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ORIGINAL ARTICLE

RP-HPLC Method Development and Validation of Lopinavir and Ritonavir in Bulk and Pharmaceutical Dosage Form

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ABSTRACT
An accurate, precise, rugged, reproducible Reverse Phase-High Pressure Liquid Chromatographic (RP-HPLC) method has been developed and validated for the simultaneous estimation of lopinavir and ritonavir in bulk and pharmaceutical dosage form of anti-retroviral protease inhibitor class of compounds. The method was achieved by using the mixed organic mobile phases of water and acetonitrile in the ratio of 60:40% v/v and chromatography was carried out on Sunfire C_{18} (4.6×250mm) 5µ column. Detection was carried out at 220nm.The flow rate was set as 0.9ml/min has provided a good peak shape of ritonavir and lopinavir. The retention time of the lopinavir and ritonavir was 3.0, 3.8±0.02min respectively. The method produces linear responses in the concentration range of 5-25µg/ml of lopinavir and 10-50µg/ml of ritonavir. The method precision for the determination of assay was below 2.0 %RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.
Keywords: *Ritonavir, lopinavir, accuracy, precision, ICH guidelines, method validation.*

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INTRODUCTION

Pharmaceutical analysis is a branch of practical chemistry that involves a series of process for identification, determination, quantification and purification of a substance, separation of the components of a solution or mixture, or determination of structure of chemical compounds. Ritonavir (trade name Norvir) is an antiretroviral drug used to treat HIV infection and AIDS. It is also known as 10-hydroxy-2-methyl-5-(1-methyl ethyl)-1-[2-(1-methyl ethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenyl methyl)-2,4,7,12-tetra azatridecan-13-oic acid, 5-thiazolyl methyl ester. Ritonavir is a protease inhibitor class and it inhibits the same host enzyme that metabolizes other protease inhibitors. This inhibition of the proteases results in increased plasma concentrations of these drugs, thus allowing the clinician to lower their dose and frequency and improving their clinical efficacy. So, the simultaneous determination with the other HIV protease inhibitors like lopinavir been shown to be effective against drug-resistant HIV. These drugs are metabolized by cytochrome P-450 (CYP) 3A in the liver. Freely soluble in methanol and ethanol, soluble in isopropanol, and practically insoluble in water [1].

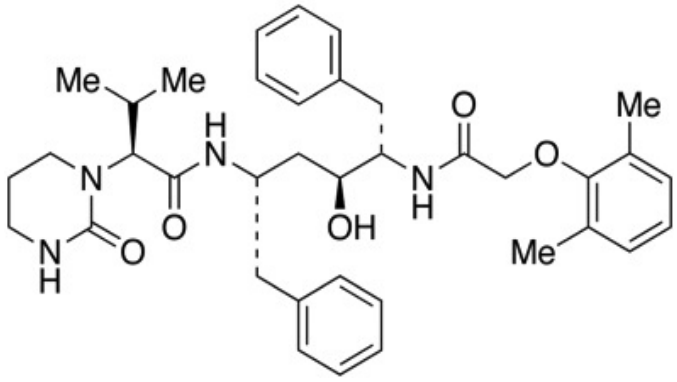

Figure 1: Structure of ritonavir

Lopinavir (ABT-378) is an antiretroviral of the protease inhibitor class. It is known as (2S)-N-[(2S,4S,5S)-5-[2-(2,6-dimethyl phenoxy) acetamido]-4-hydroxy-1,6-diphenyl hexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl) butanamide. 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, when lopinavir is administered with ritonavir as Kaletra, ritonavir inhibits the CYP 3A-mediated metabolism of lopinavir, thereby providing increased plasma levels of lopinavir. It is freely soluble in methanol and ethanol, soluble in isopropanol, and practically insoluble in water [2].

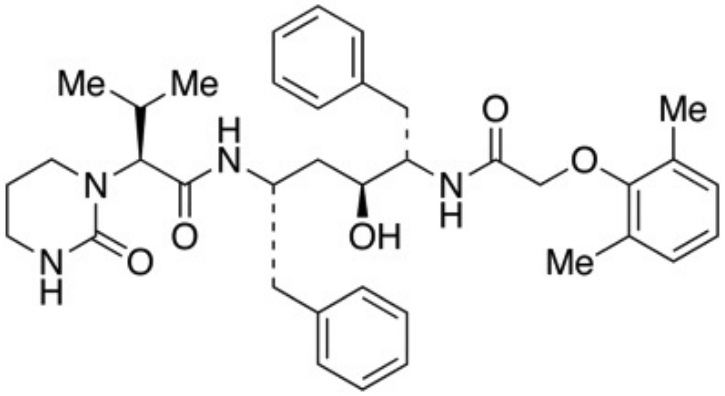

Figure 2: Structure of ritonavir

Various analytical methods have been reported for the assay of lopinavir and ritonavir individually or combination with other drugs in biological samples/formulations. They include HPLC [3-6], HPTLC[7], derivative UV spectrophotometry [8]. Simultaneous determinations of lopinavir and ritonavir dosage form were also reported by using HPLC, LC-MS, HPTLC and UV Spectroscopy. So, our aim is to develop a new rapid and sensitive RP-HPLC of simultaneous determination and to perform the validation as per ICH guidelines.

MATERIAL AND METHODS

Reagents and instruments

Reference standards of lopinavir and ritonavir were received as gift samples from Sura Labs, Hyderabad, Telangana. HPLC grade acetonitrile and methanol were purchased from Merck, India. HPLC grade water was obtained from LICHROSOLV (Merck) and it is used for the present HPLC research work. The LC system consists of Alliance 2965 separation module, software: Empower 2996 PDA detector, isocratic pump, auto sampler. The output signal was monitored and integrated by LC solutions chromatography manager software (Prominence HPLC, Shimadzu, Japan).

Preparation of mobile phase

Accurately measured 600ml (60%) of water, 400ml of acetonitrile (40%) were mixed and degassed in digital Ultrasonicator for 10min and then filtered through 0.45µ filter under vacuum filtration.

Diluent preparation

The mobile phase was used as the diluent.

Preparation of standard stock solution of lopinavir and ritonavir

Accurately weighed and transferred 10mg of lopinavir and ritonavir working standard into a 10ml of clean and dry volumetric flasks separately. Added about 7ml of methanol and sonicated to dissolve and remove the air completely and made volume up to the mark with the same methanol.

Preparation of standard working solution of lopinavir and ritonavir

Further pipette out 0.15ml of the lopinavir and 0.3ml of the ritonavir stock solutions into a 10ml volumetric flask and diluted up to the mark with methanol.

Preparation of sample stock solution

Taken an average weight of tablet and crush in a mortar by using pestle and weighed 10mg equivalent weight of lopinavir and ritonavir sample into a 10mL clean and dry volumetric flask and added about 7mL of diluent and sonicated to dissolve it completely and make volume up to the mark with the same solvent.

Preparation of sample working solution

Further pipetted 0.3ml of the Sample stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Procedure:

Injected the samples by changing the chromatographic conditions and recorded the chromatograms, noted the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Method validation

As per ICH guidelines [9-12] the method was validated and the parameters like linearity, specificity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness and stability were assessed.

System suitability

The system suitability parameters were determined by preparing standard solutions of lopinavir and ritonavir and the solutions were injected five times and the parameters like peak tailing, resolution and USP plate count were determined. The %RSD for the area of five standard injections results should not be more than 2%. The %RSD for the area of five replicate injections was found to be within the specified limits.

Specificity

Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. three replicate injections of standard and sample solutions of lopinavir and ritonavir were injected and this method was said to be specific.

Precision [13, 14]

Repeatability

Preparation of standard working solution of lopinavir and ritonavir

Further pipetted 0.15ml of the Lopinavir and 0.3ml of the Ritonavir stock solutions into a 10ml volumetric flask and diluted up to the mark with methanol. The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Intermediate precision:

To evaluate the intermediate precision (also known as ruggedness) of the method, precision was performed on different days by maintaining same conditions.

Procedure:

Day 1: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Day 2: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Preparation of drug solutions for linearity

Preparation of level – I (5ppm of lopinavir & 10ppm of ritonavir): Pipetted out 0.05ml of lopinavir and 0.1ml of ritonavir stock solutions into a 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of level – II (10ppm of lopinavir& 20ppm of ritonavir): Pipetted out 0.1ml of lopinavir and 0.2ml of ritonavir stock solutions was take into a 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of level – III (15ppm of lopinavir& 30ppm of ritonavir): Pipetted out 0.15ml of lopinavir and 0.3ml of ritonavir stock solutions into a 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of level – IV (20ppm of lopinavir& 40ppm of ritonavir): Pipetted out 0.2ml of lopinavir and 0.4ml of ritonavir stock solutions into a 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of level – V (25ppm of lopinavir& 50ppm of ritonavir): Pipetted out 0.25ml of lopinavir and 0.5ml of ritonavir stock solutions into a 10ml of volumetric flask diluted up to the mark with diluent.

Procedure: Inject each level into the chromatographic system and measure the peak area and data was shown in Table 7 & 8.

Accuracy

Preparation of standard stock solution of lopinavir and ritonavir

Accurately weigh and transfer 10 mg of lopinavir and ritonavir working standard into a 10ml of clean dry volumetric flasks separately. Add about 7ml of methanol and sonicate to dissolve and remove the air completely and make volume up to the mark with the same methanol.

Preparation of 50% standard stock solution: Further pipette 0.075ml of the lopinavir and 0.15ml of the ritonavir stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 100% standard stock solution: Accurately weigh and transfer 10 mg of lopinavir and 10mg of ritonavir working standard into a 10ml of clean dry volumetric flasks add about 7ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (stock solution). Further pipette 0.15ml of the lopinavir and 0.3ml of the ritonavir stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 150% standard stock solution: Accurately weigh and transfer 10 mg of lopinavir and 10mg of ritonavir working standard into a 10ml of clean dry volumetric flasks, add about 7mL of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (stock solution). Further pipette 0.225ml of the lopinavir and 0.45ml of the ritonavir stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure: Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the amount found and amount added for lopinavir and ritonavir and calculate the individual recovery and mean recovery values. Data was shown in the Table 9 and 10.

Robustness

Small deliberate changes in method like flow rate, mobile phase ratio, and temperature are made but there was no recognized change in the result and are within range as per ICH Guide lines. Robustness conditions like flow minus (0.8ml/min), flow plus (1ml/min), variation of mobile phase i.e., acetonitrile and water were taken in the ratio and 35:65, and 45:55 was maintained and samples were injected in duplicate manner. System suitability parameters were not much effected and all the parameters were passed. %RSD was within the limit. Data was shown in the Table 11 & 12.

Limit of detection (LOD)

The limit of detection of a compound is defined as the lowest concentration of analyte that can be detected. LOD value of lopinavir and ritonavir was found to be 0.7 and 1.8µg/mL respectively.

Limit of quantification (LOQ)

The limit of quantification is the lowest concentration of a compound that can be quantified with acceptable precision and accuracy. LOQ value of lopinavir and ritonavir was found to be 2.1 and 5.5 µg/mL respectively.

RESULTS AND DISCUSSION

Optimization of Chromatographic Condition

It was found from the UV spectra that lopinavir and ritonavir have a significant absorption at 260 nm. Therefore, 220 nm was chosen as the detection wavelength. Initially methanol and Water; Acetonitrile and methanol with varying proportions used as mobile phase. According to preliminary investigations, using various ratios of acetonitrile with methanol or methanol with water did not separate the lopinavir and ritonavir peaks or produce the desired retention times and peak symmetry. Acetonitrile and water used as mobile phase was used to obtain satisfactory peak symmetry and separation with good resolution. In order to achieve adequate resolution and satisfactory peak symmetry, a mobile phase made up of Acetonitrile and water in a 60:40%v/v ratio and Sunfire C18 (4.6×250mm) 5µ column particle size was chosen. The flow rates tested ranged from 0.5 to 1.2 ml/min. It was found that a flow rate of 0.9 ml/min was sufficient to elute both medicines in less than 10 minutes. Figure 3&4 displays the optimized chromatograms of lopinavir and ritonavir.

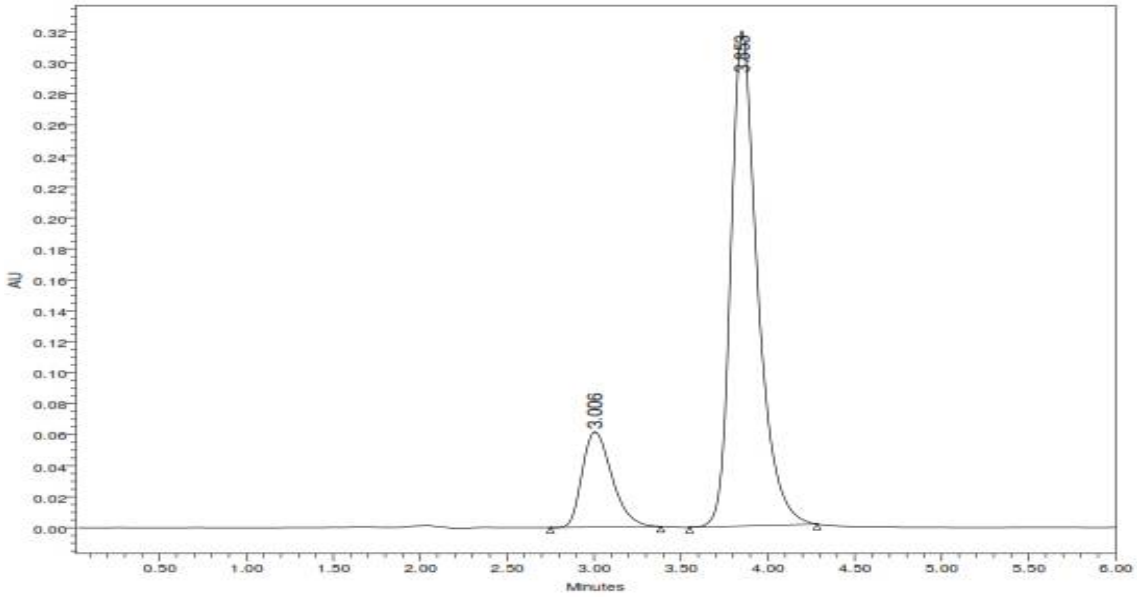

Figure 3: Optimized chromatogram (standard)

Table 1: Optimized chromatogram (standard)

S. No.	Name	RT	Area	Height	USP tailing	USP plate count
1	Lopinavir	3.006	731322	61677	1.2	8574
2	Ritonavir	3.853	3421257	319786	1.1	9664

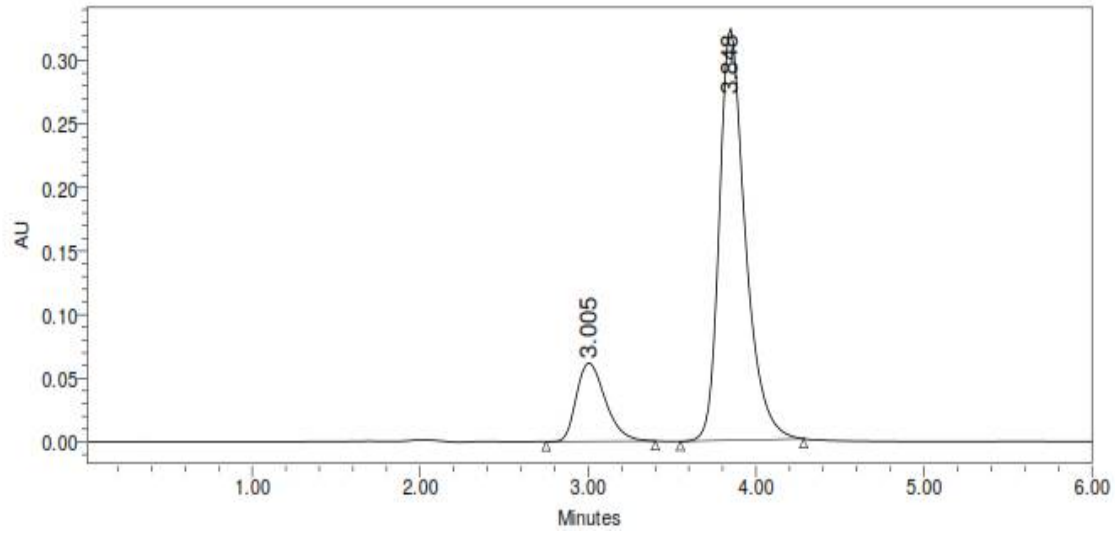

Figure 4: Optimized chromatogram

Table 2: Optimized chromatogram (sample)

S. No.	Name	RT	Area	Height	USP tailing	USP plate count
1	Lopinavir	3.005	658995	61772	1.1	7442
2	Ritonavir	3.848	3096188	324054	1.2	7331

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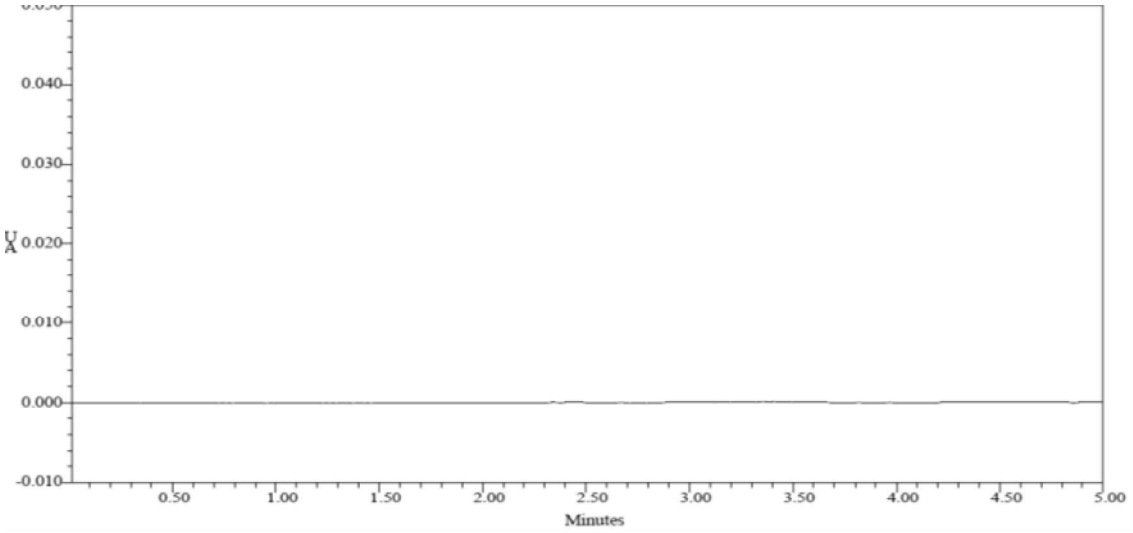

Figure 5: Chromatogram showing blank (mobile phase preparation)

Table 3: Peak results for assay standard of lopinavir

S. No.	Peak	RT	Area (µV*sec)	Height (µV)	USP plate count	USP tailing
1	Lopinavir	3.008	658263	61335	7462	1.2
2	Lopinavir	3.009	658264	61947	8264	1.1
3	Lopinavir	3.008	653426	61049	6627	1.2
4	Lopinavir	3.010	653058	61141	7264	1.1
5	Lopinavir	3.006	657393	61735	6645	1.1
Mean			656080.8			
Std.			2618.946			
% RSD			0.39918			

Table 4: Peak results for assay standard of ritonavir

S. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP plate count	US tailing
1	Ritonavir	3.857	3028176	381011	9583	1.1
2	Ritonavir	3.859	3018373	381645	8927	1.2
3	Ritonavir	3.857	3018462	381663	8465	1.1
4	Ritonavir	3.861	3081711	381746	9222	1.2
5	Ritonavir	3.853	3075143	381193	8462	1.1
Mean			3044373			
Std. Dev.			31427.07			
% RSD			1.0323			

Table 5: Peak results for Assay sample of Lopinavir

S. No.	Name	RT	Area	Height	USP tailing	USP plate count
1	Lopinavir	3.008	651712	61173	1.2	8563
2	Lopinavir	3.005	657635	61936	1.1	7462
3	Lopinavir	3.007	658917	61196	1.1	9264

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Table 6: Peak results for assay sample of ritonavir

S. No	Name	RT	Area	Height	USP tailing	USP plate count
1	Ritonavir	3.854	3029472	361938	1.1	6476
2	Ritonavir	3.853	3017462	361746	1.1	7264
3	Ritonavir	3.855	3028171	371864	1.2	6545

Linearity

It was discovered that the linearity ranges for Lopinavir and Ritonavir were 5-25 µg/ml and 10-50µg/ml, respectively. The correlation coefficient was found to be 0.999. Table 7 & 8 displays the Lopinavir and Ritonavir linearity data. Figure 6 & 7 depicted the calibration curve for Lopinavir and Ritonavir. Data from the calibration curve that were subjected to linear regression showed a linear response over the range of both medications' concentrations. As a result, the curve can be used to calculate Lopinavir and Ritonavir in pharmaceutical formulations.

Table 7: Chromatographic data for linearity study for lopinavir

Concentration level (%)	Concentration µg/ml	Average peak area
33.3	5	230247
66.6	10	462332
100	15	659905
133.3	20	892989
166.6	25	1101075

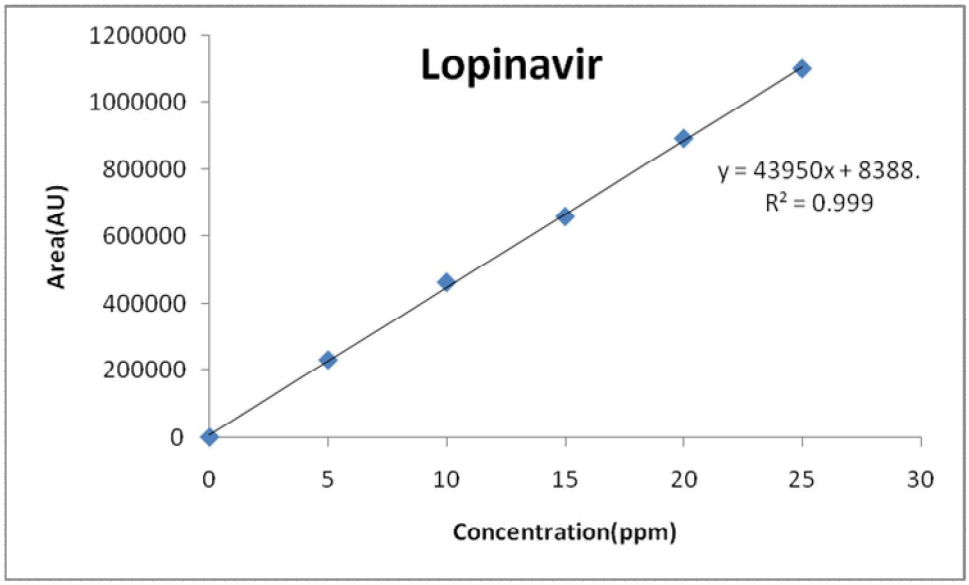

Figure 6: Chromatogram showing linearity level

Table 8: Chromatographic data for linearity study for ritonavir

Concentration level (%)	Concentration µg/ml	Average peak area
33.3	10	1215225
66.6	20	2135937
100	30	3020839
133.3	40	4078841
166.6	50	5058145

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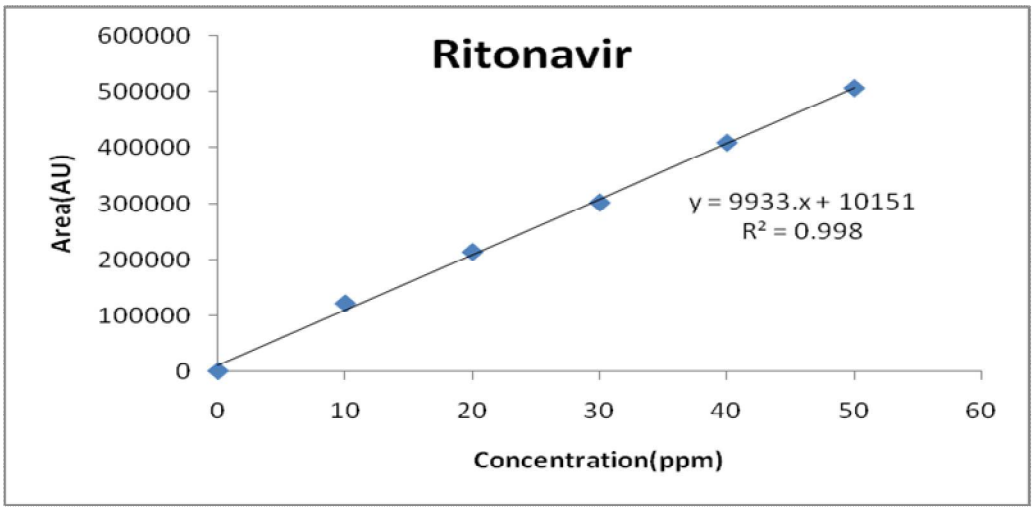

Figure 7: Chromatogram showing linearity level

Accuracy

Recovery studies were used to perform accuracy using the standard addition approach. The sample solution had standard drugs added at concentrations of 50, 100, and 150% of the sample concentration. In triplicate, each concentration was examined. According to the findings of the recovery investigations, both Lopinavir and Ritonavir had recovery rates between 99 and 101%, as indicated in Table 9 & 10.

Table 9: The accuracy results for lopinavir

%Concentration (at specification level)	Area	Amount added (ppm)	Amount found (ppm)	% Recovery	Mean recovery
50%	331938	7.5	7.3	99.88	**100.166**
100%	658274	15	14.7	98.89	
150%	970963	22.5	22.2	101	

Table 10: The accuracy results for ritonavir

%Concentration (at specification level)	Area	Amount added (ppm)	Amount found (ppm)	% Recovery	Mean recovery
50%	209357	7.5	7.49	99.7%	**99%**
100%	420697.7	15	14.9	99%	
150%	631550.7	22.5	22.48	99%	

Robustness

The study's technique parameters included flow rate variation (1ml/min,0.8ml/min), and mobile phase organic content (5 % v/v). Recovery values for analyte solutions were used to evaluate the results. According to a robustness investigation, changes in flow rate and mobile phase composition do not significantly affect the analyte response. The data is presented in Table 11 & 12.

Table 11: Results for robustness of lopinavir

Parameter used for sample analysis	Peak Area	Retention time	Theoretical plates	Tailing factor
Actual flow rate of 0.9mL/min	658211	3.006	8793	1.2
Less flow rate of 0.8mL/min	621077	3.441	7269	1.3
More flow rate of 1.0mL/min More Flow rate of 0.9mL/min	642190	2.663	9446	1.2
Less organic phase	542402	3.185	8126	1.1
More organic phase	642112	2.867	5854	1.3

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Table 12: Results for robustness of ritonavir

Parameter used for sample analysis	Peak Area	Retention time	Theoretical plates	Tailing factor
Actual flow rate of 0.9mL/min	429069	3.853	5224	1.59
Less flow rate of 0.8mL/min	472673	4.426	6328	1.58
More flow rate of 1.0mL/min	392497	3.415	6217	1.54
Less organic phase	391379	4.291	6996	1.61
More organic phase	391703	3.583	6120	1.50

CONCLUSION

The proposed method was found to be simple, fast, robust, more precise and accurate under the present experimental conditions. Therefore, the developed method can be used for routine analysis for simultaneous estimation of lopinavir and ritonavir in bulk and pharmaceutical dosage form. The present successfully validated method with excellent selectivity, linearity, sensitivity, precision and accuracy was applicable for the assay of lopinavir and ritonavir in bulk drug substance and pharmaceutical dosage forms.

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AUTHOR CONTRIBUTIONS

All authors contributed to experimental work, data collection, drafting or revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

COMPETING INTEREST STATEMENT

All authors declare that there is no conflict of interests regarding publication of this paper.

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