International Journal of Research and Development in Pharmacy and Life Sciences
Available online at http//www.ijrdpl.com
June - July, 2014, Vol. 3, No.4, pp 1074-1079
ISSN: 2278-0238

Research Article

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LOPINAVIR AND RITONAVIR BY RP-HPLC

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(Received: April 21, 2014; **Accepted:** May 22, 2014**)**

ABSTRACT

A simple, precise, rapid, selective, and economic reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed for the simultaneous estimation of Lopinavir and Ritonavir in marketed formulations. Chromatographic separation was achieved isocratically for the combination was done with a C18 column [ODS UG column. 250mm× 4.5 mm] utilizing mobile phase of composition Acetonitrile and Phosphate buffer (60:40v/v, pH 3) the flow rate was 1.5ml/min and the eluates was monitored at 220nm. Lopinavir and Ritonavir were eluted with retention times of 2.1min and 4.0min respectively. The method was found to be linear over a range of 20-100 g/ml for Lopinavir and Ritonavir. The method was validated according to the guidelines of International Conference on Harmonisation (ICH) and was successfully employed in the estimation of commercial formulations.

Keywords: Lopinavir and Ritonavir, RP-HPLC, Method validation.

INTRODUCTION

Lopinavir is chemically known as (2S)-N- [(2S, 4S, 5S)-5- [2-(2,6dimethylphenoxy) acetamido] 4-hydroxy-1, 6-diphenylhexan -2-yl] – 3 - methyl-2-(2-oxo-1,3-diazinan-1-yl) butanamide and its empirical formula is C37H48N4O5, with a molecular weight of 628.80. Lopinavir inhibits the HIV viral protease enzyme. This prevents cleavage of the gagpolpolyprotein and therefore, improper viral assembly results.

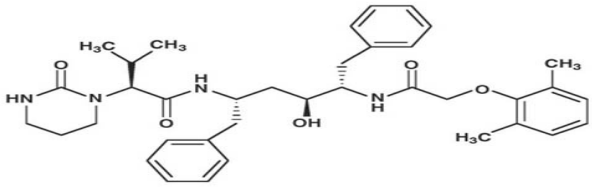

Fig. 1(a) Chemical structure of Lopinavir

This subsequently results in non-infectious, immature viral particles. The chemical structure was shown in Figure 1a.

Ritonavir is (5S, 8S, 10S, 11S)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3, 6-dioxo-8, 11-bis (phenyl methyl)-2, 4, 7, 12-tetraazatridecan-13-oic acid 5-thiazolyl methyl ester Figure.1b.

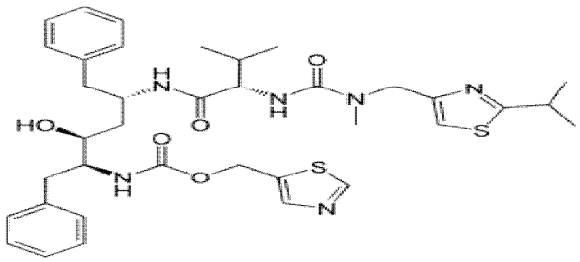

Fig. 1(b) Chemical structure of Ritonavir

It is official in Indian Pharmacopoeia and United States Pharmacopoeia. Ritonavir is an antiretroviral drug from the protease inhibitor class used to treat HIV infection and AIDS. Ritonavir is frequently prescribed with Highly Active Anti-Retroviral therapy, not for its antiretroviral action, but as it inhibits the same host enzyme that metabolizes other protease inhibitors. The lower than therapeutic doses of ritonavir are commonly given in combination with agents such as Lopinavir, Indinavir, or Amprenavir to reduce the risk of resistance by increasing the time of drug exposure. Combination therapy with the HIV protease inhibitors lopinavir and ritonavir (Sustained release capsule with combination of lopinavir 133.3 mg and ritonavir 33.3 mg is available in market by brand name kaletra®) has been shown to be effective against drug-resistant HIV. These agents 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]

Extensive literature survey revealed that very few methods were reported for the simultaneous estimation of Lopinavir and Ritonavir by RP-HPLC4-10. So, an attempt has been made to develop an accurate, precise and economically viable RP-HPLC method for the simultaneous estimation of combination of interest in the current research.

MATERIALS AND METHODS

Equipment used

The chromatographic separation was performed on Agilent 1120 compact liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20 l fixed loop. A reverse phase C18 [Agilent ODS UG 5 column, 250mm × 4.5 mm] was used. ELICO SL 218 double beam UV visible spectrophotometer and Axis AGN204-PO electronic balance were used for Spectrophotometric determinations and weighing purposes respectively.

Reagents and chemicals

Pharmaceutical grade pure Lopinavir and Ritonavir gift samples were procured from (Hetero Laboratories (I) Pvt.Ltd.Hyderabad.). Marketed formulationTablets with dose of 200 mg of Lopinavir and 50mg of Ritonavir (Kaletra) were procured from local market. (Mfd. By Abbott ltd). HPLC grade Acetonitrile and HPLC grade Water were procured from Merck specialities private limited, Mumbai.

Chromatographic conditions

C18 [Agilent ODS UG 5 column, 250mm × 4.5 mm] was used for the chromatographic separation at a detection wave length of 220nm. Acetonitrile, Phosphate buffer pH 3, in a ratio of 60:40v/v was selected as mobile phase for elution and same mixture was used in the preparation of standard and sample solutions. The elution was monitored by injecting the 20 l and the flow rate was adjusted to 1.5 ml/min.

Preparation of Mobile phase

Preparation of Phosphate buffer pH 3: Dissolve 1.36g of Potassium dihydrogen orthophosphate & 2ml of triethylamine in 800ml of HPLC water, adjust the pH to 3 with orthophosphoric acid and add sufficient HPLC water to produce 1000ml.The mobile phase was sonicated for 15 min and filtered through a 0.45 µm membrane filter paper.

Preparation of Standard solutions

25mg each Lopinavir and Ritonavir were accurately weighed and transferred into two 25ml volumetric flasks, dissolved using mobile phase and the volume was made up with the same solvent to obtain primary stock solutions A (Lopinavir) B (Ritonavir) of concentration 1000 g/ml of each drug. (Working stock solution A &B).

Preparation of Sample Solution

20 tablets (Kaletra) were initially powdered and an amount equivalent to 25mg of Ritonavir and 100mg of Lopinavir was accurately weighed into a 25ml volumetric flask, mixed with 25ml of mobile phase. The solution was made up to the volume with mobile phase and sonicated for 5 minutes. The solution was then filtered through 0.45µm Millipore membrane filter. The solution contains 1000µg/ml of Ritonavir and 4000 µg/ml of Lopinavir (Stock solution-'A').

From the above stock solution-'A' 1ml aliquot was transferred in to a 10 ml volumetric flask, volume was made up to the mark with mobile phase to obtain a final concentration of 100 µg/ml Ritonavir and 400 µg/ml Lopinavir. This solution was used as the sample stock solution- 'B'.

2ml of the sample stock solution -'B' was transferred in to a 10 ml volumetric flask, volume was made up to the mark with mobile phase to obtain a final concentration of 20µg/ml Ritonavir and 80µg/ml Lopinavir.

Optimization of RP-HPLC method

The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of Lopinavir and Ritonavir. For the method optimization, different mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with Acetonitrile, Phosphate buffer pH 3 (60:40 v/v) using C18 column [Agilent ODS UG 5 column, 250mm × 4.5 mm] figure 2.

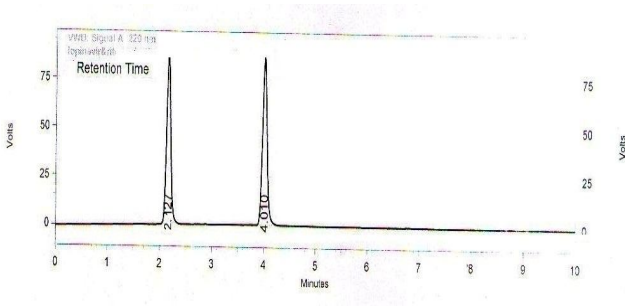

Fig 2: Optimised chromatogram of Lopinavir and Ritonavir

Validation of the RP-HPLC method

Validation of the optimized method was performed according to the ICH Q2 (B) guidelines.

System suitability

System suitability was carried out with six injections of solution of 100% concentration having 100µg/ml of Lopinavir and Ritonavir in to the chromatographic system. Number of theoretical plates (N) obtained and calculated tailing factor (T) was reported in table 1.

Linearity

For the determination of linearity, appropriate aliquots were pipetted out from 1000µg/ml (working stock solution A &B). 0.2 - 1 ml was pipetted out in to a series of 10ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 20-100 g/ml of Lopinavir and Ritonavir. Each solution was injected in triplicate. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficients. The calibration curves for Lopinavir and Ritonavir were shown in figure 3 & 4 and their corresponding linearity parameters were given in table 2.

Precision

The repeatability of the method was verified by calculating the % RSD of six replicate injections of 100% concentration (100 g/ml of Lopinavir and Ritonavir) on the same day and for intraday precision % RSD was calculated from repeated studies. The results were given in table 3.

Accuracy

To ensure the reliability and accuracy of the method recovery studies were carried out by standard addition method. A known quantity of pure drug was added to pre-analysed sample and contents were reanalysed by the proposed method and the percent recovery was reported. The results were given in table 4.

Specificity

Specificity of a method was determined by testing standard substances against potential interferences. The method was found to be specific when the test solution was injected and no interferences were found because of the presence of excipients.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s. The results were given in table 2.

Robustness

Robustness of the method was verified by altering the chromatographic conditions like mobile phase composition, flow rate, detection wave length, etc. and the % RSD should be reported.

Small changes in the operational conditions were allowed and the extent to which the method was robust was determined. A deviation of ± 2 nm in the detection wave length and ± 0.2 ml/min in the flow rate, were tried individually. Solutions of 100% test concentration with the specified changes in the operational conditions were injected to the instrument in triplicate. % RSD was reported in the table 5.

Assay of Marketed Formulations

20 l of sample solution of concentration 20 g/ml of Ritonavir and 80 g/ml of Lopinavir was injected into chromatographic system and the peak responses were measured. The solution was injected three times in to the column. The amount of drug present and percentage purity was calculated by comparing the peak areas of the standards with that of test samples figure 5, table 6.

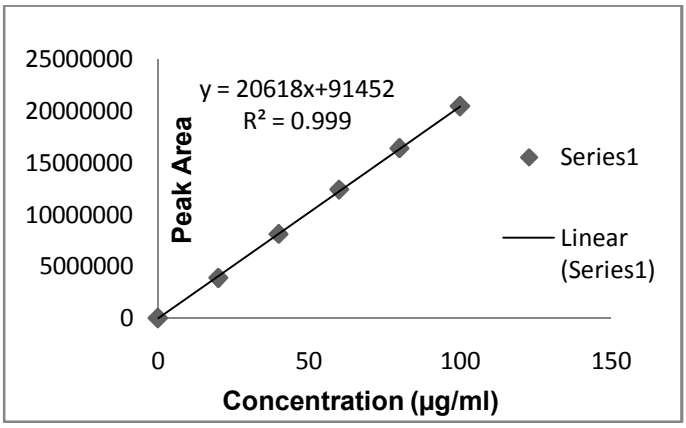

Fig 3: Calibration Curve of Lopinavir

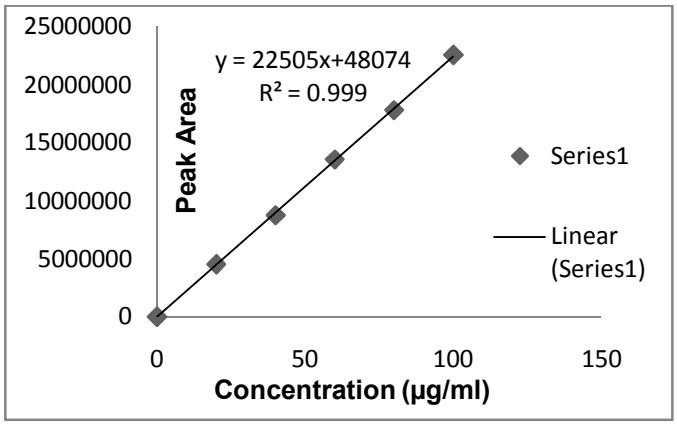

Fig 4: Calibration curve of Ritonavir

Table 1: System suitability parameters

Parameters	Lopinavir	Ritonavir
Retention Time (min)	2.1	4.01
Resolution (R_s)	2	
Tailing Factor (T)	1.2	1.4
Theoretical Plates (N)	11456	10366

Table 2: Results for Linearity (n=6)

Parameter	Lopinavir	Ritonavir
Linearity Range (µg/ml)	20-100	20-100
Regression Equation	Y=20618x+91452	Y=22505x+48074
Slope (m)	20618	22505
Intercept (c)	91452	48074
Regression Coefficient (r^2)	0.999	0.999
Limit of Detection (µg/ml)	0.316	0.433
Limit of Quantitation (µg/ml)	0.949	1.28

***n= No.of determinants**

Table 3: Results of precision (n=6)

Drug	Intraday Precision (%RSD)	Interday Precision (%RSD)
Lopinavir	0.14	0.87
Ritonavir	0.93	1.16

***n= No.of determinants**

Table 4: Results for Accuracy (n=3)

Recovery level	Lopinavir					Ritonavir				
	Amount added (µg/ml)		Conc. (µg/ml)	Amount found (µg/ml)	% Recovery	Amount added (µg/ml)		Conc. (µg/ml)	Amount found (µg/ml)	% Recovery
	std	test				std	Test			
50%	4	16	20	19.8	99.1	16	4	20	19.6	98.01
100%	24	16	40	39.9	99.7	36	4	40	39.5	98.75
150%	44	16	60	61.02	101.6	56	4	60	60.02	100.3
Mean recover	99.1-101.6%					98.01-100.3%w/w				

Table 5: Results for Robustness

Parameters (n=3)	%RSD	
	Lopinavir	Ritonavir
Detection wavelength at 218nm	0.38	0.66
Detection wavelength at 222nm	0.56	0.38
Flow rate 1.2ml/min	0.36	0.66
Flow rate 1.7ml/min	0.31	0.38

***n= No.of determinants**

Table 6: Results for Assay (n=3) of Marketed formulation

S. No	Amount Present in (mg/tab)		Amount Obtained in (mg/tab)		% Purity(w/w)	
	LOP	RIT	LOP	RIT	LOP	RIT
1	200	50	198.96	49.35	99.48	98.7

***n= No. of determinants**

RESULTS AND DISCUSSION

After a number of trials with mobile phases of different composition, Acetonitrile, Phosphate buffer pH 3 in the ratio 60:40v/v was selected as mobile phase because of better resolution and symmetric peaks. Lopinavir and Ritonavir were found to appreciable absorbance at 220 nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Lopinavir and Ritonavir at different Rt was shown in figure 2.

System suitability was carried out by injecting six replicate injections of 100% test concentration, number of theoretical plates, HETP and resolution were satisfactory. The chromatograms confirm the presence of Lopinavir and Ritonavir at 2.1min and 4.0 min respectively without any interference.

Concentration range of 20-100µg/ml for Lopinavir and Ritonavir were found to be linear with correlation coefficients 0.999 and 0.999 for Lopinavir and Ritonavir respectively. The parameters were given in table 2.

The proposed method was found to be precise and reproducible with %RSD of 0.14 and 0.93 for Lopinavir and Ritonavir respectively. %RSD was reported in table 3.

Accuracy of the method was verified by performing recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 99.1 to 101.6% w/w and 98.01-100.3%w/w for Lopinavir and Ritonavir respectively. This indicates that the method was accurate. Values obtained were given in table 4.

The limits of detection for Lopinavir and Ritonavir were found to be 0.16µg/ml and 0.33µg/ml respectively and the limits of quantitation were 0.49µg/ml and 1.01µg/ml respectively.

The method was found to be specific for the combination of interest after verifying the chromatograms showing no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination. Values obtained were given in table 6.

The method was found to be robust after changing the conditions like detection wavelength (± 2 nm) and flow rate (± 0.2 ml). %RSD was calculated for each variation and reported. Values obtained were given in table 5.

CONCLUSION

The RP-HPLC method developed and validated allows a simple and fast quantitative determination of Lopinavir and Ritonavir from their formulations. The low solvent consumption (1.5ml/min), along with short analytical run time of less than 10.0 minutes lead to an environmental friendly chromatographic procedure that allows the analysis of a large number of samples in a short period of time. All the validation parameters were found to be within the limits according to ICH guidelines. The proposed methods were found to be specific for the drugs of interest irrespective of the excipients present and the methods were found to be simple, accurate, precise, rugged and robust and can be involved in the routine analysis of the marketed formulations.

Acknowledgments

The authors are thankful to the Hetero Laboratories, Hyderabad for providing the gift samples of Lopinavir and Ritonavir respectively. And also to the management of Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur for providing facilities and great support to carry out the research work.

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