Annals of Phytomedicine 12(1): 573-580, 2023

DOI: http://dx.doi.org/10.54085/ap.2023.12.1.45

Annals of Phytomedicine: An International Journal

http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885

Original Article : Open Access

Analytical method development and validation for the simultaneous estimation of lopinavir and ritonavir by RP-HPLC method in tablet dosage form

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Article Info

Article history
Received 12 March 2023
Revised 28 April 2023
Accepted 29 April 2023
Published Online 30 June-2023

Keywords
Lopinavir
Ritonavir
RP-HPLC
Dosage form
ICH validation

Abstract

Lopinavir and ritonavir are protease inhibitor category of antiretroviral drugs. Both are used for the treatment of HIV/AIDS and COVID. A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validation of lopinavir and ritonavir in its bulk form as well as in tablet dosage form. Chromatography was carried out on a waters reliant C8, 250 × 4.6 mm, 5 µ column using a mixture of monobasic potassium phosphate buffer, and acetonitrile in proportion 55:45 v/v as the mobile phase at a flow rate of 1.5 ml/min. The detection was carried out at 215 nm. The retention time of the lopinavir and ritonavir was found to be 30.887 and 24.087 min, respectively. The method produces linear responses in the concentration range of 12.5-37.5 µg/ ml and 3.125-9.375 µg/ml, respectively, for lopinavir and ritonavir. The method precision for the determination of assay was below 2.0% RSD. The method is useful in the quality control of bulk and pharmaceutical formulations. The optimized method was validated and proved to be suitable for the quality control of the mentioned drugs in the tablet pharmaceutical dosage form, according to ICH guidelines. The developed method was found to be fairly precise, rapid and economical for simultaneous estimation of lopinavir and ritonavir when compared with the reported method.

1. Introduction

Lopinavir and ritonavir are human immunodeficiency virus (HIV) protease inhibitor. The combination has improved pharmacokinetic activity and reduced HIV resistance (Alka Rani and Wamik Azmi, 2021; Priti Vihol *et al*., 2021). Ritonavir is an antiretroviral drug used to treat HIV infection and AIDS. Ritonavir is a protease inhibitor class of drug and it inhibits the same host enzyme that metabolizes other protease inhibitors (Beckett, 2002; Carolina Trajano Velozo *et al*., 2021). This inhibition of the proteases results in elevated plasma concentrations of these drugs, thus allowing the physician to lower their dose and frequency and improving their clinical efficacy (Dhulipalli *et al*., 2016; Fathima Qurratul Ayeen *et al*., 2019). So, the simultaneous administration with the other HIV protease inhibitors like lopinavir has been shown to be effective against drug-resistant HIV. It was used as a candidate drug in the treatment of COVID during clinical trials but not found to be very effective (Fegade *et al*., 2012; Habler *et al*., 2021; Natchiappan Senthilkumar *et al*., 2021). These drugs are metabolized in the liver by cytochrome P-450 (CYP) 3A. When lopinavir is administered with ritonavir, ritonavir inhibits the CYP 3A-mediated metabolism of lopinavir, thereby providing increased plasma levels of lopinavir (Killi *et al*., 2014; Prasanthi *et al*., 2022 ; Lunn, 1996).

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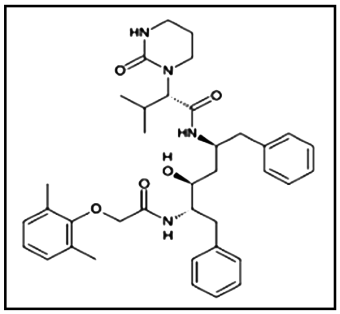
Lopinavir is known as N-[4-[[(2,6 dimethyl phenoxy) acetyl]amino]-3-hydroxy-5-phenyl-1-(phenylmethyl) pentyl] tetrahydroalpha (1methylethyl)-2-oxo 1(2H) pyrrolidine acetamide. Ritonaviris known as 10-hydroxy-2methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4thiazolyl]-3,6-dioxo-8,11-bis (phenylmethyl)-2,4,7,12 tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester (Punagoti *et al*., 2014; Rathnasamy *et al*., 2018). Both are freely soluble in methanol, ethanol and isopropanol. They are practically insoluble in water (Varaprasad *et al*., 2012; The Merck Index, 2001). A survey of literature reveals that there are few methods reported for the simultaneous determination of lopinavir and ritonavir in pharmaceutical preparations using HPLC. Several analytical methods have been reported for the assay of lopinavir and ritonavir individually or combination with other drugs in biological samples as well as formulations (Sunitha *et al*., 2015; Skoog, 2005). So our aim is to develop a new rapid and sensitive RP-HPLC for simultaneous estimation of both the drugs and to perform the validation as per ICH guidelines.



Lopinavir (a)

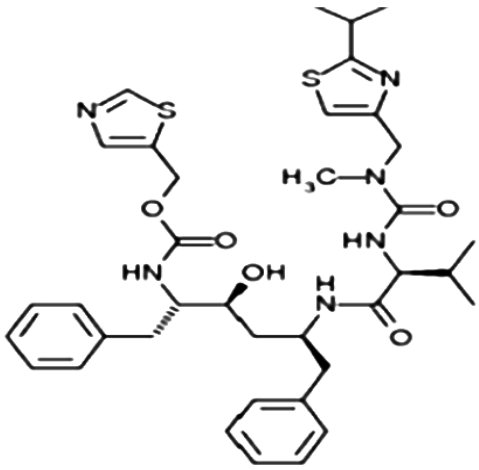


Figure 1: Structures of lopinavir and ritonavir.

There are several methods developed in the past for estimation that were found to have longer retention times, higher consumption of organic solvents, and poor resolution. Thus, there is a need to develop an analytical technique with less interference, good resolution, along with less organic solvent consumption. The present research work aimed to develop and validate a stability-indicating HPLC method for simultaneous estimation of the drugs in combined tablet solid oral dosage form by using parameters like specificity, linearity and range, accuracy, system precision, method precision, intermediate precision, robustness, solution stability and filter integrity as per ICH guidelines.

2. Materials and Methods

2.1 Chemicals and reagents

Distilled water, HPLC Water (Milli Q Grade), Acetonitrile (HPLC Grade), Monobasic potassium phosphate (AR Grade) used were of Merck AR grade and HPLC grade. Lopinavir and ritonavir working reference standards were obtained as gift samples from Green tree testing laboratories, Chennai, India.

2.2 Instruments and apparatus required

Uni Bloc-Analytical balance, Shimadzu-1601 (UV Probe Software) Double Beam UV-Visible spectrophotometer with pair of 10 mm matched quartz cells, Shimadzu1700 (UV Probe Software), Double Beam UV-Visible spectrophotometer with pair of 10 mm matched quartz cells, Shimadzu-LC-2010 AHT, HPLC System, Lab solution, Waters 2487, Dual absorbance detector, IR Affinity 1, Shimadzu with IR solution, ELICO pH meter (Model LI-120), REMI-Centrifuge apparatus and CYBERLAB-Micropipette.

2.3 Instrumentation and chromatographic conditions

The HPLC system waters reliant C_8, 250 ×4.6 mm, 5 µ equipped with UV/Visible dual absorbance detector, was used to achieve chromatographic separation. Mobile phase was composed of monobasic potassium phosphate buffer pH 5.3 and acetonitrile in a 55:45 v/v ratio. The mobile phase was filtered through 0.45 µ membrane filter, degassed and injected onto the column at 1.5 ml/ min flow rate. Injection volume of the drug solution was 50 µl, and the detection was recorded at 215 nm.

2.4 Preparation of mobile phase

Buffer was prepared by weighing 4.1 g of monobasic potassium phosphate and dissolved in 1 liter of water. The pH was 5.3. The mixture of buffer and acetonitrile was mixed in the ratio of 55:45 v/ v and degassed in the ultrasonic water bath for few minutes. The solution was filtered under vacuums using a 0.45 µ filter and used.

2.5 Preparation of standard solution

50.0 mg of lopinavir WRS and 12.5 mg of ritonavir WRS were accurately weighed and transferred into a 200 ml volumetric flask, dissolved in mobile phase to obtain the concentration of 0.25 mg/ ml for lopinavir and 0.0625 mg/ml for ritonavir. 5 ml of the above solution was pipetted into a 50 ml volumetric flask and made upto volume with the mobile phase (25 µg/ ml of lopinavir and 6.25 µg/ml of ritonavir).

2.6 Preparation of sample solution

Twenty tablets of formulation (200 mg of lopinavir and 50 mg of ritonavir) were weighed accurately and the average weight of each tablet was found. The tablets were ground to a fine powder. The tablet powder equivalent to 250 mg of ritonavir was weighed and transferred to 250 ml volumetric flask. About 120 ml of mobile phase was added to dissolve the substance and sonicated for 15 minutes. Then, it was made up to volume with mobile phase (1 mg/ ml of lopinavir and 4 mg/ml of ritonavir). The above solution was filtered through 0.45 µm PVDF filter. From the clear solution, 5 ml was pipetted out into a 100 ml volumetric flask and the solution was made upto the volume with the mobile phase. Six replicates of 50 µl of the sample solution was injected and the chromatograms were recorded. The amount of drugs in tablet formulation was calculated using the slope and intercept values from the calibration graph.

2.7 Chromatographic method development

Different mobile phases were used to run the standard drugs at various pH levels, along with organic mobile phases modifiers such as acetonitrile, methanol, and water. Additional trials were carried out to minimize tailing by altering pH, however, these changes caused the peaks to split or the resolution to diminish between the two drugs. It was also attempted to modify the organic phase, however, this led to peaks merging or to no changes in the drug's tailing. The peak forms were observed to be symmetrical under the specified chromatographic conditions.

2.8 Selection of wavelength

The standard stock solution was further diluted with diluent to get the concentration of 25 µg/ ml of lopinavir and 6.25 µg/ml of ritonavir and the solution was scanned between 200 nm to 400 nm using mobile phase as blank. The spectrum was overlain. From the overlain spectrum 215 nm was selected as the detection wavelength.

3. Results

3.1 Method validation

The analytical method was optimized and validated in accordance with the current ICH guidelines and to accomplish the vision of specificity, accuracy, linearity, precision, robustness, filter validation, solution stability.

3.1.1 System suitability

Inject 50 µl of the standard preparation in 6 replicates and check the system suitability. If system suitability is found satisfactory, proceed with the injection of sample preparations. The order of elution will be as follows:

(i) Ritonavir

(ii) Lopinavir

System suitability tests were carried out to ascertain the adequate resolution and repeatability of the developed method. Investigations were done on the following parameters: column efficiency, resolution and relative standard deviation. It was reported that the column efficiency: Not less than 5000 theoretical plates for both lopinavir and ritonavir peaks. Resolution: Not less than 4.0 between lopinavir and ritonavir peaks. Relative standard deviation: NMT 2.0 % for the 6 areas of lopinavir and ritonavir peaks from 6 replicate standard injections. The above-mentioned parameters were all within acceptable ranges.

3.1.2 Specificity

By comparing the test sample's retention time to that of reference drugs, the lopinavir and ritonavir peaks of the test drugs were assessed. The retention times of the standard and test samples showed a good correlation. It was noted that the peaks were unaffected by the diluent or excipient peaks. Table 1 represents the observations of the specificity samples. The chromatogram of standard and test sample without any interference and no interference of the placebo was observed which is shown in Figure 2.

Table 1: Specificity of lopinavir and ritonavir

S.No.	Solution preparation	Observation
1.	Blank	No peak observed
2.	Placebo	No peak observed
3.	Lopinavir standard solution	One peak observed at 30.863 min
4.	Ritonavir standard solution	One peak observed at 24.060 min
5.	Standard solution	Two peaks observed at 24.057 and 30.844 min correspond to ritonavir and lopinavir, respectively.
6.	Sample Solution	Two peaks observed at 24.087 and 30.887 min correspond to ritonavir and lopinavir, respectively.

Remarks: There is no interference of the placebo. Hence, the method is specific.

Table 2: Specificity of standard

Standard	Retention time	Area
Ritonavir	24.057	351947
Lopinavir	30.844	1810569

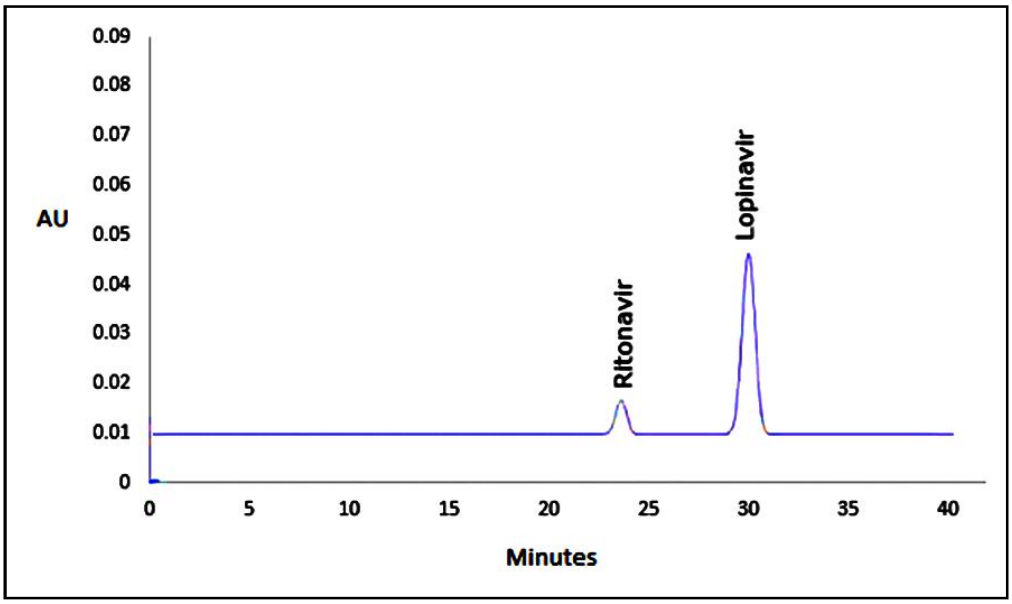


Figure 2: Chromatogram of lopinavir and ritonavir standard.

3.1.3 Linearity

Determined the linearity of lopinavir and ritonavir by plotting a graph between concentration of the test solution on X-axis and response of the corresponding solutions on Y-axis, from 50 % to 150 % as shown in Figures 3, 4 and determine the correlation coefficient square and y-intercept which is shown in Table 3.

Acceptance: Correlation Coefficient square is not less than (NLT) 0.995, y-intercept is not more than (NMT) ± 2.0 %.

Table 3: Linearity of response of lopinavir and ritonavir

Linearity of lopinavir		Linearity of ritonavir		
S.No	**Concentration (µg/ml)**	**lopinavir area**	**Concentration (µg/ml)**	**ritonavir area**
1	12.231	863181	3.123	177398
2	19.570	1423206	4.996	275770
3	24.463*	1830846	6.245*	339462
4	29.355	2103298	7.494	427961
5	36.694	2662731	9.368	525075
Slope		73003	56394	
Intercept		-9198.29	-3068.00	
Correlation coefficient		0.9988	0.9987	
Correlation coefficient square		0.9977	0.9973	
y- intercept		-0.50%	-0.90%	

* Operating concentration

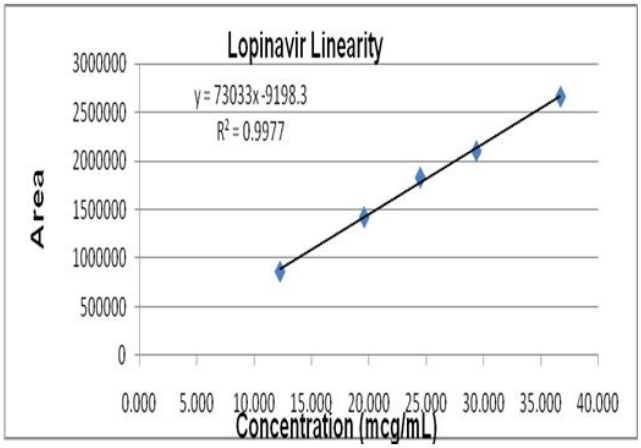


Figure 3: Lopinavir linearity.

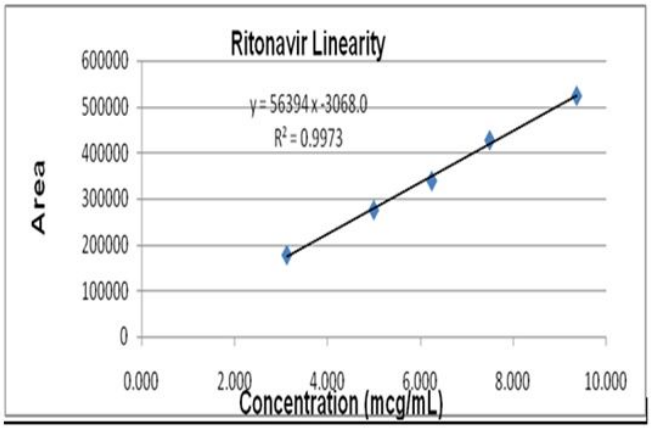


Figure 4: Ritonavir linearity.

3.1.4 Range

One solution with higher concentration and one with lower concentration (as prepared under linearity) in 6 replicates each was injected and the peak areas were recorded. Calculate the related standard deviation for the 6 areas which is shown in Table 4. Acceptance: Criteria for range is percentage relative standard deviation (% RSD) for 6 areas at two linearity levels-NMT 2.0 %

Table 4: Range of least and highest concentration peak area

	lopinavir area		Ritonavir area	
	50% Standard	150% Standard	50% Standard	150% Standard
Average area	868869	2626895	164822	519117
SD	7665.73	40193.50	2812.15	3263.25
% RSD (NMT 2.0%)	0.88%	1.53%	1.71%	0.63%

Remarks: Range was observed to be 12.231-36.694 µg/ml for lopinavir and 3.123-9.368 µg/ml for ritonavir. % RSD was within limits.

3.1.5 Accuracy

The accuracy of the assay method was determined by adding known amounts of lopinavir and ritonavir to the placebo at 50 %, 100 % and 150 % of actual concentration. The standard preparations and test preparations were injected separately in 6 replicates. The chromatograms were recorded, responses were measured which is shown in Tables 5 and 6. Percentage recovery was calculated.

Acceptance: The recovery at various levels is between 98.0 % and 102.0 %, The % RSD for recovery of triplicate samples at various levels is not more than 2.0 %.

Table 5: Accuracy of lopinavir

Sample con.	Peak response	Amount obtained (mg)	Amount added (mg)	Mean and %RSD
50%	898293	96.0949	95.9820	Mean = 100.25% RSD = 0.17%
100%	1801024	192.6645	191.9640	Mean = 100.19% RSD = 0.34%
150%	2704263	289.2885	287.9460	Mean = 100.31% RSD = 0.21%

Table 6: Accuracy of ritonavir

Sample con.	Peak response	Amount obtained (mg)	Amount added (mg)	Mean and %RSD
50%	175231	24.5070	24.8379	Mean = 98.44% RSD = 0.30%
100%	353629	49.4570	49.6758	Mean = 99.76% RSD = 0.20%
150%	531036	74.2684	74.5137	Mean = 99.64% RSD = 0.20%

Remarks: The recovery and % RSD for recovery at each level meets the acceptance criteria.

Table 7: Mean value of % of drug obtained in method precision and % RSD

	Content of lopinavir (mg/tablets)	Content of lopinavir of (% label claim)	Content of ritonavir (mg/tablets)	Content of ritonavir (% of label claim)
Average	199.79 mg	99.89%	49.50 mg	99.00%
RSD (NMT 2.0%)	1.05%	1.05%	0.73 %	0.73%

Remarks: The % RSD for 6 assay values is within acceptable limits.

3.1.6 Precision

3.1.6.1 System precision

Acceptance criteria for system precision is capacity factor is 15.00 (15-24 for the ritonavir peak). Tailing factor is 0.8-1.2 for the lopinavir and ritonavir peaks. Theoretical plates is more than 5000 for ritonavir peak. Relative standard deviation is NMT 2.0% for the lopinavir and ritonavir peaks.

Remarks: Relative standard deviation for peak response. Number of theoretical plates and resolution are within acceptable limits.

3.1.6.2 Method precision

Acceptance: The % RSD for the six assay determinations is NMT 2.0 % .

3.1.6.3 Intermediate precision

Analyst, instrument and day variability test was performed, and overall % RSD of lopinavir is 1.11% and overall RSD of ritonavir is 1.00 % .

Acceptance**:** The % RSD for the six assay determinations shall be NMT 2.0 %.

3.1.7 Robustness

By making minor, purposeful modifications to the wavelength, flow rate, and mobile phase, the method's robustness was demonstrated. The samples were injected in 6 replicates and % RSD was calculated which is within acceptable limits.

Acceptance: It should pass the system suitability under each variable parameter.

Table 8: Intermediate precision

	Content of lopinavir (mg/tablets)	Content of lopinavir of (%label claim)	Content of ritonavir (mg/tablets)	Content of ritonavir (% of label claim)
Average	198.66 mg	99.33%	49.46 mg	98.92%
%RSD (NMT 2.0%)	1.11%	1.11%	1.00%	1.00%
Cumulative %RSD (NMT 2.0%)	0.88%		0.83%	

3.1.8 Filter integrity

After passing through 0.45 µm PVDF, 0.45 µm nylon and 0.45 µm PTFE filters, the filtered samples were injected. The areas obtained for the filtered samples were compared against the centrifuged sample as shown in Table 9.

Acceptance: The % deviation in area of the filtered solution from the area obtained in centrifuged solution is NMT 2.0 %.

3.1.9 Solution stability

The sample solutions were prepared and their stability was to be tested for the initial hour, 6 h, 12 h, 23 h, and 34 h. The percentage of deviation was also measured which is shown in Tables 10 and 11. Acceptance: The % deviation from the initial area at each time is NMT 2.0 %.

Table 9: Filter integrity

Test	lopinavir test area	% Deviation from the centrifuged area	Ritonavir test area	% Deviation from the centrifuged area
Centrifuged sample	1808261	-	350532	-
Filtration through PVDF filter	1792836	0.85%	349298	0.35%
Filtration through Nylon filter	1822665	0.80%	349899	0.18%
Filtration through PTFE filter	1825343	0.94%	348997	0.44%

Remarks: The areas obtained for the solutions filtered through PVDF, Nylon and PTFE filters are well within specified limits. PVDF, Nylon and PTFE filters are suitable for filtration.

Table 10: Solution stability of lopinavir standard and test at different time period

S.No	Time (h)	Lopinavir % deviation from the initial area			
		Standard area	Deviation	Test area	Deviation
1.	Initial	1763392	-	1854584	-
2.	6	1781216	1.01%	1833979	1.11%
3.	12	1786162	1.29%	1869315	0.79%
4.	23	1791614	1.60%	1884271	1.60%
5.	34	1802055	2.19%	1868346	0.74%

Table 11: Solution stability of ritonavir standard and test at different time period

S.No.	Time (h)	Ritonavir % deviation from the initial area			
		Standard area	Deviation	Test area	Deviation
1.	Initial	349856	-	344932	-
2.	6	351060	0.34%	346192	0.37%
3.	12	352579	0.78%	339284	1.64%
4.	23	354297	1.27%	341316	1.05%
5.	34	359087	2.64%	3600616	1.57%

3.1.10 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were calculated according to ICH recommendations where the approach is based on the signal-to-noise ratio. A signal-to-noise ratio of 3:1 and 10:1 was considered for calculating LOD and LOQ respectively. For lopinavir, LOD was 11.73 and LOQ was 12.22. For ritonavir LOD was 1.053 and LOQ was 3.122. The result obtained was within the limits.

4. Discussion

The system suitability test was applied to chromatograms taken under optimum conditions to check various parameters such as

theoretical plates (5266), capacity factor (15), asymmetry (1.24) and signal-to-noise ratio (10.483). Suitable test results were achieved for the proposed method. All these results indicate the suitability of the instrument for the developed method.

For the study of precision six replicates of the standard solution were injected into the HPLC system in inter-day and intraday intervals. The % RSD values of day 1 and day 2 for inter-day intervals were found to be 1.11 % and 0.83 % for lopinavir and 0.57 % and 1.57 % for ritonavir. Therefore, the % RSD values for precision studies are within the accepted limits of 2 %.

Linearity was performed using standard solutions in the concentration range of 10-40 mcg/ml. The calibration curve was constructed for the standards by plotting the concentrations versus peak area and evaluated by linear regression analysis. The correlation coefficient (R) was found to be 0.9977, which is within the accepted limits.

Accuracy was performed by spiking a pre-quantified sample with standard at 50 %, 100 % and 150 %. The solutions were prepared in triplicates and analyzed through the developed method. The mean recovery values of obtained for 3 trials were 100.25 %, 100.19 % and 100.31 % for lopinavir and 98.44 %, 99.76 % and 99.64 % for ritonavir, respectively, which indicates that there is an extremely less interference coming from matrix components.

Robustness and filter integrity are within acceptable limits.

A signal-to-noise ratio of 3:1 and 10:1 was considered for calculating LOD and LOQ, respectively. For lopinavir, LOD was 11.73 and LOQ was 12.22. For ritonavir LOD was 1.053 and LOQ was 3.122. The results obtained were within the limits.

5. Conclusion

A simple and efficient RP-HPLC method for simultaneous estimation of lopinavir and ritonavir in tablet dosage form was developed and validated. Chromatography was carried out on a waters reliant C8, 250 × 4.6 mm, 5 µ column using a mixture of monobasic potassium phosphate buffer, and acetonitrile in proportion 55:45 v/v as the mobile phase at a flow rate of 1.5 ml/min, the detection was carried out at 215 nm. The retention time of the lopinavir and ritonavir were found to be 30.887 and 24.087 min, respectively. The method produces linear responses in the concentration range of 12.5-37.5 µg/ ml and 3.125-9.375µg/ml, respectively for both lopinavir and ritonavir. The method precision for the determination of assay was below 2.0 % RSD. The linear regression coefficient was not more than 0.999 for both lopinavir and ritonavir. For lopinavir LOD was 11.73 µg/ml and LOQ was 12.22 µg/ml. For ritonavir LOD was 1.053 µg/ml and LOQ was 3.122 µg/ml. The results obtained were good and found within the limit, proving that the developed method can be used for estimation of lopinavir and ritonavir tablets. Most of the methods used in the past for estimating lopinavir and ritonavir were tedious. Thus, a reverse-phase HPLC method was developed and validated. The results of the system suitability and applicability indicated that the proposed method is suitable and applicable for routine laboratory analysis. With less retention time, the approach offers good resolution between the drugs. The proposed technique is linear, accurate, precise, robust, specific, and selective. The results obtained from the validation parameters meet the pre-established acceptance criteria. Therefore, the study results confirm that the developed method is a suitable technique for simultaneous estimation of lopinavir and ritonavir in tablet dosage form.

Acknowledgements

The authors thank the management and staffs, Department of Pharmaceutical Chemistry and Analysis, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS) and Green Tree laboratory Pvt Ltd, Chennai for providing us the instrumentation facilities to carry out this work.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Citation **Akella Anuradha, M. Vijey Aanandhi and Afroz Patan (2023). Analytical method development and validation for the simultaneous estimation of lopinavir and ritonavir by RP-HPLC method in tablet dosage form. Ann. Phytomed., 12(1):573-580. http://dx.doi.org/10.54085/ap.2023.12.1.45.**