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STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LOPINAVIR AND RITONAVIR BY USING RP-HPLC

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ABSTRACT

A simple, Accurate, precise method was developed for the simultaneous estimation of the Lopinavir and Ritonavir in Pharmaceutical dosage form. Chromatogram was run through Kromasil C18(250mm 4.6mm, 5µ). Mobile phase containing Orthophosphoric acid buffer and Acetonitrile in the ratio of 48:52 was pumped through column at a flow rate of 1.0 ml/min. Temperature was maintained at 30°C. Optimized wavelength for Lopinavir and Ritonavir was 310nm. Retention time of Lopinavir and Ritonavir were found to be 2.214 min and 3.163 min. %RSD of the Lopinavir and Ritonavir were and found to be 0.6 and 0.3 respectively. %Recover was Obtained as 99.64% and 99.56% for Lopinavir and Ritonavir. LOD, LOQ values were obtained from regression equations of Lopinavir and Ritonavir were 0.20ppm, 0.61ppm and 0.08ppm, 0.24ppm respectively. Regression equation of Lopinavir y = 7745x+22310, and of Ritonavir is y = 11440x + 7469. Retention times were decreased and that run time was also decreased so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

KEYWORDS: Lopinavir, Ritonavir, RP-HPLC.

INTRODUCTION

Lopinavir1, 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 C37H48N4O5, having a molecular weight of 628.80.

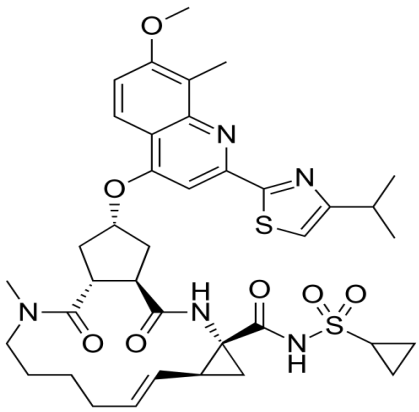

Figure 1: Structure of lopinavir.

Ritonavir2, 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- thiazol methyl ester and its empirical formula is C37H48N6O5S2 with a molecular weight of 720.9.Both the drugs were used as antiretroviral agents.

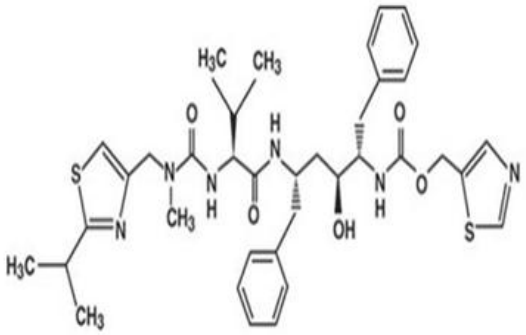

Figure 2: Structure of Ritonavir.

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 HPLC3-6, high performance thin layer chromatography 7, derivative UV spectrophotometry8. Literature survey reveals that no analytical method for determination of lopinavir and ritonavir in combine dosage forms is reported. So it is felt worthwhile 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.

2. Experimental work

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-66membrane was used for the HPLC work. The LC system consisted ofisocratic pump, auto sampler and UV detector. The output signal was monitored and integratedusing LC solutions chromatography Manager Software (Prominence HPLC, Shimadzu, Japan).

Mobile phase

A mixture of ortho phosphoric acid phosphate buffer, acetonitrile in the ratio (48:52 v/v) was used as mobile phase 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 weighedaccurately 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.

Preparation of sample solution of Lopinavir and Ritonavir

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 Kromasil C18 analytical column with UV detection 310 nm.

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 (70:30:, 48:52,50:50, 60:40v/v) of buffer: acetronitrile 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 (48:52v/v) potassium hydrogen phosphate buffer: acetonitrile 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 inUV region (310nm) 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 2.213 and 3.163 min, respectively (Fig.1).

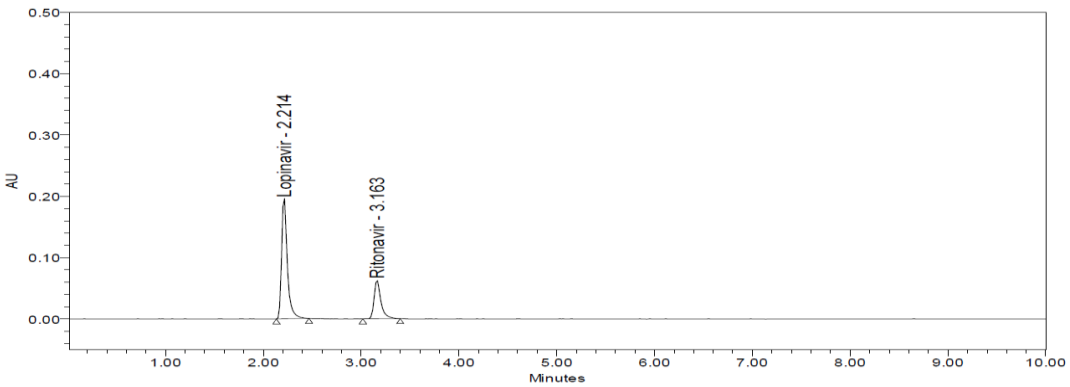

Figure 3: Chromatogram for lopinavir and ritonavir.

System suitability

Table 1: System suitability parameters.

S.No	Name	Retention time	Peak area	Resolution	Tailing factor	USP plate count
1	Lopinavir	2.214	800110	8.3	1.35	7444
2	Ritonavir	3.163	303033		1.44	10664

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 (Table 2) 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 3, which indicated that the method is precise.

Table 2: Recovery study of Lopinavir and Ritonavir using the proposed HPLC method.

Sample	Amount added (µg/ml)	Recovery (%)	%RSD
Lopinavir	50	99.59	0.4
	100	100.34	0.9
	150	99.00	0.5
Ritonavir	12.5	99.54	0.3
	25	99.30	0.3
	37.5	99.85	0.4

Table 3: Method precision for Lopinavir and Ritonavir in combined dosage form.

s.no	Drug name	Lablled amount	Amount found in mg	
			Inter day	Intra day
1	Lopinavir	100 mg	99.8±0.02	99.6±0.03
2	Ritonavir	25 mg	24.90±0.03	24.75±0.08

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 25-150µg mL-1 of lopinavir and 12.5 -7.5 µg mL-1 of ritonavir (Fig.2). Slope, intercept and the correlation coefficient were determined and the regression statistics are shown in Table 3.

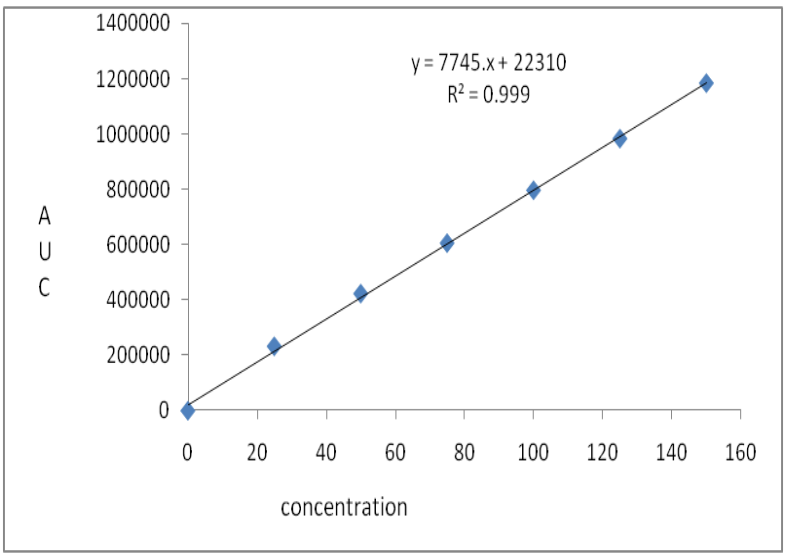

Figure 4: Linearity graph of lopinavir.

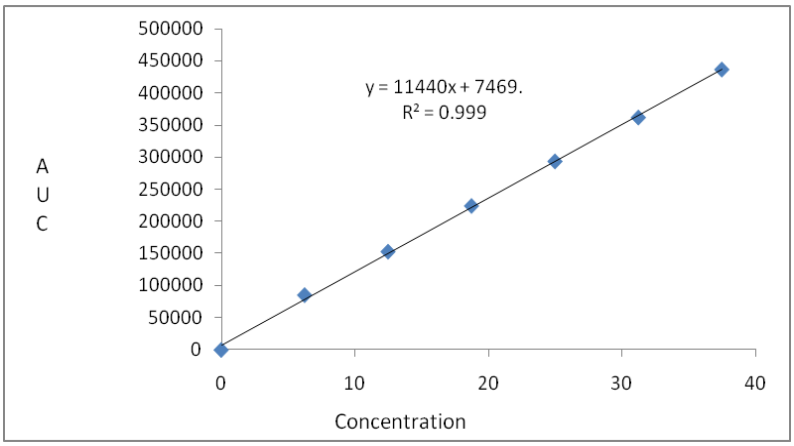

Figure 5: Linearity curve of Ritonavir.

Table 4: Analytical Performance Parameters for Lopinavir and Ritonavir.

Parameters	Lopinavir	Ritonavir
Linearity Range	25ppm-150ppm	12.5ppm-75ppm
Regression equation	y = 7745.x + 22310	y = 11440.x + 7469
Correlation coefficient	0.999	0.999
Slope	7745	11440
Intercept	22310	7469

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 calibrationcurve. The limit of detection of a compound is defined as the lowest concentration of analyte that can be detected. The results were tabulated below in table-

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.

Table 5: LOD and LOQ.

Drug Name	LOD	LOQ
Lopinavir	0.20	0.61
Ritonavir	0.08	0.24

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.

Table 6: Robustness.

S.NO	Degradation Condition	Lopinavir			Ritonavir		
		% Drug Degraded	Purity Angle	Purity Threshold	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	4.52	0.206	0.415	4.89	0.427	0.612
2	Alkali	2.99	0.739	0.896	2.94	0.402	0.589
3	Peroxide	1.18	0.214	0.321	1.67	0.492	0.717
4	Thermal	0.91	0.820	1.850	0.75	0.253	0.448
5	UV	0.68	0.766	1.555	0.98	0.372	0.715
6	Water	0.59	0.764	1.548	0.51	0.345	0.687

Table 7: Summary table for validation parameters.

Validation Parameters	Method developed	
	LOPI	**RITO**
Range	25-150 µg/ml	6.25-37.5 µg/ml
Regression Coefficient	0.999	0.999
Slope (m)	7745	11440
Intercept (c)	22310	7469
Regression equation	Y=7745.x+ 22310	y = 11440x + 7469
Assay	99.54%	99.035
Retention time	2.214	3.163
System Precision (%RSD)	0.8	1.1
Method Precision (%RSD)	0.6	0.3
Accuracy	99.64%	99.56%
LOD	0.20	0.08
LOQ	0.61	0.24
Robustness	0.2%-1.2%	0.7%-1.2%

CONCLUSION

The new stability indicating RP-HPLC method was developed and validated as per guidelines for the simultaneous determination of Lopinavir and Ritonavir in combined pharmaceutical dosage form. The proposed method was found to be accurate, precise, simple, economic, rapid and having good specificity. The developed method can be applied for the assay of commercial tablets containing Lopinavir and Ritonavir in routine quality control analysis.

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