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Research Article

Analytical Method Development and Validation for Lopinavir and Ritonavir in Bulk and Dosage Form

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ABSTRACT

Objective: A simple, accurate and precise RP-HPLC method for simultaneous determination of Lopinavir and Ritonavir in bulk and dosage forms.

Methods: A reversed phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the quantitative determination of lopinavir (LPV) and ritonavir (RTV) on Agilent C18 (2) 250 × 4.6 mm, 5 µ column as a stationary phase and mobile phase composition of Acetonitrile: 0.1% Ortho-phosphoric acid (pH 3.5) (80:20, v/v) at a flow rate of 1ml/min.

Results: Quantification was achieved with UV detection at 217 nm. The retention times of Lopinavir and Ritonavir was 7.38 and 2.98 min respectively. The result of linearity was obtained in the concentration range of 0-20µg/ml for both Ritonavir and Lopinavir. %Recovery was Obtained as 99.25% and 99.72% for Ritonavir and Lopinavir respectively. LOD, LOQ values are obtained from regression equations of Ritonavir and Lopinavir were 4.6ng/spot, 1.5ng/spot, 5.10ng/spot, 21.00ng/spot respectively. Regression equation of Ritonavir and Lopinavir Y= 0.1828x - 0.0236 and y = 0.1639x - 0.0261. Retention times are decreased and that run time was decreased so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

INTRODUCTION

Ritonavir is belongs to a class of medications called protease inhibitors. It's IUPAC name is (2S)-N-[(2S,4S,5S)-5-{[2-(2,6-dimethylphenoxy)acetyl]amino}-4-hydroxy- 1,6 diphenyl-hexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide. It has molecular formula C37H48N6O5S2 and molecular weight 720.312 g/mole [1]. The chemical structure of ritonavir is shown as in fig. 1.

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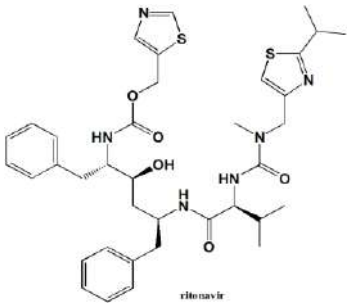
Lopinavir is an antiretroviral protease inhibitor used in combination with other antiretroviral in the treatment of HIV-1 infection[2]. It has molecular formula, C37H48N4O5 and molecular weight as 628.80 g/mole. IUPAC name of Lopinavir is 1,3-thiazol-5-ylmethyl [3-hydroxy-5- [3-methyl-2-[methyl- [(2-propan-2- yl1,3- thiazol- 4-yl)methyl] carbamoyl] amino-butanoyl] amino-1,6-diphenyl-hexan-2-yl] aminoformate. It is white to light tan powder and freely soluble in methanol and ethanol[3,4]. The chemical structure of ritonavir is shown as in figure 2.

Combination therapy with the HIV protease inhibitors Lopinavir and ritonavir 200 mg of Lopinavir and 50 mg of Ritonavir is available in market by brand name Lopimune) has been shown to be effective against drug-resistant HIV-1[5]. These agents are metabolized by cytochrome P-450 (CYP) 3A in the liver 4-6. When Lopinavir is administered with ritonavir as Lopimune®, ritonavir inhibits the CYP 3A- mediated metabolism of lopinavir, thereby providing increased plasma levels of lopinavir4-6[6,7]. In this work new HPLC method is developed, optimized and validated for the assay of two drugs viz., lopinavir and ritonavir in combined dosage forms [8].

A survey of literature reveals that there are few methods reported for the simultaneous determination of lopinavir and ritonavir in pharmaceutical preparations using HPLC [9]. In this communication we report a new simple, rapid and precise HPLC method for simultaneous determination of lopinavir and ritonavir in combination tablet, which can be used for its routine analysis in ordinary laboratories.



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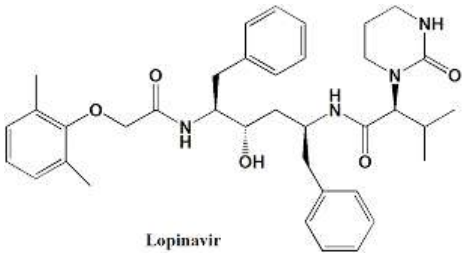

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MATERIALS AND METHODS

Chemicals

The bulk drugs of lopinavir and ritonavir were obtained as gift samples from Mylan Laboratories Ltd. Aurangabad, Maharashtra, India. Combination tablets of Lopinavir 200 mg and Ritonavir 50 mg form were purchased from local market. All the solutions for analysis were prepared and analyzed freshly.

Instrumentation and analytical conditions

Chromatography was performed using Agilent 1100 and 1200series equipped with HPLC and equipped with G1311A (Quaternary gradient type) pump and SPD-10A UV-Vis detector. The methods were conducted using a gradient reverse phase technique. The analytical conditions (mobile phase composition, flow rate and analytical wavelengths) for the two drugs have been summarized in Table 1. The mobile phases were prepared freshly, filtered through 0.45 µ membrane filter and sonicated for 10 min before use in order to desecrate.

Preparation of standard stock solution of Lopinavir

Weighed 25.0 mg of Ritonavir standard and transferred into 50 mL volumetric flask, added 40 mL of diluent and sonicated to dissolve then made up to volume with diluent.

Preparation of standard stock solution of Ritonavir

Weighed 20.0 mg of Lopinavir standard and transferred into 25 mL volumetric flask, added 20 mL of diluent and sonicated to dissolve then made up to volume with diluent.

Mobile phase preparation:

The mobile phase was prepared by mixing 80 mL acetonitrile and 20 mL 0.1% ortho-phosphoric acid buffer (pH 3.5) filtered through nylon 0.45µm membrane filter. The mobile phase was degassed for 15 minutes before use.

DETECTION WAVELENGTH FOR LOPINAVIR AND RITONAVIR

The detection wavelength was determined by scanning the solution in the range 200-400 nm using blank. The overlain spectra were scanned and the wavelength was detected as 217nm which was selected for analysis see figure 3.

Preparation of standard solution:

2mL of standard stock solution Lopinavir and 5 mL of standard stock solution Ritonavir was pipetted in a 10 mL volumetric flask and then made up to volume with diluent.

Preparation of Sample solution:

Weighed powdered tablets (Equivalent to 200 mg of Lopinavir) 83 mg and transferred into 1000 mL volumetric flask, added 800 mL of diluent and sonicated for 10 minutes then made up to volume with diluent. Kept the flask for 5 minutes and then filtered the supernatant liquid. Discarded first few mL of filtrate, further 5 mL of the filtrate was diluted to 25 mL with diluent

The optimized chromatographic conditions for analysis see table 1

METHOD OPTIMIZATION

A RP-HPLC method was developed for lopinavir and ritonavir, which can be used easily with pharmaceutical dose 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. Here the Drugs pKa were known from literature as 1.5 (Strongest Basic) and 2.84 (Strongest Basic) for Lopinavir and Ritonavir respectively. We have selected the Ortho-phosphoric acid (pH 3.5) as buffer for mobile phase composition. And C18 (250mm X 4.6mm) column was chosen because it is suitable for low to high pH range, good lifetime, good efficiency and good selectivity. Drugs were eluted with good retention time, resolution, all the system suitable parameters like plate count and tailing factor were within the limits. See table 1

Trial are runs to get well separated chromatograms see table 2

The well separated chromatogram was found in trial 5 see figure 4

METHOD VALIDATION PARAMETERS

The developed method was validated as per the International Conference on Harmonization (ICH) 14, 15 guidelines with respect to linearity and range, specificity, precision, accuracy, robustness, limit of detection and limit of quantification.

RESULT AND DISCUSSION

System suitability

To verify that the analytical system is working properly and can give accurate and precise results, the system suitability parameters are to be set. See table 3

Linearity:

The linearity of the responses of the drugs was verified at six concentration levels, ranging from 0-20µg/ml for both LPV and RTV. The calibration graph was plotted as peak area Vs the concentration. The linear regression equation of the calibration curve found for RTV Y= 0.1828x - 0.0236 and for LPV y = 0.1639x - 0.0261 respectively. The calibration graphs were found to be linear in the plotted concentrations. The

coefficient of determination was 0.9998 for LPV and 0.9997 for RTV. (See figure 5and 6)

The results obtained with the detector response were found to be linear in the concentration range of 0-20µg/ml for both LPV and RTV.

Precision

A homogenous sample of a single batch was analyzed six times. The method was found to be precise as indicated by % RSD which is less than 2 for both LPV and RTV. See table 4

Accuracy

Accuracy of the method was determined by Recovery studies. To the pre-analyzed sample, the reference standards of the drugs were added at the level of 50%, 100%, 150%. Aliquots of sample solutions containing LPV and RTV 0.60 mL, 0.80 mL respectively were transferred to three volumetric flasks (10 mL) containing 0.48, 0.60, 0.72 mL LPV and 0.64, 0.80 and 0.96 mL RTV working standard solution. The contents were mixed and diluted to volume in order to obtain final concentrations of 10.8, 12.0, 13.2 µg/mL LPV and14.4, 16.0, 17.6 µg/mL RTV, respectively. The recoveries were verified by estimation of drugs in triplicate preparations at each specified concentration level. See table 5 and 6

The % RSD values less than 2 indicative of accuracy of the method

Range

The range of analytical method is the interval between the upper and lower levels of analyte that has been demonstrated to be determined with a suitable accuracy, precision and linearity. The specified range is derived from linearity and accuracy studies.

From the above results, it can be concluded that the method is linear, precise and accurate between 50% and 150% levels of target concentration. Hence, the range of the method is 50% to 150%.

Robustness

Robustness of the method was checked by making slight deliberate changes in chromatographic conditions like flow rate and wavelength. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed RP-HPLC method is robust. see table 7

LOD and LOQ studies

The Limit of detection and quantification were calculated using standard deviation of the response and slope of calibration curve. LOD was found to be 1.5ng/spot for Lopinavir and 4.6ng/spot for Ritonavir. LOQ was found to be 21.00ng/spot for Lopinavir and 5.10ng/spot for Ritonavir.

Assay

The drug content of marketed dosage form (LOPIMUNE - Ritonavir 50 mg and Lopinavir 200 mg) were calculated six times using proposed method. The average % assay was calculated and found to be 97.91 % and 96.59 % for Lopinavir and Ritonavir respectively. Assay results shown in Table 9 indicates that method is suitable for analysis of marketed formulation. See table 8

CONCLUSION

The developed RP-HPLC method provides simple, accurate, precise, rapid and reproducible quantitative analysis for simultaneous determination of LPV and RTV in bulk and tablets. The method was validated as per ICH guidelines and can be employed for routine analysis and can be adopted in regular Quality control test in Industries.

Table 1: The Optimized Chromatographic Conditions For Analysis

Column	C18 (250mm X 4.6mm)
Detection wavelength	217nm
Flow rate	1ml/min
Temperature	25°C
Sample volume	20µl
Mobile phase	Acetonitrile: 0.1% OPA(pH 3.5), 80:20

Table 2: Trial Runs To Get Well Separated Chromatograms

Trial	Mobile phase	Flow (ml/min)	Conclusion
Trial 1	MeOH: Water(90:10)	1ml/min	Peaks are not well separated
Trial 2	MeOH: Water(80:20)	1ml/min	Single separated peak seen
Trial 3	ACN: 0.1%OPA pH 6.2 (90:10)	1ml/min	Peaks get separated too close
Trial 4	ACN: 0.1%OPA pH 6.2 (80:20)	1ml/min	Peaks are not well separated
Trial 5	ACN: 0.1%OPA pH 3.5 (80:20)	1ml/min	Peaks are well separated.

Table 3: Results System Suitability

Sr. No.	System suitability parameters	Results	
		Lopinavir	Ritonavir
1	Tailing factor for both Ritonavir and Lopinavir peaks should be NMT2.0	1.21	1.00
2	Theoretical plate number	2776.2	6439.3
3	Retention time	7.38	2.98
4	Peak area	60987	20976

Table 4: Results Of Method Precision

	Assay in %	
Set no.(n=6)	**Lopinavir**	**Ritonavir**
Mean	97.91	96.59
%RSD	0.2%	0.4%

Table 5: Results Of Accuracy And Linearity Of Lopinavir

Lopinavir		
Level (% Concentration)	**Mean Area of Linearity**	**Mean Area of 3 sets for Accuracy**
50%	568.83	581.20
100%	1142.95	115.90
150%	1661.20	172.25
Correlation coefficient		**0.998**

Table 6: Results Of Accuracy And Linearity Of Ritonavir

Ritonavir		
Level (% Concentration)	**Mean Area of Linearity**	**Mean Area of 3 sets for Accuracy**
50%	121.390	143.658
100%	227.407	237.857
150%	390.539	343.604
Correlation coefficient		**0.998**

Table-7: Results Of Robustness

Robustness					
The system suitability Parameters should pass for all the conditions.		**Lopinavir**		**Ritonavir**	
		Tailing Factor	**%RSD**	**Tailing Factor**	**%RSD**
Original condition		1.045	0.1%	1.043	0.2%
Flow Change	**-0.2ml/min**	1.062	0.2%	1.062	0.1%
	+0.2ml/min	1.101	0.3%	1.096	0.4%
Temperature	**-5°C**	0.976	0.2%	0.96	0.2%
	+5°C	0.925	1.3%	0.919	1.0%
Organic phase ratio change	**-2.0 %**	1.076	0.2%	1.073	0.1%
	+2.0%	1.072	0.1%	1.069	0.2%

Table 8: Assay Result Of Marketed Dosage Form

Dosage form	Active ingredients	Labelled amount(mg/tab)	Mean ± S.D	Assay	%RSD
LOPIMUNE tablets	Lopinavir	200mg	200.02 ±0.50	100.05	0.37
	ritonavir	50mg	49.97 ±0.36	99.85	0.46

Table 9: Summary Of Validation Parameters Of HPLC

Parameters	Lopinavir	Ritonavir
Calibration range	0-20µg/ml	0-20µg/ml
Optimized wavelength	217nm	217nm
Retention time	7.38min	2.98min
Regression equation (Y*)	y = 0.1639x - 0.0261	Y= 0.1828x - 0.0236
Correlation coefficient(r2)	0.9998	0.9997
Precision (% RSD*)	0.2%	0.4%
% Recovery	99.7222	99.2591
LOD	1.5ng/spot	4.6ng/spot
LOQ	21.00ng/spot	5.10ng/spot

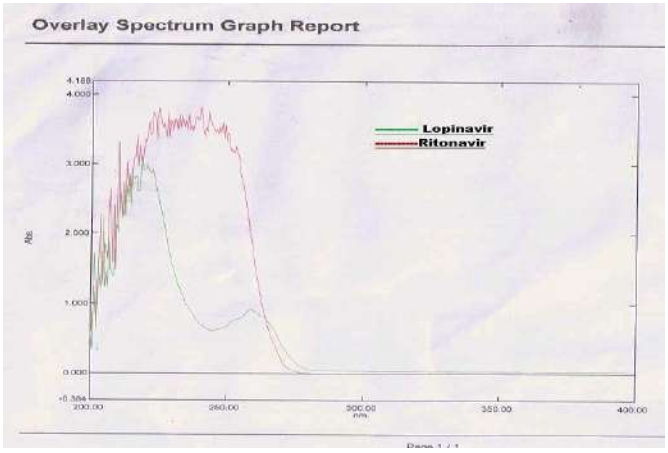

Figure 3: Overlay Spectrum Of Lopinavir And Ritonavir

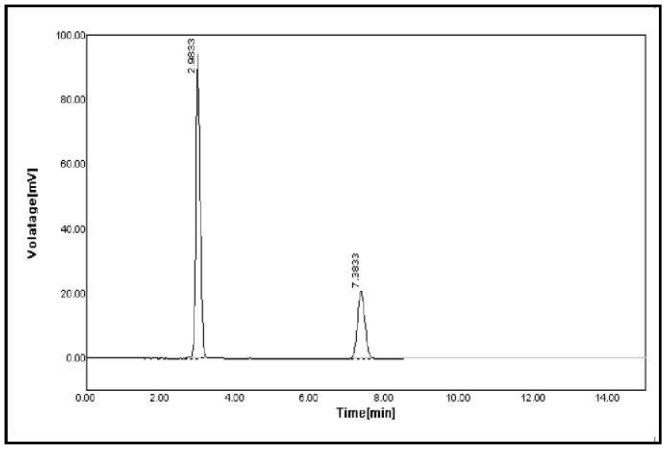

Figure 4: well separated chromatogram of trial 5ritonavir+ lopinavir; 80% ACN+ 20% (0.1 % OPA; pH 3.5)

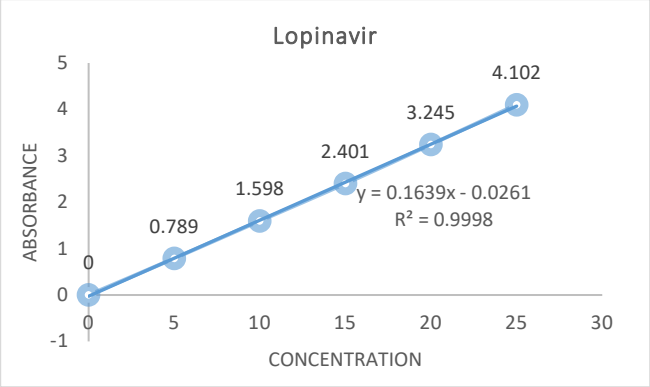

Figure 5: calibration curve data of lopinavir

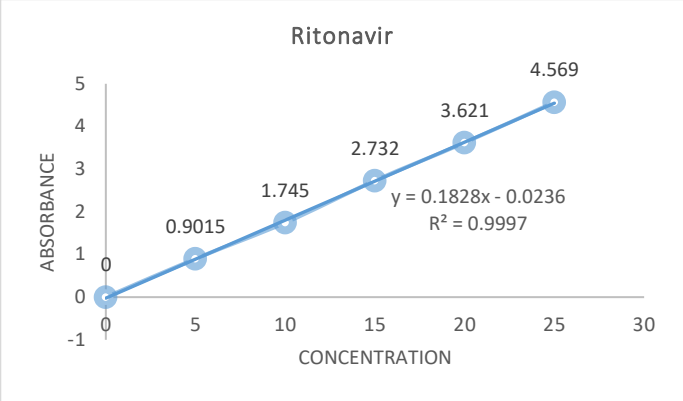

Figure 6: calibration curve data of ritonavir

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