Research Article

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METHOD DEVELOPMENT AND VALIDATION OF RITONAVIR AND LOPINAVIR IN BULK AND DOSAGE FORM BY RP-HPLC

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Abstract

A reversed phase high-performance liquid chromatography method was developed and validated for the quantitative determination of two antiviral drugs ritonavir and lopinavir. Chromatography was carried out on a reversed phase zorbxs, C18column,of(250x4.6mm,5µ) with mobile phase mixture of buffer and solvent(40:60). Acetonitrile and methanol was used as mobile phase solvent (80:20) and the pH was adjusted to 3 with O-phosphoric acid, at a flow rate 1ml/min .The retention time for ritonavir is 2.2min and lopinavir is 3.68min. The UV range was detected at 240nm for lopinavir and ritonavir respectively. The different analytical performance parameters such as linearity, precision, accuracy, and specificity, limit of detection (LOD) and limit of quantification (LOQ) were determined according to International Conference on Harmonization ICH Q2B guidelines. The linearity of the calibration curves for each analyte in the desired concentration range is good (r^2>0.9). Limits for repeatability and intermediate prescision individual assay must be within 98% to102%. Hence the proposed method is highly sensitive, precise and is successfully applied for the reliable quantification of API content in the commercial formulations of lopinavir and ritonavir.

Keywords: Lopinavir, Ritonavir, UV spectrophotometry;RP-HPLC.

Introduction

One of the deadliest and unmanageable chronic health catastrophes is HIV/AIDS. It requires lifelong treatment with potent life saving essential drugs that include nucleoside reverse transcriptase inhibitors, non nucleoside reverse transcriptase inhibitors. Amongst these lopinavir and ritonavir drug combinations is a protease inhibitor used as a second line regimen to treat patients with HIV. Lopinavir is chemically designated as [1S-[1R*,(R*)3R*,4R*]]-N-[[(2,6-dimethyl phenoxy acetyl]amino]-3-hydroxy-5-phenyl-1-(phenyl methyl) pentyl] tetra hydro-alpha-(1-methyl ethyl)-2-oxo-1(2H) - pyramidine acetamide with molecular weight 628.80. Emperical formula $C_{37}H_{28}N_4O_5$.

Ritonavir is chemically designated as 10-Hydroxy-2-methyl-5-(1-methyl ethyl)-1-[2-(1-methyl ethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenyl methyl)-2,4,7,12-tetraazatridecan-13-oic acid,5-thiazolyl methyl ester,[5S-(5R*,8R*,10R*,11R*)]. With molecular weight720.95.Emperical formula $C_{37}H_{48}N_6O_5S_2$.Literature survey revealed several analytical methods for the determination of

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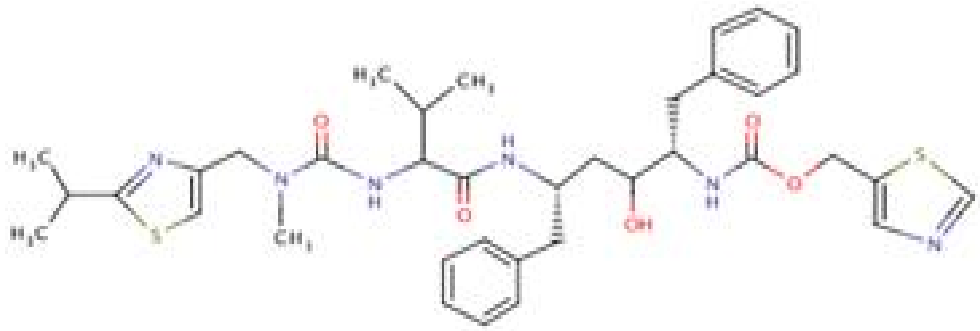
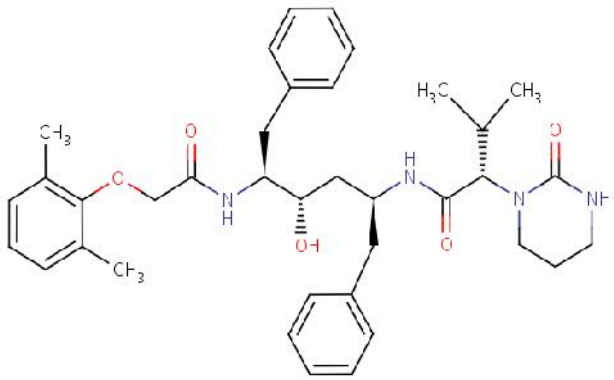
ritonavir and lopinavir in tablets, capsules and syrups which employ technique such as high performance liquid chromatography (HPLC), Ultra performance liquid chromatography(UPLC), and high performance thin layer chromatography (HPTLC). In biological fluids, the active principles as well as their metabolites have been quantitatively determined by HPLC with UV-Visible detection. LC/MS/MS, Spectroscopic method, Micellar electro kinetic chromatography method and Tandem mass spectrometry.

The proposed method was optimized and validated in accordance with International Conference on Harmonization (ICH) guidelines .The aim of present work is to develop a simple, rapid, precise, accurate and selective reversed phase chromatographic method and to estimate the lopinavir and ritonavir in bulk and its solid dosage forms.Lopinavir and ritonavir is a well known omposition used as anti-HIV drugs and was developed by Abott Laboratories to improve pharmaceuticals and to reduce HIV resistance.Different methods were reported in estimation of individual as well as combination of Lopinavir and ritonavir.Administered alone, lopinavir has insufficient bioavailability; however, like several HIV protease inhibitors, its blood levels are greatly increased by low doses of ritonavir, a potent inhibitor of cytochrome P450 3A4Abbott therefore pursued a strategy of co-administering lopinavir with sub-therapeutic doses of ritonavir, and lopinavir is only marketed as a co-formulation with ritonavir.

It is the first multi-drug capsule to contain a drug not available individually. Lopinavir/ritonavir was approved by the USA FDA on 15 September 2000 and in Europe in April 2001. Its patent will expire in the US on June 26, 2016.Abbott Laboratories was one of the earliest users of the Advanced Photon Source, a national synchrotron-radiation light source at Argonne National Laboratory. One of the early research projects undertaken at the Advanced Photon Source was the Human Immunodeficiency Virus. Using X-ray crystallography, researchers found the points of attack of the HIV protease inhibitors – agents that block the breakdown of proteins. Protease inhibitors stop HIV from making new copies of itself by blocking the last step in the process, when the virus attempts to replicate – and out of that discovery came the drug Kaletra.



Structure of Ritonavir



Structure of Lopinavir

Experimental

Instrument

HPLC Water, Model no.2695 Empower 2 software.

Chromatographic parameters

column:-zorbxsb, C18, 250X4.6mm,5µ
Wavelength:- 240nm
Flow rate:-1ml/min
Injection volume:-20µl
Mobile phase:- buffer : solvent (40:60)
Solvent: - Acetonitrile : methanol (80:20)
Run time:-7min
Retention time:-2.2 min for Ritonavir and 3.68 min for Lopinavir

Reagents

Ritonavir ,Lopinavir, potassium di-hydrogen O-phosphate, Water, Methanol, O-phosphoric acid.

Materials and methods

Drugs and instruments

HPLC water, model no 2695 Empower 2 software was the instrument used for this experiment connected with UV-Visible detector. Acetonitrile HPLC grade, Methanol, O-phosphoric acid,potassium di-hydrogen O-phosphate purchased from Aman chemicals Hyderabad. Drugs Ritonavir and Lopinavir purchased from Matrix Laboratories limited, Maharastra, India.

Standard preparation

Accurately weighted 200mg of lopinavir ,25mg ritonavir is transfer into 100ml volumetric flask and make up with diluents, sonicate for 10mintues kept 5mintues filtered 0.45micrometers filter paper .

Sample preparation

Accurately weighed 10tablets and calculate average weight of those tablets and crushed with motor to take tablet powder equal to single tablet weight and transfer into (equivalent weight 200mg) 100ml volumetric flask add 25ml of diluents and sonicate 15mintues then filter through 0.45micrometers filters and make up with diluents further concentration add diluent as per test method.

Preparation of buffer

6.8gm of potassium di-hydrogen O-phosphate was taken in 1000ml of water and pH was adjusted to 3 with O-phosphoric acid.

System suitability

System suitability is performed by six replicate standards inject into HPLC .it can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision .the USP defines parameters that can used to determine the system suitability prior to analysis .these parameters are retention time plate count, resolution, tailing and %RSD.

Selectivity

Selectivity of the method was carried by out standards of lopinavir and Ritonavir were inject into HPLC after that commercial product and placebo, excipients are one after one .it determines interference excipients peaks with analyte peaks.

Linearity

Method linearity was determined by prepare five replicate standard solutions of those drugs in different (50%, 100%,150%)concentration were inject in to the HPLC. plot the graph standard areas verses concentration levels.

Accuracy (recovery studies)

Recovery studies were carried out by prepare triplicate standard solutions in 50%, 100%, 150% concentrations levels and pre analyse the amount of samples.

Precision

Method precision was performed by prepare six replicate samples from single formulation and inject into HPLC at the same manner after 24 hours or day to day variation prepare six replicate samples from same formulation and inject into HPLC observe uniformity of test result and calculate the %RSD .

Robustness

Method robustness was determined by the small changes in chromatographic conditions like as 0.2ml flow rate $+5^0$ temperature and inject the sample observe the results there were no marked changes compare to the other analysis.

Results

System suitability parameters of standard 1 and standard 2 five replicate injection results are given below table1 and 2 also chromatogram figure 1. Those result all are within the limit and also uniform % RSD is 0.5 so it proves method is

suitable for analysis. Result of selectivity was proved by the figure 1 and 2. These figures are standard chromatogram of lopinavir and Ritonavir second one is market formulation of lopinavir and Ritonavir they were not observed excipients and placebo peaks interference with analyte peaks so method is highly selective. Linearity of the results were given tables 2, 3 and calibration curves shown in fig 3,4 Three different concentration levels of six replicate samples area was very linear and correlation coefficient was 1.000 it proves method is linear. Method accuracy results of lopinavir and Ritonavir are given table 4 and 5. Three spiked level (50%,100%,150%) known amount of drugs were compare to recovery of lopinavir99.6% and Ritonavir 100.3% as per ICH acceptance criteria of accuracy was 98% and 102% so it proves method is highly accurate. Inter day and intraday of those runs parameters like retention time , tailing , resolution and plate count all are uniform and area %RSD was less than 1. Robustness results were given table 6. And the peaks were shown in figure5,6. They were no significant changes observed at deliberate changes in temperature and flow rate trails then method is robust.

Discussion

The methods were specific as none of the excipients interfered with the analytes of interest. Hence, the methods were suitably employed for validating the commercial anti-retroviral individual formulations.

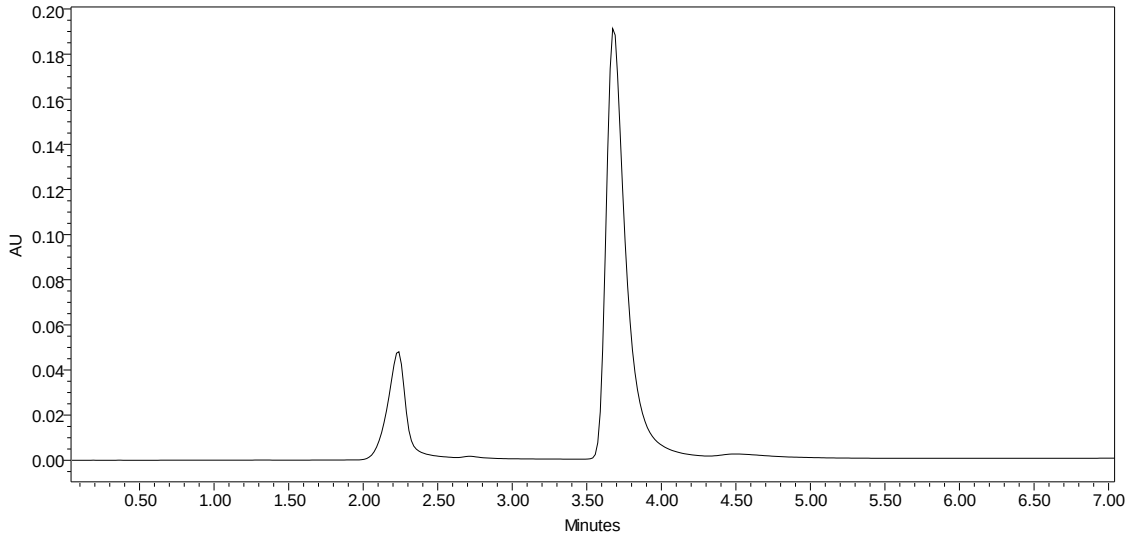


Fig. No. 01: Typical chromatogram of standard solution

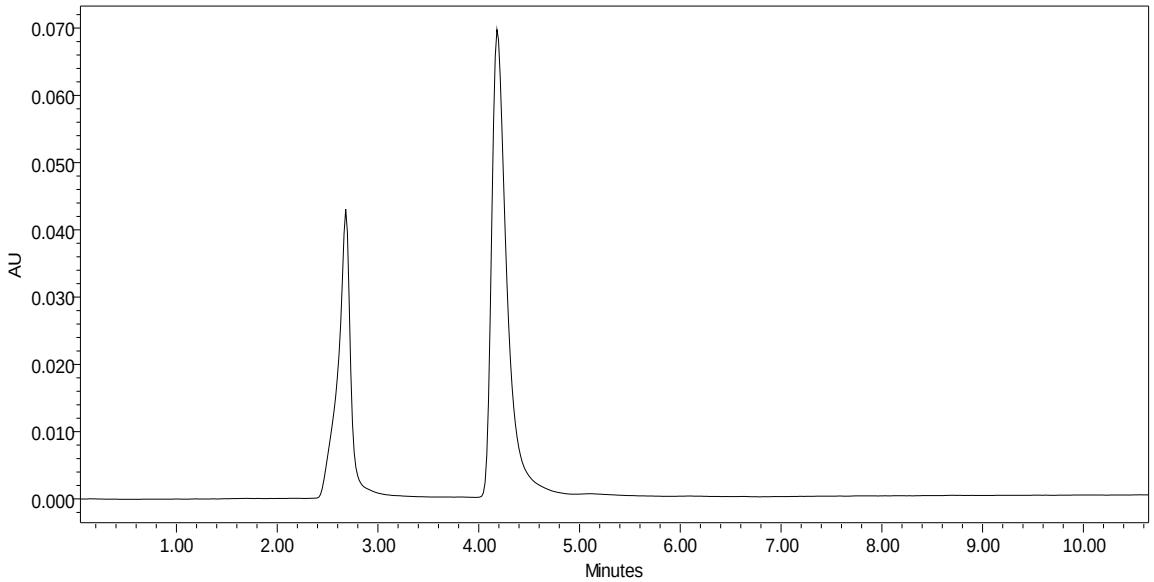


Fig. No. 02: Typical chromatogram of Sample

Table No. 01: Standard results

Injection no.	Retention time	Area response	Retention time	Area response Ritonavir
	Lopinavir	Lopinavir	Ritonavir	
1	3.678	11149.9	2.211	2085.84814
2	3.680	11187.2	2.218	2089.01636
3	3.681	11162.5	2.221	2086.30176
4	3.678	11161.1	2.222	2083.90869
5	3.680	11150.4	2.226	2093.15918
6	3.679	11155.2	2.232	2080.01294
Mean	3.679	11161.1	2.221	2086.4
RSD	0.5%	0.1%	0.5%	0.2%
Standard deviation		1.0%to2.0%	1.0%to2.0%	

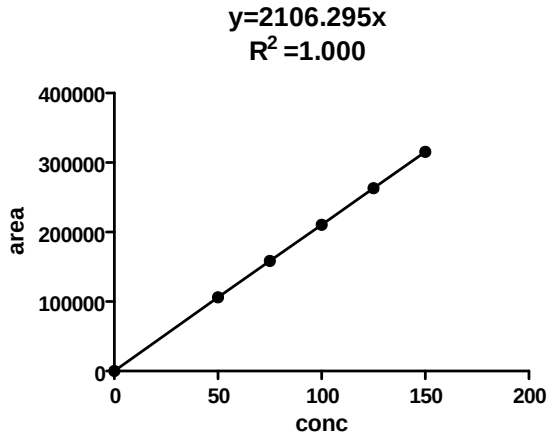


Fig. No. 03: Calibration curve of lopinavir

Table N0. 02: Linearity results of lopinavir

S.No.	Concentration	Area
1	0	0
2	50	106332
3	75	158636
4	100	210448
5	125	263039
6	150	315604

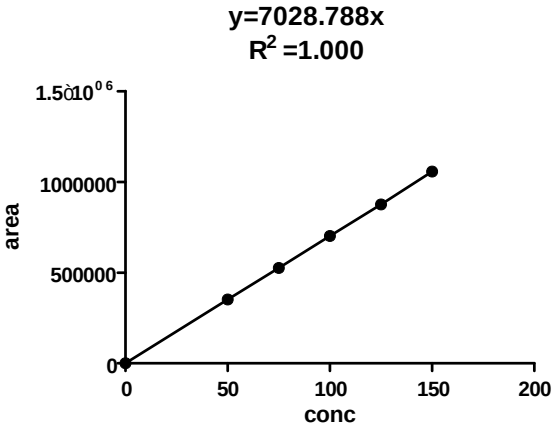
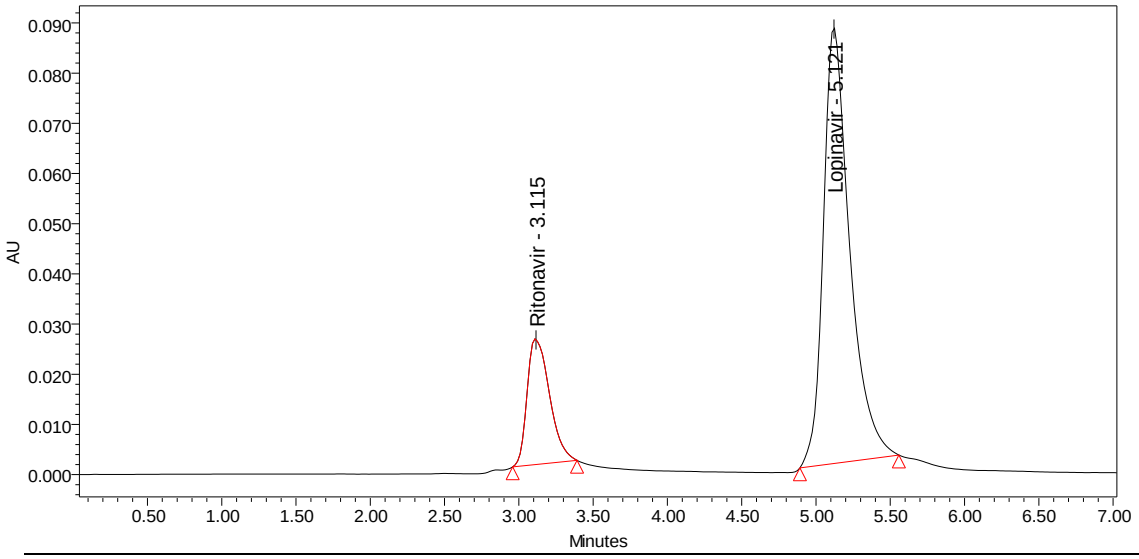


Fig. No. 04: Calibration curve of Ritonavir

Table No. 03: Linearity results of ritonavir

S.No.	Conc.	Area
1	0	0
2	50	352105
3	75	526232
4	100	701595
5	125	876101
6	150	1057496



	Name	Retention Time	Area	% Area	Height	USP Resolution	USP Tailing	USP Plate Count
1	Ritonavir	3.123	284948	19.99	26189		1.386926	9030.797116
2	Lopinavir	5.122	1140300	80.01	91179	6.543734	1.295989	10230.934423

Fig. No. 05: Shows the robustness results with a flow change of -0.2mi/min

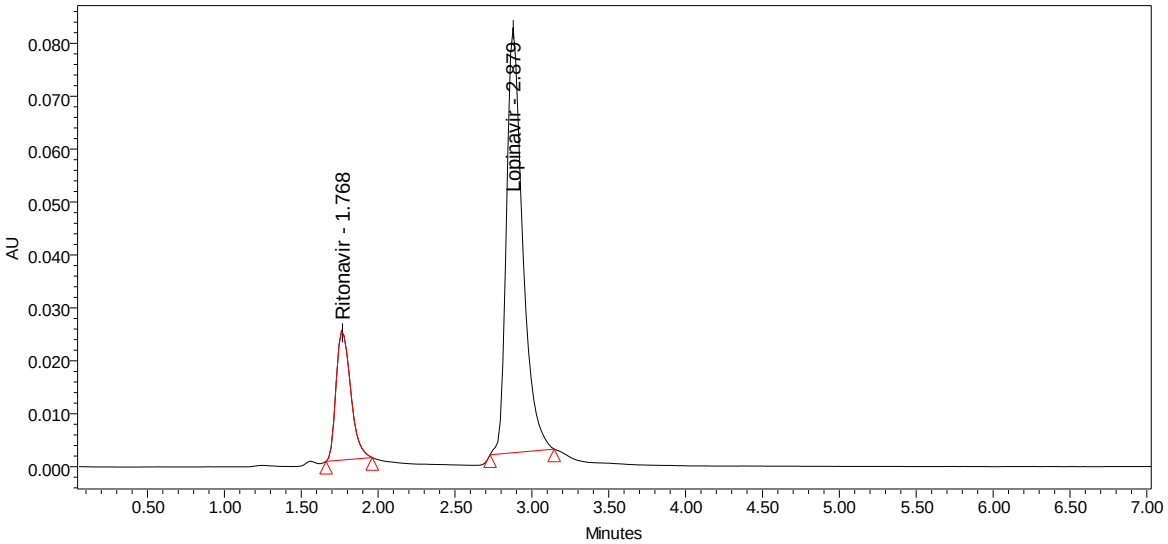


Fig. No. 06: Shows robustness results with flow change of +0.2ml/min.

Limits

Limits for system suitability

%RSD of retention times of the peaks of all the 5 injections is NMT 2%

%RSD of peak area NMT 2%

No. of theoretical plates NLT 3000

Tailing factor NMT 2

Limits for repeatability and intermediate precision:-

Individual assay must be with in 98% to 102%

%RSD of assay NMT 2%

Table No. 04: Shows accuracy results of lopinavir

Spiked level	Sample weight	Area response	*mg added	mg recovered	%recovery	Mean
50%	100	5836	96.35	96.37	100	99.6
50	100	5790	96.26	95.62	99.3	99.6
50	100	5776	95.39	95.39	99.6	99.6
100	100	11661	192.60	192.60	100.0	99.9
100	100	11678	192.61	192.61	100.1	99.9
100	100	11331	187.72	187.72	99.7	99.9
150	300	17193	282.18	283.18	100.6	100.6
150	300	16700	274.46	275.77	100.5	100.6
150	300	17919	293.51	295.89	100.8	100.6

Table No. 05: Accuracy results of ritonavir

Spiked level	Sample weight	Area response	*mg added	mg recovered	%recovery	mean
50%	100	1148	24.93	25.15	100.9	100.6
50%	100	1142	24.92	25.03	100.4	100.6
50%	100	1139	24.82	24.97	100.6	100.6
100%	100	2307	49.72	50.55	101.7	100.6
100%	100	2305	49.81	50.50	101.4	100.6
100%	100	2233	49.57	48.91	98.7	100.6
150%	300	3398	74.65	74.45	99.7	99.5
150%	300	3299	73.65	72.27	98.9	99.5
150%	300	3542	73.09	77.60	100.0	99.5

Table No. 06: Results of lopinavir and ritonavir robustness

		Robustness			
		Lopinavir Tailing factor	RSD	Ritonavir Tailing factor	RSD
Original conditions	$+5^0$c -5^0c	1.045	0.1%	1.0403	0.2%
Flow change	-0.2ml/min	1.377	0.2%	1.403	0.1%
	+0.2ml/min	1.43	.3%	1.274	0.4%

Conclusion

The method described enables to the method development and validation of Ritonavir and Lopinavir. We had run various trails runs at different chromatographic conditions finally we founded the above conditions are suitable for method development and validation of Lopinavir and Ritonavir in bulk and formulation dosage forms. The advantages lie in the simplicity of sample preparation and low cost of reagents used. The proposed analytical method using HPLC ensure proper resolution and precise quantification of the compounds and also we observed validation parameters all are within the limits and %RSD is very low as it will be use full for routine analysis of quality control, stability, and further studies. Results from statistical analysis of the experimental results were indicative of satisfactory precision and reproducibility. Hence, this HPLC method can be used for routine drug analysis of Ritonavir and Lopinavir.

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