solution, 150 mg of Lopinavir and 100 mg of Ritonavir were accurately weighed, dissolved in the diluent, and sonicated to ensure complete dissolution before being diluted to volume (stock solution). Five millilitres of this stock were further diluted to 50 mL to obtain a working standard containing 150 ppm of Lopinavir and 100 ppm of Ritonavir. Similarly, the sample solution was prepared by dissolving 247 mg of Lopinavir and 199 mg of Ritonavir in the diluent, sonicated for 30 minutes, centrifuged, filtered through a 0.45 µm membrane filter, and diluted in the same manner to achieve the same working concentration.

Under these optimized conditions, Lopinavir and Ritonavir showed sharp, well-resolved peaks at retention times of 2.938 min and 3.886 min, respectively, with peak responses of 3,581,428 and 2,464,821, tailing factors of 1.19 and 1.07, and a resolution of 4.79. System suitability parameters met the acceptance criteria, with tailing factors <2.0, theoretical plates >2000, and resolution >2, confirming the suitability of the developed RP-HPLC method for simultaneous estimation of Lopinavir and Ritonavir.

III. RESULTS AND DISCUSSION

Optimized Chromatographic Method

The chromatographic method was optimized in Trial-6, demonstrating suitability for validation as all parameters were within limits. The analysis was performed using a Waters HPLC system equipped with an autosampler and PDA detector, employing a Waters Symmetry Shield RP-18 column (150 × 4.6 mm, 3.5 µm) in isocratic mode. The mobile phase consisted of Methanol and Heptane Sulfonic acid (pH 2.5, adjusted with OPA) in a 10:90 ratio. The flow rate was 1 mL/min, injection volume 10 µL, detection wavelength 236 nm, runtime 6 min, and temperature maintained at ambient (25°C). The method provided precise and efficient separation of Lopinavir and Ritonavir within a short runtime, suitable for routine quality control.

Table 3: Optimized Chromatographic Conditions

Parameter	Observation
Instrument	Waters HPLC with autosampler & PDA detector
Column	Waters Symmetry Shield RP-18 (150 × 4.6 mm, 3.5 µm)
Mobile Phase	Methanol: Heptane Sulfonic acid pH 2.5 (10:90)
Flow Rate	1 mL/min
Injection Volume	10 µL
Wavelength	236 nm
Runtime	6 min
Temperature	Ambient (25°C)
Mode	Isocratic

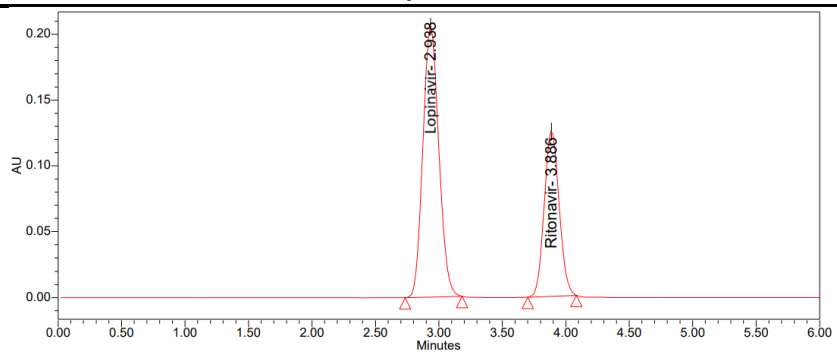

Figure 1: Optimized chromatogram of Lopinavir & Ritonavir (Trial-6)

System Suitability

System suitability parameters for Lopinavir and Ritonavir met ICH requirements with tailing factors <2, plate counts >2000, and resolution >2. Lopinavir and Ritonavir exhibited retention times of 2.938 and 3.886 min, respectively, with tailing factors of 1.19 and 1.07, resolution of 4.79, and %RSD <0.5.

Table 4: System Suitability Parameters

Parameter	Lopinavir	Ritonavir
Retention Time (min)	2.938	3.886
Plate Count	12844	15574
Tailing Factor	1.19	1.07
Resolution	—	4.79
%RSD	0.34	0.27

Analytical Method Validation

Precision: System precision and repeatability were confirmed with %RSD values for Lopinavir (0.34–0.69%) and Ritonavir (0.27–0.87%), meeting acceptance criteria (≤2%). Intermediate precision across two days also showed consistent results (%RSD ≤0.75%).

Linearity: Linearity was established over 25–150% of the target concentration. Lopinavir and Ritonavir exhibited correlation coefficients (R^2) of 0.99917 and 0.99978, respectively, confirming a strong linear relationship.

Accuracy: Recovery studies at 50%, 100%, and 150% concentration levels showed mean recoveries of 99.6% for Lopinavir and 99.9% for Ritonavir, within the acceptable range of 98–102%.

Robustness: Minor changes in flow rate (0.9–1.1 mL/min) and organic phase composition (9:91–11:89) did not significantly affect retention time, resolution, tailing factor, theoretical plates, or %RSD, indicating robustness.

Sensitivity: LOD and LOQ were determined as 0.45 µg/mL and 1.50 µg/mL for Lopinavir, and 0.30 µg/mL and 1.00 µg/mL for Ritonavir, meeting S/N ratio requirements of 3:1 and 10:1, respectively.

Discussion

The optimized RP-HPLC method for simultaneous estimation of Lopinavir and Ritonavir demonstrated high precision, accuracy, and robustness, confirming its suitability for routine quality control. The system suitability parameters, including retention times, plate counts, tailing factors, and resolution, met ICH criteria, ensuring reliable peak separation and consistent chromatographic performance. Precision studies, including system, method, and intermediate precision, showed %RSD well below 2%, indicating excellent repeatability and reproducibility across different days. Linearity assessment revealed strong correlation coefficients ($R^2 > 0.999$) over a wide concentration range, validating the method's proportional response. Accuracy studies through recovery experiments at multiple concentration levels yielded recoveries within 98–102%, confirming the method's reliability. Robustness testing showed minimal effect of minor variations in flow rate and mobile phase composition on chromatographic performance. The method's sensitivity, with

low LOD and LOQ values, allows detection and quantification of trace concentrations. Overall, this validated method is precise, accurate, robust, and suitable for routine pharmaceutical analysis of Lopinavir and Ritonavir.

IV. CONCLUSION

The developed RP-HPLC method for simultaneous estimation of Lopinavir and Ritonavir is precise, accurate, and robust, meeting all ICH validation criteria. Optimized chromatographic conditions ensured sharp, well-resolved peaks with acceptable retention times, tailing factors, plate counts, and resolution. System, method, and intermediate precision studies demonstrated excellent repeatability and reproducibility, while linearity and accuracy assessments confirmed reliable quantification over a wide concentration range. Robustness testing indicated the method's resilience to minor variations in chromatographic parameters. Additionally, low LOD and LOQ values highlight its sensitivity. This method is rapid, reliable, and suitable for routine quality control and analytical applications.

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