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RESEARCH ARTICLE

METHOD DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF RITONAVIR AND LOPINAVIR IN PURE AND PHARMACEUTICAL DOSAGE FORM

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Abstract

A simple, accurate, precise method was developed for the simultaneous estimation of the ritonavir and lopinavir in tablet dosage form. Chromatogram was run through Std ODS 250mm x 4.6 mm, 5µ. Mobile phase containing buffer and acetonitrile in the ratio of 60:40A was pumped through column at a flow rate of 1ml/min. Buffer used in this method was 0.02N KH_2PO_4 buffer at pH 4.6. Temperature was maintained at 30°C. Optimized wavelength for ritonavir and lopinavir was 250 nm. Retention time of ritonavir and lopinavir were found to be 2.27min and 3.74min. %RSD of the ritonavir and lopinavir were and found to be 0.46 and 0.37 respectively. % Recover was obtained as 99.91 to 100.14 for Ritonavir and 99.78 to 100.06 for lopinavir respectively. LOD, LOQ values are obtained from regression equations of ritonavir and lopinavir were 0.25ppm, 0.74ppm, 0.50ppm and 1.51ppm respectively. Regression equation of ritonavir and lopinavir y = 10321x + 767.6 and y = 3625x + 545.7

INTRODUCTION

Lopinavir is chemically (2S)-N-[(2S, 4S, 5S)-5-[2-(2, 6-dimethylphenoxy)acetamido]- 4-hydroxy-1, 6-diphenylhexan -2-yl]-3- methyl-2-(2-oxo-1,3-diazinan-1- yl) butanamide**.** Ritonavir is chemically 1,3-thiazol-5-ylmethyl *N*-[(2*S*,3*S*,5*S*)-3-hydroxy-5-[(2*S*)-3- methyl-2-{[methyl({[2-(propan-2-yl)-1,3 thiazol4yl]methyl})carbamoyl]amino} butanamido] -1,6-diphenylhexan-2-yl] carbamate [1-3]. The mechanism of action of lopinavir and ritonavir is to inhibit the HIV viral protease enzyme. This prevents cleavage of the gag-pol polyprotein and, therefore results in improper viral assembly and subsequently results in non-infectious, immature viral particles.

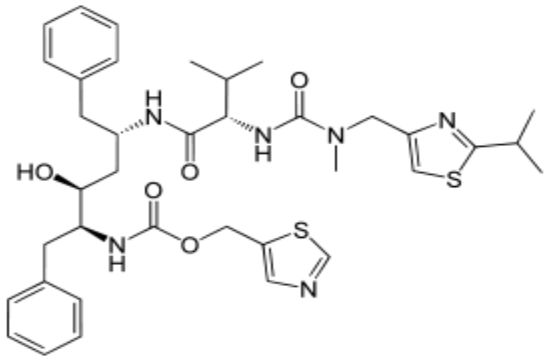


Fig.1:Molecular structure of ritonavir

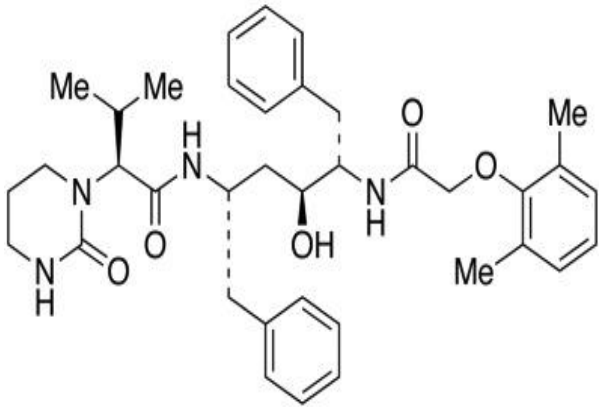


Fig.2:Molecular structure of lopinavir

Literature survey revealed that only few analytical methods are reported for both the drugs in alone. Very few analytical methods have been reported for simultaneous estimation of ritonavir and lopinavir like, HPLC [4-7]and HPTLC [8-11] methods.

EXPERIMENTAL:

Reagents and chemicals:

Analytically pure samples of ritonavir and lopinavir were procured from Hetero HC Pvt.Ltd.,Hyderabad. Pharmaceutical dosage forms used in the study was ritocom tablets (Hetero HC Pvt.Ltd.,Hyderabad) labeled to contain 50mg of ritonovir and 200mg of lopinavir were were procured from local market. Acetonitrile, methanol, distilled water, potassium dihydrogen phosphate, ortho-phosphoric acid (AR grade) were obtained from qualigens, Mumbai..

Instrument:

HPLC instrument used was of WATERS HPLC 2965 SYSTEM with auto injector and PDA detector. Software used is Empower 2. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz was be used for measuring absorbance for ritonavir and lopinavir solutions.

Method development:

Preparation of Buffer:

Accurately weighed 1.36 gm of potassium dihydrogen ortho phosphate in a 1000ml of volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water, then pH adjusted to 4.8 with orthophosphoric acid.

Preparation of mobile phase

The mobile phase was prepared by mixing of buffer and acetonitrile (60:40 v/v) and the pH adjusted to 4.8 by using orthophosphoric acid. The mobile phase was sonicated for 15min and then it was filtered through 0.45µ Whatman filter paper.

Preparation of standard stock solution:

About 50 mg of each reference standard of ritonavir and lopinavir were accurately weighed & transferred to 50 ml volumetric flasks. Both the drugs were dissolved in 50 ml of mobile phase with shaking and then volume was made up to the mark with mobile phase to get 1000 µg/ml of standard stock solution of each drug. Then it was ultra sonicated for 10 minutes and filtered through 0.20 µ membrane filter. For calibration curve, stock solutions of ritonavir and lopinavir were appropriately diluted to obtain working standard solutions in the increasing concentration range.

Sample preparation:

Twenty tablets were weighed accurately and finely powdered. A quantity of powder equivalent to 50 mg of ritonavir and 200 mg of lopinavir was transferred into a 50 ml volumetric flask, dissolved in mobile phase. Solution was ultrasonicated for 20 min. Filtered through Whatman filter paper No. 42. Filtrate was diluted with mobile phase to obtain final concentration within linear range. From the filtered solution 0.2ml was pipeted out into a 10 ml volumetric flask and made up to 10ml with diluents. Chromatogram was recorded at 250 nm. Content of drugs in sample solution was calculated by comparing mean peak area of sample with that of the standard.

Validation parameters:

Method development:

Optimized Method: Drugs were eluted with good retention time, resolution, all the system suitable parameters like plate count and tailing factor were within the limits.

Column used	:	ODS 250mm x 4.6 mm, 5µ
Buffer used	:	0.1% OPA
Mobile phase	:	Buffer and acetonitrile in the ratio of 60:40A
Flow rate	:	1ml/min
Wavelength	:	250 nm
Temperature	:	30°C
Injection volume	:	10µl

System suitability: All the system suitability parameters are within range and satisfactory as per ICH guidelines

Table 1: Data for system suitability parameters of ritonavir and lopinavir

Property	Ritonavir	Lopinavir
Retention time (Rt)	2.27min	3.74min
Theoretical plates (N)	5347 ± 63.48	9868 ± 63.48
Tailing factor (T)	1.28 ± 0.117	1.18± 0.117

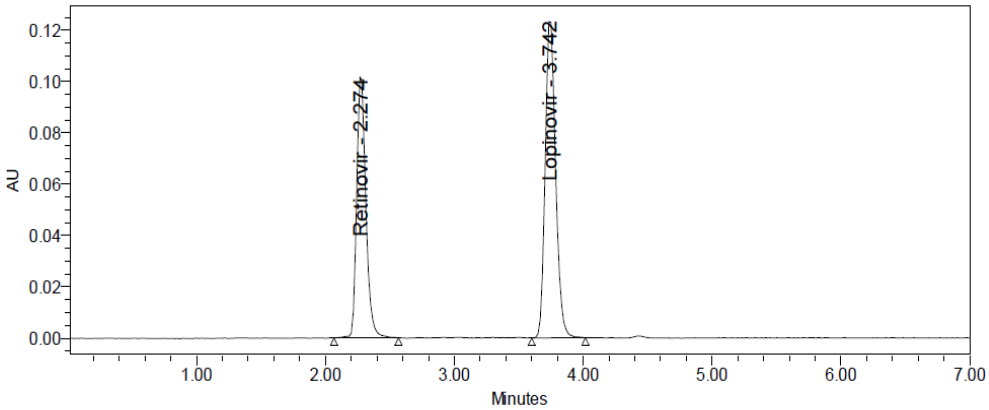
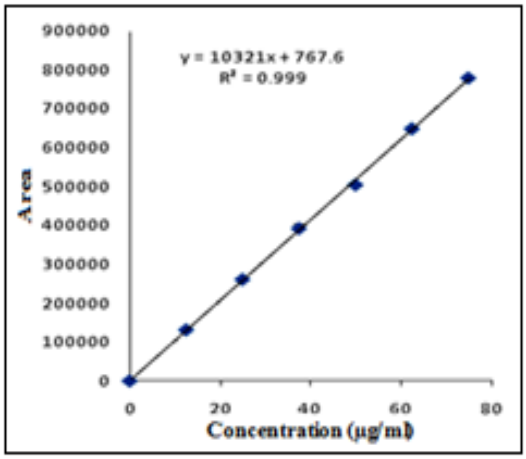


Fig.3:Typical chromatogram of ritonavir and lopinavir

Linearity:

Accurately measured volumes of working standard solution of ritonavir and lopinavir were transferred into a series of 10ml volumetric flasks and diluted appropriately with mobile phase. 20 µl of each solution was injected under operating chromatographic conditions described above. Calibration curves were obtained by plotting the response (area of drug peak) on Y-axis versus concentration of drug on X-axis. Regression equation of the ritonavir and lopinavir are found to be, y = 10321x + 767.6, y = 3625x +545.7 and regression co-efficient was 0.999. The method was found linear over a concentration range of 12.5 - 75 µg/mL and 50 - 300 µg/mL for ritonavir and lopinavir respectively.



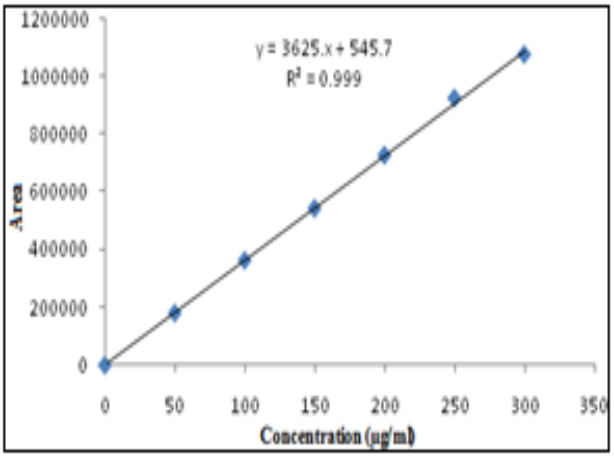


Fig.4: Calibration curve of ritonavir **Fig.5: Calibration curve of lopinavir**

Precision (Repeatability):

In the intraday studies, solutions of standard and sample were repeated six times in a day and percent relative standard deviation (%RSD) for response factor was calculated. The intraday %RSD of ritonavir and lopinavir were found to be 0.46 and 0.37 respectively.

Inter day precision:

In the interday variation studies, injections of standard and sample solutions were made on three consecutive days and %RSD was calculated. The interday %RSD for ritonavir and lopinavir were found to be 0.96 and 0.68 respectively. From the data obtained the developed RP-HPLC method was found to be precise.

Table 2: Precision studies of ritonavir and lopinavir

S. No.	Intraday precision		Inter day precision	
	Ritonavir	**Lopinavir**	**Ritonavir**	**Lopinavir**
1	496582	681536	486191	678382
2	493896	676111	492152	679145
3	497311	680108	495803	674446
4	497629	675749	485284	670943
5	492102	678115	485840	668686
6	493740	680931	485283	674443
Mean	**495210**	**678758**	**489054**	**674320**
S.D	2266.9	2479.3	4687.4	4553.6
%RSD	0.46	0.37	0.96	0.68

Accuracy:

The accuracy of the method was determined by recovery experiments. Known concentration of working standard was added to the fixed concentration of the pre-analyzed solution. Percent recovery was calculated by comparing the area before and after the addition of working standard. For both the drugs, recovery was performed in the same way. The recovery studies were performed in triplicate. This standard addition method was performed at 50%, 100%, 150% level and the percentage recovery was calculated. Percent recovery was within the range of 99.91 to 100.14 for ritonavir and 99.78 to 100.06 for lopinavir which indicates that the method was accurate.

Table 3:Accuracy results of ritonavir and lopinavir

Sample	Amount added (µg/ml)	Amount recovered (µg/ml)	Recovery (%)	% RSD
Ritonavir	50	24.97	99.91	0.68
	100	50.07	100.14	0.62
	150	75.03	100.05	0.78
Lopinavir	50	99.96	99.96	0.31
	100	200.12	100.06	0.49
	150	299.34	99.78	1.01

Limit of detection and limit of quantification

The Limit of detection and quantification were calculated using standard deviation of the response and slope of calibration curve. The LOD for ritonavir and lopinavir was found to be 0.25 µg/ml and 0.50 µg/ml respectively. The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified. The LOQ was 0.74 µg/ml and 1.51 µg/ml for ritonavir and lopinavir respectively.

Robustness:

Robustness of the method was checked by making slight deliberate changes in chromatographic conditions like flow rate and wavelenght. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed RP-HPLC method is robust.

Table 4: Robustness data for ritonavir and lopinavir

S.No	Parameter	Modification	% RSD		Tailing factor	
			Ritonavir	Lopinavir	Ritonavir	Lopinavir
1	Flow rate (ml/min)	0.8	0.31	0.45	1.19	1.16
		1.2	0.28	0.26	1.17	1.15
2	Wavelength	248nm	0.97	0.49	1.16	1.12
		252 nm	0.97	0.43	1.17	1.19

Assay:

The drug content of marketed dosage form (RITOCOM- Ritonavir 50 mg and Lopinavir 200 mg) were calculated six times using proposed method. The average % assay was calculated and found to be 99.84 % and 100.05% for ritonavir and lopinavir respectively. Assay results shown in **Table 5** indicates that method is suitable for analysis of marketed formulation.

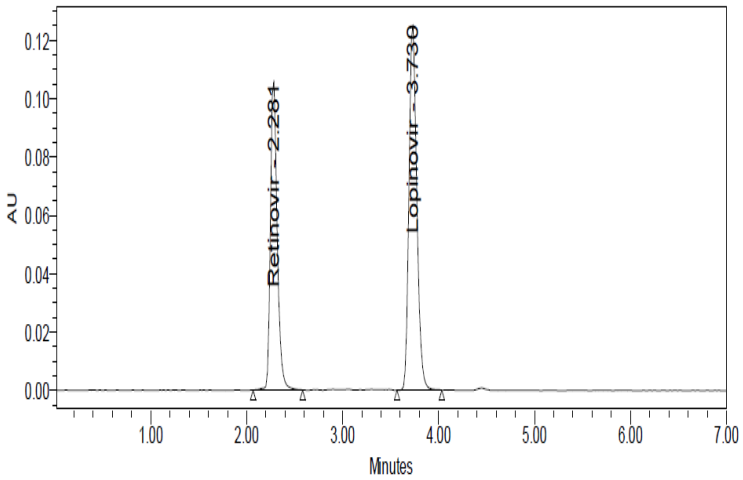


Fig6: Assay chromatogram for Ritonavir and Lopinavir

Table 5: Assay of tablet

Dosageform	Active ingredients	Labeled amount (mg/tab)	Mean%±SD	Assay	%RSD
RITOCOM Tablets	**Ritonavir** **Lopinavir**	**50 mg** **200 mg**	**49.99 ±0.36** **200.02 ±0.60**	**99.84** **100.05**	**0.46** **0.37**

Degradation Studies:

In order to demonstrate the stability of both standard and sample solutions during analysis, both solutions were analyzed over a period of 24hr at room temperature. The results show that for both solutions, the retention time and peak area of ritonavir and of lopinavir remained almost similar (% R.S.D. less than 2.0) and no significant degradation within the indicated period, thus indicated that both solutions were stable for at least 24hr, which was sufficient to complete the whole analytical process. Further forced degradation studies were conducted indicating the stability of proposed method. The results are summarized in **Table 6**.

Acid degradation sample

10 tablets were accurately weighed and powdered. Weight equivalent to 50mg of ritonavir and 200 mg of lopinavir was taken in a 50mL clean dry volumetric flask add about 30mL of mobile phase and sonicate to dissolve it for about 30minutes with intermittent shaking at controlled temperature. Then add 5mL of 0.1N acid (Hydrochloric acid), refluxed for 60minutes at 80°C, then cooled to room temperature, neutralize with 0.1N base (Sodium hydroxide) and dilute to volume with mobile phase. Filter about 5mL of the above sample solution through 0.45µ membrane filter. Pipette 1mL of the above filtered sample solution into a 10 mL volumetric flask and dilute to volume with mobile phase.

Base degradation sample

10 tablets were accurately weighed and powdered. Weight equivalent to 50mg of ritonavir and 200 mg of lopinavir were taken in a 50mL clean dry volumetric flask add about 30mL of mobile phase and sonicate to dissolve it for about 30minutes with intermittent shaking at controlled temperature. Then add 5mL 0.1N base (Sodium hydroxide), refluxed for 60minutes at 80°C, then cooled to room temperature, neutralize with 0.1N acid (Hydrochloric acid) and dilute to volume with mobile phase. Filter about 5mL of the above sample solution through 0.45µ membrane filter. Pipette 1mL of the above filtered sample solution into a 10mL volumetric flask and dilute to volume with mobile phase.

Thermal degradation sample

10 tablets were accurately weighed and powdered. This powder is exposed to 105^0C for 5 days. Weight equivalent to 50mg of ritonavir and 200 mg of lopinavir were taken in a 50mL clean dry volumetric flask add about 30mL of mobile phase and sonicate to dissolve it for about 30minutes with intermittent shaking at controlled temperature. And dilute to volume with mobile phase. Filter about 5mL of the above sample solution through 0.45µ membrane filter. Pipette 1mL of the above filtered sample solution into a 10mL volumetric flask and dilute to volume with mobile phase.

Peroxide degradation sample

10 tablets were accurately weighed and powdered. Weight equivalent to 50mg of ritonavir and 200 mg of lopinavir were taken in a 50mL clean dry volumetric flask add about 30mL of mobile phase and sonicate to dissolve it for about 30minutes with intermittent shaking at controlled temperature. Then add 2ml of 30% peroxide and refluxed for 60minutes at 80°C, then cooled to room temperature. The volume was made up to mark with mobile phase. Filter about 5mL of the above sample solution through 0.45µ membrane filter. Pipette 1mL of the above filtered sample solution into a 10mL volumetric flask and dilute to volume with mobile phase.

Table 6:Results of degradation studies

S.NO	Degradation condition	Ritonavir			Lopinavir		
		% Drug degraded	Purity angle	Purity threshold	% Drug degraded	Purity angle	Purity threshold
1	Acid	7.54	14.291	0.346	7.18	0.361	0.321
2	Alkali	6.89	0.792	1.296	6.34	0.174	0.304

3	Oxidation	5.84	8.789	0.304	5.34	0.265	0.320
4	Thermal	4.83	0.615	1.287	4.52	0.294	0.419
5	UV	1.52	0.925	2.296	1.02	0.925	0.304
6	Water	0.60	0.946	2.300	0.75	0.368	1.308

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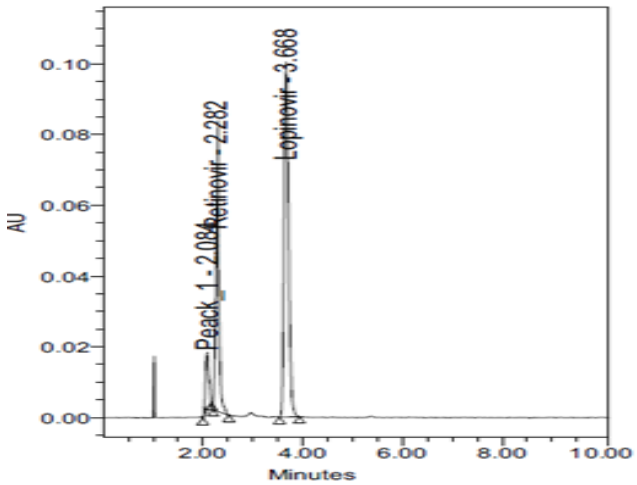


Fig 7:Acid degradation chromatogram

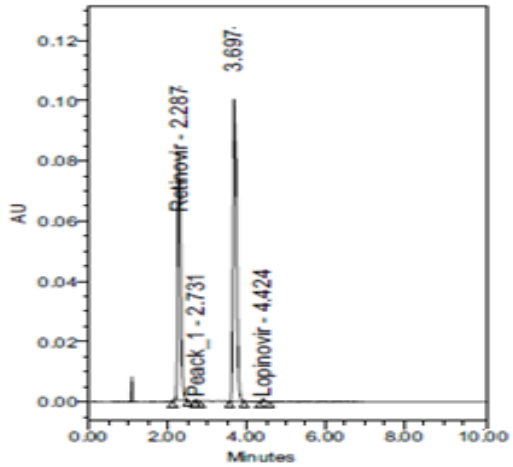


Fig 8:Base degradation chromatogram

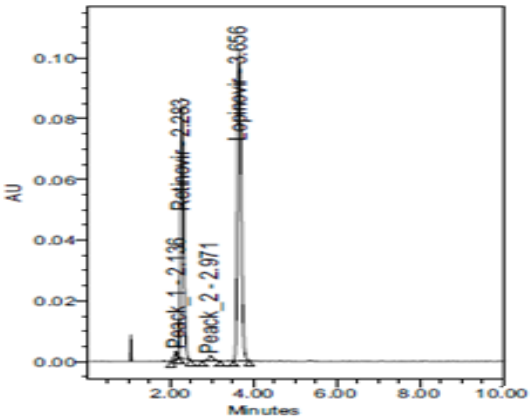


Fig 9:Peroxide degradation chromatogram

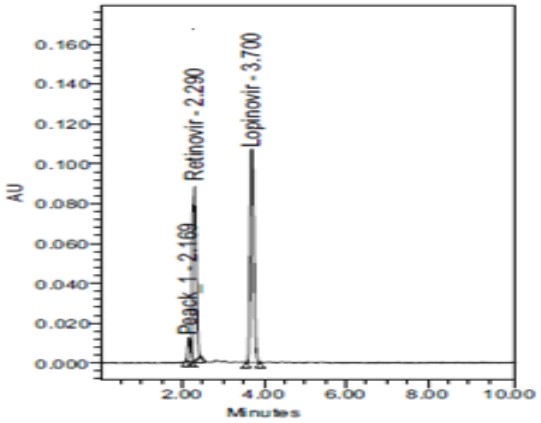
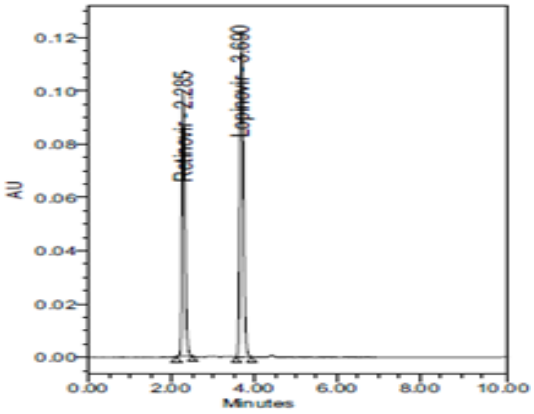


Fig 10. Thermal degradation chromatogram



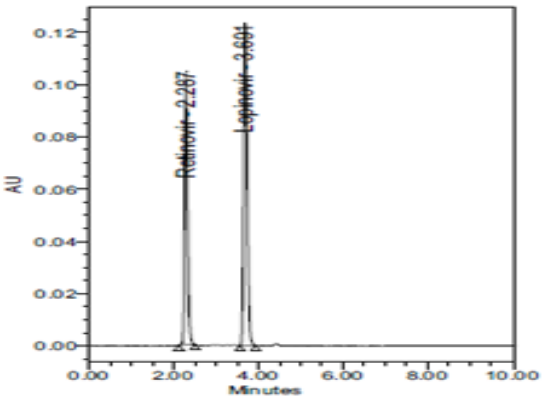


Fig11: UV degradation chromatogram **Fig12: Water degradation chromatogram**

Table 7: Summary of validation parameters of HPLC

Parameters	Ritonavir	Lopinavir
Calibration range (mcg / ml)	12.5-75	50-300
Optimized wavelength	250nm	250nm
Retention time	2.27min	3.74min
Regression equation (Y*)	y = 10321x + 767.6	y = 3625x + 545.7
Correlation coefficient(r^2)	0.999	0.999
Precision (% RSD*)	0.46	0.37
% Recovery	99.85%	100.05%
Limit of detection (mcg / ml)	0.25	0.50
Limit of quantitation (mcg / ml)	0.74	1.51

CONCLUSION:

A simple, accurate, precise method was developed for the simultaneous estimation of the Ritonavir and Lopinavir in tablet dosage form. Retention time of Ritonavir and Lopinavir were found to be 2.27min and 3.74min. %RSD of the Ritonavir and Lopinavir were and found to be 0.46 and 0.37 respectively. %Recover was Obtained as 99.85% and 100.05% for Ritonavir and Lopinavir respectively. LOD, LOQ values are obtained from regression equations of Ritonavir and Lopinavir were 0.25ppm, 0.74ppm and 0.50ppm, 1.51ppm respectively. Regression equation of Ritonavir is of Lopinavir y = 10321x + 767.6 and y = 3625x + 545.7.Retention times are decreased and that run time was decreased so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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