ejpmr, 2025, 12(7), 188-195

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

SJIF Impact Factor 7.065

Research Article

ISSN (O): 2394-3211

ISSN (P): 3051-2573

METHOD DEVELOPMENT AND METHOD VALIDATION BY RP-HPLC OF LOPINAVIR AND RITONAVIR

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Article Received on 11/05/2025 **Article Revised on 01/06/2025** **Article Accepted on 22/06/2025**

ABSTRACT

A reverse phase liquid chromatographic method for estimation of Lopinavir and Ritonavir in bulk drugs and marketed pharmaceutical dosage form was developed and validated. The chromatographic conditions to achieve the highest performance parameters using Sunfire C $_{18}$ (4.6×250mm) 5µm Column with guard filter were optimized. The separation was carried out using a mobile phase containing Acetonitrile: Water (40:60% v/v) in the ratio of 40:60% v/v pumped at a flow rate of 0.9 mL/min with detection at 220 nm. The method was shown to be linear in 5–25 µg/mL and 75–375 µg/mL concentration range (regression coefficients of 0.9995 and 0.9982) for Lopinavir and Ritonavir respectively. The limit of detection (LOD) and limit of quantification (LOQ) was found to be 0.7µg/ml and 2.1µg/ml & 13.8µg/ml and 41.8µg/ml for Lopinavir and Ritonavir respectively. The accuracy of the method was assessed by adding fixed amount of pre- analyzed sample to different standard solutions (50%, 100%, and 150% of the tested concentration) in triplicate. The percentage mean recoveries were found to 98%-102%. The method was found to be precise with %RSD value was found to be within the limits for intraday and interday precision study, respectively. The method specificity and robustness were also established. New and sensitive RP-HPLC method for estimation of Lopinavir and Ritonavir has been developed, in respect to the reviewed analytical methods.

KEYWORDS: Lopinavir and Ritonavir, RP-HPLC, Accuracy, Precision, Robustness.

INTRODUCTION

Ritonavir is an HIV protease inhibitor that interferes with the reproductive cycle of HIV. Although it was initially developed as an independent antiviral agent, it has been shown to possess advantageous properties in combination regimens with low-dose ritonavir and other protease inhibitors. It is now more commonly used as a booster of other protease inhibitors and is available in both liquid formulations and as capsules.

While ritonavir is not an active antiviral agent against hepatitis C virus (HCV) infection, it is added in combination therapies indicated for the treatment of HCV infections as a booster. Ritonavir is a potent CYP3A inhibitor that increases peak and trough plasma drug concentrations of other protease inhibitors such as Paritaprevir and overall drug exposure. American Association for the Study of Liver Diseases (AASLD) and the Infectious Diseases Society of America (IDSA) guidelines recommend ritonavir-boosted combination therapies as first-line therapy for HCV Genotype 1a/b and 4 treatment-naïve patients with or without cirrhosis.
Lopinavir is an antiretroviral protease inhibitor used in combination with other antiretrovirals in the treatment of HIV-1 infection. Lopinavir is marketed and administered exclusively in combination with ritonavir - this combination, first marketed by Abbott under the brand name Kaletra in 2000, is necessary due to lopinavir's poor oral bioavailability and extensive biotransformation. Ritonavir is a potent inhibitor of the enzymes responsible for lopinavir metabolism, and its co-administration "boosts" lopinavir exposure and improves antiviral activity.[7] Like many other protease inhibitors (e.g. saquinavir, nelfinavir), lopinavir is a peptidomimetic molecule - it contains a hydroxyethylene scaffold that mimics the peptide linkage typically targeted by the HIV-1 protease enzyme but which itself cannot be cleaved, thus preventing the activity of the HIV-1 protease.

Lopinavir was previously under investigation in combination with ritonavir for the treatment of COVID-19 caused by SARS-CoV-2.

EXPERIMENTAL WORK

INSTRUMENTS USED

Instruments used

S.No.	Instruments and Glasswares	Model
1	HPLC	Shimadzu LC- AT VP With SPD-10A VP UV-Visible Detector, Software: Autochro data module
2	pH meter	Labindia
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital Ultra Sonicator	Labman

CHEMICALS USED

Materials Used

S.No.	Chemical	Brand Names
1	Lopinavir	AR labs
2	Ritonavir	AR labs
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck
5	Triethylamine	Merck

HPLC METHOD DEVELOPMENT

Preparation of standard solution

Accurately weigh and transfer 10 mg of Lopinavir and Ritonavir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.15ml of the Lopinavir and 2.25ml of the Ritonavir stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization

Initially the mobile phase tried was Methanol: Water and Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Acetonitrile: Water in proportion 40:60 v/v respectively.

Optimization of Column

The method was performed with various columns like Symmetry, C18 (4.6×150mm, 5µ) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Instrument used	:	Shimadzu LC- 10 AT VP.
Temperature	:	Ambient
Column	:	C18 (4.6×250mm) 5µ
Mobile phase	:	Acetonitrile: Water (40:60v/v)
Flow rate	:	0.9ml/min
Wavelength	:	220nm
Injection volume	:	10ml
Run time	:	6min

VALIDATION

PREPARATION OF MOBILE PHASE

Preparation of mobile phase

Accurately measured 600ml (60%) of Water, 400ml of Acetonitrile (40%) were mixed and degassed in digital ultra sonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

VALIDATION PARAMETERS SYSTEM SUITABILITY

Accurately weigh and transfer 10 mg of Lopinavir and 10mg of Ritonavir working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the Lopinavir and 2.25ml of the Ritonavir stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

SPECIFICITY STUDY OF DRUG

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Lopinavir and 10mg of Ritonavir working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the Lopinavir and 2.25ml of the Ritonavir stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution

Take average weight of Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Lopinavir and Ritonavir sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 2.25ml of the Sample stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

PREPARATION OF DRUG SOLUTIONS FOR LINEARITY

Accurately weigh and transfer 10 mg of Lopinavir and 10mg of Ritonavir working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (5ppm of Lopinavir & 75ppm of Ritonavir)

Pipette out 0.05ml of Lopinavir and 0.75ml of Ritonavir stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (10ppm of Lopinavir& 150ppm of Ritonavir)

Pipette out 0.1ml of Lopinavir and 1.5ml of Ritonavir stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (15ppm of Lopinavir& 225ppm of Ritonavir)

Pipette out 0.15ml of Lopinavir and 2.25ml of Ritonavir stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (20ppm of Lopinavir& 300ppm of Ritonavir)

Pipette out 0.2ml of Lopinavir and 3.0ml of Ritonavir stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (25ppm of Lopinavir& 375ppm of Ritonavir)

Pipette out 0.25ml of Lopinavir and 3.75ml of Ritonavir stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

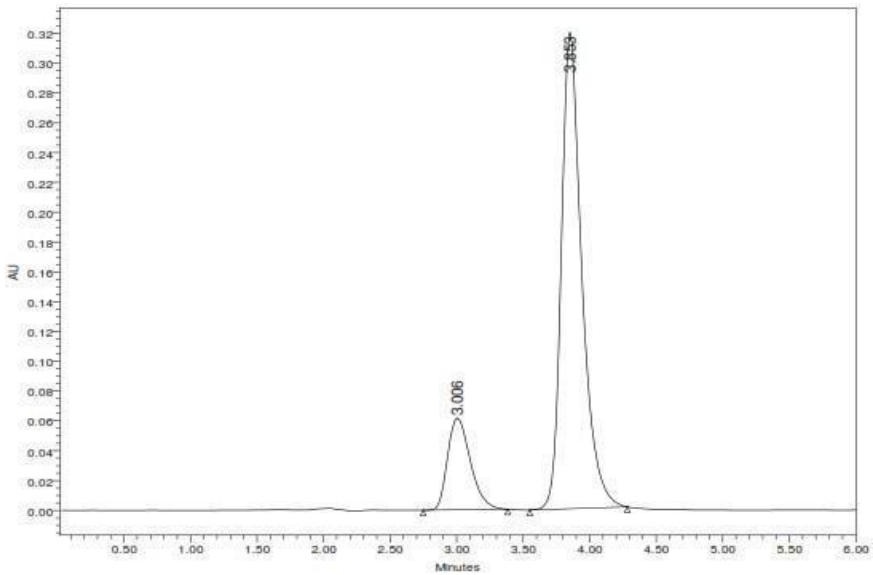
Procedure

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

PRECISION REPEATABILITY

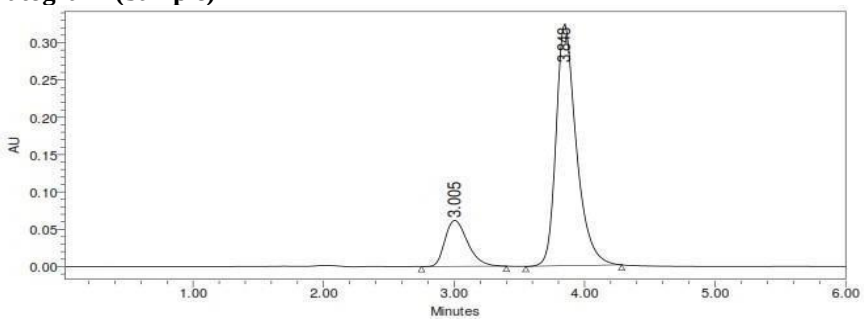
RESULTS AND DISCUSSION



Optimized Chromatogram (Standard) Optimized Chromatogram (Standard)
Optimized Chromatogram (Sample)

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Lopinavir	3.006	732514	62563	1.4	8685
2	Ritonavir	3.853	3421532	321452	1.3	9696

Optimized Chromatogram (Sample)



Optimized Chromatogram (Sample)

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Lopinavir	3.005	658995	61772	1.1	7442
2	Ritonavir	3.848	3096188	324054	1.2	7331

METHOD VALIDATION

S.No.	Peak Name	RT		Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lopinavir	3.008		658263	61335	7462	1.2
2	Lopinavir	3.009		658264	61947	8264	1.1
3	Lopinavir	3.008		653426	61049	6627	1.2
4	Lopinavir	3.010		653058	61141	7264	1.1
5	Lopinavir	3.006		657393	61735	6645	1.1
Mean				656080.8			
Std. Dev.				2618.946			
% RSD				0.39918			

Peak Results for Assay Standard of Ritonavir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Ritonavir	3.857	3028176	381011	9583	1.1
2	Ritonavir	3.859	3018373	381645	8927	1.2
3	Ritonavir	3.857	3018462	381663	8465	1.1
4	Ritonavir	3.861	3081711	381746	9222	1.2
5	Ritonavir	3.853	3075143	381193	8462	1.1
Mean			3044373			
Std. Dev.			31427.07			
% RSD			1.0323			

Peak results for Assay sample of Lopinavir

S.No	Name		RT	Area	Height	USP Tailing	USP Plate Count
1	Lopinavir		3.008	651712	61173	1.2	8563
2	Lopinavir		3.005	657635	61936	1.1	7462
3	Lopinavir		3.007	658917	61196	1.1	9264

Peak results for Assay sample of Ritonavir

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Ritonavir	3.854	3029472	361938	1.1	6476
2	Ritonavir	3.853	3017462	361746	1.1	7264
3	Ritonavir	3.855	3028171	371864	1.2	6545

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Lopinavir and Ritonavir in pharmaceutical dosage form was found to be 100.1%.

LINEARITY

Results of Repeatability for Lopinavir

S. No.	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lopinavir	3.003	654426	61521	8474	1.1
2	Lopinavir	3.005	659862	61937	8262	1.2
3	Lopinavir	3.007	650837	62018	8117	1.1
4	Lopinavir	3.008	651433	61893	7917	1.2
5	Lopinavir	3.005	652752	61867	8011	1.1
6	Lopinavir	3.010	653698	62547	8154	1.3
Mean			653834.6667			
Std Dev			3244.17248			
%RSD			0.496176273			

Results of Repeatability for Ritonavir

S. No.	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Ritonavir	3.851	3028371	381736	6881	1.1
2	Ritonavir	3.852	3009188	380138	9363	1.2
3	Ritonavir	3.854	3067464	386615	7844	1.1
4	Ritonavir	3.853	3076611	380183	9746	1.2
5	Ritonavir	3.851	3011912	379471	7883	1.2
6	Ritonavir	3.861	3023456	385642	7475	1.3
Mean			3036167			
Std Dev			28822.69227			
%RSD			0.949311822			

Results of Intermediate Precision day1 for Lopinavir

Mean			655482.8			
Std. Dev.			4258.945			
% RSD			0.649742			

Results of Intermediate Precision day1 for Ritonavir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPPlate count	USPTailing
1	Ritonavir	3.851	3021731	369771	8564	1.1
2	Ritonavir	3.848	3019183	372746	9227	1.1
3	Ritonavir	3.848	3029847	371866	7565	1.2
4	Ritonavir	3.850	3028471	369017	7726	1.1
5	Ritonavir	3.849	3088641	376453	6746	1.2
6	Ritonavir	3.860	3056633	386621	5977	1.1
Mean			3040751			
Std. Dev.			26990.09			
% RSD			0.887613			

Results of Intermediate Precision Day 2 for Lopinavir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPPlate count	USPTailing
1	Lopinavir	3.006	648822	61847	6983	1.1
2	Lopinavir	3.008	640863	59882	7728	1.2
3	Lopinavir	3.008	643382	60774	9576	1.1
4	Lopinavir	3.007	641884	58928	8275	1.2
5	Lopinavir	3.007	647822	61483	9837	1.1
6	Lopinavir	3.005	649181	60928	8744	1.2
Mean			645325.7			
Std. Dev.			3711.009			
% RSD			0.57506			

Results of Intermediate Precision Day 2 for Ritonavir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPPlate count	USPTailing
1	Ritonavir	3.853	3075833	389911	7039	1.1
2	Ritonavir	3.857	3029583	379019	9857	1.2
3	Ritonavir	3.854	3021991	381875	7881	1.1
4	Ritonavir	3.855	3022485	391099	7902	1.2
5	Ritonavir	3.854	3085833	389222	9285	1.1
6	Ritonavir	3.853	3019482	391184	8955	1.2
Mean			3042535			
Std. Dev.			30022.42			
% RSD			0.986757			

The Accuracy Results for Lopinavir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	331938	7.5	7.3	99.88	100.166
100%	658274	15	14.7	98.89	
150%	970963	22.5	22.2	101	

The Accuracy Results for Ritonavir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	209357	112.5	112.3	99.7%	99%
100%	420697.7	225	224.7	99%	
150%	631550.7	337.5	337.4	99%	

Limit of Detection for Lopinavir and Ritonavir
Result
Lopinavir
= 3.3×9373/43950
= 0.7µg/ml

Ritonavir
= 3.3×55482/13244
= 13.8µg/ml

QUANTITATION LIMIT
The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$$LOQ=10\times\sigma/S$$

Where σ = Standard deviation of the response
S = Slope of the calibration curve

Lopinavir
= 10×9373/43950
= 2.1µg/ml

Ritonavir
= 10×55482/13244
= 41.8µg/ml

Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs□ 2	6.57
2	Asymmetry	T □ 2	Lopinavir = 0.46 Ritonavir = 0.77
3	Theoretical plate	N □ 2000	Lopinavir = 6946 Ritonavir = 5076

Repeatability Analysis for Lopinavir and Ritonavir

HPLC Injection Replicates	AUC for Lornoxicam	AUC for Thiocolchicoside
Replicate – 1	652546	3056284
Replicate – 2	652395	3012546
Replicate – 3	663254	3048757
Replicate – 4	651298	3059865
Replicate – 5	661025	3027849
Replicate – 6	656258	3014789

Average	656129.3	3036682
Standard Deviation	4997.445	21011.27
% RSD	0.761655	0.691915

Results for Robustness -Lopinavir

Parameter used for sample Analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 0.9mL/min	658211	3.006	8793	1.2
Less Flow rate of 0.8mL/min	621077	3.441	7269	1.3
More Flow rate of 1.0mL/min	642190	2.663	9446	1.2
Less organic phase	542402	3.185	8126	1.1
More organic phase	642112	2.867	5854	1.3

Results for Robustness-Ritonavir

Parameter used for sample analysis	Peak Area	Retention Time	heoretical plates	Tailing factor
Actual Flow rate of 0.9mL/min	429069	3.853	5224	1.59
Less Flow rate of 0.8mL/min	472673	4.426	6328	1.58
More Flow rate of 1.0mL/min	392497	3.415	6217	1.54
Less organic phase	391379	4.291	6996	1.61
More organic phase	391703	3.583	6120	1.50

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative Simultaneous estimation of for Lopinavir and Ritonavir in bulk drug and pharmaceutical dosage forms.

Lopinavir was found to be slightly soluble in water, freely soluble in ethanol, soluble in HPLC grade water and methanol on ultrasonication, insoluble in hexane. Ritonavir was found to be slightly soluble in DMSO, Methanol and Water, ethanol and sparingly soluble in Chloroform.

Acetonitrile: Water was taken in the ratio of 40:60% v/v was chosen as the mobile phase. The solvent system used in this method was economical.

The %RSD values were within 2 and the method was found to be precise.

SUMMARY

The analytical method was developed by studying different parameters.

First of all, maximum absorbance was found to be at 220nm and the peak purity was excellent. Injection volume was selected to be 10µl which gave a good peak area.

The column used for study was Sunfire C_{18} (4.6×250mm) 5µm Column because it was giving good peak.

35ºC temperatures was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time.

Mobile phase is Acetonitrile: Water was taken in the ratio of 40:60% v/v was fixed due to good symmetrical peak. So, this mobile phase was used for the proposed study.

Water and Acetonitrile were selected because of maximum extraction sonication time was fixed to be 15min at which all the drug particles were completely soluble and showed good recovery.

Run time was selected to be 6.0min because analyze gave peak around 3.006 and 3.853min for Lopinavir and Ritonavir respectively and also to reduce the total run time.

The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision were found to be accurate and well within range.

The analytical method was found linearity over the range of 5 -25µg/ml and 75-375µg/ml for Lopinavir and Ritonavir respectively target concentration.

ACKNOWLEDGMENT

In forwarding this thesis entitled "**Method Development and Method Validation by RP-HPLC of Lopinavir and Ritonavir**'' I would like to express my sincere thanks to my guide Rizwana Begum, M.Pharm, Assistant Professor, Department of Pharmaceutical Analysis, Dhanvanthri College of Pharmaceutical Sciences, Mahabubnagar, Telangana. For his remarkable guidance, constant encouragement and philosophical suggestions made us possible to submit my dissertation. My deep sincere of gratitude to our Chairman Sri.P. Yadaiah Guptha and Secretary Sri. P. Narendra Guptha for their constant encouragement throughout this work at Dhanvanthri College of Pharmaceutical Sciences, Mahabubnagar, Telangana.I extended my sincere thanks to our Professor cum Principal, Dr. A. Yasodha, Department of Pharmaceutical Analysis, Dhanvanthri College of Pharmaceutical Sciences, Mahabubnagar, Telangana, for providing facilities and permitting me to get carry out all my work successfully.

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