



## Stability Indicative and Cost Effective Creation and Validation of Analytical Method of Lopinavir and Rilpivirine by High Performance Liquid Chromatography

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Validation**ABSTRACT**

We have developed a completely unique and reliable HPLC technique for simultaneous quantification of Lopinavir and Rilpivirine. Chromatographic detachment was attained on a X-bridge phenyl column (150x4.6mm, 3.5  $\mu$ ) using isocratic elution with a buffer containing buffer and acetonitrile with the proportion of 70:30 as movable phase with a flow of 1 ml/min at room temperature and UV detection was carried out at 250 nm. Dissolve 1ml of tri ethylamine in 1 lt of HPLC grade water and filter through 0.45  $\mu$  filter paper, this solution was used as buffer. 8 min. run time was used to separate Lopinavir and Rilpivirine. Analysis was achieved within 15 min over an honest linearity within the concentration range from 20-300  $\mu$ g/ml of Lopinavir and 2.5-37.5  $\mu$ g/ml of Rilpivirine. By injecting the standard six times, system suitability parameters were studied and the outcomes were under the acceptable limit. Precision and recovery study results were found to be within the suitable limit. By using the above technique, assay of Lopinavir and Rilpivirine was performed and found to be within the limit. Degradation studies were carried out on Lopinavir and Rilpivirine, with a purity threshold greater than purity angle in all conditions and within the allowable range. The above mentioned technique was validated according to ICH guidelines.

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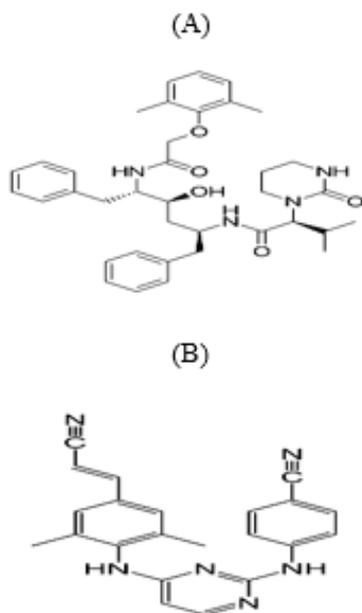
inhibitor, ritonavir (lopinavir/ritonavir). Lopinavir is 98-99 percent strongly bound to plasma proteins. There are contradictory reports concerning the penetration of Lopinavir into the cerebrospinal fluid (CSF) (Orešković and Klarica, 2014; Kwong *et al.*, 2020). According to anecdotal reports (Vening, 1982), Lopinavir cannot be recognized in the CSF, however a review of paired CSF- plasma samples from 26 patients receiving Lopinavir/Ritonavir found Lopinavir CSF levels above the IC50 in 77% of samples (Capparelli *et al.*, 2005). Only the medication combination Lopinavir/Ritonavir was side effects, interactions and contraindications assessed.

**INTRODUCTION**

Lopinavir is an anti retroviral product (Gardner *et al.*, 2009) in the class of protease inhibitors. It is used as a fixed dose formulation against HIV infection (Eisinger *et al.*, 2019) with another protease

Rilpivirine (TMC 278, trade name Edurant) is a prescription medication, for the treatment of HIV infection (Stellbrink, 2007). It is a non-nucleoside reverse transcriptase (NNRTI) (Boyer *et al.*, 2001; Ren *et al.*, 2001) second generation inhibitor with

higher potency, extended half life and decreased side effect profile contrast with older NNRTIs, such as Efavirenz (Goebel *et al.*, 2006). Like Etravirine a second generation NNRTI accepted in 2008, Rilpivirine is a diarylpyrimidine (DAPY) (Mordant *et al.*, 2007). In patients with baseline HIV viral loads greater than 100,000 copies/mm<sup>3</sup>, Rilpivirine in union with Emtricitabine and Tenofovir has been shown to have higher virologic failure rates than Atripla (Brignone *et al.*, 2019). Figure 1 shows the chemical structures of Lopinavir and Rilpivirine.



**Figure 1: Structural representations of (A) Lopinavir and (B) Rilpivirine**

## Experiment

### Chemicals and Reagents

Acetonitrile and Ortho phosphoric acid, tri ethyl amine, water (HPLC mark) were acquired from Merck (India) Ltd, Worli, Mumbai, India. The reference standards of Lopinavir and Rilpivirine APIs were taken from Spectrum Pharma Research Solutions Pvt. Ltd., Hyderabad.

### Equipment

An HPLC system (Waters alliance e2695 model) consisting of quaternary pump, PDA detector-2998 was used. Data processing was performed with Empower 2.0 software.

### Chromatographic Conditions

Chromatographic detachment was achieved in isocratic mode at room temperature using X-bridge phenyl (150x4.6 mm, 3.5  $\mu$ ). A mixture of acetonitrile and 0.1 percent triethylamine (TEA) in 70:30 v/v at a stream of 1 ml/min was used as movable

phase. The injection volume was 10  $\mu$ l and the run time was 10.0 min.

### Preparation of Buffer

1 mL of tri ethylamine is dissolved in 1 liter of HPLC grade water and filtered through 0.45  $\mu$  filter paper.

### Diluent

Movable phase was used as diluent.

### Standard Preparation

Carefully weigh and transfer 200 mg of Lopinavir and 25 mg of Rilpivirine in a volumetric flask of capacity 100 ml and add app. 70 ml of diluent, sonicated to melt it for 30 min. and add diluent up to mark. Take 5 ml of the above solution and dilute it to 50 ml and filtered through 0.45  $\mu$  nylon syringe filter.

### Method Validation

#### System Suitability

In order to confirm the system performance, system suitability parameters were measured. The parameters including USP plate count, USP tailing and %RSD were calculated and found to be within the limit.

#### Specificity

Specificity is the capacity to evaluate the analyte unambiguously the analyte in the presence of other constituents that may be expected to be combined in the sample and standard solution. It is checked by examining the chromatograms of blank samples and samples spiked with Lopinavir and Rilpivirine.

#### Accuracy

Accuracy is the closeness of the test findings gained by the technique to the real value. It is assessed by the recovery studies will be evaluated at three different concentration levels. In each level a minimum of three injections were administered at each level and quantity of the drug present, percentage recovery and the associated standard deviations were calculated.

#### Precision

Precision of the analytical technique is the degree of agreement among independent test results. It was studied by investigation of multiple sampling of homogeneous sample. The precision of the present technique was assessed in terms of repeatability, intra-day and inter-day variations. It was checked by analyzing the samples at different time intervals of the same day as well as on different days.

#### Linearity

Linearity of an analytical process is its ability to produce results within a given range directly propor-

tional to the concentration of the analyte in the sample within a definite range. The six series of standard solutions were selected for the evaluation of the linearity range, six series of standard solutions were selected. Using peak area versus concentration of the regular solution, the calibration curve was plotted and the regression equations were calculated. The least squares method was used to calculate the slope, intercept and coefficient of correlation.

### Stress Degradation

Stress Degradation should not have any interference between the peaks of forced degradation preparations obtained for the chromatogram. Stress degradation studies were conducted in accordance with ICH guidelines Q1 (A) R2. The peaks of degradation should be well spaced and the resolution between the peaks should be at least 1.0. The peak purity of the principle peaks will pass only when there is separation. Forced degradation studies were achieved to obtain the degradation of about 20 percent by different types of stress conditions.

### Robustness

An analytical procedure's robustness is a measure of its ability to remain unchanged by small but intentional differences in method parameters and gives sign of its reliability during normal use. Robustness study was carried out by injecting standard solution into the HPLC system and modifying chromatographic settings such as flow rate ( $\pm 0.2$  ml/min), organic content in the mobile phase ( $\pm 10\%$ ). By establishing the effect of the modified parameters the separation factor, retention time and peak asymmetry were estimated.

## RESULTS AND DISCUSSION

The present study was designed to develop a simple, accurate and rapid analytical RP-HPLC method that can be used for the simultaneous estimation of Lopinavir and Rilpivirine in bulk and pharmaceutical dosage form for the analysis of the assay technique. In order to provide good assay performance the chromatographic conditions were optimized. To optimize mobile phase, various combinations were tried for Lopinavir and Rilpivirine. The final working mobile phase is (0.1%) triethylamine and acetonitrile in the composition of (30:70 v/v). Mobile phase for each drug was selected on the basis of its polarity. In order to obtain sufficient sensitivity for the two APIs in smaller proportions (Lopinavir and Rilpivirine) detection was carried out in several wavelengths. Finally, the 250 nm wavelength was selected where the two drugs showed good absorption. The flow rate was 1.0 ml/min. For Lopinavir

and Rilpivirine the retention time was 6.482 min, 2.925 min respectively. The proposed method is confirmed by all the outcomes within the limits in accordance with the ICH guidelines. The detection was carried out with a total runtime of 8.0 min. Optimized chromatographic conditions were showed in Table 1.

### System Suitability

The System Suitability was performed by injecting standard solution containing 200  $\mu\text{g/ml}$  of Lopinavir and 25  $\mu\text{g/ml}$  of Rilpivirine in six replicates. The results indicate that the system suitability parameter is within the limit. Standard chromatogram was showed in Figure 2 and system suitability results were represented in Table 2.

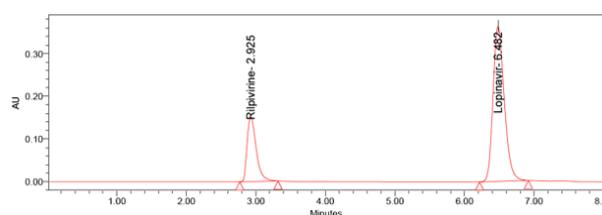


Figure 2: Chromatogram of System Suitability

### Specificity

There was no interference from blank at the retention time of Lopinavir and Rilpivirine. Figure 3 shows the blank chromatogram of Lopinavir and Rilpivirine.

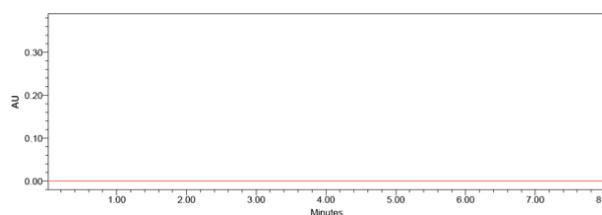


Figure 3: Chromatogram of Blank

### Linearity

By plotting a calibration curve of the peak area against its respective concentration, linearity was determined. From this calibration curve, it was found that the curve was linear in the range of 20-300  $\mu\text{g/ml}$  of Lopinavir and 2.5-37.5  $\mu\text{g/ml}$  of Rilpivirine. The regression equations for calibration curve of Rilpivirine was  $Y = 27717.07x + 28136.70$  ( $R^2 = 0.9992$ ) and  $Y = 8151.63x + 11034.55$  ( $R^2 = 0.9998$ ) for Lopinavir respectively. Linearity results were showed in Table 3 and the calibration plots of Rilpivirine and Lopinavir were represented in Figure 4.

### Precision

In terms of intraday and intermediate precision variations, the accuracy of this method was assessed.

**Table 1: Optimized Chromatographic Conditions**

Parameter	Proposed method
Stationary Phase	X-bridge phenyl (150x4.6 mm, 3.5 $\mu$ )
Mobile Phase	0.1% Tri ethyl amine: Acetonitrile (30:70)
Injection Volume	10 $\mu$ l
Flow Rate	1.0 ml/min
Column Temperature	Ambient
Wave Length	250 nm
Run Time	8.0 min
Retention time of Rilpivirine	2.925 min
Retention time of Lopinavir	6.482 min

**Table 2: Results of System Suitability**

Parameter	Rilpivirine	Lopinavir
Theoretical plate count	2497	7815
Tailing factor	1.52	1.23
Resolution	-	13.23
Retention time	2.925	6.482

**Table 3: Results of Linearity**

S. No	Rilpivirine		Lopinavir	
	Concentration ( $\mu$ g/ml)	Area	Concentration ( $\mu$ g/ml)	Area
1	2.5	104265	20	178924
2	6.25	212452	50	419862
3	12.5	389575	100	851729
4	25	723139	200	1609432
5	31.25	905367	250	2043458
6	37.5	1049622	300	2473340

**Table 4: Results of Method Precision**

S. No.	Area of Rilpivirine	Area of Lopinavir
1	723139	1609432
2	723457	1610984
3	723654	1610894
4	723689	1609538
5	724587	1609421
6	724791	1609649
Mean	723886	1609986
Std. dev	654.99	743.08
% RSD	0.09	0.05

**Table 5: Results of Intermediate Precision**

Area of Rilpivirine	Relative standard Deviation	Area of Lopinavir	Relative standard Deviation
723143	0.05	1609568	0.07
723471		1610984	
723661		1610868	
723673		1609548	
724576		1608421	
724785		1608648	

**Table 6: Results of Accuracy**

Accuracy	Amount of Rilpivirine	% Recovery	Amount of Lopinavir	% Recovery
50	12.5	98.4	100	100.1
100	25	99.3	200	99.8
150	37.5	99.1	300	99.6

**Table 7: Results of Robustness**

Parameter	% RSD of Rilpivirine	% RSD of Lopinavir
Flow (0.8 ml/min)	0.64	0.43
Flow (1.2 ml/min)	0.71	0.77
Organic phase (63:37)	0.35	0.51
Organic phase (77:23)	0.98	0.87

**Table 8: Results of Forced Degradation**

Stress Parameter	% of Degradation	
	Rilpivirine	Lopinavir
Acid degradation (1N HCl)	13.7	13.6
Alkali degradation (1N NaOH)	14.2	13.0
Peroxide degradation (30% Peroxide)	14.3	14.9
Reduction degradation (30% sodium bisulphate)	15.3	14.7
Thermal (sample, 70°C, 6 Hrs)	14.8	12.7
Photo (sample, sunlight, 12 Hrs)	15.4	13.6
Hydrolysis (5ml HPLC water)	10.9	11.2

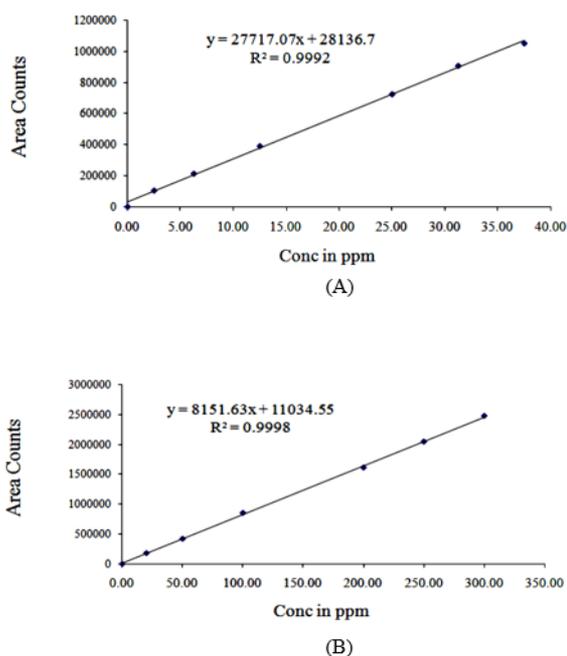
Six repeated analyses of the sample solution of Rilpivirine and Lopinavir on the same day under the same experimental conditions were used to determine the intraday studies. In the same laboratory, the intermediate precision of the method was carried out by studying the analysis with different analysts and with different instruments. As percentage RSD values were found to be <2 percent, the method is highly precise. At each added concentration, good recoveries of the drug were obtained, indicating that the method was exact. Method precision results were showed in Table 4.

#### Intermediate Precision (Ruggedness)

Intermediate precision results were showed in Table 5.

#### Accuracy

By calculating the recovery experiments at three levels, the accuracy of the method was achieved (50%, 100% and 150%). APIs were prepared with a concentration of 12.5, 25, 37.5  $\mu\text{g}/\text{ml}$  of Rilpivirine and 100, 200, 300  $\mu\text{g}/\text{ml}$  of Lopinavir. For each spike level, the test solution was injected three times and the assay was performed as per test method. The recovery outcomes were close to 100% and also the RSD values were less than  $\pm 2\%$  as well. The recovery percentage, mean and relative standard deviations have been calculated. Recovery values showed within the desired range, the method was accurate. Table 6 shows the accuracy results of Lopinavir and Rilpivirine.



**Figure 4: Calibration plots of (A) Rilpivirine and (B) Lopinavir**

### Robustness

Robustness of the chromatographic technique was determined by changing flow rate and movable phase composition. % RSD was found to be within the acceptable limit. Table 7 shows the robustness results of Rilpivirine and Lopinavir.

### Forced Degradation

The proposed method can be used for release and stability studies for effective evaluations and can be considered as stability preferable technique. The forced degradation study carried out according to the ICH requirements includes acid, base, oxidation, reduction, thermal, photo and hydrolysis degradation. From the chromatograms, it is evident that the selected drugs were stable under the applied stress conditions though degraded peaks were observed. Results of forced degradation were shown in Table 8.

### CONCLUSION

In this study, a novel, rapid, economical, sensitive and easily available HPLC method was evolved for the simultaneous estimation of Rilpivirine and Lopinavir in API form. The main advantages of this method are no HPLC methods were reported. Other merits are shorter run time, low price, accessibility, sensitivity, reliability and reproducibility. These properties are important to analyze the sample. The validation of all the parameters like linearity, accuracy, specificity, robustness, method precision were done and found to be within the acceptable limit.

The related standard deviation values for all the parameters were found to be less than 2%, which indicates the validity of the method and the results gained by this technique are in fair agreement. So the proposed method could be easily applied for the routine analysis of Rilpivirine and Lopinavir in quality control laboratories without any preliminary separation.

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The authors declare that they have no funding support for this study.

### Conflict of Interest

The authors declare that there is no conflict of interest for this study.

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