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Research Article

A VALIDATED RP HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LOPINAVIR AND RITONAVIR IN COMBINED DOSAGE FORM

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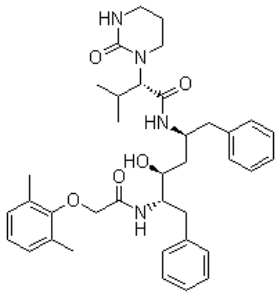
ABSTRACT

An accurate, sensitive, precise and robust reverse phase high performance liquid chromatographic method for the simultaneous estimation of lopinavir and ritonavir in combined dosage form has been developed and validated. Chromatographic separation was conducted on Phenomenex Gemini C18 (250 mm×4.6 mm, 5µ) column at room temperature using Potassium hydrogen phosphate buffer (pH adjusted to 6.0 ± 0.1 with diluted potassium hydroxide solution), acetonitrile and methanol in the ratio of 50:35:15v/v and at a flow rate of 1.0 ml / min, while UV detection was performed at 254 nm. The retention time for lopinavir and ritonavir was found to be 6.0±0.2 and 3.7±0.1 min, respectively. The method was found to be linear in the range of 400-600µg/ml for lopinavir and 100-150 µg/ml for ritonavir. The developed method was validated in terms of accuracy, precision, LOD, LOQ, robustness and solution stability. The proposed method can be successfully used for the estimation of lopinavir and ritonavir in bulk and combined dosage forms.

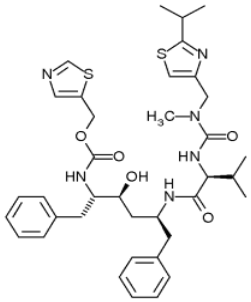
Keywords: Lopinavir; Ritonavir ; RP-HPLC; Formulations.

INTRODUCTION

Lopinavir[1], is chemically known as (2H) – pyrimidine acetamide N-[[4-(2,6- methyl phenoxy)acetyl]amino]-3-hydroxy 5- phenyl- 1-(phenyl methyl) pentyl, tetrahydro-α- (1- methyl ethyl)- 2- oxo and its empirical formula is $C_{37}H_{48}N_4O_5$, having a molecular weight of 628.80.



Lopinavir



Ritonavir

Ritonavir[2], is chemically known as 2,4,7,12- tetra azatridecan- 13oic acid, 10 hydroxy- 2- methyl- 5- (1- methyl ethyl)- 1- [2- (1- methyl ethyl)- 4- thiazolyl]- 3,6- dioxo- 8,11- bis(phenyl methyl)- 5-thiazolmethyl ester and its empirical formula is $C_{37}H_{48}N_6O_5S_2$ with a molecular weight of 720.90. Both the drugs were used as antiretroviral agents.

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], high performance thin layer chromatography [7], derivative UV spectrophotometry[8]. Literature survey reveals that no analytical method for determination of lopinavir and ritonavir in combined dosage forms is reported. So it is felt worth while to develop a simple, rapid, accurate, precise and more economical high performance liquid chromatographic method for simultaneous estimation of lopinavir and ritonavir in bulk and its combined dosage form.

EXPERIMENTAL

Materials and instruments

Reference standards of lopinavir and ritonavir were obtained as gift samples from Aurobindo Pharmaceuticals, Hyderabad. Market formulation of this combination Emletra and Ritocom were procured from the local market. HPLC grade acetonitrile and methanol were obtained from Merck (India). Analytical grade potassium hydrogen phosphate buffer and potassium hydroxide were purchased from SD Fine chemicals, India. Water obtained from Millipore with milli Q system, filtered through 0.45 µ nylon-66 membrane was used for the HPLC work. The LC system consisted of isocratic pump, auto sampler and UV detector. The output signal was monitored and integrated using LC solutions chromatography Manager Software (Prominence HPLC, Shimadzu, Japan).

Mobile phase

A mixture of Potassium hydrogen phosphate buffer (pH adjusted to 6.0 ± 0.1 with diluted potassium hydroxide solution), acetonitrile and methanol in the ratio (50:35:15v/v) was used as mobilephase which was filtered through a 0.45µ nylon membrane filter.

Preparation of mixed standard solution of Lopinavir and Ritonavir

About 100 mg of lopinavir and 25 mg of ritonavir were weighed accurately and transferred to 100 ml standard volumetric flask. It was dissolved in mobile phase then the solution was sonicated for about 10 min and the volume was made up to the mark with mobile phase to give a stock solution containing 1mg/ml of lopinavir and 0.25 mg/ml of ritonavir.

Procedure for recording chromatograms

Different aliquots of 4.0, 4.5, 5.0, 5.5 and 6.0 ml of mixed standard solution of lopinavir and ritonavir were transferred into a series of 10ml standard volumetric flasks and were made up to 10ml with mobile phase. The standard solutions prepared as above were filtered through 0.45µ membrane filter. Under the optimized chromatographic conditions a steady base line was recorded. After stabilization for 30 min all the standard solutions were injected separately using rheodyne injector and the chromatograms were recorded.

Preparation of sample solution

To determine the content of the drugs in pharmaceutical formulations, twenty tablets were weighed and pulverized using a mortar and pestle. An amount equivalent to 100 mg of lopinavir and 25 mg of ritonavir, was transferred to a 100ml standard volumetric flask, about 60ml of mobile phase was added and sonicated for about 10minutes. Then volume was made up to the mark with mobile phase and filtered through a 0.45 µ nylon membrane filter. An aliquot portion of the filtrate was further diluted to get final

concentration of 500 µg/ml of lopinavir and 125 µg/ml of ritonavir. All the determinations were conducted six times to ensure repeatability of the method. The mean peak area of the each drug was calculated.

RESULTS AND DISCUSSION

The purpose of the present study was to develop a rapid and sensitive RP-HPLC method for the simultaneous estimation of lopinavir and ritonavir in combined dosage form using Phenomenex Gemini C_{18} analytical column with UV detection.

Method optimization

To optimize the operating conditions for isocratic RP-LC detection of analytes, a number of parameters such as the mobile phase composition, pH and flow rate were varied. Various ratios (70:15:15, 60:20:20, 50:30:20, 50:35:15v/v) of buffer: acetronitrile: methanol was tested as starting solvent for system suitability study. The variation in the mobile phase led to considerable changes in the chromtographic parameters like symmetry, capacity factor and retention time. The pH effect showed that optimized conditions are reached when the pH value was 6.0, producing well resolved and sharp peaks for lopinavir and ritonavir. Henceforth, in the present method the ratio of (50:35:15v/v) potassium hydrogen phosphate buffer: acetonitrile: methanol, pH adjusted to 6.0 ±0.1 with 10% sodium hydroxide as a mobile phase, at a flow rate of 1.0 $mLmin^{-1}$ was chosen as optimal conditions. The appropriate wavelength in UV region (254nm) was selected for the measurement of active ingredients in the proposed method. For quantitative determination of lopinavir and ritonavir in formulations, initially mixed standard solution was injected into the column five times and the retention time of lopinavir and ritonavir was found to be 6.1 ± 0.05 and 3.7 ± 0.05 min, respectively (Fig.1).

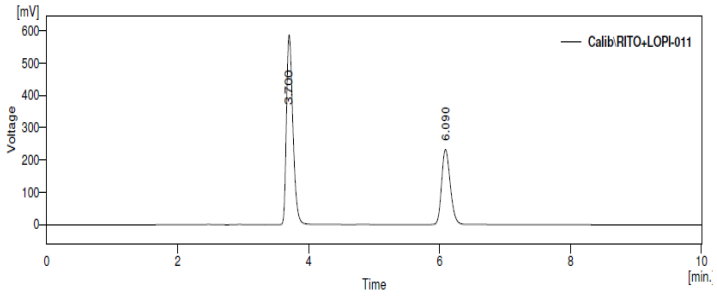


Fig.1: Standard chromatogram of Ritonavir and Lopinavir

Validation[8]

The described method has been validated for the simultaneous estimation of lopinavir and ritonavir using following parameters.

Accuracy

Accuracy of the method was demonstrated at three different concentration levels (80-120%) by spiking a known quantity of standard drugs into a analyzed sample in triplicate. The results of accuracy (Table1) revealed that the method was more accurate.

Precision

For the precision of the method, three replicate were injected into the system on two different non consecutive days, in each case %RSD was calculated. Results of precision are given in Table 2, which indicated that the method is precise.

Table 1: Recovery study of Lopinavir and Ritonavir using the proposed HPLC method

S. No.	Spiked level	% Recovery		% RSD	
		Lopinavir	Ritonavir	Lopinavir	Ritonavir
1.	50%	100.05	99.42	0.566	0.645
2.	100%	99.98	98.35	0.752	0.578
3.	150%	97.96	99.56	0.834	0.783

Table 2: Method precision for Lopinavir and Ritonavir in combined dosage form

S.No	Drug name	Labelled amount	Amount found (mg)*	
			Intra day	Inter day
1	Lopinavir	200 mg	198.8±0.64	197.5±0.84
2	Ritonavir	50 mg	49.6±0.58	98.35±0.56

*average of six determinations

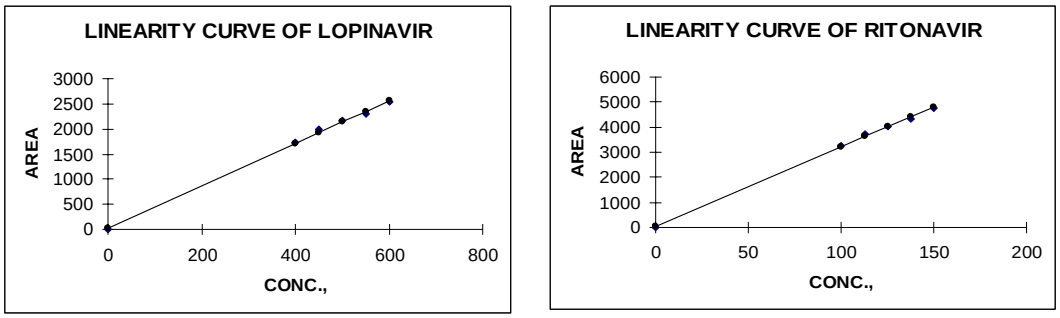


Fig. 2: Calibration curve of Lopinavir and Ritonavir

Linearity

To establish linearity of the proposed method, five different sets of drug solution was prepared and analyzed. Standard curves were constructed in the concentration range of 400-600 µg mL^{-1} of lopinavir and 100-150 µg mL^{-1} of ritonavir (Fig.2). Slope, intercept and the correlation coefficient were determined and the regression statistics are shown in Table 3.

Limit of detection (LOD) and limit of quantization (LOQ)

The limit of detection and limit of quantification for lopinavir and ritonavir were calculated from the linearity data using relative standard deviation of the response and slope of the calibration curve. 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 34 and 7.4 µg mL^{-1},

respectively. 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 103 and 22 µg mL^{-1}, respectively.

Specificity

No interference of peaks were found in the chromatogram indicating that excipients used in the tablet formulation did not interfere with the estimation of the drugs by the proposed method for the simultaneous determination of lopinavir and ritonavir in the combined dosage form, hence the method is specific.

Robustness

In order to demonstrate the robustness of the method, system suitability parameters were verified by making deliberate changes in the chromatographic conditions, viz. change in flow rate by ±0.05 mL min^{-1}, change in pH of the buffer by ±0.1 unit and change in the ratio of mobile phase (±2% absolute). The method was demonstrated to be robust over an acceptable working range of its HPLC operational parameters.

To ascertain the system suitability for the proposed method a number of statistical values such as theoretical plates, HETP, peak asymmetry, resolution have been calculated with the observed readings and the results are tabulated in Table 3.

Table 3: System Suitability Parameters

S. No.	Parameters	Lopinavir	Ritonavir
1.	Retention time (min)	6.1	3.7
2.	Thoretical plates	8535	9375
3.	Tailing factor	1.25	0.48
4.	Resolution	11.35	-
5.	Range (µg/ml)	200 - 800	50 – 200
6.	Slope	4.238	31.907
7.	Intercept	20.554	35.86
8.	Correlation Coefficient	0.9999	0.9998
9.	LOD(µg/ml)	34	7.4
10.	LOQ(µg/ml)	103	22

The HPLC method developed in the present study has been used to quantify lopinavir and ritonavir in the combined dosage form (Fig.3). The average drug content of lopinavir and ritonavir was found to be 196.54 and 49.59 mg of the labeled amount mention in Table 4.

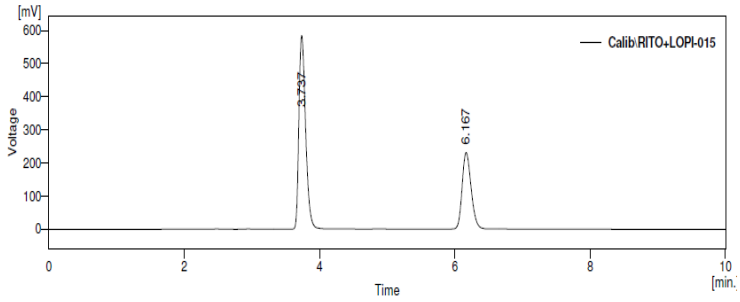


Fig. 3: Sample chromatogram of Ritonavir and Lopinavir in formulation

Table 4: Mean (±S.D.) amount of Lopinavir and Ritonavir in tablet dosage forms by proposed HPLC method

S. No.	Drug Name	Label claim	Amount found*	% Purity *
1.	Lopinavir	200 mg	196.65	98.32
2.	Ritonavir	50 mg	49.71	99.42

* Average of six determinations

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.

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