American Journal of Clinical Microbiology and Antimicrobials

Research Article
Published: 10 Jan, 2018

RP-HPLC Method Development and Method Validation of Lopinavir and Ritonavir in 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 pharmaceutical dosage form of anti-retroviral protease inhibitor class of compounds. The method was achieved by using the mixed organic mobile phases of Acetonitrile: Methanol with the buffer of potassium dihydrogen phosphate (pH 3.5) and Xbridge C_{18} (250 mm × 4.6 mm i.d., 3.5 µm. The flow rate was set as 1.1 ml/min has provided a good peak shape of Ritonavir and Lopinavir were found well separated at 5.7 and 6.6 min respectively.

Keywords: RP-HPLC; Lopinavir; Ritonavir; Xbridge C_{18}

Introduction

Ritonavir (trade name Norvir) is an antiretroviral drug used to treat HIV infection and AIDS. 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. When Lopinavir is administered with Ritonavir as kaletra, ritonavir inhibits the CYP 3A- mediated metabolism of Lopinavir, thereby providing increased plasma levels of Lopinavir [1-3]. Lopinavir is known as N-[4-[[(2,6 dimethylphenoxy) acetyl] amino]-3-hydroxy-5-phenyl-1-(phenylmethyl) pentyl] tetrahydro-alpha-(1methylethyl)-2-oxo 1(2H) pyrrolidine acetamide. Ritonavir is known as 10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12 tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester. Both are freely soluble in methanol and ethanol, isopropanol and practically insoluble in water. A survey of literature reveals that there are two methods reported for the simultaneous determination of lopinavir and ritonavir in pharmaceutical preparations using HPLC [1,4-6]. There are several analytical methods have been reported for the assay of Lopinavir and Ritonavir individually or combination with other drugs in biological samples as well as formulations [7-9].

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 [10-17].

Materials and Methods

Reagents and materials

The drug samples of Lopinavir and Ritonavir was procured from Dr. Reddy's Pharma, India. Methanol, Acetonitrile, water (HPLC grade LICHROSOLV, Merck.), 0.45 µm filter (Millipore, Bangalore). Pharmaceutical formulation of Lopinavir and Ritonavir were purchased from a local pharmacy.

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Received Date: *10 Oct 2017*
Accepted Date: *31 Dec 2017*
Published Date: *10 Jan 2018*

Citation:
Rathnasamy R, Karuvalam RP, Pakkath R, Kamalakannan P, Sivasubramanian A. RP-HPLC Method Development and Method Validation of Lopinavir and Ritonavir in Pharmaceutical Dosage Form. Am J Clin Microbiol Antimicrob. 2018; 1(1): 1002.

Table 1: RP-HPLC isocratic method optimization by different mobile phase composition and flow rate.

Trail No	Column	Mobile phase (Buffer: Methanol: Acetonitrile)	Flow Rate (mL/min)
1	X-Bridge	40:10:50	1
2	X-Bridge	60:00:40	1
3	X-Bridge	40:10:50	1
4	X-Bridge	40:10:50	0.8
5	X-Bridge	50:10:40	1.3
6	X-Bridge	40:20:40	0.9
7	X-Bridge	40:50:10	1.1

Instruments

A Waters RP-HPLC instrument equipped with software (Empower 2,695 separation module) and an UV-Visible or DAD detector, manual injector with 100 µl loop, and X-Bridge C_{18} (150 mm × 4.6 mm i.d., 5 µm particle size). The UV/VIS spectrophotometer used was LABINDIA UV 3,000+. The pH meter was of Adwa-AD1020 make and pipettes and burettes were of Borosil make.

Preparation of standard stock solution

Accurately weighed amount of 10 mg Ritonavir and 10 mg Lopinavir were taken to a 10 mL cleaned and dried volumetric flask. This was then diluted with 7 mL of mobile phase and was sonicated to obtain stock solution of 1,000 µg/mL. Further, an amount of 0.75 mL of this solution was pipette into a 10 mL volumetric flask and diluted up to the mark with mobile phase.

Preparation sample solution

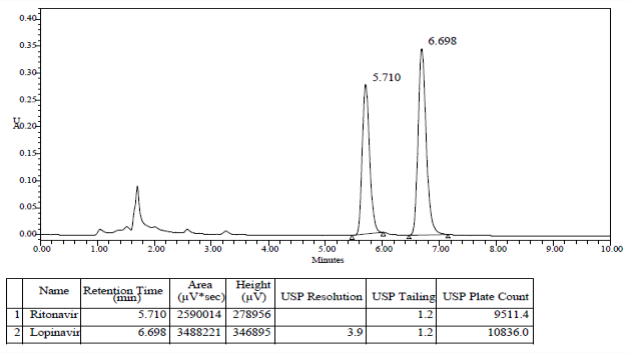
Twenty tablets were finely powdered and weighed the sample of powdered tablets equivalent to Lopinavir (200 mg) and Ritonavir (50 mg) were transferred to a 100 mL volumetric flask and dissolved in Mobile Phase. The solution was sonicated for 15 min to ensure solubility of drug. The contents were made up to the mark with Mobile Phase and filtered through a 0.45 µ nylon membrane filter.

Result and Discussion

Method optimization

A RP-HPLC method was developed for two anti-retroviral drugs, which can be conveniently employed in pharmaceutical dosage forms. The method development consists of some important parameters like selection of buffer, column, flow rate and etc. So the selection of buffer should to be reasonable conditions like; if acid group present in the compound the buffer pH should be two units below the pKa is recommended and for the basic compounds pH should be two units above the pka is recommended. Drugs pKa were known from literature as 1.5 (Strongest Basic) and 2.84 (Strongest Basic) for Lopinavir and Ritonavir respectively. So the pH of the mobile phase adjusted to 3.5. We have selected the potassium dihydrogen phosphate (pH 3.5) as buffer for mobile phase composition. In addition to that the pH of buffers should not affect the stability of the drugs as well as the stationary phase lifetime. Hence XBridge C-18 column was chosen because it is suitable for low to high pH range, good lifetime, good efficiency and good selectivity. The pH of 5 mM ammonium acetate buffer was investigated in the pH range of 3-8. Both Lopinavir and Ritonavir possess low pKa value, less than 3.7. As a result, it was found that an increase in the value of pH decreased retention time on both drugs.

We intended to develop a simple isocratic HPLC method for the simultaneous determination of lopinavir and ritonavir by using the different mobile phase composition based on its polarity and different flow rate based on peak resolution are summarised in (Table 1). Finally we achieved a very good resolution by using Xbridge C18 (250 mm × 4.6 mm i.d., 3.5 µm particle size), potassium dihydrogen phosphate buffer (pH 3.5): Acetonitrile: Methanol (40:50:10) with flow rate was set as 1.1 mL/min. Detection wavelength was 220 nm and the injection volume as 20 µL, and Temperature: 25°C ± 30°C and the peaks of ritonavir and lopinavir were found well separated at 5.7 and 6.6 min respectively (Figure 1).



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Resolution	USP Tailing	USP Plate Count
1	Ritonavir	5.710	2590014	278956		1.2	9511.4
2	Lopinavir	6.698	3488221	346895	3.9	1.2	10836.0

Figure 1: Chromatogram of Ritonavir & Lopinavir.

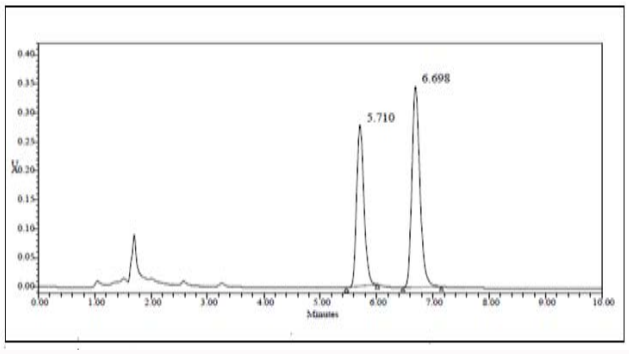


Figure 2: Chromatogram for system suitability.

Method validation

Validation is a process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce meeting, its predetermined specifications and quality attributes.

System suitability: It is defined as tests to measure the method that can generate result of acceptable accuracy and precision. The system suitability was carried out after the method development with optimized method. Six replicate injection of standard stoke solution was performed and measured the % Related Standard Deviation (RSD) of peak area was less than 2.0 and the % Related Standard Deviation of Retention Time (RT) was less than 1.0. The system suitability parameters satisfying the USP requirement as the Resolution between Ritnovir and Lopinavir peaks were not less than 2.0, the theoretical plates were not less than 2,000 and the tailing

Table 2: System suitability parameters for Ritonavir and Lopinavir.

Tailing factor	1.2	1.2
Retention time	5.7	6.6
Theoretical plates	9511.4	10836

Table 3: Table for precision of Ritonavir and Lopinavir.

Parameter	Method precision		Intermediate precision	
Average	Ritonavir	Lopinavir	Ritonavir	Lopinavir
Area	1687657	2098765	1531512	2074884
SD	2390.2	2598.3	2437.4	2566.2
%RSD	0.18	0.16	0.16	0.12

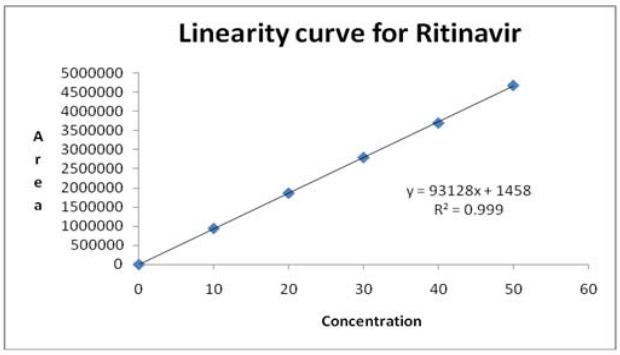


Figure 3: Linearity curve for Ritinavir.

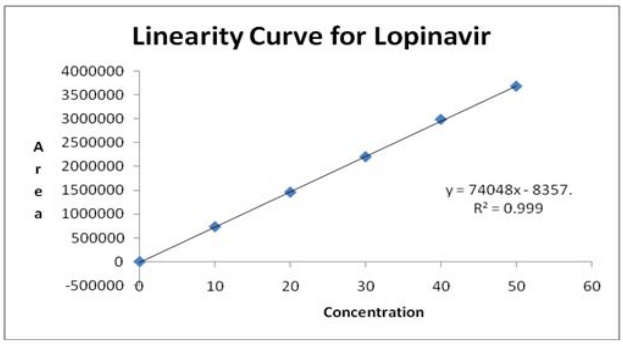


Figure 4: Linearity curve for Lopinavir.

factor was not less than not more than 2.0 shown in Figure 2 and data summarised in Table 2 thus indicating the suitability.

Precision: The Precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeated to multiple samples. The precision of the analytical method is determined by Assay for six homogeneous samples.

Method Precision: Method precision or reproducibility was demonstrated by analysing with six sample preparations and results are tabulated in the Table 3.

Intermediate Precision: Intermediate precision was performed with same batch of the samples in a same lab but with different analyst in different days and results are tabulated in the Table 3.

%RSD values of peak area found to be less than 2.0. Hence the optimized method was found to be precise as per ICH guidelines Q2 (R1).

Accuracy (% Recovery): Accuracy is the closeness of the test results obtain by the method to the true value. Accuracy may often be expressed as recovery. Accuracy was performed with the test solution of 50%, 100% and 150% and injected as triplicate of each concentration.

For preparation of 50% solution (With respect to target Assay concentration): Accurately weigh and transfer 4.8 mg of Ritnovir and 5.0 mg of Lopinavir working Standard in to a 10 mL clean dry volumetric flask add about 7 mL of Diluent and Sonicate to dissolve it completely and make volume up to the mark with the same solvent. Pipette out 0.75 ml of Ritnovir & Lopinavir of the above stock solution in to a 10 ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% solution (With respect to target Assay concentration): Accurately weight and transfer 9.7 mg of Ritnovir and 10.0 mg of Lopinavir working standards into a 10 mL clean dry volumetric flask add about 7 mL of Diluents and sonicated to dissolve it completely and make volume up to the mark with the same solvent. Pipette out 0.75 ml of Ritnovir & Lopinavir of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

For preparation of 150% solution (With respect to target Assay concentration): Accurately weigh and transfer 15.0 mg of Ritnovir and 15.0 mg of Lopinavir working standards into a 10 mL clean dry volumetric flask add about 7 mL of Diluents and sonicated dissolve it completely and make volume up to the mark with the same solvent. Pipette out 0.75 ml of Ritnovir and Lopinavir of the above stock solution in to a 10 ml volumetric flask and dilute up to the mark with diluents (Table 4).

$$\%\text{recovery} = \frac{(\text{Amount Recovered})}{(\text{Actual amount added})} \times 100$$

Linearity and range: The ability of the method to produce result is directly proportional to the concentration of the analyte in the samples within a given range. A calibration curves were plotted over a concentration range 10 ppm to 50 ppm for Ritnovir and Lopinavir. These solutions were injected into the HPLC under the optimized conditions. Recorded the chromatograms and measured the peak responses. The spectrum and area of Ritnovir and Lopinavir were analysed and summarized in Table 5.

Table 4: Accuracy (recovery) data.

Inj. sample	Amount present	Amount recovered	% recovered	Mean recovery	Acceptance Criteria
Ritonavir	4.8 mg	4.76 mg	99.30%	98.70%	100 ± 2.0%
	9.7 mg	9.51 mg	98.10%		
	15.0 mg	14.8 mg	98.70%		
Lopinavir	5.0 mg	4.92 mg	98.40%	98.5%**.5**	100 ± 2.0%
Inj. sample	**Amount present**	**Amount recovered**	**% recovered**	**Mean recovery**	**Acceptance Criteria**
Ritonavir	4.8 mg	4.76 mg	98.10%	98.70%	100 ± 2.0%
	9.7 mg	9.51 mg	97.89%		
	15.0 mg	14.8 mg	97.68%		
Lopinavir	5.0 mg	4.92 mg	97.47%	98.5%**.6**	100 ± 2.0%

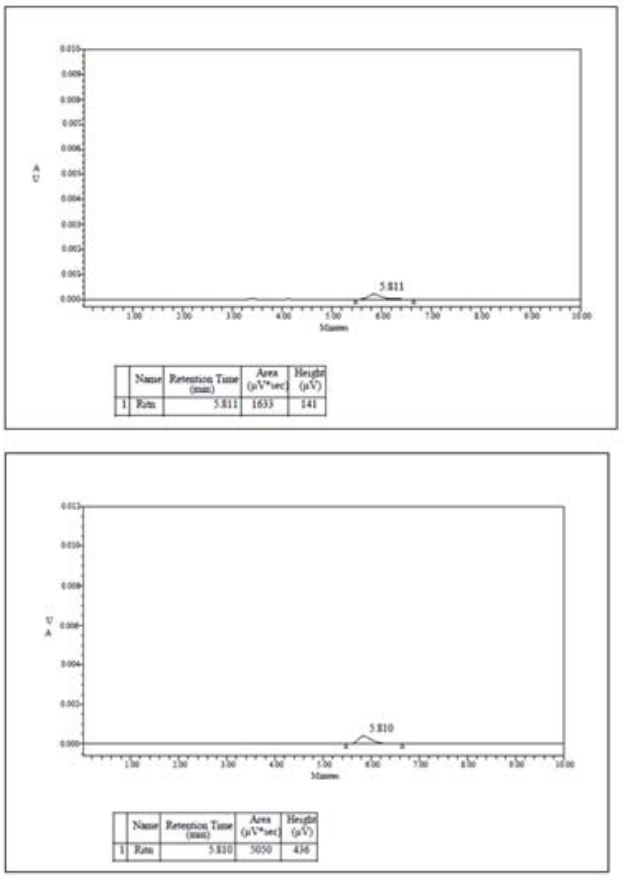


Figure 5: Chromatogram of LOD and LOQ for Ritinavir.

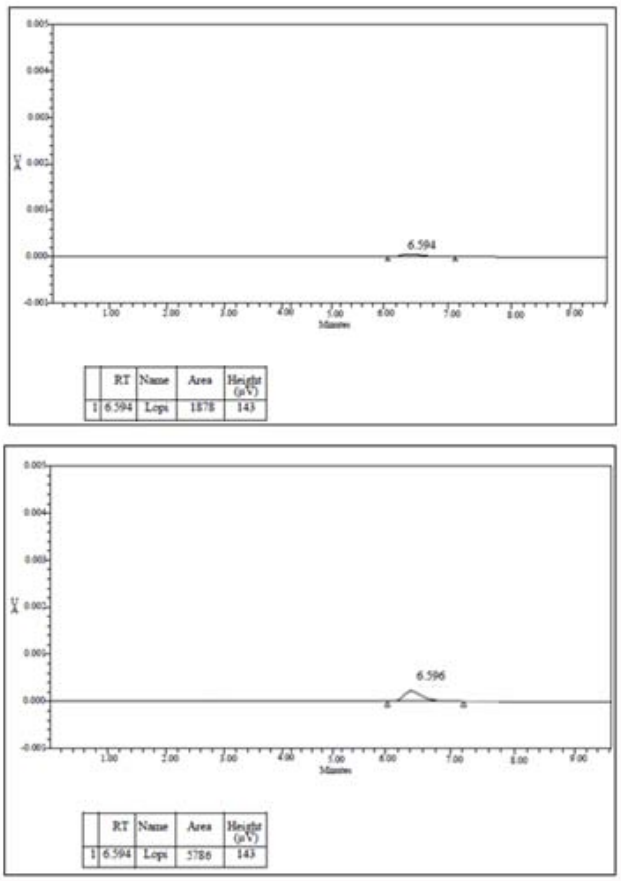


Figure 6: Chromatogram of LOD and LOQ for Lopinavir.

Table 5: Area of different concentration of Ritonavir and Lopinavir.

Concentrations (mg/ml)	Ritonavir area	Lopinavir area
10	942992	731788
20	1862897	1456684
30	2794602	2200558
40	3697638	2986015
50	4679842	3682081
Correlation Coefficient r^2	0.99995	0.99991
Intercept	1458.048	-8357.1
y-Intercept	0.052174	-0.37977

Table 6: LOD for Ritonavir and Lopinavir.

Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
Ritonavir	52	152	2.92
Lopinavir	52	158	3.03

Drawn a plot between the concentration (mg/mL) against area and reported the slope, intercept. 10 ppm to 50 ppm of Ritonovir and Lopinavir the chromatograms are shown in (Figure 3 and 4). The linearity range was found to lie from 10 ppm to 50 ppm. The correlation coefficient and y-intercept also found to be within the acceptance criteria of ICH guidelines Q2 (R1).

Limit of detection and limit of Quantitation (LOD and LOQ): The limits of detection and quantification were also done and shown in (Figures 5 and 6). Based on the calibration curves for Ritonavir and Lopinavir and based on the signal to noise ratios, The LOD was found to be less than 3:1 for Ritonavir and Lopinavir the LOQ was also found to be 10:1 respectively (Table 6).

Conclusion

An attempt has been made to develop the RP-HPLC method optimization for simultaneous estimation of Lopinavir and Ritonavir in combined dosage form. As the literature survey revealed that few methods were available for their simultaneous estimation, but there is a need of a simple, economical and proper method for estimation of above combination in combined dosage form.

$$\%\text{recovery} = \frac{(\text{Amount Recovered})}{(\text{Actual amount added})} \times 100$$

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