STABILITY INDICATING ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF LOPINAVIR AND RITONAVIR IN BULK AND TABLET DOSAGE FORM BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC)

Paduri Amani[1*], Narender Malothu[2], N. Karnakar[3], Ramya Sri S[4]

Abstract

Developed an accurate, precise and reproducible ultra performance liquid chromatographic method (UPLC)for simultaneous estimation of Lopinavir and Ritonavir in bulk and tablet dosage forms. Chromatographic separations of the drugs were achieved on a Gemini C18 (4.6×50mm, 5.0 µm) using Isocratic elution of Water: methanol (30:70) pH 2.8 adjusted by using ortho phosphoric acid at flow rate of 0.5 ml/min. UV (Ultra Violet) detection was performed at 215 nm. The retention time obtained for the lopinavir was 0.502min and for the ritonavir was 1.583min. The calibration curves were linear over the range of 0-100 µg/mL and 0-25 µg/mL for lopinavir and ritonavir respectively. The method is validated as per ICH guidelines by determining its specificity, accuracy, precision, linearity and range, ruggedness, robustness and system suitability.

Keywords: Lopinavir, Ritonavir, Ultra performance liquid chromatographic method (UPLC)

[1*,2]Department of Pharmaceutical Analysis, KL College of Pharmacy, KL Deemed to be Universitty, Koneru Lakshmaiah education Foundation, Vaddeswaram, Guntur, AP, India.
[3]Department of Pharmaceutics, Venkateshwara Institute of Pharmaceutical sciences, Nalgonda,Telangana, India.
[4]Department of Pharmacy, University College of Technology, Osmania University, Hyderabad, Telangana, India.

***Corresponding Author:** Paduri Amani
*****Department of Pharmaceutical Analysis, KL College of Pharmacy, KL Deemed to be Universitty, Koneru Lakshmaiah education Foundation, Vaddeswaram, Guntur, AP, India.
Email: Id- amanipaduri0002@gmail.com.

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INTRODUCTION

Ultra Performance Liquid Chromatography (UPLC) is a relatively new modern technique which gives a new direction for liquid chromatography and it is applicable for particle having less than 2 µm in diameter to acquire better resolution, speed and sensitivity as compared with High-Performance Liquid Chromatography (HPLC)[1].

Lopinavir is an antiretroviral of the protease inhibitor class. Lopinavir is chemically designated as [1S- [1R*, (R*), 3R*, 4R*]]-N- [4- [[(2,6-dimethyl phenoxy) acetyl] amino]-3-hydroxy-5-phenyl-1-(phenylmethyl) pentyl] tetrahydro-alpha-(1-methylethyl)-2-oxo-1(2H) pyrimidine acetamide. Its molecular formula is $C_{37}H_{48}N_4O_5$, and its molecular weight is 628.80. Lopinavir was formulated with another protease inhibitor, ritonavir. When administered alone, lopinavir has insufficient bioavailability[2].

Ritonavir is an antiretroviral drug from the protease inhibitor class used to treat HIV infection and AIDS. Ritonavir is frequently prescribed with highly active anti-retroviral therapy, not for its antiretroviral action, but as it inhibits the same host enzyme that metabolizes other protease inhibitors. The chemical name of Ritonavir is (5S, 8S, 10S, 11S) - 10- hydroxy- 2- methyl- 5- (1- methylethyl) -1- [2- (1-methylethyl) - 4- thiazolyl] - 3, 6-dioxo-8, 11- bis (phenylmethyl)-2, 4, 7, 12- etraaz-atridecan- 13-oic acid 5-thiazolyl methyl ester. It is official in Indian Pharmacopoeia and United States Pharmacopoeia[3].

A literature survey reveals analytical methods like UV spectrophotometric[6], HPTLC[7], HPLC[8], LC-MS for simultaneous determination of lopinavir and ritonavir in pharmaceutical dosage forms and biological fluids[9-11] are reported. However, no references are reported so far for the simultaneous determination of said drugs by UPLC method. It is needed to determine the intrinsic stability of a drug substance in formulation and to establish degradation pathways of drug substances and drug products.

MATERIALS AND METHODS:

Lopinavir and Ritonavir were obtained as gift samples from Sura Pharma Labs, Dilsukhnagar. All the reagents used were of UPLC grade were purchased from MERCK, India. The commercially available tablets(Lopimune) containing a combination of Lopinavir 200 mg and Ritonavir 50mgwere procured from pharmacy. All the solutions for analysis were prepared and analyzed freshly.

Instruments

Waters UPLC with a PDA detector processed by Empower software. Sartorius analytical micro-balance (Gottingen, Germany), an ultra-sonic cleaner (Spincotech Pvt. Ltd), pH meter LI 610 ELICO (Mumbai, Maharashtra, India) were also used.

UPLC Method development and validation for simultaneous determination of Lopinavir and Ritonavir in drug products Chromatographic conditions

The separation was achieved on Waters Acquity UPLC BEH C18 column (2.1 × 50mm i.d., 1.7 µm particle size). The mobile phase water: methanol (30:70) pH 2.8 (adjusted by using ortho phosphoric acid) used throughout the analysis. The flow rate of mobile phase was 0.5 ml/min and the detection was monitored at UV-visible PDA detector (215nm). The mobile phase was filtered through a nylon 0.22 µm membrane filter and was degassed before use. The column temperature was maintained at 30°C and injection volume was 5 µL.

Standard preparation

Standard stock solution containing Lopinavir (1000µg/mL) and Ritonavir(1000µg/mL) was prepared by transferring 100mg Lopinavir100mg Ritonavir working standard into a 100 mL volumetric flask. A 40 mL portion of diluent (methanol: 1N HCL, 1:1v/v) was added, sonicated and cooled to room temperature. The solution was diluted to the mark with diluent. Standard solution containing Lopinavir(100µg/mL) and Ritonavir(100 µg/mL) was prepared by pipetting 10 mL stock solution into a 100mL volumetric flask and diluted up to the mark with diluent.

Test preparation

Twenty tablets of marketed formulation Lopimune Tablet containing Lopinavir 200 mg and Ritonavir 50mg were weighed and the average weight was calculated. The tablets were crushed with a mortar and pestle for 10 min. A portion of powder equivalent to the weight of 5 mg of Lopinavir and 5 mg of Ritonavir was accurately weighed and transferred to a 100 ml volumetric flask. Approximately 50 ml diluent was added and the mixture was sonicated for 15 min with intermittent shaking. The contents were restored to room temperature and diluted to volume with diluent to furnish stock test solution. The stock solution was filtered through 0.45µm membrane filters and 10 ml of the filtered solution was transferred to a 100

ml volumetric flask and diluted to volume with diluents to give test solution containing 1000µg/ml Lopinavir and 1000µg/ml Ritonavir.

Parameters for method validation are listed below:

Linearity

Linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in samples within a given range.

Determination - To establish linearity of the proposed method, separate series of solutions were prepared from the stock solutions and analyzed. Linearity was determined by a series of five to six estimation. The responses were found directly proportional to the concentrations of the analytes.

Range

ICH defines the range of an analytical procedure as the interval from the upper to the lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Accuracy

ICH defines the accuracy of an analytical procedure as the closeness of agreement between the conventional true value or an accepted reference value and the value found. Accuracy may be estimated from the recovery of a known standard solution ‘‘spiked’’ or added into the sample. That is, a known amount of the same substance that is to be tested is added to an aliquot of the sample, usually as a solution, prior to the analysis. The concentration of the analyte in the spiked solution of the sample is then measured. The percent spike recovery is then calculated.

$$\% \text{ Recovery} = \frac{100\,(Xs - Xu)}{K}$$

Where, **Xs**= Measured value for the spiked sample
Xu= Measured value for the unspiked sample adjusted for the dilution of the spike
K= Known value of spike in the sample.
To check the degree of accuracy of the method, recovery studies were performed in triplicate by standard addition method at 80%, 100% and 120%. known amounts of standard were added to pre-analyzed samples and were subjected to the proposed method.

Acceptance criterion

Recovery need not be 100% but all result should fall between ±20% of the expected value at lower concentration and ±15% at higher concentration and the percent relative standard deviation should be less than 2.

Precision

The precision of analysis is usually determined by running duplicate or replicate testing on one of the samples in a given batch of samples. It is expressed statistically as standard deviation, relative standard deviation (RSD), coefficient of variance (CV), standard error of the mean (*M*), and relative percent difference (RPD). The standard deviation in measurements, however, can vary with the concentrations of the analytes. On the other hand, RSD, which is expressed as the ratio of standard deviation to the arithmetic mean of replicate analyses and is given as a percent, does not have this problem and is a more rational way of expressing precision:

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

Arithmetic mean of replicate analysis

The precision of the method, as intra-day repeatability was evaluated by performing six independent assays of the test sample preparation and calculating the % RSD. The intermediate (interday) precision of the method was checked by performing same procedure on different days by another person under the same experimental conditions.

Robustness

The standard solution was prepared and injected for 3 times with following condition
1) Aq. Phase ratio of mobile phase has changed ± 1%
2) Flow rate has changed ± 10%
3) Change of wavelength

Forced degradation studies

The stability of the developed method was established by performing forced degradation studies of the drug in the presence of acid, alkali, H_2O_2, temperature, light.

Acid degradation

Degradation under acidic condition was evaluated by treating 1 ml of standard stock solution of Lopinavir and Ritonavir with 1 ml of 2N HCl and refluxed for 30 min at 60 ± 2 °C. The resulting solution was diluted to 10 ml with the diluent.

Alkali degradation

Under alkaline conditions, degradation was studied by refluxing 1 ml of standard stock solution of Lopinavir and Ritonavir with 1 ml of 2N NaOH for 30 min at 60 ± 2 °C. The stressed solution was made up to 10 ml with the diluent.

Oxidative degradation

About 1 ml of standard stock solution of Lopinavir and Ritonavir was subjected to oxidative degradation by refluxing with 20% *v/v* H_2O_2 in a 10-ml volumetric flask for 30 min at 60 ± 2 °C and made up with the diluent.

Thermal degradation

Thermal stability of the drugs was evaluated by placing the standard stock solution in the oven at 105 ± 2 °C for 6 h. About 1 ml of the stressed solution was diluted to 10 ml with the diluent.

Photolytic degradation

Photolytic degradation was studied by exposing the standard solution of Lopinavir and Ritonavir to sun light for 7 days. The resulting stressed solution was diluted to 10 ml with the diluent.

About 10 µl of each of the solutions exposed to different stress conditions were injected separately into the column, and the chromatograms were recorded to evaluate the stability of the drugs.

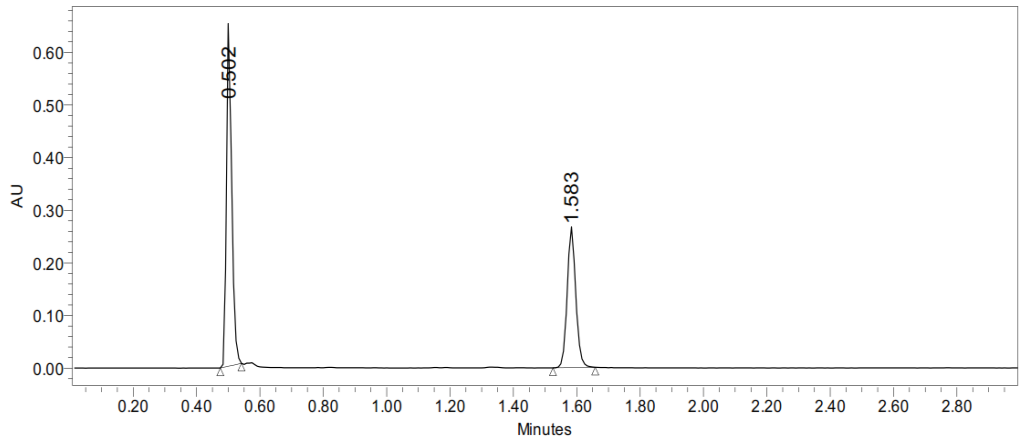
RESULTS AND DISCUSSION

Optimized Chromatogram (Standard)

Mobile phase: water: methanol (30:70) pH 2.8 adjusted by using ortho phosphoric acid
Column: Gemini C18 (4.6×50mm, 5.0 µm)
Flow rate: 0.5 ml/min
Wavelength: 215 nm
Column temp: 30ºC
Injection Volume: 5 µl
Run time: 3 minutes

Table: - peak results for optimised

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Lopinavir	0.502	752559	662646		1.3	4559
2	Ritonavir	1.583	502263	264186	22.6	1.1	12031



Observation: From the above chromatogram it was observed that the Lopinavir and Ritonavir peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Optimized Chromatogram (Sample)

Mobile phase : water: methanol (30:70) pH 2.8 adjusted by using ortho phosphoric acid
Column : Gemini C18 (4.6×50mm, 5.0 µm)
Flow rate : 0.5 ml/min
Wavelength : 215 nm
Column temp : 30ºC
Injection Volume : 5 µl
Run time : 3 minutes

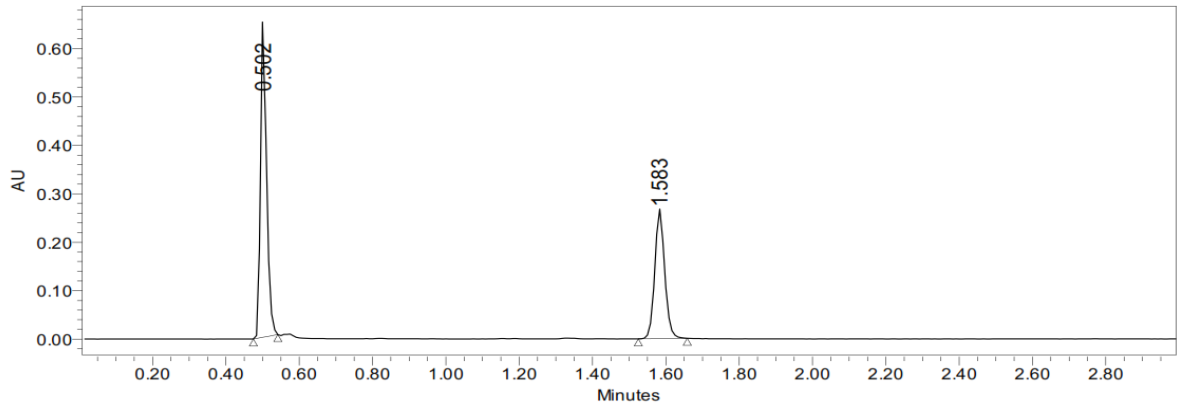


Table: Optimized Chromatogram (Sample)

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Lopinavir	0.502	752653	662786		1.1	6523
2	Ritonavir	1.583	502278	264225	24.2	1.2	11179

Acceptance criteria:

- Resolution between two drugs must be not less than 2
- Theoretical plates must be not less than 2000
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

System suitability

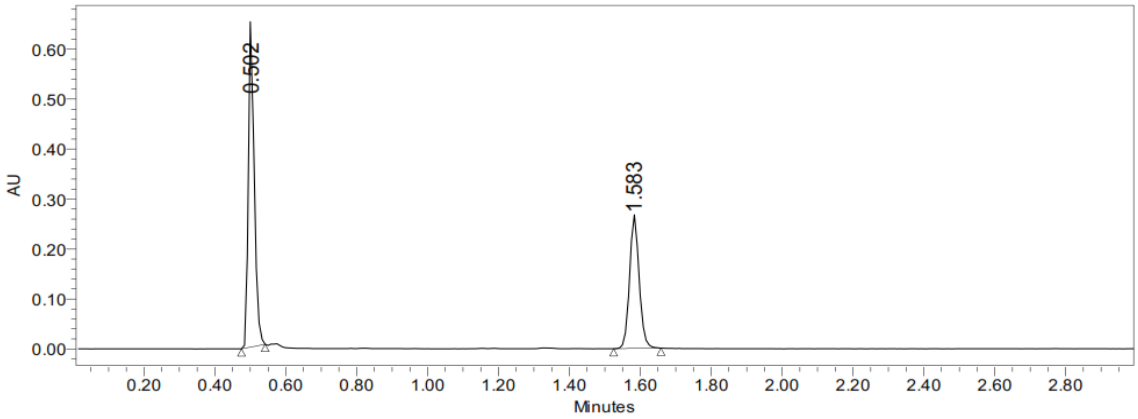


Fig: Chromatogram showing injection -1

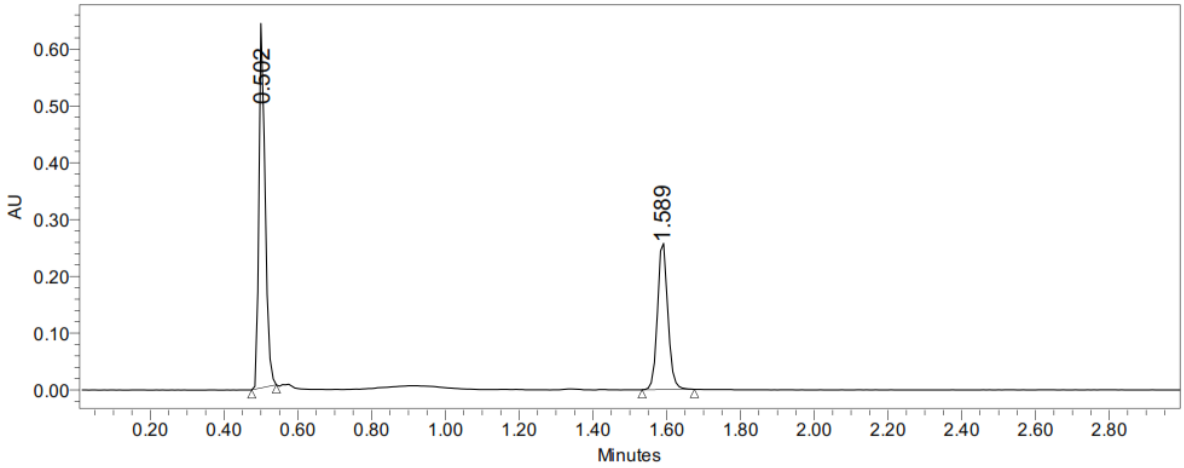


Fig: Chromatogram showing injection -2

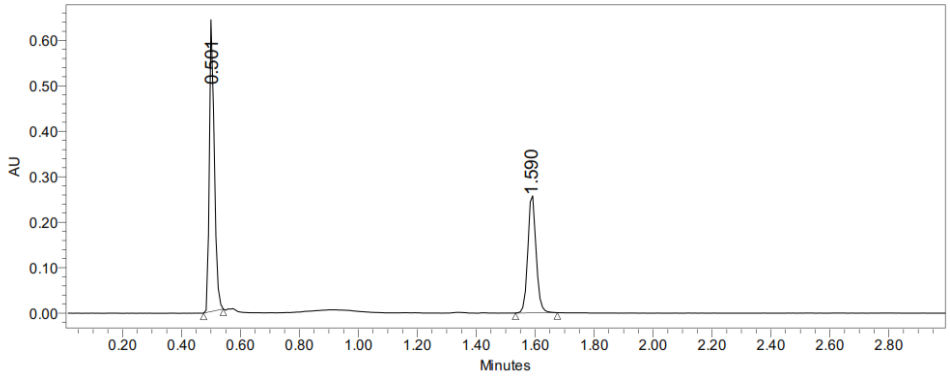


Fig: Chromatogram showing injection -3

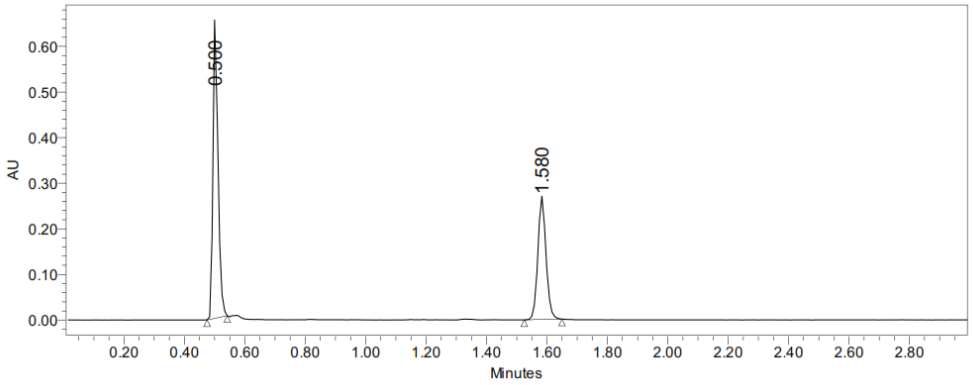


Fig: Chromatogram showing injection -4

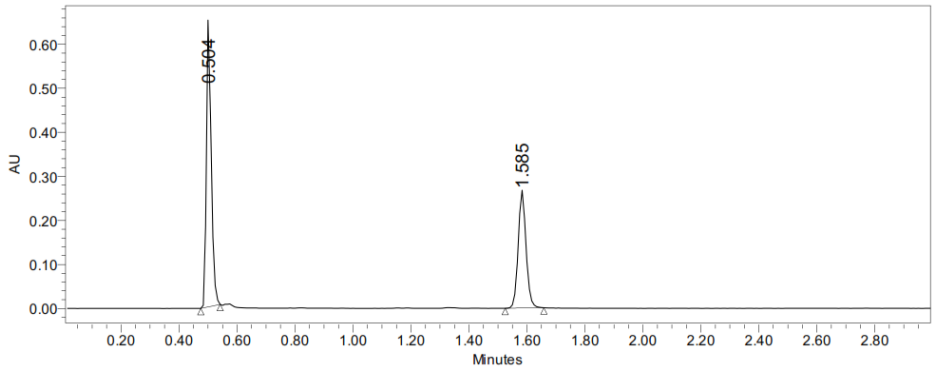


Fig: Chromatogram showing injection -5

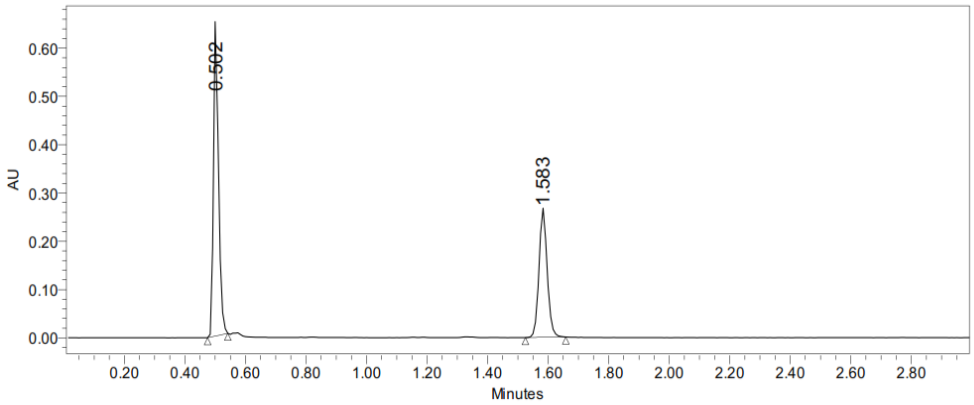


Table: Results of system suitability for Lopinavir

S No	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Lopinavir	0.502	743386	662289	4736	1.3
2	Lopinavir	0.502	752476	662745	4895	1.3
3	Lopinavir	0.501	753691	663722	4497	1.3
4	Lopinavir	0.500	751187	653579	4771	1.3
5	Lopinavir	0.504	745963	653921	4963	1.3
Mean			749340.6			
Std. Dev			4444.979			
% RSD			0.593185			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

Table: Results of system suitability for Ritonavir

S No	**Name**	**Rt**	**Area**	**Height**	**USP plate count**	**USP Tailing**	**USP Resolution**
1	Ritonavir	1.583	501245	259386	11179	1.1	22.1
2	Ritonavir	1.589	501174	266605	12875	1.1	22.5
3	Ritonavir	1.590	501488	265529	12999	1.1	22.9
4	Ritonavir	1.580	502163	265531	12874	1.1	22.8
5	Ritonavir	1.585	514571	266615	12872	1.1	22.7
Mean			504128.2				
Std. Dev			5850.745				
% RSD			1.160567				

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Assay (Sample):

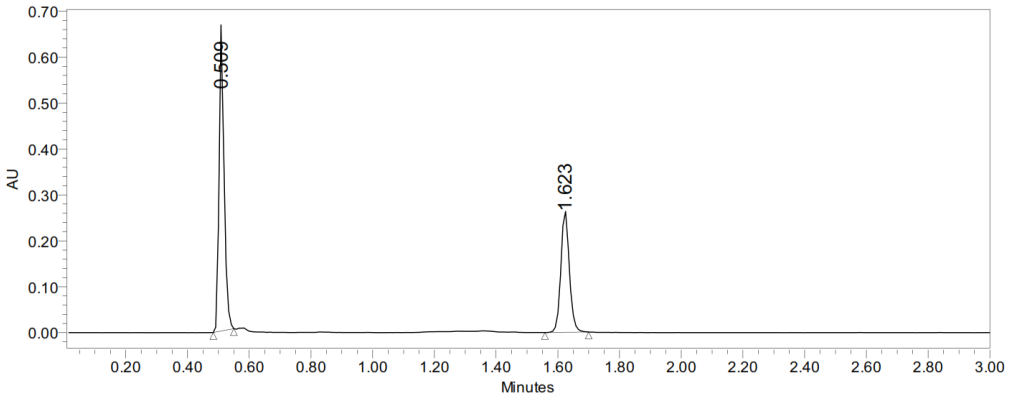


Fig: Chromatogram showing assay of sample injection-1

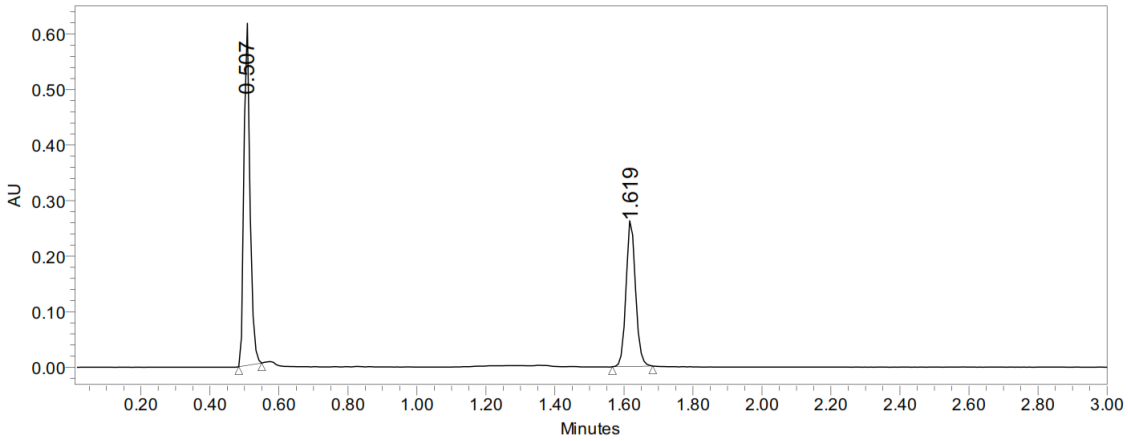


Fig. Chromatogram showing assay of sample injection-2

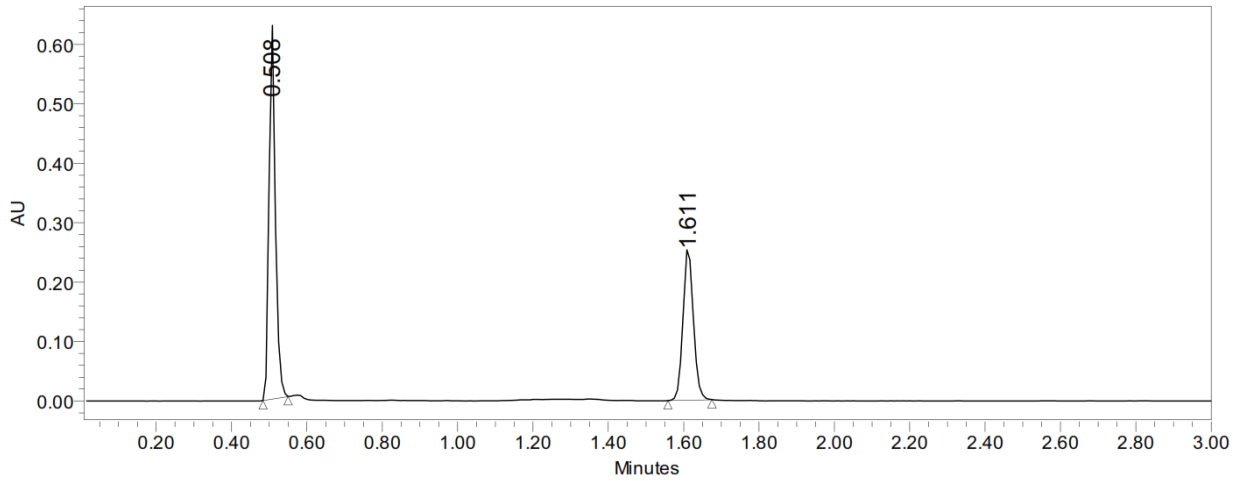


Table: Peak results for Assay sample

S no	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Lopinavir	0.509	749667	623257		1.3	5122.5	1
2	Ritonavir	1.623	491382	259385	22.3	1.1	11492.4	1
3	Lopinavir	0.507	734254	630844		1.3	4821.5	2
4	Ritonavir	1.619	504286	266601	23.5	1.2	12925.7	2
5	Lopinavir	0.508	750068	672058		1.3	4883.4	3
6	Ritonavir	1.611	506964	265525	23.1	1.2	13952.7	3

%ASSAY =

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

=734254/750068×10/60×60/0.0305×99.7/100×0.6285/200×100 (Lopinavir)

=100.55%

=504286/507457×10/15×15/0.125×99.8/100×0.6285/50×100 (Ritonavir)

=99.73%

The % purity of Lopinavir and Ritonavir in pharmaceutical dosage form was found to be 100.55%, 99.73%.

LINEARITY

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY:

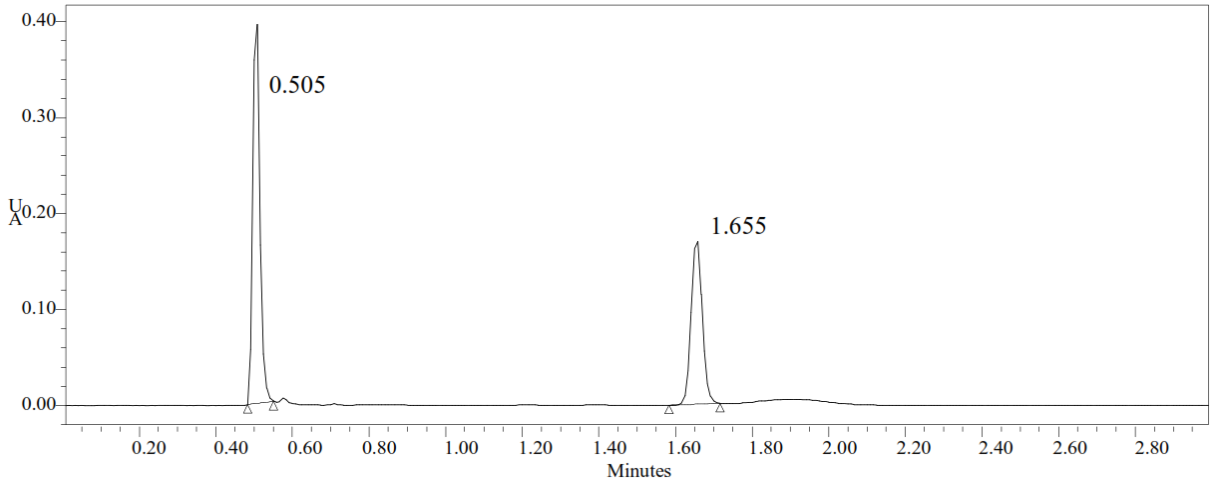


Figure: Chromatogram for linearity concentration-20 µg/ml of Lopinavir &5 µg/ml of Ritonavir

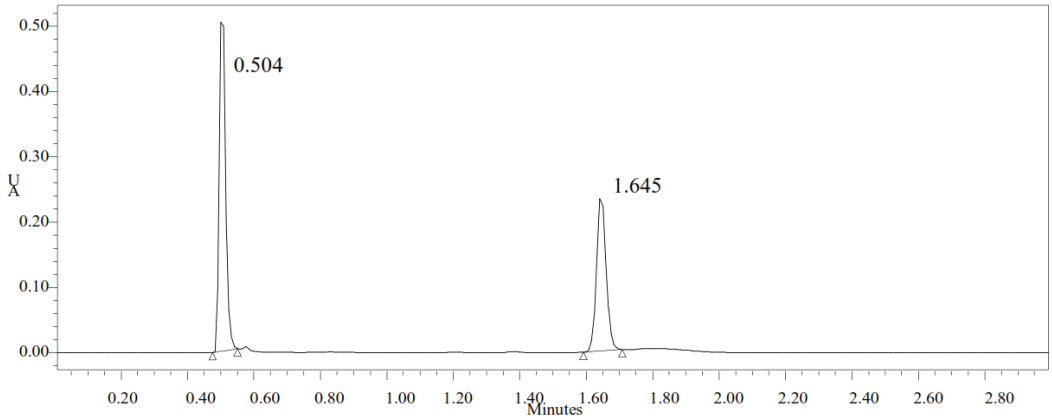


Figure: chromatogram for linearity concentration-40 µg/ml of Lopinavir &10µg/ml of Ritonavir

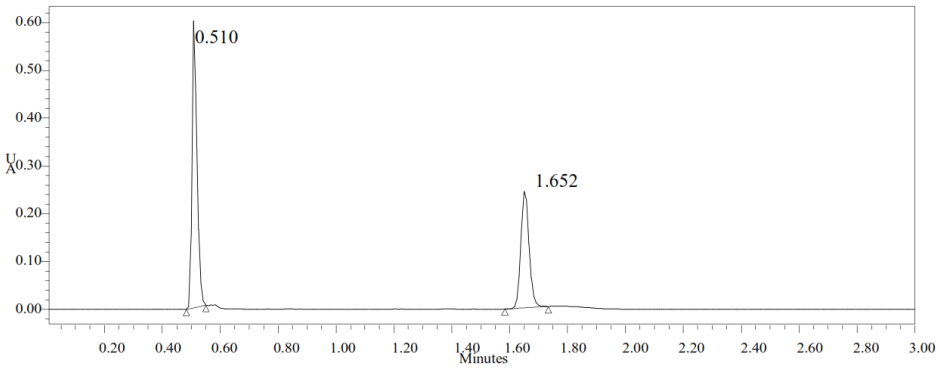


Figure: chromatogram for linearity concentration-60 µg/ml of Lopinavir &15 µg/ml of Ritonavir

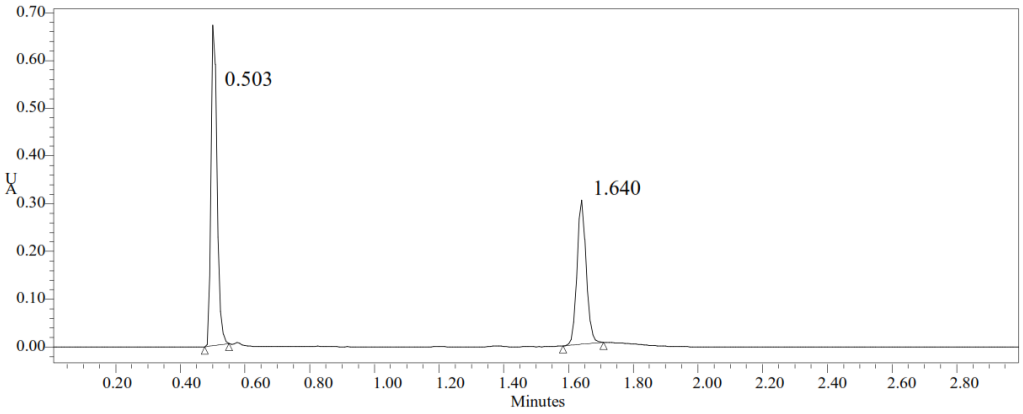


Figure: chromatogram for linearity concentration-80 µg/ml of Lopinavir&20 µg/ml of Ritonavir

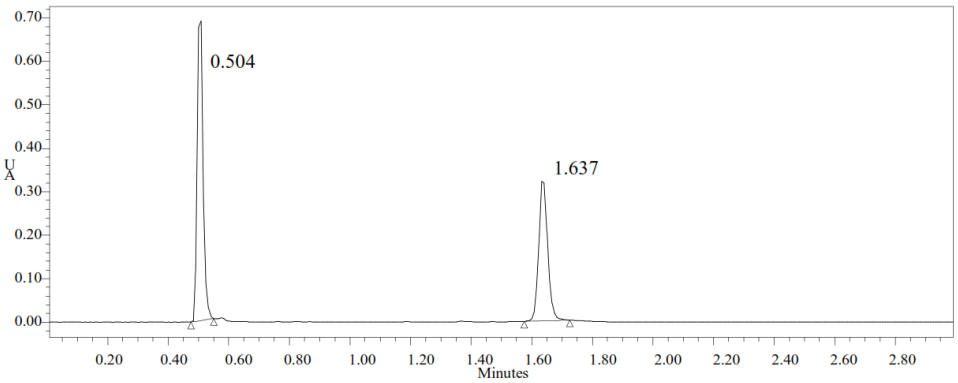


Figure: chromatogram for linearity concentration-100 µg/ml of Lopinavir&25 µg/ml of Ritonavir

Lopinavir:

Concentration µg/ml	Average Peak Area
0	0
20	521793
40	620803
60	713828
80	827261
100	932646

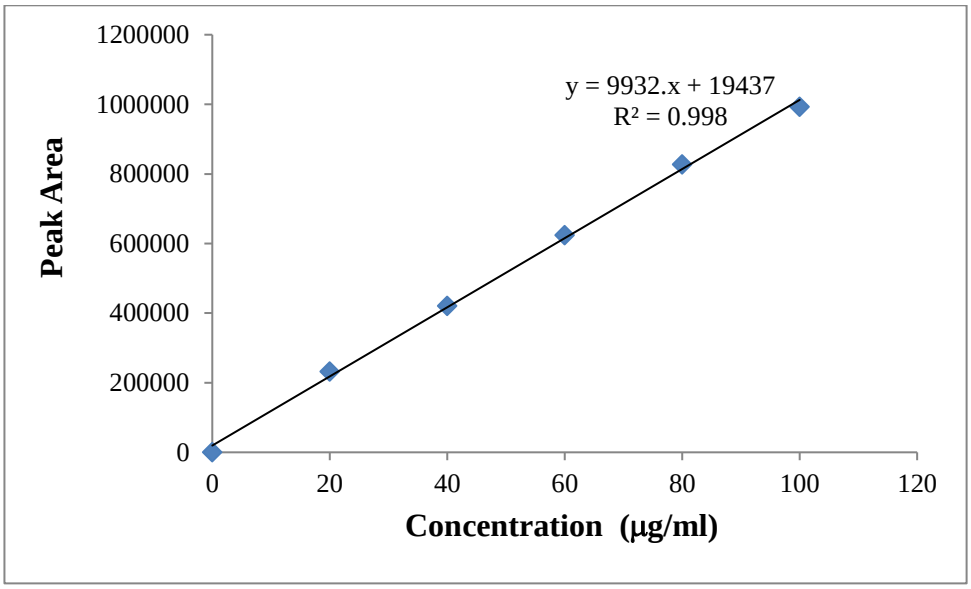


Figure 6.3.4 calibration graph for Lopinavir

LINEARITY PLOT:

The plot of Concentration (x) versus the Average Peak Area (y) data of DRUG is a straight line.

Y = mx + c

Slope (m) = 9932

Intercept (c) = 19437

Correlation Coefficient (r) = 0.998

VALIDATION CRITERIA:

The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION:

Correlation Coefficient (r) is 0.99, and the intercept is 19437. These values meet the validation criteria.

Ritonavir

Concentration µg/ml	Average Peak Area
0	0
5	189013
10	364022
15	529213
20	696276
25	856691

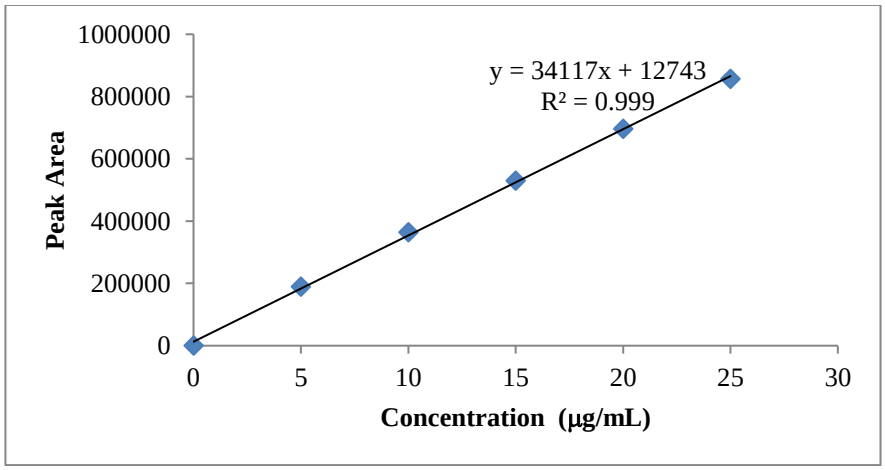


Figure 6.3.4 calibration graph for Ritonavir

LINEARITY PLOT:

The plot of Concentration (x) versus the Average Peak Area (y) data of drug is a straight line.

$$Y = mx + c$$

Slope (m) = 34117

Intercept (c) = 12743

Correlation Coefficient (r) = 0.999

VALIDATION CRITERIA:

The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION:

Correlation Coefficient (r) is 0.99, and the intercept is 12743. These values meet the validation criteria.

PRECISION:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

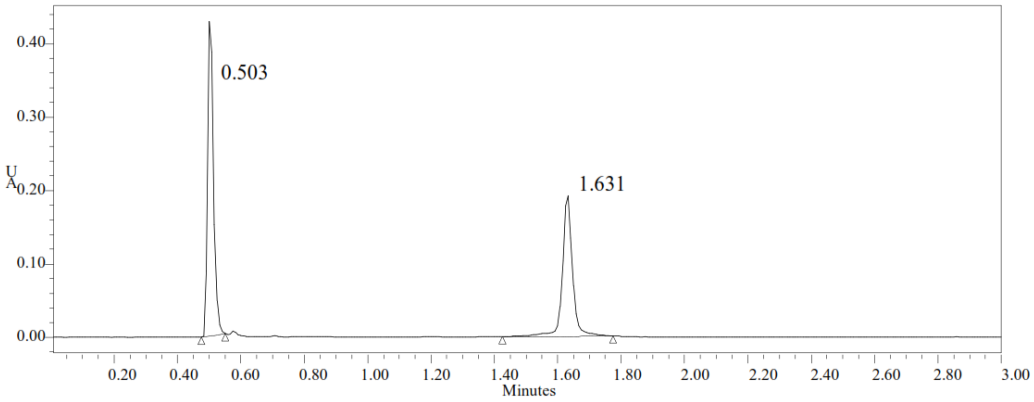


Fig. Chromatogram showing accuracy-50% injection-1

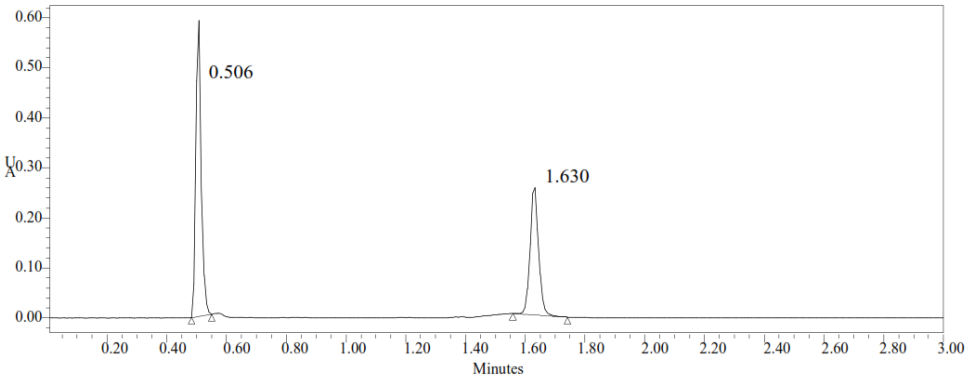


Fig Chromatogram showing accuracy-100% injection-1

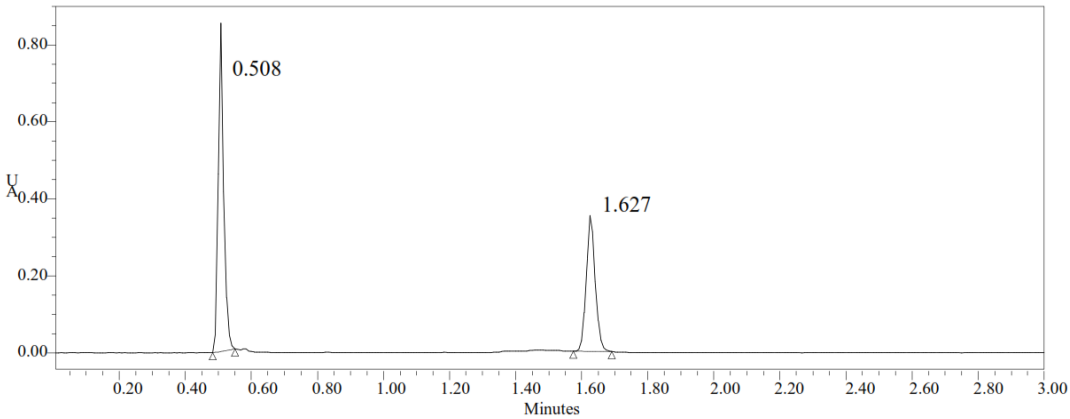


Fig Chromatogram showing accuracy-150% injection-1

The accuracy results for Lopinavir

%Concentration (at specification Level)	Area	Amount Added(ppm)	Amount Found(ppm)	% Recovery	Mean Recovery
50%	375416	30	30	100	99.9%
100%	743771	60	60.06	100.08	
150%	1119706	90	89.8	99.8	

The accuracy results for Ritonavir

%oncentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	259579	7.5	7.5	100	99.8%
100%	512032	15	14.9	99.3	
150%	772662	22.5	22.54	100.1	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Table: Results for Robustness
Variation in flow

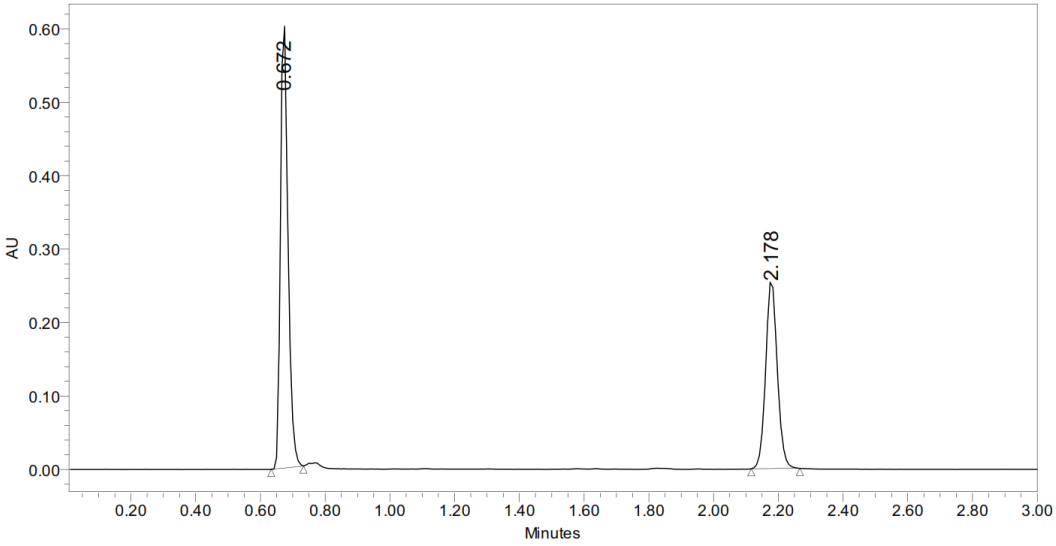


Figure: chromatogram showing less flow of 0.4 ml/min

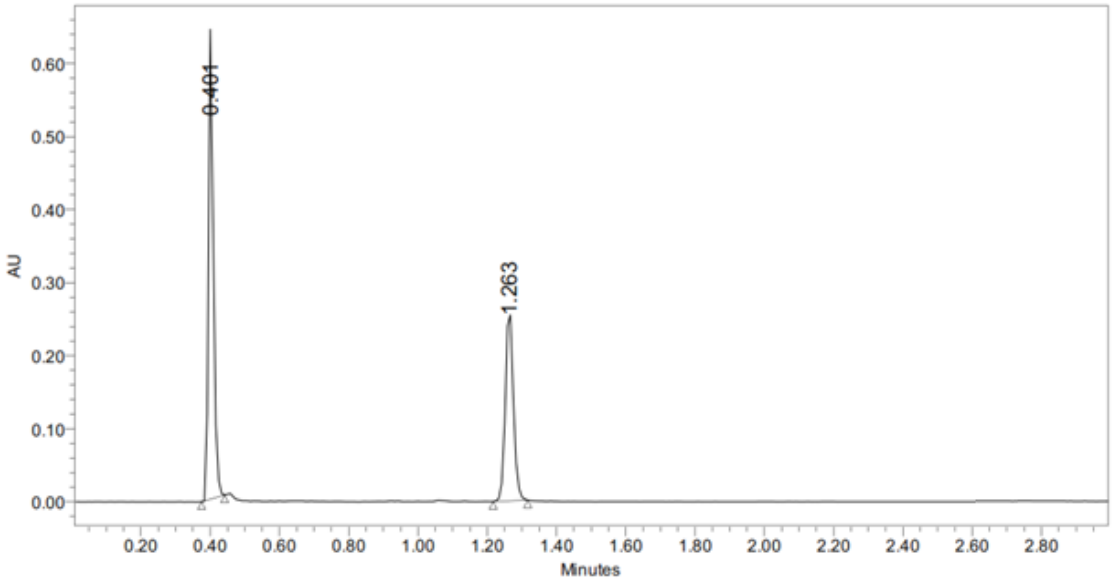


Figure: chromatogram showing more flow of 0.6 ml/min

Variation of mobile phase organic composition

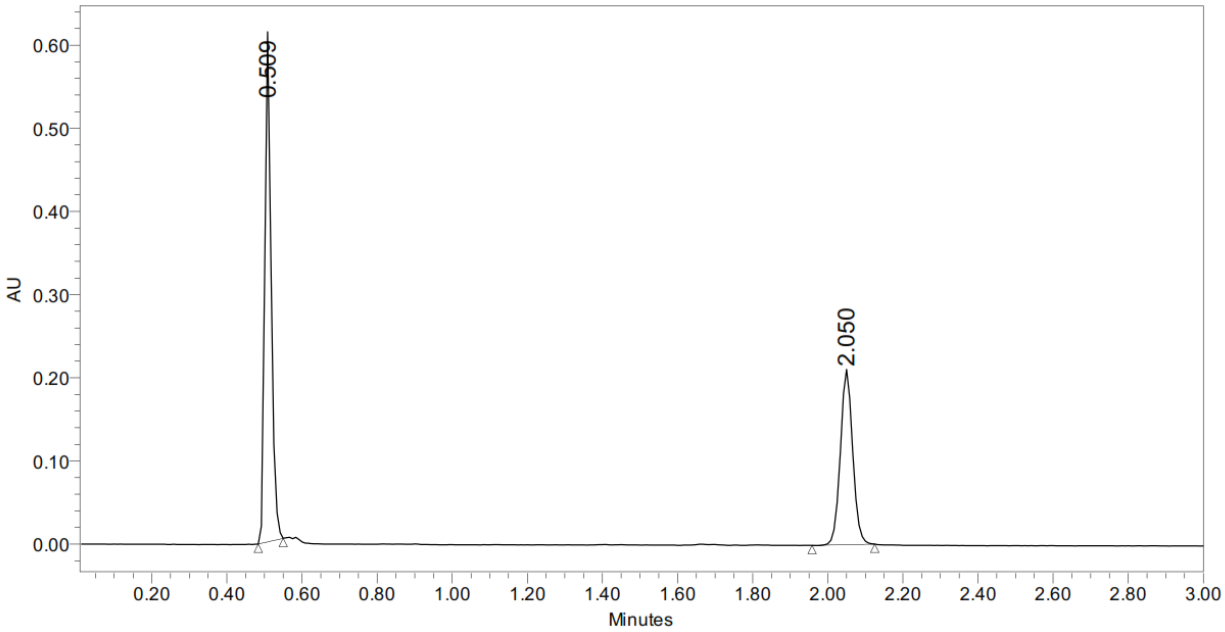


Figure: chromatogram showing less organic composition

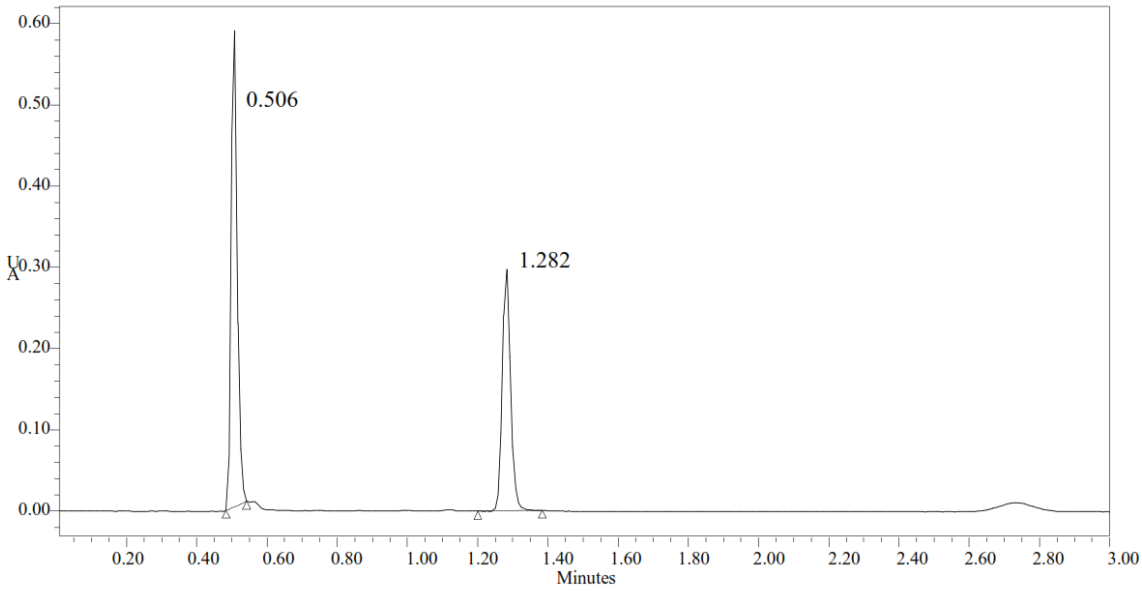


Figure: chromatogram showing more organic composition

Lopinavir:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical	Tailing factor
Actual Flow rate of 0.5 mL/min	745308	0.506	4623	1.3
Less Flow rate of 0.4 mL/min	976844	0.672	3178.8	1.3
More Flow rate of 0.6 mL/min	629107	0.401	2118.2	1.2
Less organic phase	719402	0.509	2847.3	1.2
More organic phase	709077	0.506	2341.5	1.1

Ritonavir:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 0.5 mL/min	511409	1.630	11031	1.1
Less Flow rate of 0.4 mL/min	637649	2.178	15759.1	1.1
More Flow rate of 0.6 mL/min	422762	1.263	11131.1	1.1
Less organic phase	480018	2.050	2847.3	1.2
More organic phase	486433	1.282	11186.5	1.1

RESUTS

Linearity:

The linearity of lopinavir and ritonavir was formed over the concentrations of 20-100ug /ml and 5-25ug/ml respectively with the regression equation and co-efficient of correlation Y=9932x+19437,0.998lopin and Y=34117x+12743,0.999 for ritonavir.

Accuracy: Accuracy of the method was studied through recovery studies at different spiking levels i.e 50%,100%and 150%.the mean percentage recovery of lopinavir 99.9%and 99.8%.

Robustness: As a part of robustness with small and deliberate changes in mobile phase ratio, column temperature and flow rate there are no significant variations in peak area and resolution between lopina and ritona .

Forced degradation studies: forced degradation studies were performed by exposing the samole solution at various stress conditions Degradation studies revealed that lopinavir was degraded in acidic, alkaline, peroxide, photo and thermal conditions but no effect was shown on ritonair.

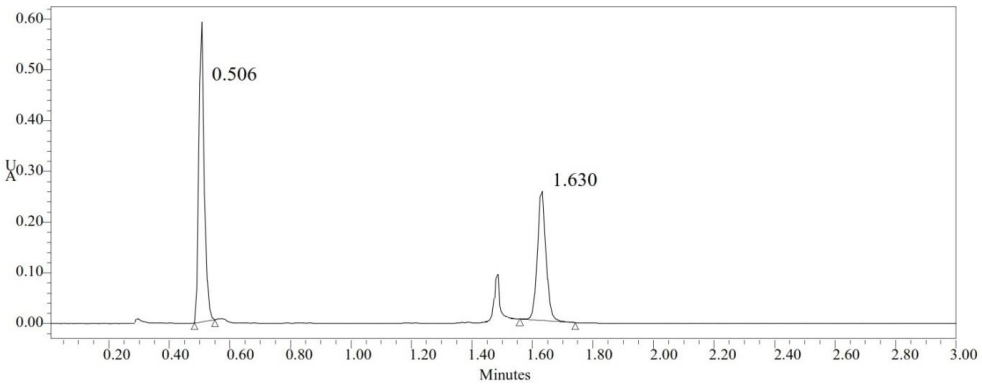


Figure: Chromatogram of acid degradation solution

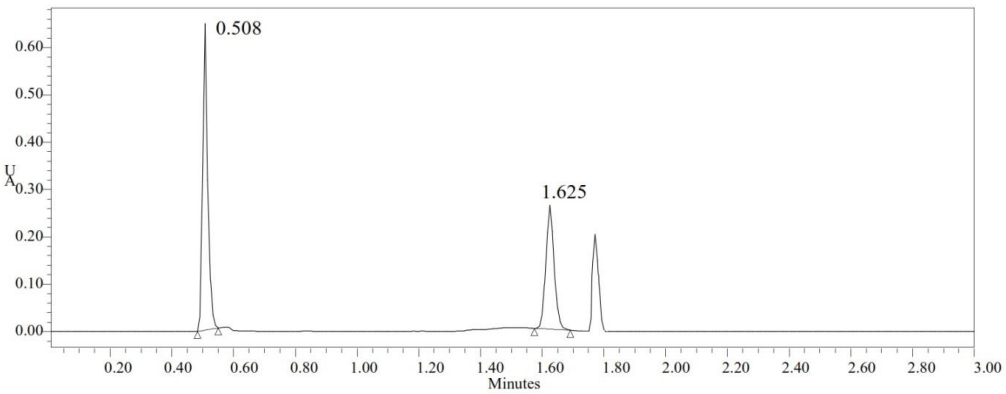


Figure: Chromatogram of alkaline degradation solution

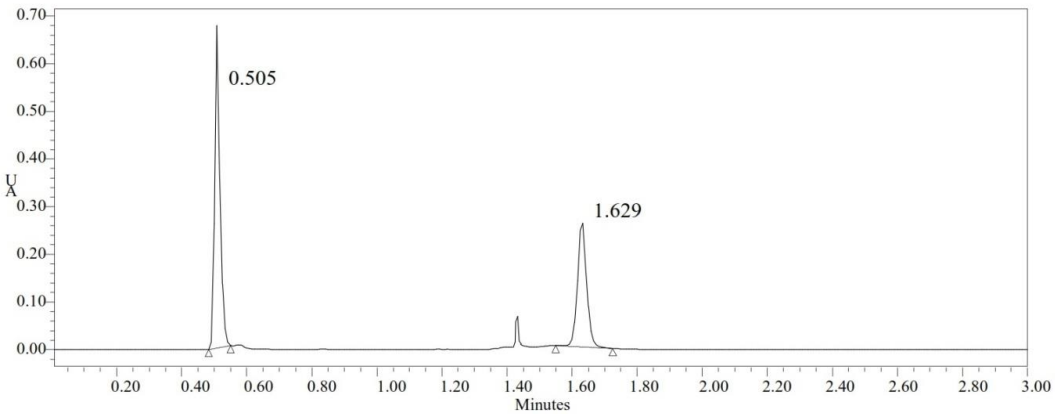


Figure: Chromatogram of oxidative degradation solution

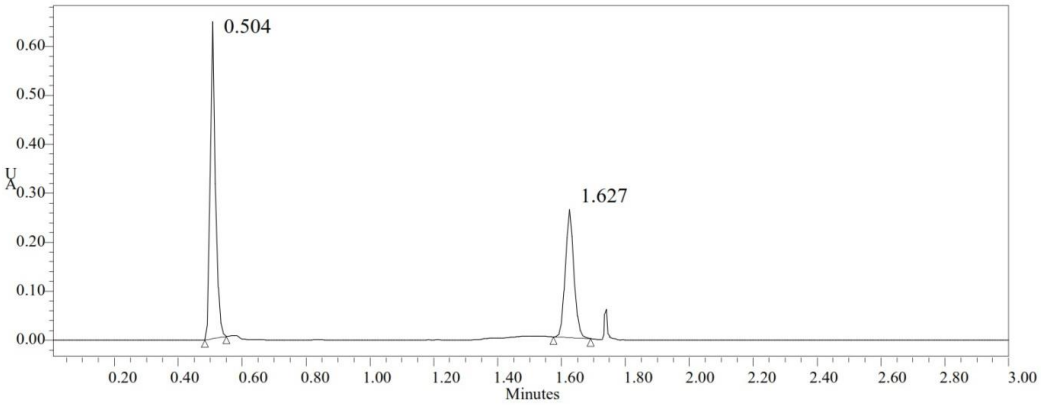


Figure: Chromatogram of Photo degradation solution

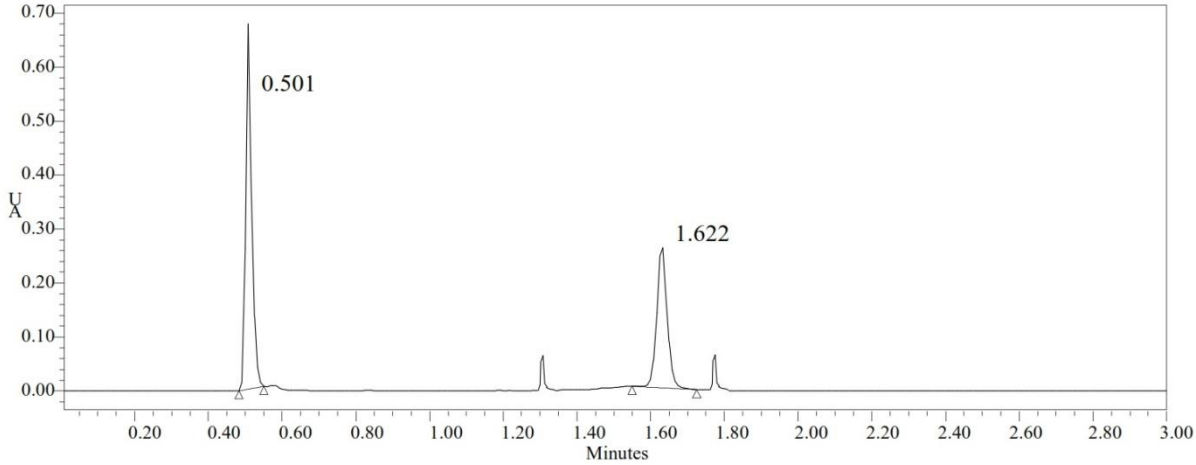


Figure: Chromatogram of thermal degradation solution

From the forced degradation conditions, it was observed that no degradation of Lopinavir peak. As per ICH guidelines, the limit of acceptable forced degradation is less than 20%. In the proposed method, the degradation of Lopinavir and Ritonavir was less than 20%, which represents the stability-indicating method.

Table Summary of forced degradation studies of Lopinavir and Ritonavir

Stressor	%degradation of Lopinavir	%degradation of Ritonavir	% of Ritonavir recovery
Acid	Nil	0.117 %	99.88%
Base/Alkaline	Nil	3.31 %	96.69%
Oxidative	Nil	0.052 %	99.94%
Photolytic	Nil	0.055 %	99.94%
Thermal	Nil	0.156 %	99.84%

CONCLUSION

Ultra Performance Liquid Chromatography (UPLC) method was developed and validated for the simultaneous determination of lopinavir and ritonavir in tablets. The chromatographic separation was performed on acquity UPLC, Gemini C18 (4.6×50mm, 5.0 µm) using Isocratic elution of Water: methanol (30:70) pH 2.8 adjusted by using ortho phosphoric acid at flow rate of 0.5 ml/min. UV (Ultra Violet) detection was performed at 215 nm. Total run time was 3 min within which main compounds were separated. The method was validated for accuracy, repeatability, reproducibility and robustness. Linearity, Limit of Quantification (LOQ) and Limit of Detection (LOD) was also established. At last From the forced degradation studies, it reveals that there is no degradation of Lopinavir and Ritonavir peak As per ICH guidelines, the limit of acceptable forced degradation is less than 20%. and results getting for Lopinavir is Nil and Ritonavir is below 20%, which represents the stability-indicating method. This method was successfully applied for content determination of lopinavir and ritonavir in pharmaceutical formulations. This method can be conveniently used in quality control

laboratory for routine analysis for assay as well as for evaluation of bulk drugs and pharmaceutical formulations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

UPLC: Ultra performance liquid chromatography; ML: Milli Liter; °C: Degree Centigrade; µg: Microgram; µm: Micrometer; nm: Nanometer.

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