VALIDATED RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LOPINAVIR IN PURE FORM AND MARKETED PHARMACEUTICAL TABLET DOSAGE FORM

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ABSTRACT: *A Novel, simple, rapid, specific and accurate reverse phase high performance liquid chromatographic method has been developed for the validated of Lopinavir in bulk as well as in marketed pharmaceutical dosage form. This separation was performed on a Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size column with Methanol: Acetonitrile in the ratio of 70: 30 v/v as mobile phase at a flow rate of 1.0 mL min−1 with UV detection at 245nm; the constant column temperature was Ambient. The retention time of Lopinavir was found to be 2.768min. The calibration plot was linear over the concentration range of 6–14µg mL−1 with limits of detection and quantification values of 0.507 µg mL−1 and 1.539µg mL−1 respectively. The % recovery was found to be within the limits. The proposed method was simple, precise, specific, accurate and rapid, making it suitable for estimation of Lopinavir in bulk and marketed pharmaceutical dosage form dosage form.*

Keywords: *Lopinavir, RP-HPLC, ICH Guidelines, Accuracy, Precision.*

I. INTRODUCTION

Lopinavir is an antiretroviral protease inhibitor used in combination with other antiretrovirals in the treatment of HIV-1 infection. Lopinavir[1] is marketed and administered exclusively in combination with ritonavir - this combination, first marketed by Abbott under the brand name Kaletra in 2000, is necessary due to lopinavir's poor oral bioavailability and extensive biotransformation. Ritonavir is a potent inhibitor of the enzymes responsible for Lopinavir metabolism, and its co-administration "boosts" Lopinavir exposure and improves antiviral activity. Like many other protease inhibitors (e.g. Saquinavir, Nelfinavir), Lopinavir[2] is a peptidomimetic molecule - it contains a hydroxyethylene scaffold that mimics the peptide linkage typically targeted by the HIV-1 protease enzyme but which itself cannot be cleaved, thus preventing the activity of the HIV-1 protease. Lopinavir is currently under investigation in combination with ritonavir for the treatment of COVID-19 caused by SARS-CoV-2. Lopinavir is an antiretroviral protease inhibitor used in combination with ritonavir in the therapy and prevention of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS). Lopinavir can cause transient and usually asymptomatic elevations in serum aminotransferase levels and, rarely, clinically apparent, acute liver injury. In HBV or HCV coinfected patients, highly active antiretroviral therapy with Lopinavir may result of an exacerbation of the underlying chronic hepatitis B or C. Lopinavir is a peptidomimetic HIV protease inhibitor that retains activity against HIV protease with the Val 82 mutation. Lopinavir is less affected by binding to serum proteins than the structurally-related drug ritonavir. The IUPAC Name of Lopinavir[3] is (2S)-N-[(2S, 4S, 5S)-5-[[2-(2, 6-dimethyl phenoxy) acetyl] amino]-4-hydroxy-1, 6-diphenyl hexan-2-yl]-3-methyl-2-(2-oxo-1, 3-diazinan-1-yl) butanamide. The Chemical Structure of Lopinavir is

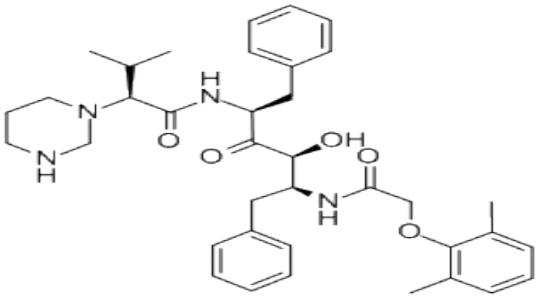

Fig.1. Chemical Structure of Lopinavir

A literature search revealed very few papers on analytical methods of this drug. One research paper has reported a UV method of analysis and another one has reported a HPLC method of analysis for Lopinavir using UV detector [27-31]. Hence the aim of this study was to develop a simple, accurate and precise RP-HPLC analytical method for the estimation of Lopinavir in tablet formulation.

II.MATERIALS AND METHODS

Pharmaceutical grade Lopinavir was supplied as a gift sample by Syncorp labs, Hyderabad. Methanol, Orthophosphoric acid (OPA), Acetonitrile and HPLC grade water were obtained from Merck. All solvents used in this work are HPLC grade. RP-HPLC-HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector, T60-LABINDIA UV – Vis spectrophotometer, High Precision Electronic Balance, Ultra Sonicator (Wensar wuc-2L), Thermal Oven, PH Analyser (ELICO), Vaccum Filtration Kit (Labindia), was employed in this method. Analytical column[4] used for the separation of analytes is Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size.

Method Development

HPLC Instrumentation & Conditions: The HPLC system employed was **HPLC WATERS** with Empower2 Software with Isocratic[5] with UV-Visible Detector[6].

Standard preparation for UV-spectrophotometer analysis:

The standard stock solutions – 10 mg of Lopinavir standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with Methanol. Further dilutions were done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with methanol to get 10ppm concentration.

It scanned in the UV spectrum[7] in the range of 200 to 400nm. This has been performed to know the maxima of Lopinavir, so that the same wave number can be utilized in HPLC[8] UV detector for estimating the Lopinavir.

Preparation of Mobile Phase:

The mobile phase[9] used in this analysis containing of a mixture of Methanol and Acetonitrile in the ratio of 70:30 v/v was prepared in the volume of 1000ml in which 700ml of Acetonitrile was mixed with 300ml of Methanol.

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Lopinavir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette out 0.1ml of Lopinavir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Diluent

The mobile phase was used as diluent[10].

Method Validation

The developed RP-HPLC method was validated for parameters like system suitability, linearity, accuracy, precision, limit of detection(LOD), limit of quantitation (LOQ) and robustness according to ICH guidelines[11].

System Suitability

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability[12] parameters like theoretical plates, resolution and asymmetric factor were evaluated. The system suitability parameters were tabulated in table 1. All the parameters were found to be within the limits.

Precision

Method precision

The precision of the method was verified by precision method studies. The sample solution was prepared at working concentration and analysis was carried out at replicating. The sample solutions of Lopinavir were prepared as per the test method and injected 6 times into the column. The results of precision[13] were tabulated in table 3. The average was taken, and % RSD was calculated and reported. % RSD values were within the limits, and the method was found to be precise.

Linearity

The linearity[14] of the test solutions for the assay method was prepared from Lopinavir standard stock solution at five concentration levels from 80% to 120% of assay concentration. The peak area versus concentration data was treated by least-squares linear regression[15] analysis (fig-4). The results have shown an excellent correlation between peak areas and concentration within the concentration range of 6–14µg/ml for Lopinavir

(table-6). The correlation coefficients were found to be 0.9997 for the drug, which meet the method validation[16] acceptance criteria and hence the method was said to be linear for the drug.

Accuracy

The accuracy[17] of the method was determined by recovery studies[18] by the determination of % mean recovery of both the drugs at three different levels (80 %, 100 % and 120%). At each level, three determinations were performed. The percentage recovery and mean percentage recovery were calculated for the drug was shown in table 2. The observed data were within the required range, which indicates good recovery values and hence the accuracy of the method developed.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered, and the system suitability parameters were evaluated. The solutions prepared as per the test method and injected at different variable conditions like flow rate (0.9, 1.1 ml/min.) and wavelength (250nm, 240nm), system suitability parameters were compared with that of method precision. The results were tabulated in table 7. At the flow rate of 1.0 ml/min shows, a sharp peak with good resolution and rest of the flow rates were found to be not satisfactory. The method passed all system suitability parameters indicating that the method was robust[19].

Detection Limit and Quantification Limit (LOD and LOQ)

The detection limit[20] of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit[21] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision[22] and accuracy.

$$LOD = 3.3 \times \sigma/S \text{ and } LOD = 10 \times \sigma/S$$

Where, = the standard deviation of the response, Slope = slope of the calibration curve.

III. RESULTS AND DISCUSSION

Method Development

Determination of Wavelength:

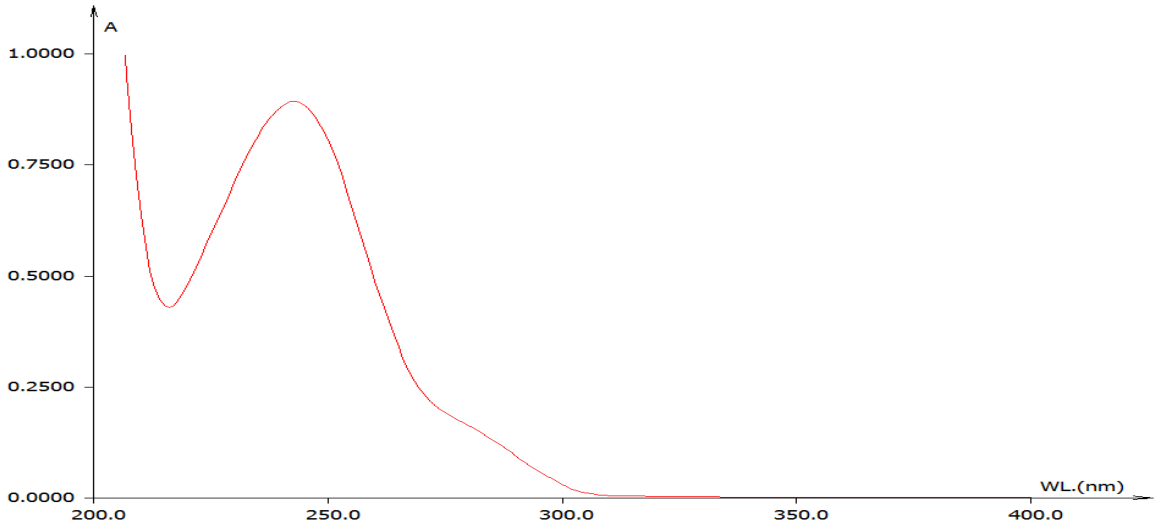

Fig.2. UV-Spectrum for Lopinavir

Observation: While scanning the Lopinavir solution we observed the maxima at 245nm.

Optimized Chromatographic Conditions:

Column	: Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size
Mobile Phase	: Methanol: Acetonitrile (70: 30% v/v)
Flow Rate	: 1.0ml/minute
Wave length	: 245 nm
Injection volume	: 10 µl
Run time	: 7 minutes
Column temperature	: Ambient

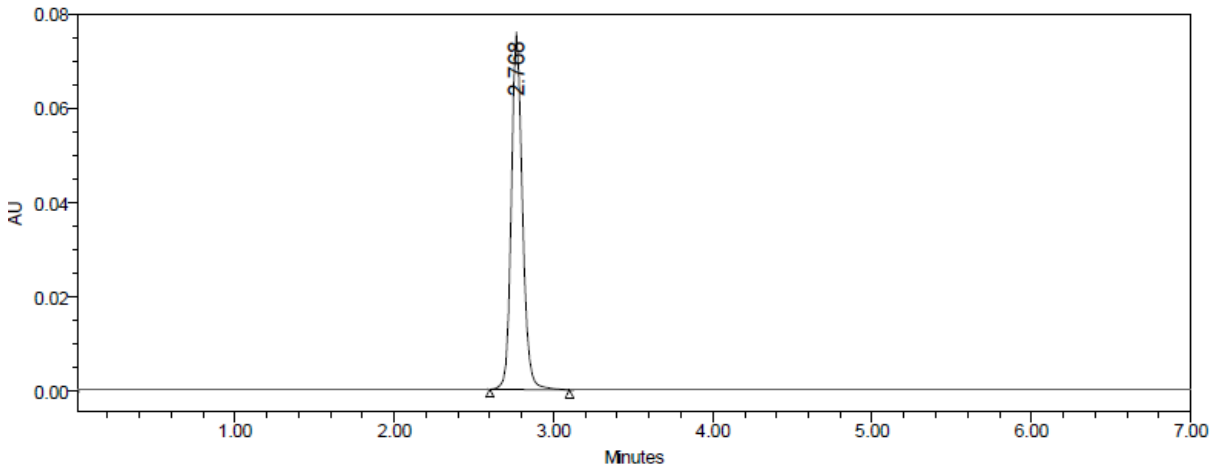

Fig.3. Optimized Chromatogram for Lopinavir

Method Validation

System Suitability Test

System suitability testing[23] is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system[24] that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-1.

Table-1: Data of System Suitability Test

S.No.	Injection No.	RT	Area	Height	USP Plate Count	USP Tailing
1	Injection 1	2.786	715268	47844	5857	1.36
2	Injection 2	2.784	716584	46985	5986	1.38
3	Injection 3	2.768	715364	47258	5784	1.35
4	Injection 4	2.789	714895	47152	5896	1.34
5	Injection 5	2.784	716587	47258	5749	1.36
6	Injection 6	2.781	718549	47985	5657	1.39
Mean			**716207.8**		**5821.5**	**1.36**
S.D			**1347.976**			
%RSD			**0.18821**			

Accuracy:

Recovery Study:

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Lopinavir were taken and 3 replications of each has been injected to HPLC system[25]. From that percentage recovery values were calculated from the linearity equation y = 74143x + 7294.9. The results were shown in table-2.

Table-2: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Mean % Recovery	
	Amount Injected	Amount Recovered				
S_1 : 80 %	8	8.013	601425	100.162	Mean = 100.195%	

S_2 : 80 %	8	8.012	601396	100.150		% Mean Recovery = 100.364 %
S_3 : 80 %	8	8.022	602123	100.275		
S_4 : 100 %	10	10.038	751584	100.380	Mean = 100.356	
S_5 : 100 %	10	10.039	751642	100.390		
S_6 : 100 %	10	10.030	750969	100.300		
S_7 : 120 %	12	12.057	901253	100.475	Mean = 100.541	
S_8 : 120 %	12	12.073	902431	100.608		
S_9 : 120 %	12	12.065	901864	100.541		

Observation: From the Accuracy Method, we observed that the mean %Recovery of the drug is 99.686 which are within the range[26] of 98-102%.

Precision:

Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug Lopinavir (API). The percent relative standard deviation was calculated for Lopinavir.

Table-3: Results of Repeatability readings

HPLC Injection Replicates of Lopinavir	Retention Time	Peak Area	Theoretical Plates	Tailing Factor
Replicate – 1	2.777	716984	5986	1.36
Replicate – 2	2.795	715698	5897	1.37
Replicate – 3	2.789	716859	5869	1.39
Replicate – 4	2.797	718548	5967	1.37
Replicate – 5	2.797	714895	5984	1.35
Replicate – 6	2.799	715986	5879	1.38
Average		**716495**	**5930.333**	**1.37**
Standard Deviation		**1268.126**		
% RSD		**0.17699**		

Observation: From the Precision method, we observed that the %RSD of the Peak Area is 0.176 which are within the acceptable range as per ICH guidelines.

Intermediate Precision:

The Intermediate Precision consists of two methods:-

Intra Day: In Intra Day process, the 80%, 100% and 120% concentration are injected at different intervals of time in same day.
Inter Day: In Inter Day process, the 80%, 100% and 120% concentration are injected at same intervals of time in different days.

Table-4: Peak results for Intra-Day Precision

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Lopinavir	2.784	716587	48685	1.38	5954	1
2	Lopinavir	2.768	717845	48698	1.39	5935	2
3	Lopinavir	2.786	716857	46989	1.36	5798	3
4	**Average**		**717096.3**	**48124**	**1.376**	**5895.66**	
5	**S.D**		**662.2698**				
6	**% RSD**		**0.092354**				

Table-5: Peak results for Inter-Day Precision

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Lopinavir	2.780	716987	49867	1.34	5968	1
2	Lopinavir	2.794	718695	48574	1.33	5998	2
3	Lopinavir	2.775	718542	48569	1.39	5859	3
4	**Average**		**718074.7**	**49003.33**	**1.353333**	**5941.667**	
5	**S.D**		**945.0483**				
6	**% RSD**		**0.131609**				

Observations: The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Lopinavir revealed that the proposed method is precise.
Linearity & Range:
To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 6-14µg/ml. The prepared solutions were sonicated. From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Table-6: Linearity Concentrations of Lopinavir

S.No.	Concentration (in ppm)	Peak Area
1	0	0
2	6	457896
3	8	607574
4	10	752268
5	12	896587
6	14	1036579

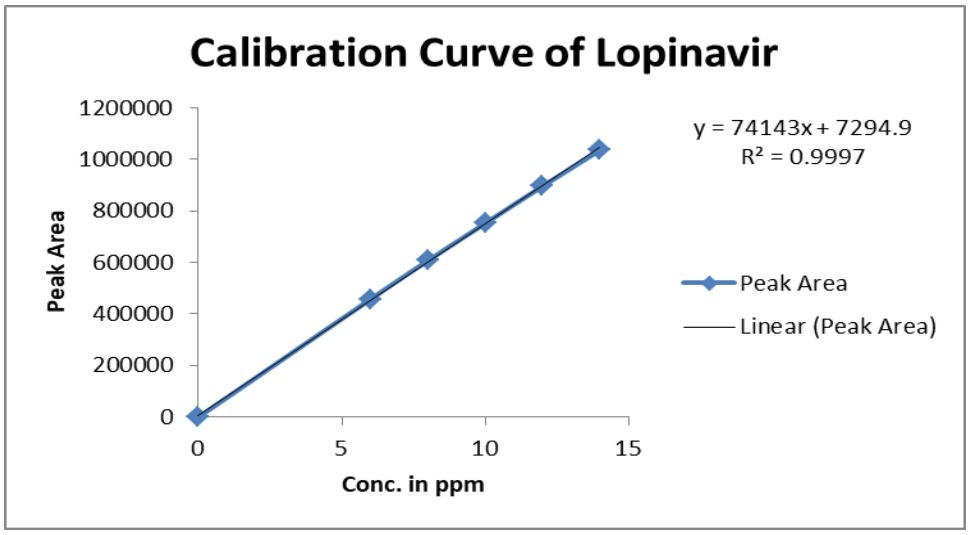

Fig.4. Calibration Curve of Lopinavir

Observation: We observed that the calibration curve showed good linearity in the range of 6-14 µg/ml, for Lopinavir with correlation coefficient (R^2) of 0.9997. A typical calibration curve has the regression equation of y = 74143x + 7294.9 for Lopinavir.

Method Robustness: Influence of small changes in chromatographic conditions such as change in flow rate 1ml (± 0.1ml/min), Wavelength of detection 245nm (±2nm) & organic phase content in mobile phase 60 (±5%) studied to determine the robustness of the method are also in favour of (Table-7, % RSD <2%) the developed RP-HPLC method for the analysis of Lopinavir (API).

Table-7: Results of Method Robustness Test

Change in Parameter	Theoretical Plates	Tailing Factors
Flow (1.1 ml/min)	5954	1.35
Flow (0.8 ml/min)	6188	1.39
More Organic (70+5)	5748	1.41
Less Organic (70-5)	6185	1.48
Wavelength of Detection (250 nm)	6184	1.69
Wavelength of detection (240nm)	6247	1.47

LOD & LOQ: The detection limit (LOD) and quantization limit (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S).

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.507 & 1.539 µg/ml respectively.

IV. CONCLUSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Lopinavir, different chromatographic conditions were applied & the results observed are presented in previous chapters.Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size Column was preferred because using this column peak shape, resolution and absorbance were good. Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, Acetonitrile, water, 0.1N NaOH, 0.1NHCl). The drug was found to be more sol in aqueous solution of sodium hydroxide; sol in warm ethanol; slightly sol in ether. Lopinavir is practically insoluble in water, in ether and in chloroform; soluble in methanol; slightly soluble in alcohol. Using these solvents with appropriate composition newer methods can be developed and validated. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Lopinavir it is evident that most of the HPLC work can be accomplished in the wavelength range of 245 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 10µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay and stability related impurity studies which can help in the analysis of Lopinavir in different formulations. A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Lopinavir API. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. The result shows the developed method is yet another suitable method for assay, purity which can help in the analysis of Lopinavir in different formulations.

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