Rp-hplc method development and validation for simultaneous estimation of lopinavir and ritonavir in bulk and tablet dosage form

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***Abstract*: A simple, Accurate, precise method was developed for the simultaneous estimation of the ritonavir and lopinavir in bulk and tablet dosage form. Chromatogram was run through schimadzu C_{18} (250 x 4.6 mm, 5µ).Mobile phase containing Acetonitrile: phosphate Buffer taken in the ratio 60:40v/v was pumped through column at a flow rate of 1.2 ml/min. Buffer used in this method was 0.1% OPA buffer. Optimized wavelength selected was 226 nm. Retention time of ritonavir and lopinavir were found to be 6.428min and 7.325 min. %RSD of the ritonavir and lopinavir were and found to be 0.7 and 0.8 respectively. %Recovery was obtained as 100.16% and 98.96% for ritonavir and lopinavir. LOD, LOQ values obtained from regression equations of ritonavir and lopinavir were 4.08, 3.49 and 12.3, 10.5 respectively.Regression equation of ritonavir is y =13739x+11111, and y = 3713x+3510 of lopinavir.Retention times were decreased and run time was decreased, so the method developed was simple and economical that can be adopted in regular quality control test in Industries.**

***Keywords:* Ritonavir, lopinavir, Method development, RP-HPLC**

INTRODUCTION

Genotype 1a/b and 4 treatment-naïve patients with or without cirrhosis.. 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 formulation 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 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 a first-line therapy for HIV(Fig.1)

Lopinavir is an antiretroviral belonging to the *protease inhibitor* class. It is marketed by Abbott as Kaletra, a co-formulation with a sub-therapeutic dose of ritonavir, as a component of combination therapy to treat HIV/AIDS.(Fig.2)

From the literature review i found that more usage of buffer and I found 2-3 methods.But there are less 1-2 methods for assay determination of ritonavir and lopinavir in bulk and tablet dosage forms.

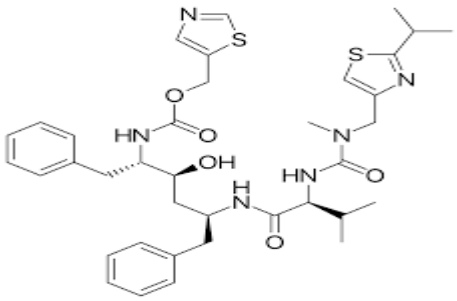


Figure 1 Ritonavir structure

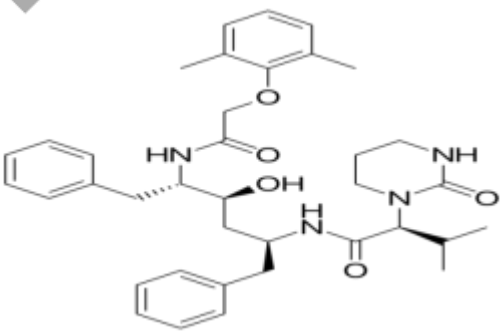


Figure 2 Lopinavir structure

MATERIALS AND METHODS

Materials: Ritonavir and lopinavir pure drugs (API), Combination ritonavir and lopinavir(Ritocom 30S tablet), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid.

Methods:

- **Diluent:** Based up on the solubility of the drugs, diluent was selected i.e methanol.
- **Preparation of standard stock solutions:** Weighed 50.4mg of ritonavir and 200.2mg lopinavir of and transferred to 100ml volumetric flask. And 3/4 th of diluent was added to these flask and sonicated for 10 minutes and volume were made upto mark with diluent and labeled as standard stock solution.
- **Preparation of standard solution:** 10ml from each stock solution was pipetted out and taken into a 100ml volumetric flask and made up with diluent.
- **Preparation of sample stock solutions:** 20 tablets were weighed and calculated average weight of tablets. Weighed 405.52mg powder and transferred into a 100 ml volumetric flask. 70ml of diluent was added, sonicated for 30 min with intermediate shaking and volume diluted with diluent and mixed wellup and filtered through 0.45µ millipore nylon filter.
- **Preparation of sample solution:** From stock solution 4ml was pipetted out and transferred into a 50ml volumetric flask and volume diluted with diluent and mixed well.
- **Detection of wave length:** The spectra of various diluted standard solution ritonavir and lopinavir in mobile phase were recorded using PDA detector.The peak of maximum absorbance was observed at 262nm(Fig.3)

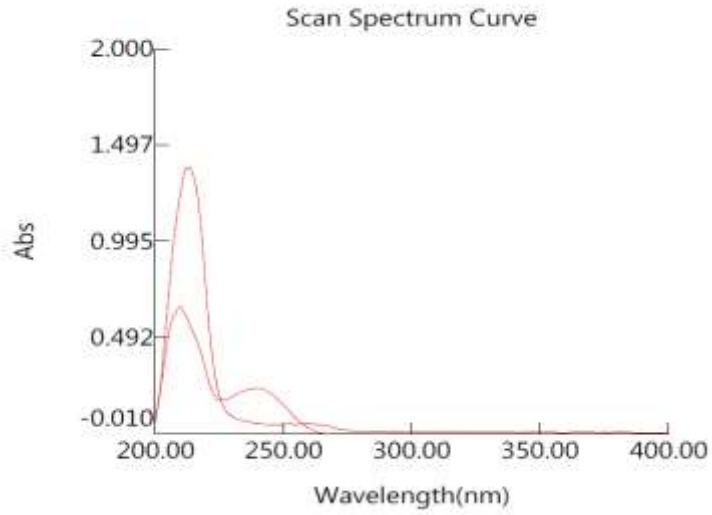


Figure 3 Uv spectrum

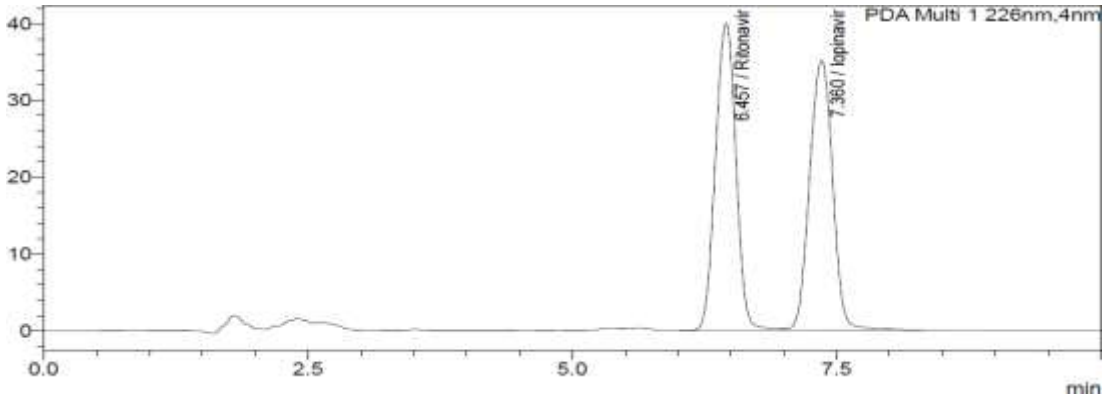
Table 1 Optimized method conditions

Mobile phase	Acetonitrile: Phosphate buffer(60:40v/v)
Flow rate	1.2ml/min
Column	Shimadzu C18(4.6 x 250mm, 5µm)
Injection volume	20□l
Run time	9 min
Detector wave length	226nm
Diluent	Methanol
Observation	Both peaks have good resolution, tailing factor, theoretical plate count and resolution.

Result: The ritonavir and lopinavir were eluted at 6.457min and 7.360 min respectively with good resolution. Plate count, tailing factor and resolution was very satisfactory. So, this method was optimized and to be validated as per ICH guidelines.

Discussion: The ritonavir and lopinavir peaks were well separated and have good resolution.

Figure 4 Optimized chromatogram



RESULTS AND DISCUSSION

Method validation:

Validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. The following parameters were evaluated.

1. System suitability:

System suitability testing is an integral part of any analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analysed constitute an integral system factor are parameters that are normally used in assessing the column performance.(Fig.5)These parameters include column efficiency, resolution, tailing factor, related standard deviation, number of theoretical plates,relative retention time and capacity factor. System suitability was performed by injecting blank followed by six injections of standards.(Table 2-3)

Table 2 System suitability data of standard chromatogram

	Ritonavir		Lopinavir	
Injection No	**Retention time**	**Peak area**	**Retention time**	**Peak area**
1	6.407	732295	7.302	741274
2	6.415	736523	7.312	747820
3	6.428	73066	7.325	743638
4	6.439	730251	7.337	743290
5	6.445	728299	7.344	742058
6	6.428	734425	7.325	742058
Mean		732077	743356	
Standard deviation		2997.533	2354.379	
%RSD		0.4	0.3	

Table 3 Data of system suitability

Parameters	Ritonavir	Lopinavir
Retention time	6.407	7.302
Tailing factor	0.998	0.990
Theoretical plates	35651	37570
%RSD	0.4	0.3

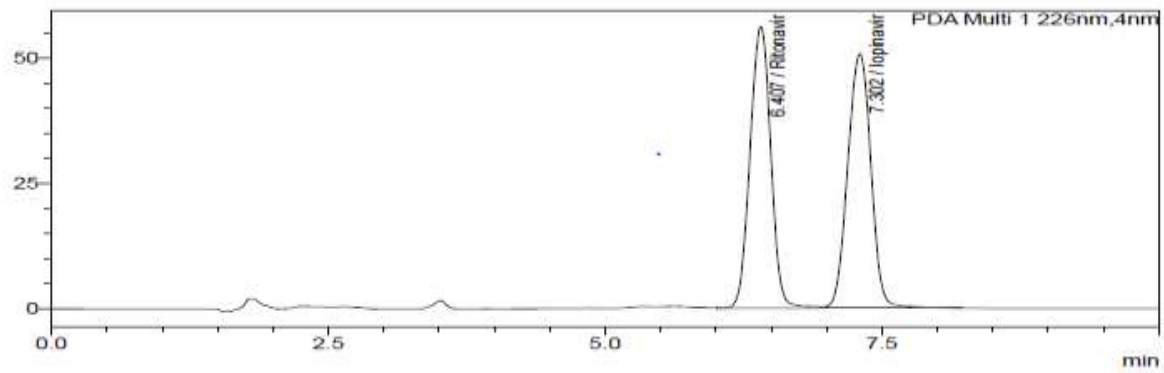


Figure 5 System suitability chromatogram

Result: From the system suitability studies it was observed that all the parameters like retention time, tailing factor, theoretical plates are within limit, hence it is concluded that the instrument and column are suitable to perform assay.
Discussion: The ritonavir and lopinavir were observed that all the parameters were within the limit.
Acceptance criteria:
The % RSD of ritonavir and lopinavir area should be NMT 2.0.
The number of theoretical plates (N) for the ritonavir and lopinavir peaks is NLT 2000.
The tailing factor (T) for the ritonavir and lopinavir peaks is NMT 2.0.

2. Specificity:
Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and excipients. Specificity was performed by injecting the blank followed by placebo and there should be no chromatogram at the retention time of API, Placebo.(Fig.6-8)

Figure 6 Specificity chromatogram of blank

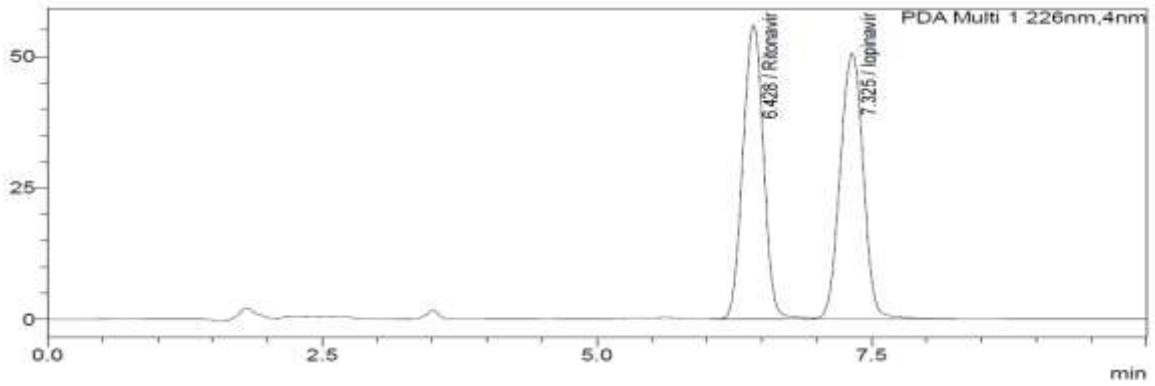
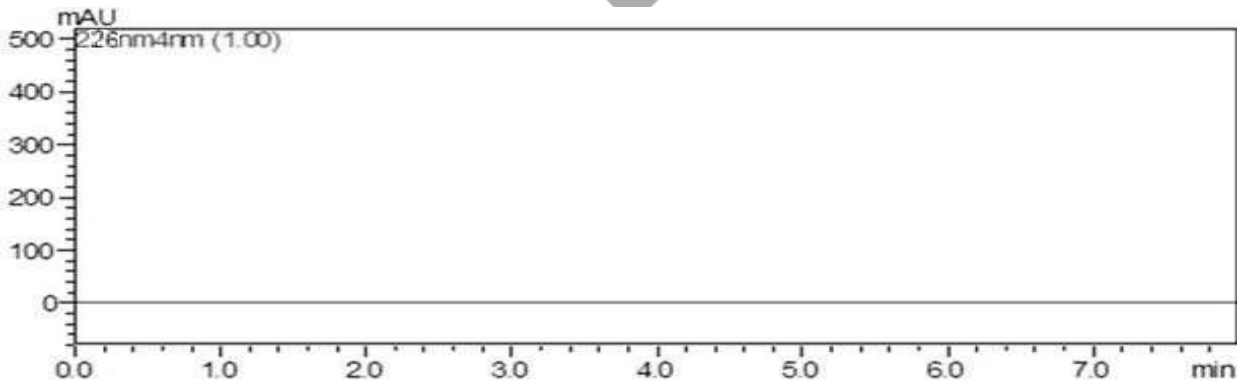


Figure 7 Chromatogram of sample



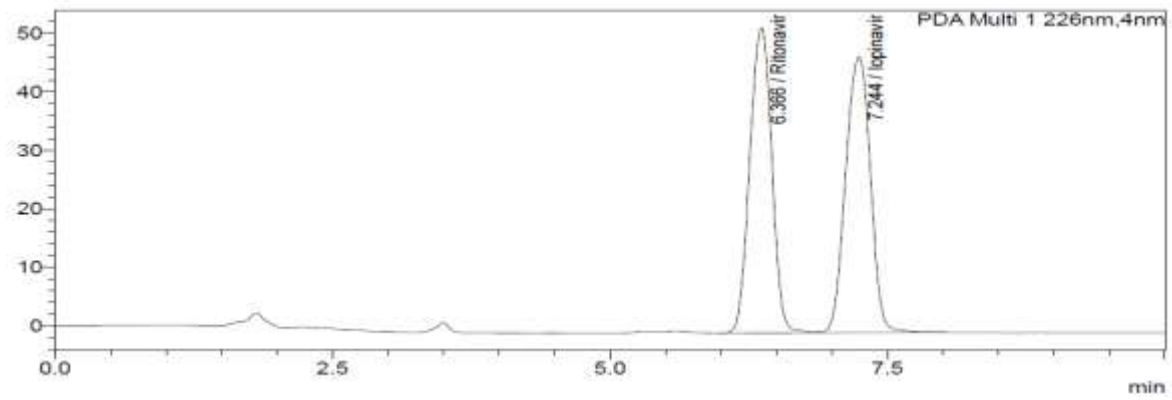


Figure 8 Chromatogram of standard

Result: No interference peak were not found at the place of ritonavir and lopinavir main analytes in blank.Retention time of ritonavir and lopinavir were 6.394 and 7.360 min.We did not find any interfering peaks in blank at retention time of these drugs ritonavir and lopinavir in this method.

Discussion: The developed method for ritonavir and lopinavir were said to be specific.

3.Linearity: Linearity of an analytical procedure is its ability to obtain test results,which are directly proportional to the concentration (amount) of analyte in the sample. (Fig.9-10) The linearity of the ritonavir and lopinavir peak area responses were determined from 25 % to 100 % and 100%-350% respectively of the nominal method concentration, 1mg/ml, by preparing 6 concentrations covering the expected range of the method.(Table 4).

Table 4 Linearity data of ritonavir and lopinavir

Ritonavir		Lopinavir	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
25	361543	100	365089
38	539523	150	554618
50	708273	200	738157
63	880460	250	922896
75	1023289	300	1107236
100	1327010	350	1486514

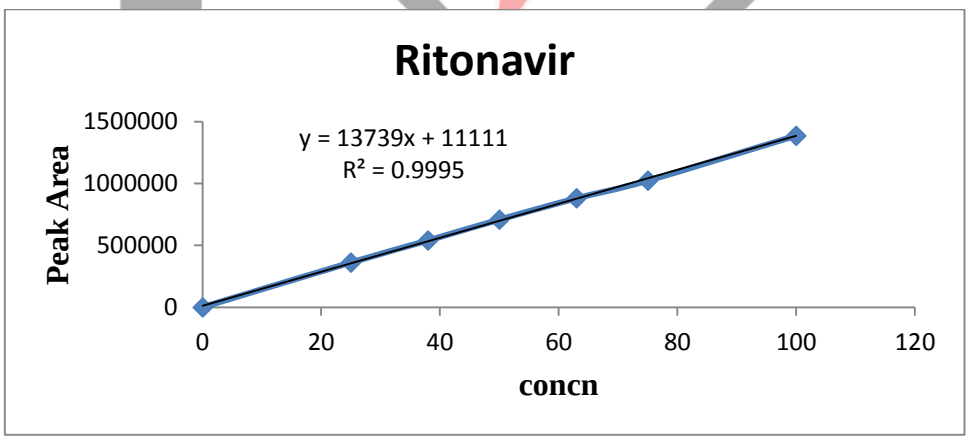


Figure 9 Calibration curve of ritonavir

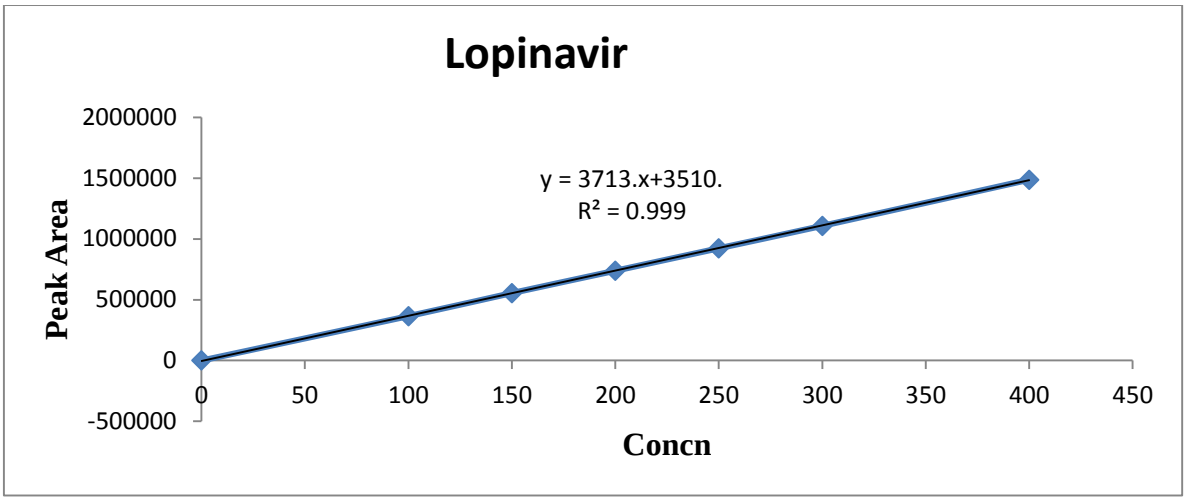


Figure 10 Calibration curve of lopinavir

Result: Six linear concentrations of ritonavir (25-100µg/ml) and lopinavir (100-350µg/ml) were injected.Average areas were mentioned above and linearity equations obtained for ritonavir was y =13739.x + 11111 and of lopinavir was y =3713.x + 3510. Correlation coefficient obtained was 0.999 for the two drugs.

Discussion: The correlation coefficient of ritonavir and lopinavir were found to be within the limit.

4. Precision:

The precision of an analytical method is a measure of the random error and is defined as the agreement between replicate measurements of the same sample. It is expressed as the percentage coefficient of variation (%CV) or relative standard deviation (RSD) of the replicate measurements.

$$\%\ \text{RSD} = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

(a)System precision:

For precision studies 6 replicate injections of ritonavir and lopinavir standard were performed. (Fig.11)% RSD was determined for peak areas of ritonavir and lopinavir.(Table 5)

Table 5 System precision data of ritonavir and lopinavir

S. No	Area of Ritonavir	Area of Lopinavir
1.	722449	734889
2.	728182	742314
3.	725449	748498
4.	729182	743214
5.	739142	738498
6.	724294	733214
Mean	728116	740105
S.D	5942.202	5696.718
%RSD	0.8	0.7

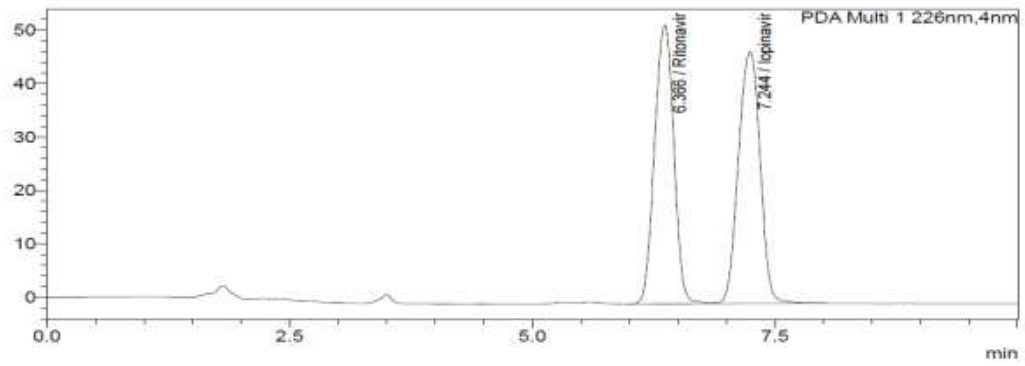


Figure 11 System precision chromatogram

Result: The sample solution for six injections were given and the obtained areas were mentioned in (Table 5).Average area, standard deviation and %RSD were calculated for ritonavir and lopinavir drugs. %RSD obtained as 0.8% and 0.7% respectively for ritonavir and lopinavir. **Discussion:** The %RSD for ritonavir and lopinavir peaks were found to be within the limit.

(b)Method precision: Method precision will be determined by Six multiple injection at 100% of nominal concentration. method precision was calculated by % RSD and it should be less than 2.

Table 6 Method precision data of ritonavir and lopinavir

S. No	Area of Ritonavir	Area of Lopinavir
1.	722549	734566
2.	723456	741324
3.	730666	749621
4.	730512	743290
5.	740123	731262
6.	721453	742058
Mean	728127	740354
S.D	7120.581	6549.921
%RSD	0.9	0.8

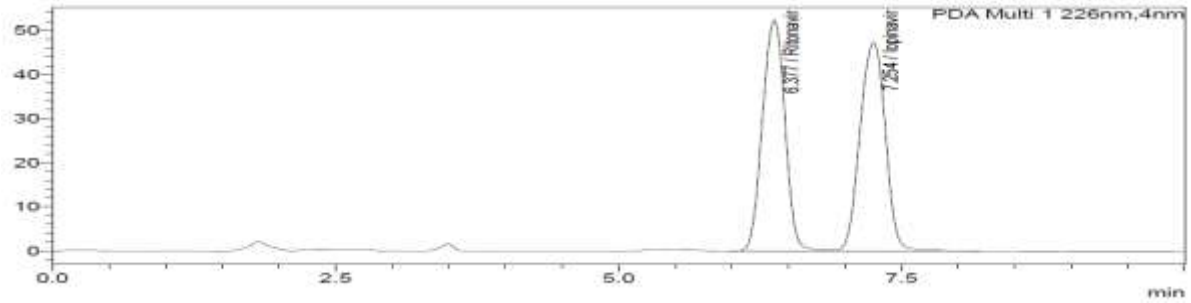


Figure 12 Method precision chromatogram

Result: The sample solutions of ritonavir and lopinavir six injections were given and the obtained areas were mentioned above (Table 6). Average area, standard deviation and %RSD were calculated for two drugs. % RSD obtained as 0.9% and 0.8% respectively for ritonavir and lopinavir.

Discussion: The %RSD for ritonavir and lopinavir peaks were found to be within the limit.

5.Accuracy:

The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or as an accepted reference value and the value found.(Table 7-8) Accuracy is calculated as the percentage of recovery by the assay of the known added amount of the analyte in the sample. (Fig.12-14)

Recovery studies were performed to assess the accuracy of the method, where known amounts of analyte were spiked into Solution and recovered. Three concentrations were prepared covering the range of the method.

Table No:7 Accuracy table of ritonavir

Sample	Accuracy	Peak area	%Recovery	%Mean recovery	Over all %recovery
Ritonavir	50	367744	100.4	100.0	100.03
	50	365428	99.3		
	50	368945	100.4		
	100	726915	98.8	99.1	
	100	725411	99.3		
	100	726840	99.3		
	150	1110247	101.1	101.0	
	150	1109145	100.9		
	150	1110421	101.0		

Table No: 8 Accuracy table of lopinavir

Sample	Accuracy	Peak area	%Recovery	%Mean recovery	Over all %recovery
Lopinavir	50	371064	98.3	98.5	98.9
	50	374048	98.6		
	50	374048	98.8		
	100	741417	98.7	98.4	
	100	738999	98.1		
	100	743017	98.5		
	150	1120201	99.0	99.9	
	150	1135204	100.6		
	150	1140404	100.1		

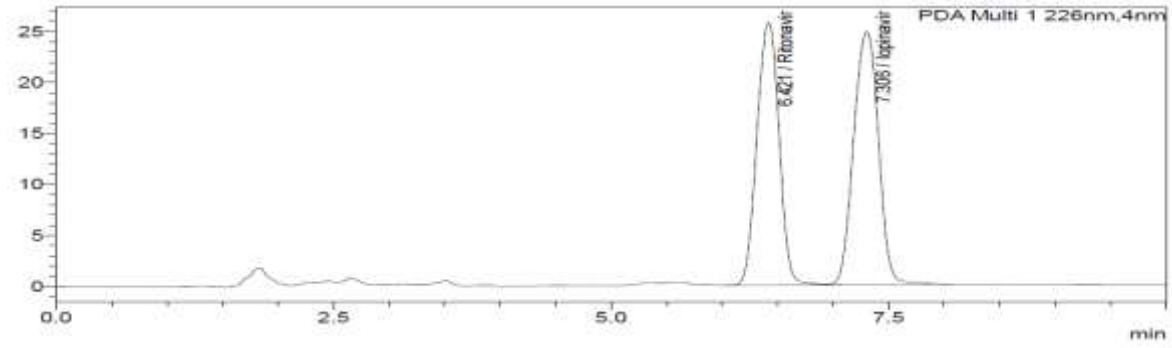


Figure 13 Accuracy 50% chromatogram of ritonavir and lopinavir

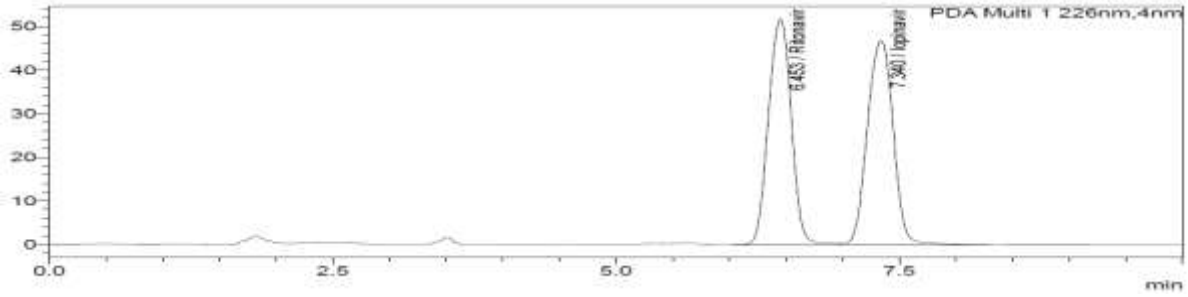


Figure 14 Accuracy 100% chromatogram of ritonavir and lopinavir

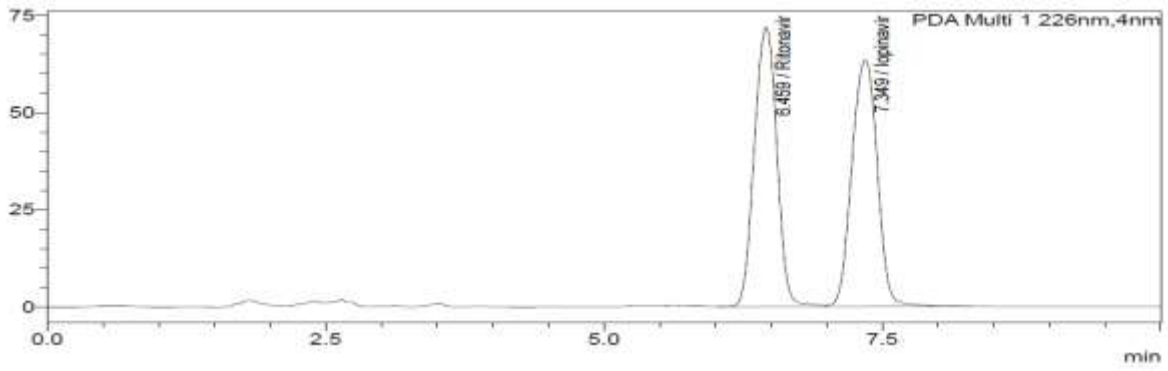


Figure 15 Accuracy 150% chromatogram of ritonavir and lopinavir

Result: Three levels of ritonavir and lopinavir accuracy samples were prepared.Triplicate injections were given for each level of accuracy and mean %recovery was obtained as 98.9% and 100.03% for ritonavir and lopinavir respectively.

Discussion: The %recovery for ritonavir and lopinavir were found to be within the limit.

6.LOD(Limit of detection):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantities.(Fig.15). The detection limit can be calculated based on the Standard Deviation of the Response and the Slope.(Table 9)

The parameter LOD was determined on the basis of response and slope of the regression equation. The Detection Limit (DL) may be expressed as:

LOD = 3.3 F/S

F = Residual standard deviation of the response,

S = Slope of the calibration curve.

7.LOQ(Limit of quantification):

parameter LOQ was determined on the basis of response and slope of the regression equation. (Fig:16).The Quantification Limit (QL) may be expressed as: **LOQ = 10 F/S**

Where, F = Residual standard deviation of the respone

S = Slope of the calibration curve.

Table 9 LOD and LOQ table of ritonavir and lopinavir

Molecule	LOD	LOQ
Ritonavir	4.08	12.3
Lopinavir	3.49	10.5

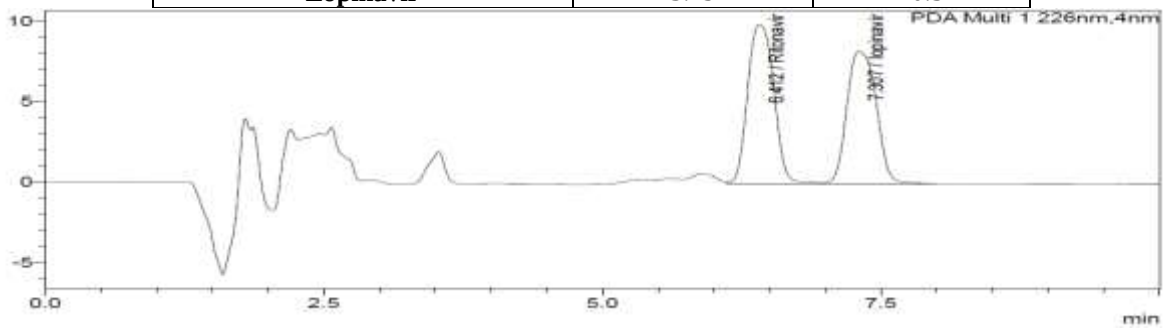


Figure 16 LOD chromatogram of standard

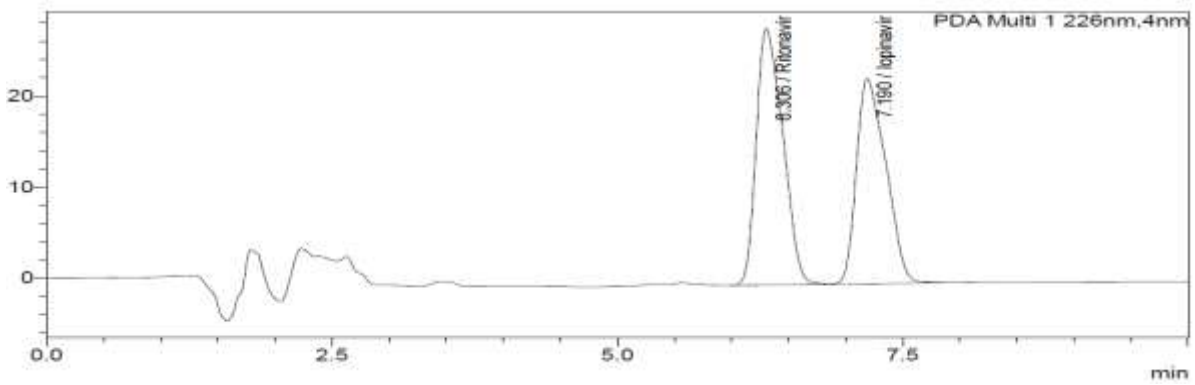


Figure 17 LOQ chromatogram of standard

Result: The LOD and LOQ for this method were found to be 4.08µg/ml, 3.49µg/ml and 12.3µg/ml,10.5µg/ml for ritonavir and lopinavir respectively.

8. Robustness: It is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and indication of its suitability during normal usage. (Table 10) (Fig.17-22).Examples of typical variations in assay, impurities and dissolution method validation by HPLC are

1. Effect of variation in mobile phase composition
2. Effect of mobile phase flow rate.
3. Effect of variation in wavelength

Table 10 Robustness data for ritonavir and lopinavir

S.no	Condition	%RSD of Ritonavir	%RSD of Lopinavir
1	Flow rate (-) 1.0ml/min	0.3	0.5
2	Flow rate (+) 1.4ml/min	0.5	0.6
3	Mobile phase (-) 50B:50A	0.2	0.5
4	Mobile phase (+) 70B:30A	0.2	0.3
5	Wavelength (-)	0.1	0.3
6	Wavelength (+)	0.3	0.2

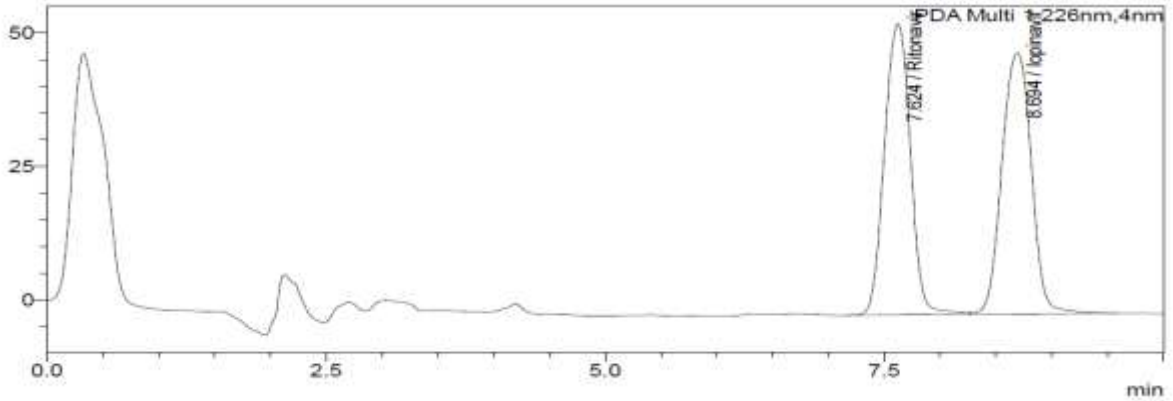


Figure 18 Flow minus chromatogram of ritonavir and lopinavir

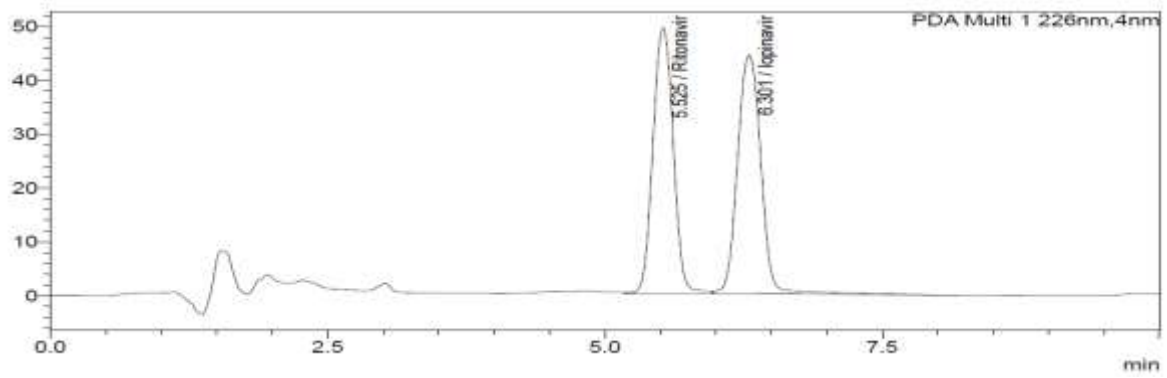


Figure 19 Flow plus chromatogram of ritonavir and lopinavir

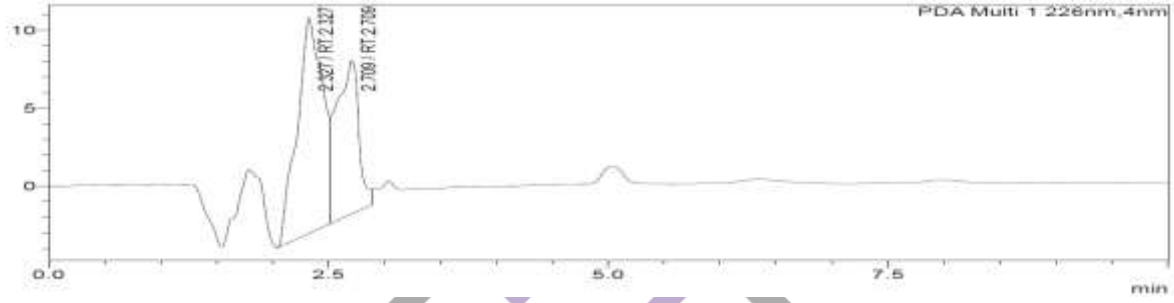


Figure 20 Mobile phase minus chromatogram of ritonavir and lopinavir

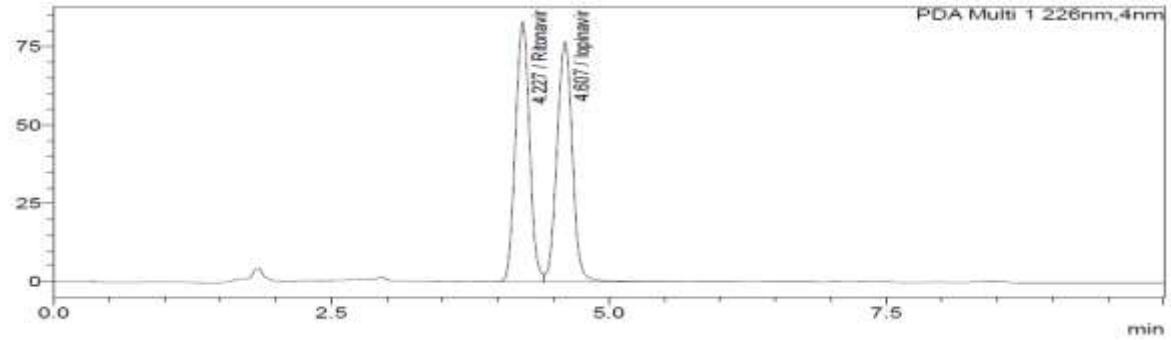


Figure 21 Mobile phase plus chromatogram of ritonavir and lopinavir

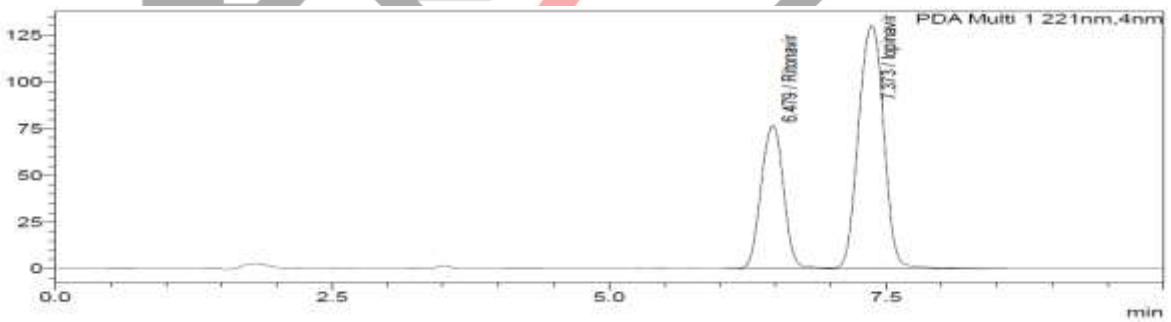


Figure 22 Wavelength minus chromatogram of ritonavir and lopinavir

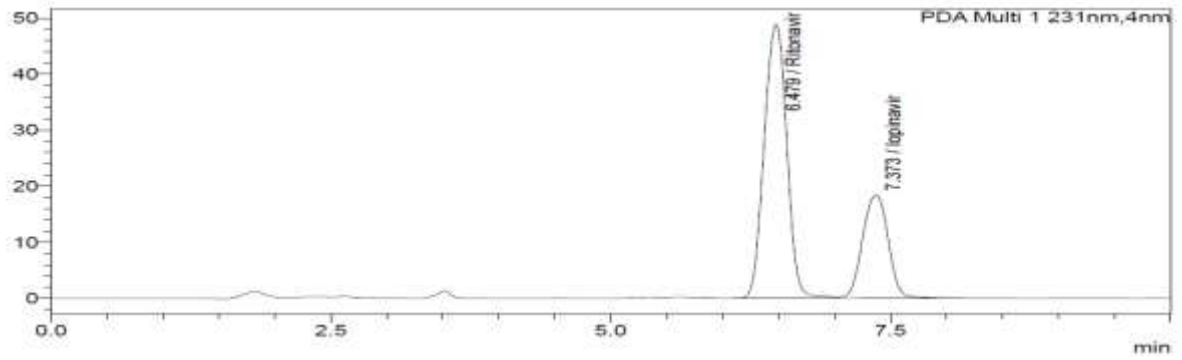


Figure 23 Wavelength plus chromatogram of ritonavir and lopinavir

Result: Robustness conditions like flow minus (1.1ml/min), flow plus (1.3ml/min), mobile phase acetonitrile:buffer(50:50v/v), mobile phase acetonitrile:buffer(70:30v/v), wavelength (231nm) and wavelength (221nm) ritonavir and lopinavir samples were injected. System suitability parameters were not much affected and all the parameters were passed. %RSD were within the limit.

Assay: The following formula used for the calculation of percentage content. (Table 11)

$$\text{Percent Assay} = \frac{\text{Practical Yield}}{\text{Therotical Yield}} \text{ X } 100$$

Table 11 Assay calculation

Tablet Sample	Label Claim (mg)	Amount Present	Assay%
Ritonavir	50	48	101.03%
Lopinavir	200	98	100.4%

Result: The %assay for ritonavir and lopinavir were found to be 101.03 and 100.4 respectively.

Discussion: The %assay for ritonavir and lopinavir were found to be within the limit.

CONCLUSION

Simultaneous analysis of lopinavir and ritonavir in their bulk and pharmaceutical formulation has been successfully achieved by the application of the developed RP-HPLC method. The drug peaks were well resolved with use of mobile phase system, Acetonitrile : Potassium dihydrogen ortho phosphate (0.02M, pH is adjusted to 3.0 with 1% Orthophosphoric acid)(60:40 v/v) with the flow rate of 1.2 ml/min and the retention time was found to be 6.428 min and 7.325 min for ritonavir and lopinavir at 226 nm (UV detector). The developed method was validated according to ICH guidelines. The linearity, precision, accuracy, reproducibility and selectivity of the method have been established. The developed method showed no interference with the formulation excipients and there was good resolution between the peaks. The developed methods are accurate and precise and hence can be applied for quality control evaluation of drugs in bulk and formulations and other matrices. The developed RP-HPLC methods are more sensitive and use economic mobile phase system, well resolved peaks and data from accuracy and precision when compared to the reported methods. Hence can be used for routine analysis in laboratory and industry.

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