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Method Development and Validation of antiviral combination as Ritonavir and Lopinavir in Bulk and Pharmaceutical Dosage Form by RP-HPLC.

Rane SS*, Chaudhari[1] RY and Patil VR[2].

* Asst. Prof., Department of Pharmaceutical Chemistry, TVES's Hon. L. M. C. College of Pharmacy, Faizpur.
[1]Vice Principle TVES's Hon. L. M. C. College of Pharmacy, Faizpur and HOD of Department of Pharmaceutical Chemistry TVES's Hon. L. M. C. College of Pharmacy, Faizpur.
[2]Dean for the faculty of Medicine and Pharmacy, North Maharashtra University, Jalgaon, Maharashtra and Principle, TVES's Hon. L. M. C. College of Pharmacy, Faizpur.

ABSTRACT

A simple, selective and sensitive high performance liquid chromatographic method has been developed and validated for the simultaneous determination of ritonavir and lopinavir both as a bulk drug and in pharmaceutical formulations. The method employed Eurosphere C18 column (250 x 4.6 mm id, 5 µm particle size) as the stationary phase while methanol and phosphate buffer pH adjusted to 7 (78: 22 v/v, pH 7) was used as mobile phase. The method showed high sensitivity with linearity range from 10 to 50 µg/ml and 40 to 200 µg/ml with correlation coefficients of 0.999 and 0.998 for ritonavir and lopinavir respectively observed at 230 nm wavelength. Best resolution was obtained retention time of 6.1000 and 7.5167 min respectively for ritonavir and lopinavir at flow rate of 1.0 ml per minute. Mean percent recovery of triplicate samples at each level for both drugs were found in the range of 98.78% to 100.15% with RSD of less than 2.0%. 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: RP-HPLC, Lopinavir, Ritonavir, Combined dosage forms, Simultaneous estimation, Validation.

Corresponding author

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INTRODUCTION

Lopinavir (ABT-378) chemically is (2S)-N-[(2S,4S,5S)-5-[2-(2,6-dimethylphenoxy)acetamido]-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide. Lopinavir is a novel protease inhibitor (PI) developed from ritonavir although having structural similarity to ritonavir, lopinavir is more potent against HIV-1 than ritonavir. Co-administration with low-dose ritonavir significantly improves the pharmacokinetic properties and hence the activity of lopinavir against HIV-1 protease. It is used against HIV infections as a fixed-dose combination with another protease inhibitor. A 2014 study indicates that lopinavir is effective against the human papilloma virus (HPV).[1-3] The chemical structure as shown in figure 1.

Ritonavir chemically is 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl]carbamate. It is a peptidomimetic Human Immunodeficiency Virus (HIV) protease inhibitor designed to complement of the enzyme active site. Ritonavir is an orally active against both HIV-1 and HIV-2. Ritonavir inhibit CYP3A4 metabolism and increase concentrations of lopinavir by preventing metabolism.[4,5] Ritonavir is official in Indian Pharmacopoeia and United States Pharmacopoeia[6,7]. The chemical structure as shown in figure 2.

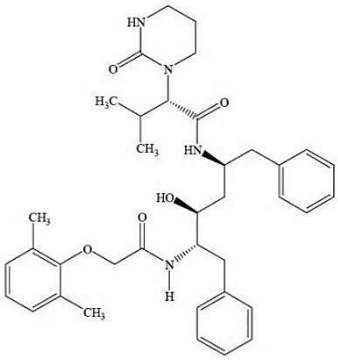

Figure 1: Lopinavir

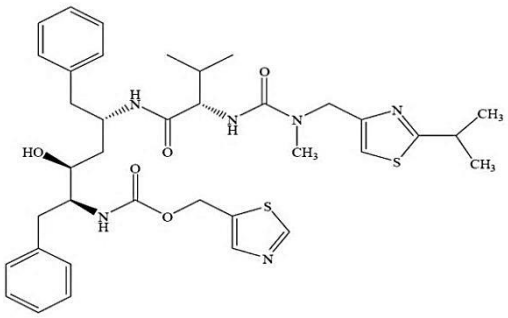

Figure 2: Ritonavir

From the literature survey, it was found that literature survey revealed that very few methods were reported for the simultaneous estimation of lopinavir and ritonavir by RP-HPLC [8-11].

So, an attempt has been made to develop an accurate, precise and economical 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 Systronic LC 6600 liquid chromatographic system integrated with a injector equipped with 20µl fixed loop. The chromatographic system operated using Chemitochrom software and separate programmable UV detector. System coupled with reverse phase C18 (ODS UG 5 column, 250mm ×4.5 mm) was used. Shimadzu UV 1800 double beam UV visible spectrophotometer and Sansui-vibra DJ-150S-S electronic balance were used for Spectrophotometric and weighing purposes respectively.

Reagents and chemicals

Pharmaceutical grade pure lopinavir and ritonavir gift samples were procured from Cipla Pvt. Ltd., Kurkumbh. Marketed formulation Tablets with dose of 200 mg of lopinavir and 50mg of ritonavir (Lopimune) were procured from local market. (Mfd. By Cipla Pvt. Ltd.). HPLC grade methanol, acetonitrile and water were procured from Merck specialities private limited and sodium dihydrogen phosphate from SD fine chem. limited, Mumbai.

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Chromatographic conditions

A reverse phase C18 (column, 250mm ×4.6 mm) was used for the chromatographic separation at a detection wave length of 224nm. HPLC grade methanol and phosphate buffer in a ratio of 78:22 v/v pH adjusted to 7.0 was selected as mobile phase for elution. The elution was monitored by injecting the 20µl and the flow rate was adjusted to 1.0 ml/min.

Standard preparations

Stock standard solutions of lopinavir and ritonavir at the concentration of 1 mg/ml were separately prepared by dissolving accurately weighed 100mg of the drugs in 100 ml methanol. Working standard solution of ritonavir was prepared by dilution of its stock solution with 50% methanol until the final concentration of 0.1 mg/ml was obtained. The solutions were filtered through 0.45µ membrane filter.

Selection of mobile phase

The pure drug of lopinavir and ritonavir were injected into the HPLC system and run in different solvent systems. Different mobile phases like methanol and buffer, acetonitrile and buffer, methanol, acetonitrile and water tried. It was found that sodium dihydrogen phosphate buffer and methanol gives satisfactory results as compared to other mobile phases. Finally by using systematic approach, the optimal composition of the mobile phase was determined to be methanol and phosphate buffer in composition of 78:22 pH adjusted to 7.0.

Selection of wavelength

Appropriate dilution was prepared using standard stock solution of each drug and both the solutions were scanned over range of 200-400 nm, using medium scan speed. Considering the overlain spectra 224nm has been selected as detection wavelength for HPLC method.

Analysis of dosage form

Twenty tablets (lopinavir-200 mg and ritonavir-50 mg) were weighed and finely powdered. Powder equivalent to 80 mg of lopinavir and 20 mg ritonavir was accurately weighed and added into a 100 ml volumetric flask, add about 60-ml methanol. Sonicate for 30 minute to dissolve and then made up to the volume with methanol and filter the solution through 0.45 membrane filter. Then 10 ml of the above filtrate was transferred into a 100 ml volumetric flask and diluted to the mark with methanol to obtain working standard solution 20 µg/ml and 80 µg/ml for ritonavir and lopinavir respectively. The chromatogram is shown in Figure 1.

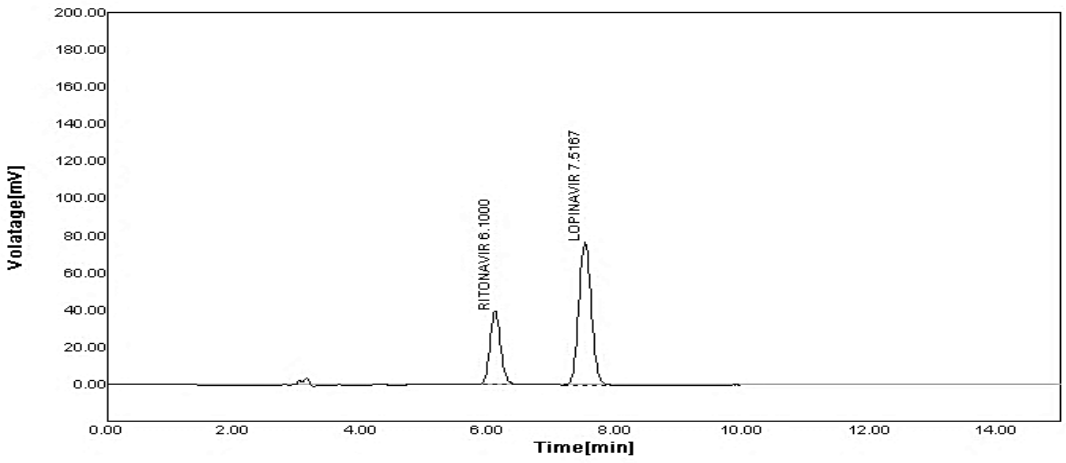

Figure 1: Typical chromatogram showing the elution of ritonavir and lopinavir at their respective retention times

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Validation Parameters:

Validation of the optimized RP HPLC method was performed as per the ICH Q2 (B) guidelines.[12]

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.

Table 1: System suitability parameters

Parameters	Lopinavir	Ritonavir
Retention Time (Rt) min	7.5167	6.1000
Resolution (Rs)	3.4000	
Tailing Factor (T)	1.0385	1.0909
Theoretical Plates (N)	5754.6	6138.9

* An average of six determinations

Linearity

Calibration curves were obtained from the peak area and concentration of the drug were subjected to regression analysis and correlation coefficients. Table 2 represents the linearity of the proposed method which shows the responses for the drugs was strictly linear (r2>0.999) in the concentration range of 5-30 µg/ml for ritonavir and 20-120 µg/ml for lopinavir respectively. The slope and intercept for lopinavir was found to be 12.737 and 3.1981 whereas for ritonavir was found to be 23.034 and 0.2013 respectively.

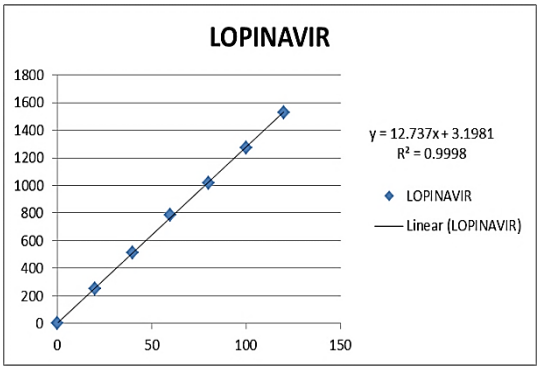

Figure 2: Calibration curve of lopinavir

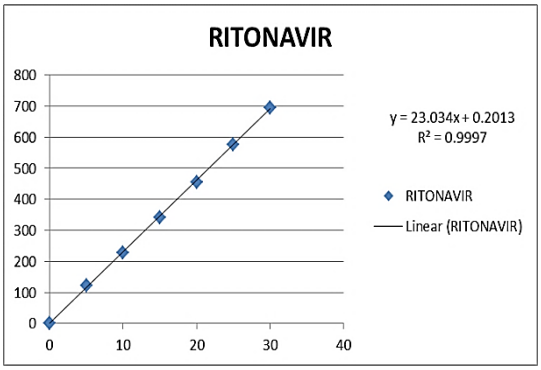

Figure 3: Calibration curve of ritonavir

Table 2: linearity of the proposed method.

Sr. No.	Parameter	Lopinavir	Ritonavir
1.	Linearity range	40-200 µg/ml	10-50 µg/ml
2.	Regression equation	y = 12.737x + 3.1981	y = 23.034x + 0.2013
3.	Slope	12.765	23.194
4.	Intercept	1.8511	1.749
5.	Regression Coefficient (R^2)	0.9998	0.9999
6	LOD	3.01214	0.72375
7	LOQ	9.12771	2.19318

* An average of six determinations

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Accuracy and precision

Accuracy and precision were determined by elaboration of three standard calibration curves, two from the same day (intra-day) and third one from a different day (inter-day). The intra-day and inter-day precisions (% RSD) at different concentration levels were found to be less than 2 % (Table 3). Moreover the % RSD (less variation) showed good precision of the developed HPLC method.

Accuracy data of analytical method in the present study ranged from 99.87 - 100.15% for lopinavir and 98.78 to 100.15% for ritonavir which (Table 3) indicates that there was no interference from excipient components of market formulation.

The LOD and LOQ were determined from the calculated standard deviations of each calibration standard. LOD was found to be 3.01214 µg/ml and 0.72375 µg/ml and LOQ was found to be 9.12771 µg/ml and 2.19318 µg/ml for lopinavir and ritonavir respectively. The calculated LOQ and LOD concentrations confirmed that the method is sensitive.

Table 3: Recovery studies by RP-HPLC

Level of Drug added (%)	Ingredient	Amount added (mg)	Amount recovered (mg)	Mean Recovery (%)
80	Lopinavir	80	79.90	99.87
	Ritonavir	40	40.11	100.27
100	Lopinavir	100	99.94	99.94
	Ritonavir	50	49.89	99.78
120	Lopinavir	120	120.18	100.15
	Ritonavir	60	59.27	98.78

* An average of three determinations

Table 4: Precision data for the analysis of lopinavir and ritonavir

	Lopinavir				Ritonavir			
	Intra-day		Inter-day		Intra-day		Inter-day	
Sr. No.	Conc. (µg/ml)	% RSD	Conc. (µg/ml)	% RSD	Conc. (µg/ml)	% RSD	Conc. (µg/ml)	% RSD
1	40	0.90	40	1.08	10	1.08	10	1.21
2	80	0.78	80	0.98	20	0.98	20	1.36
3	120	1.24	120	1.47	30	1.66	30	1.87
4	160	1.36	160	1.97	40	1.78	40	1.75
5	200	1.81	200	1.78	50	1.67	50	1.84

* An average of three determinations

Table 5: Assay of tablet dosage form

Table 4. Assay of tablet dosage form			
Ritonavir		Lopinavir	
Labeled amount (mg/tablet)	Amount found %	Labeled amount (mg/tablet)	Amount found %
50	99.19	120	99.36
	98.99		101.85
	100.89		99.36
	101		100.44
	99.04		99.24
	100.98		100.45
Mean	100.01	Mean	100.11
SD	1.034	SD	1.013
% RSD	1.034	% RSD	1.013

RESULTS AND DISCUSSION

A RP-HPLC method was developed for two anti-retroviral drugs, which can be conveniently employed for routine quality control in pharmaceutical dosage forms. The chromatographic conditions were optimized in order to provide a good performance of the assay. The mobile phase for each drug was selected based on its polarity. Different ratios of methanol: buffer compositions were tried for lopinavir and ritonavir and the fixed mobile phase was 78:22 pH adjusted to 7. The flow rate was optimized to reduce the extent of longitudinal broadening which is inversely related to flow rate of mobile phase. Best resolution was obtained retention time of 6.1000 and 7.5167 min respectively for ritonavir and lopinavir at flow rate of 1.0 ml per minute. The chromatogram shown in Figure 1 confirms that the method was specific as none of the excipients interfered with the analytes of interest. The recoveries achieved are good for both the molecules hence; the method was suitably employed for assaying the commercial anti-retroviral formulations.

CONCLUSION

The proposed RP-HPLC is simple, reliable and selective. It also provides satisfactory accuracy and precision with lower limits of detection and quantification. Moreover the shorter duration of analysis for lopinavir and ritonavir make these reported methods suitable for routine quantitative analysis in pharmaceutical dosage forms.

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