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Stability Indicating High Performance Liquid Chromatographic Estimation of Ritonavir and Lopinavir in their Combined Tablet Dosage form

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ABSTRACT

A stability indicating high performance liquid chromatography method is developed and validated for the estimation of two anti-viral drugs, ritonavir and lopinavir in tablet dosage form. Separation and quantification was performed on Agilent Eclipse XDB (150 x 4.6 mm; 3.5 µm particle size) analytical column using 0.1M KH_2PO_4 and acetonitrile (40:60 v/v) as the mobile phase. A UV detector set at 230 nm was used for evaluation. The developed method is validated according to ICH guidelines. The correlation coefficients of calibration curves were found to be 0.9999 in the concentration range of 100–300 and 400–1200 µg/ml for ritonavir and lopinavir, respectively. The method had an acceptable precision and accuracy. The method had the potential to quantify the selected drugs simultaneously from tablet dosage forms without any interference of the excipients. The selected drugs were also subjected to acid, base, oxidation, thermal and photolytic degradation studies. The degradation products obtained were well resolved from the drugs. As the method might efficiently separate the drugs from its degradation products, it can be used for stability indicating analysis.

Keywords: *High-Performance Liquid Chromatography; Ritonavir; Lopinavir; Stability- Indicating Method.*

INTRODUCTION

Ritonavir [1, 2] is a Human Immunodeficiency Virus Type 1 (HIV-1) protease inhibitor belonging to the n-carbamoyl-alpha amino acids and derivatives class. Chemically, ritonavir is described as 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl -2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl] amino}butanamido]-1,6-diphenylhexan-2-yl]carbamate. The HIV-1 protease is required for the conversion of viral polyprotein precursors into individual functional proteins found in infectious HIV-1. Ritonavir inhibits the HIV viral proteinase enzyme and thereby interfering with the reproductive cycle of HIV-1.

Lopinavir [3, 4] is a retroviral protease inhibitor belonging to the class known as amphetamines and derivatives. Chemically, lopinavir is known as (2*S*)-*N*-[(2*S*,4*S*,5*S*)-5-[2-(2,6-dimethyl phenoxy)acetamido]-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-(2-oxo-1,3 -diazinan-1-yl)butanamide. Lopinavir exerts its activity by inhibiting the HIV viral protease enzyme. This enzyme is responsible for cleavage of gag-pol polyprotein to form individual functional proteins of infectious HIV. The inhibition of this enzyme results in improper viral assembly. Fig. 1 & 2 shows the chemical structure of ritonavir and lopinavir, respectively.

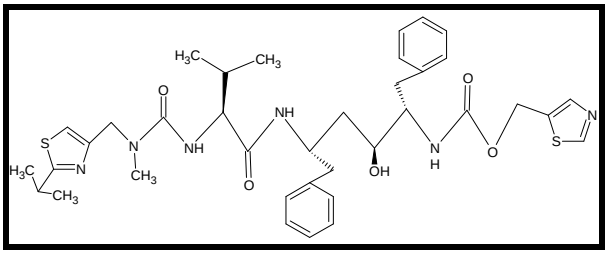

Fig. 1: Chemical Structure of Ritonavir

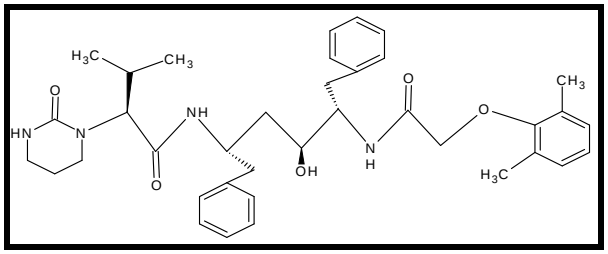

Fig. 2: Chemical Structure of Lopinavir

HIV protease inhibitors are always used in combination with at least two anti-HIV drugs. Lopinavir has a higher invitro activity against both wild-type and mutant HIV-1 proteases. However, its invivo activity is greatly attenuated due to its hepatic metabolism by enzyme cytochrome P450 3A4. This also results in the decreased concentration of lopinavir in the blood [5]. Though ritonavir was developed as an inhibitor of HIV protease, it is now not often used for its own antiviral activity. Particularly, ritonavir is used to inhibit the enzyme cytochrome P450-3A4 [6]. Therefore, ritonavir or lopinavir alone is not efficient in curing HIV infection. Combining lopinavir with low-dose ritonavir produces lopinavir concentration far exceeding those needed to suppress invitro and invivo viral replication [7]. Thus, the combination of lopinavir and ritonavir may decrease the chance of developing acquired immunodeficiency syndrome, HIV-associated illnesses and may reduce the risk of transmitting the HIV virus [8, 9]. FDA in USA and in Europe approved lopinavir/ritonavir combination on September 2000 and April 2001, respectively [10].

The literature reports, many methods for simultaneous quantitative determination of ritonavir and lopinavir in bulk, tablet dosage form, capsule dosage form and human plasma. These methods include simultaneous estimation of ritonavir and lopinavir by UV spectrophotometry [11-14], HPTLC [15-18], HPLC [19-21], UPLC–ESI-MS/MS [22] and LC-MS [23]. The summary of reported methods is shown in Table 1.

The quality of lopinavir/ritonavir pharmaceutical product, in terms of purity and stability of the active substance is essential for the efficient and safe delivery of its therapeutic values to the HIV patients. The presence of degradation products may cause changes in the chemical, pharmacological and toxicological

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properties of the active drugs and its pharmaceutical products [25-27]. Generally, pharmaceutical substances are sensitive to environmental factors such as temperature, humidity and light. These factors typically vary during manufacturing, transportation, storage and distribution. For these reasons, stability testing is necessary for providing information about degradation products and the long-term effects of the environmental factors on the active drug and its pharmaceutical products [28, 29].

Therefore, a stability-indicating method for the simultaneous determination of lopinavir/ritonavir in bulk and in pharmaceutical dosage forms is essential. However, to the best of our knowledge, there was no report in the literature on the stability-indicating simultaneous assay for lopinavir/ritonavir. The present work describes a simple, precise, accurate and reproducible stability indicating HPLC method for simultaneous estimation of ritonavir and lopinavir in tablet formulation.

Table No. 1: Reported methods for the simultaneous estimation of ritonavir and lopinavir

Reference	Method	Ritonavir				Lopinavir				Application
		Linearity (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Recovery (%)	Linearity (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Recovery (%)	
Jyoti *et al.,* [11]	**UV spectro - photometry**	10-35	NR	NR	99.51	100-500	NR	NR	98.92	Tablet dosage form
Jyoti *et al.,* [11]	**UV spectro-photometry**	10-35	NR	NR	98.5	100-500	NR	NR	99.2	-do-
Jaiprakash *et al.,* [12]	**UV spectro-photometry**	5-30	1.414	4.2	99.89	10-60	1.1	3.33	99.82	-do-
Vaishali *et al.,* [13]	**UV spectro-photometry**	5-30	0.1	0.5	98.3	20-120	0.5	10	99.9	-do-
Nagulwar *et al.,* [14]	**UV spectro-photometry**	5-30	NR	NR	99.54	20-120	NR	NR	100.07	-do-
Jaiprakash *et al.,* [15]	**HPTLC**	100-700[a]	11.52[a]	26.22[a]	100.2	100-600[a]	6.58[a]	34.11[a]	100.00	-do-
Patel *et al.,* [16]	**HPTLC**	1.5-5	1.5	5.10	101.29	6.5 - 20	4.6	21.0	99.90	Capsule dosage form
Sulebhavikar *et al.,* [17]	**HPTLC**	1.67 to 5	5.10	4.6	103.50	6.67 - 20	5.10	21.0	102.28	-do-
Patwari *et al.,* [18]	**HPTLC**	400-2400[a]	102.08[a]	476.55[a]	99.78	1600-9600[a]	306.24[a]	1429.65[a]	98.40	Tablet dosage form
Jagadeeswaran *et al.,* [19]	**RP-HPLC**	10-50	0.013	0.465	100.1	40-200	0.013	0.465	102.1	-do-
Phechkrajang *et al.,* [20]	**RP-HPLC**	0.4 - 4.4	NR	NR	100.3	2-18	NR	NR	100.5	Human plasma
Kou *et al.,* [21]	**RP-HPLC**	0.05–5	NR	NR	79.17	0.5–20	NR	NR	52.26	-do-
Shrivastav *et al.,* [22]	**UPLC–ESI-MS/MS**	2.9–1452[b]	NR	NR	97.5	29.6–14379[b]	NR	NR	96.6	-do-
Temghare *et al.,* [23]	**LC-MS-MS**	20-3000[b]	NR	20[b]	92.95	50-20000[b]	NR	50[b]	91.06	-do-

a= ng/spot; b=ng/ml; NR=not reported

MATERIALS AND METHODS

Apparatus:

A Waters 2695 alliance with binary HPLC pump equipped with Waters 2998 PDA detector and Waters Empower2 software was used in the present investigation.

Mobile phase:

The solvents and chemicals used in the preparation of mobile phase were of HPLC grade and analytical grade, respectively. The mobile phase used was 0.1M KH_2PO_4 and acetonitrile in the ratio of 40:60 *v/v*. Before use, the mobile phase was filtered through millipore membrane filter and degassed for 15 minutes by sonication.

Chromatographic conditions:

Agilent Eclipse XDB (150 x 4.6 mm; 3.5 µm particle size) analytical column was used for separation and simultaneous analysis of ritonavir and lopinavir. The column temperature was maintained at 30±1^{0}C. The separation was carried out under isocratic elution. The flow rate was maintained as 1.0 ml/min. The injection volume was 10 µl. The eluents were detected at 230 nm.

Standard solutions:

The standard stock solution was prepared by dissolving 50 mg of ritonavir and 200 mg of lopinavir in 100 ml mobile phase. Working standard solutions equivalent to 100-300 µg/ml ritonavir and 400-1200 µg/ml lopinavir was prepared from stock solution by appropriately diluting the stock standard solution with the mobile phase.

Sample Solution:

Ten tablets were weighed and crushed to a fine powder. The powder equivalent of 50 mg of ritonavir and 200 mg of lopinavir was taken in a 100 ml volumetric flask containing 20 ml of mobile phase, sonicated for 20 minute and made up to mark with the same solvent. The resultant mixture was filtered through 0.45 µm filter paper. The filtrate was diluted appropriately with the mobile phase to get a final concentration of 200 µg/ml ritonovir and 800 µg/ml lopinavir.

RESULTS AND DISCUSSION

HPLC parameters optimization:

So as to study the simultaneous elution of more than one drug under isocratic conditions, different chromatographic conditions (type of the column, mobile phase composition, flow rate and pH) have been investigated. The objective of the simultaneous HPLC method development was to achieve a peak tailing factor <2, USP plate count ≥ 2000, retention time in between 3 and 5 minutes, along with good resolution. This objective was obtained using mobile phase consisting of 0.1M potassium dihydrogen phosphate – acetonitrile in the proportion of (40/60, *v/v*). The pH of the mobile phase was adjusted to 6.2 with orthophosphoric acid. Under the above described conditions, the analyte peaks were well defined, resolved and free from tailing. The tailing factors were <2 for both the peaks. The elution orders were ritonavir (retention time- 3.088 min) and lopinavir (retention time- 4.491 min) at a flow rate of 1.0 ml/min (Fig. 3). The optimum wavelength for detection was 230 nm at which much better detector responses for the selected drugs were obtained.

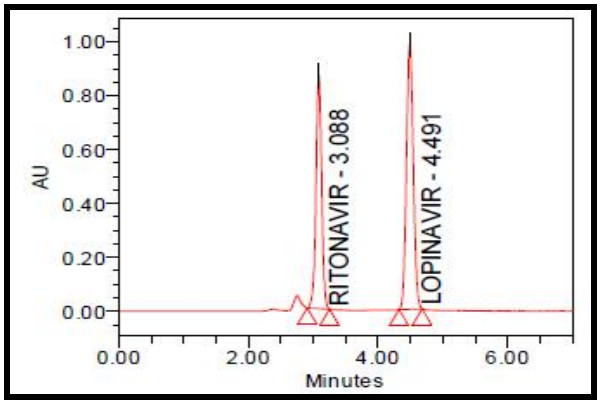

Fig. 3: Typical chromatogram of ritonavir and lopinavir

Method validation:

The optimized RP-HPLC method for simultaneous assay of ritonavir and lopinavir was validated according to ICH guidelines [24] with respect to system suitability, linearity, sensitivity, accuracy, precision and robustness.

System suitability:

In relation to U.S. Pharmacopeia, system suitability tests are an integral part of a liquid chromatographic method. System suitability tests are used to confirm that the column efficiency, resolution and reproducibility of the chromatographic system are sufficient for the analysis. System suitability test was assessed from five replicate injections of the standard solution containing 200 µg/ml ritonovir and 800 µg/ml lopinavir. The results of system suitability in comparison with the required limits are shown in Table 2. The results are found to be suitable and are within the accepted limits.

Table No. 2: System suitability test of the HPLC method

Parameters	Results		Recommended limits
	Ritonavir	Lopinavir	
Retention time	3.085	4.490	-
Peak area	5184858 (%RSD – 0.1)	7196600 (%RSD – 0.3)	RSD ≤1
USP resolution	-	7.829	> 1.5
USP plate count	6171	8488	> 2000
USP tailing factor	0.979	1.063	≤ 2

Linearity and range:

The linearity of the method was determined by analyzing five different concentrations of each drug. The calibration curve was plotted by area under the peak responses of the drugs against their corresponding concentrations. Calibration curves were linear over the concentration range of 100-300 µg/ml for ritonavir and 400-1200 µg/ml for lopinavir. The parameters such as a regression equation and regression coefficient are given in Figures 4 and 5. The results show a good correlation between the peak areas of the drugs and their corresponding concentrations.

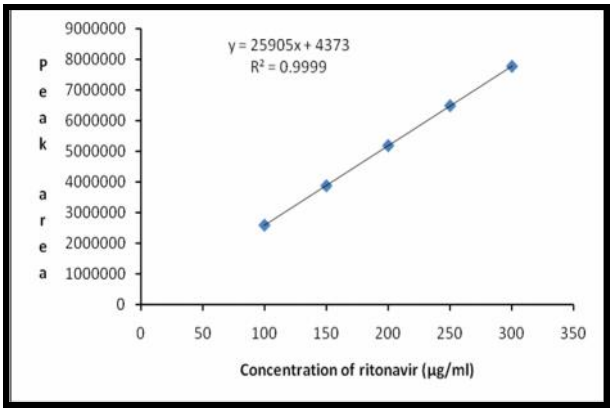

Fig. 4: Linearity curve of ritonavir

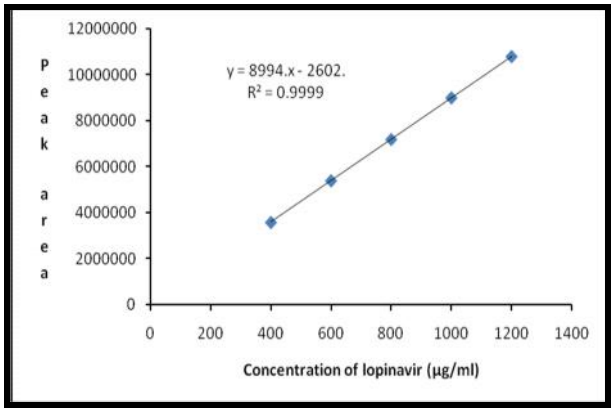

Fig. 5: Linearity curve of lopinavir

Sensitivity:

The sensitivity of the method was assessed by calculating limit of detection (LOD) and limit of quantification (LOQ) according to ICH guidelines. The results are summarized in Table 3. The low values of LOD and LOQ demonstrate the sufficient sensitivity of the method. The chromatograms of ritonavir and lopinavir at LOD and LOQ levels are presented in Fig. 6 & 7.

Table No. 3: Sensitivity of the HPLC method

Parameters	Results	
	Ritonavir	Lopinavir
LOD	0.766	2.698
LOQ	2.552	8.996

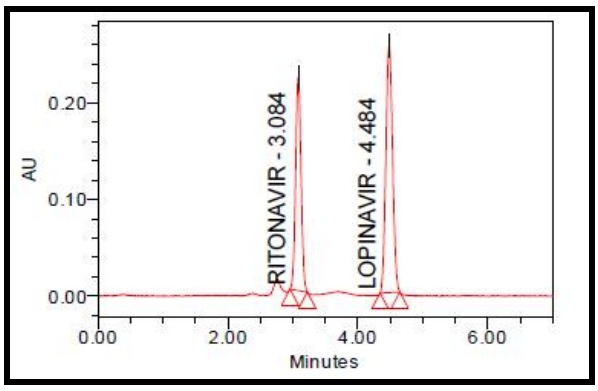

Fig. 6: Chromatogram of ritonavir and lopinavir at LOD level

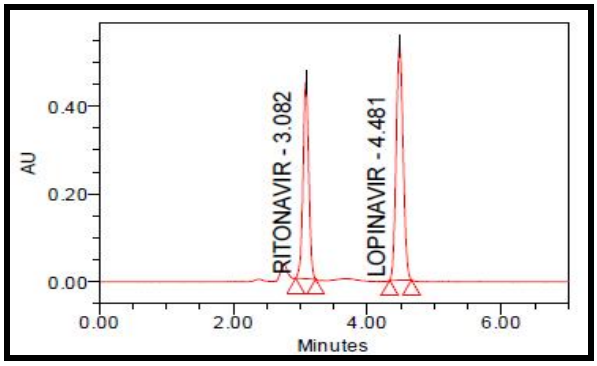

Fig. 7: Chromatogram of ritonavir and lopinavir at LOQ level

Precision:

Precision was determined by injecting six standard solutions of ritonavir (200 µg/ml) and lopinavir (800 µg/ml). The peak areas were determined. Relative standard deviation of peak areas of the two drugs was then calculated to represent precision. The results are summarized in Table 4. The low % RSD values indicated that the method was precise.

Accuracy:

Accuracy of the method was evaluated by recovery studies at three concentration (50%, 100%, and 150%) levels by standard addition method. The mean percentage recoveries obtained were shown in Table 5. The good % recovery values showed that the method was highly accurate.

Table No. 4: Precision of the HPLC method

Ritonavir		Lopinavir	
Peak area	**%RSD**	**Peak area**	%RSD
5187921	**0.05**	7193701	**0.03**
5189262		7193734	
5188179		7191824	
5183364		7190328	
5183135		7193292	
5185037		7196492	

Table No. 5: Accuracy of the HPLC method

Drug	Spiked Level	µg/ml added	µg/ml found	% Recovery	% Mean
Ritonavir	50%	99.413	99.14	100	100
	50%	99.413	99.12	100	
	50%	99.413	99.29	100	
	50%	99.413	99.24	100	
	50%	99.413	99.21	100	
	50%	99.413	99.15	100	
	100%	198.200	198.29	100	100
	100%	198.200	198.38	100	
	100%	198.200	198.34	100	
	150%	297.621	297.36	100	100
	150%	297.621	297.06	100	
	150%	297.621	297.31	100	
	150%	297.621	297.37	100	
	150%	297.621	297.26	100	
	150%	297.621	297.13	100	
Lopinavir	50%	400.062	398.33	100	100
	50%	400.062	398.57	100	
	50%	400.062	398.34	100	
	50%	400.062	398.77	100	
	50%	400.062	398.97	100	
	50%	400.062	398.20	100	
	100%	797.600	797.02	100	100
	100%	797.600	797.52	100	
	100%	797.600	797.82	100	
	150%	1197.693	1189.17	99	100
	150%	1197.693	1189.80	99	
	150%	1197.693	1188.44	99	
	150%	1197.693	1195.20	100	
	150%	1197.693	1196.00	100	
	150%	1197.693	1192.99	100	

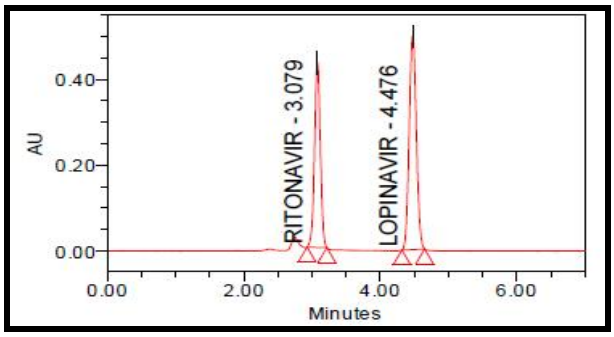

Fig. 8: Chromatogram of ritonavir and lopinavir at 50% level

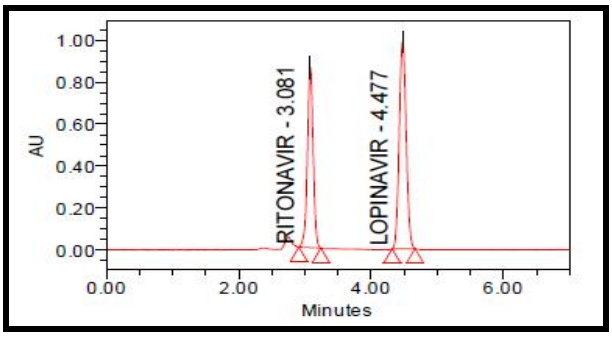

Fig. 9: Chromatogram of ritonavir and lopinavir at 100% level

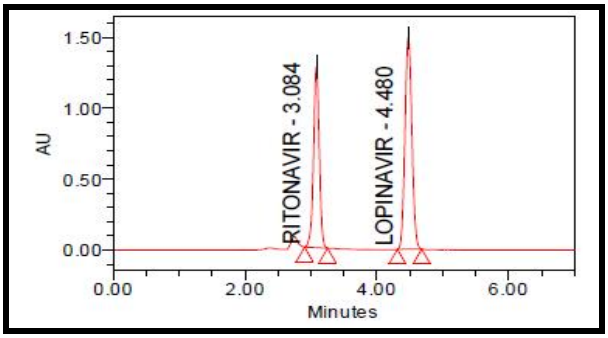

Fig. 10: Chromatogram of ritonavir and lopinavir at 150% level

Forced degradation study:

The forced degradation study was performed to prove the specificity of the method. Degradation study was performed by subjecting the tablet powder to degradations such as acid, alkaline, oxidation, thermal and photolytic conditions to evaluate the interference of degradants. Thermal degradation was performed by keeping the sample in petri dish and then placed them in an oven at 105°C for 30 minutes. The photolytic study was carried out by placing the sample in petri dish and exposed to sun light for 24 hours. Acid, base, and oxidation degradations were performed by adding 10 ml of 0.1N HCl, 10 ml of 0.1N NaOH and 10 ml of 30% peroxide solution, respectively to the sample and sonicated for 30 minutes. The acid degraded sample and base degraded sample are neutralized with 0.1 N NaOH and 0.1 N HCl, respectively. The degraded samples were injected into the HPLC system. The results are shown in Table 6. The chromatograms of degraded samples are presented in Fig. 11-15.

Table No. 6: Specificity of the HPLC method

Type of degradation	Ritonavir			Lopinavir		
	Area	% Recovered	% Degraded	Area	% Recovered	% Degraded
Acid	4186063	80	20	6292206	87	13
Base	4281275	82	18	6499272	90	10
Peroxide	4681862	89	11	6592950	91	9
Light	4981132	95	5	6794875	94	6
Heat	4784393	91	9	6793672	94	6

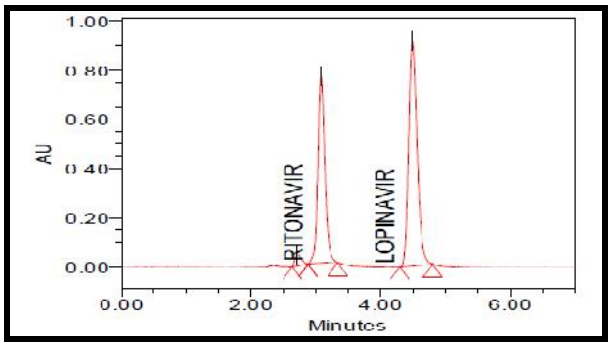

Fig. 11: Chromatogram of ritonavir and lopinavir after degradation with acid

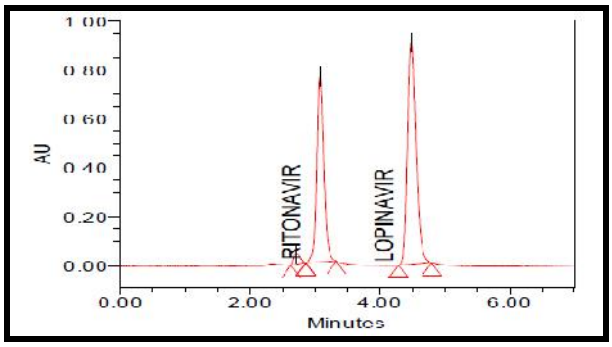

Fig. 12: Chromatogram of ritonavir and lopinavir after degradation with base

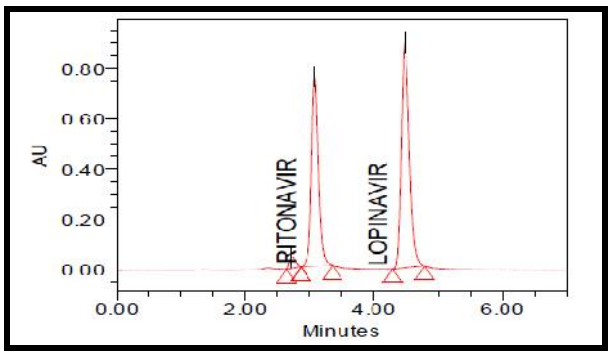

Fig. 13: Chromatogram of ritonavir and lopinavir after degradation with hydrogen peroxide

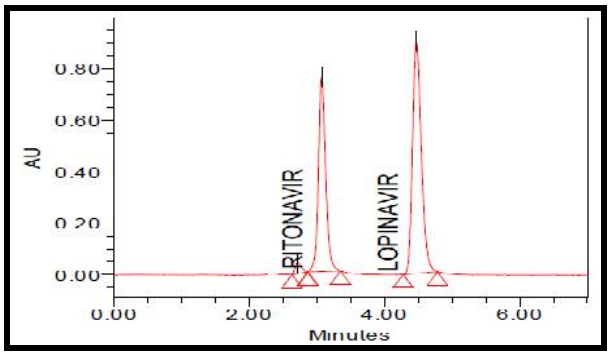

Fig. 14: Chromatogram of ritonavir and lopinavir after thermal degradation

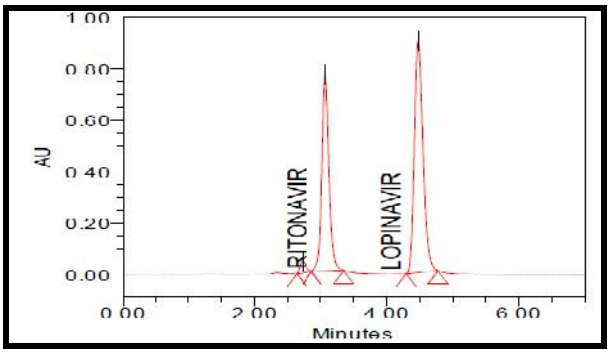

Fig. 15: Chromatogram of ritonavir and lopinavir after photolytic degradation

Robustness:

In order to show the robustness of the method, system suitability parameters were evaluated at different flow rate and different column temperature. The parameters used to define robustness are retention time, USP tailing factor and USP plate count. The results showed (Table 7) that slight variations in method parameters had a negligible effect on the analysis.

Table No. 7: Robustness of the method

Drug	Parameter	Retention time	Peak area	USP Plate Count	USP Tailing
Ritonavir	Flow 1	3.067	5194724	6920	0.946
	Flow 2	2.575	4249875	5408	0.975
	Temperature 1	3.076	5205367	6335	0.974
	Temperature 2	3.837	6659737	7027	0.968
Lopinavir	Flow 1	3.755	5951316	7487	1.064
	Flow 2	4.460	7220105	8320	1.082
	Temperature 1	4.410	7213646	9529	1.071
	Temperature 2	5.562	9255858	9744	1.076

CONCLUSION

A RP-HPLC method has been reported for simultaneous estimation ritonavir and lopinavir. The proposed method gives good resolution of the two drugs. The validation of developed method was done as per ICH guidelines and proved that method to be simple, sensitive, precise, accurate, selective and robust. The validated method was successfully applied to the determination of commercially available tablet dosage. The method can be used for the routine quality control analysis of tablet dosage forms containing ritonavir and lopinavir.

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