A NOVEL DESIGN OF EXPERIMENT RP-HPLC APPROACH FOR THE ESTIMATION OF LENACAPAVIR BY HYDROTROPY

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ABSTRACT

Background: Lenacapavir is a selective inhibitor of HIV-1 capsid protein and has demonstrated potent anti-viral activity used for the treatment of multidrug-resistant Human Immunodeficiency Virus type 1 (HIV-1) infection.

Method: A new, simple, accurate, rapid, precise, reproducible and cost-effective RP-HPLC method for the quantitative estimation of Lenacapavir in bulk and pharmaceutical dosage form. The developed RP-HPLC method for the quantitative estimation of Lenacapavir is based on measurement of absorption at maximum wavelength 240 nm using 2% Sodium Benzoate and Methanol (50:50% v/v) as a solvent. The stock solution of Lenacapavir was prepared, and subsequent suitable dilution was prepared in mobile phase to obtained standard curve. The standard solution of Lenacapavir shows absorption maxima at 240 nm.

Results: The Lenacapavir obeyed Beer Lambert's law in the concentration range of 20-100µg/ml with regression 0.999 at 240 nm. The overall % recovery was found to be 99.78% for Lenacapavir which reflects that the method was free from the interference of the impurities and other excipients, used in the bulk and marketed dosage form. The low value of % RSD was indicative of accuracy and reproducibility of the method. The % RSD for inter-day and intra-day precision was found to be 0.2 for Lenacapavir respectively which is <2% hence proved that method is precise.

Conclusion: The results of analysis have been validated as per International Conference on Harmonization (ICH) guidelines. The developed method can be adopted in routine analysis of Lenacapavir in bulk and tablet dosage form. The proposed method was found to be rapid, accurate, precise, specific, robust, rugged and economical.

Keywords: Lenacapavir, RP-HPLC, Accuracy, Precision.

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INTRODUCTION

The field of Pharmaceutical research continually seeks innovative methods to improve drug development, formulation, and analysis. Lenacapavir, a novel and potent antiretroviral agent, has shown great promise in the treatment of HIV/AIDS. Accurate and reliable estimation of this drug's concentration is crucial to ensure its efficacy and safety. High-Performance Liquid Chromatography (HPLC) is a widely employed analytical technique for drug quantification due to its high sensitivity, specificity, and reproducibility.

In recent years, researchers have explored various enhancement techniques to improve the sensitivity of HPLC methods, one of which is hydrotropy[3,4]. Hydrotropy is a unique solubilization phenomenon wherein certain hydrotropes can significantly increase the solubility of poorly water-soluble drugs. This property has been leveraged to enhance the detection and quantification of certain drug compounds by HPLC, thereby improving the method's performance.

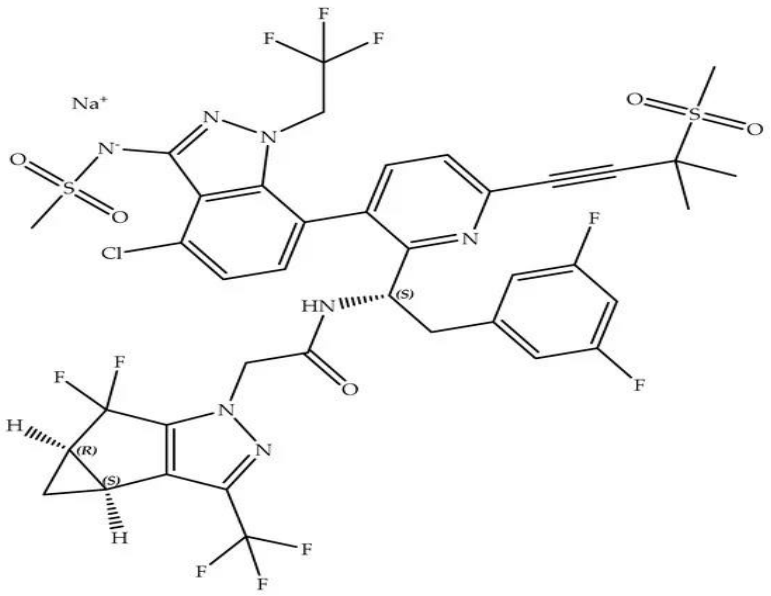

Figure 1: Structure of Lenacapavir[1]

This research presents a novel approach to estimate Lenacapavir using a Design of Experiment (DOE). RP-HPLC method in conjunction with hydrotropic solubilization. Design of Experiment is a statistical methodology that allows researchers to optimize experimental parameters systematically, leading to more robust and efficient analytical methods.

The objectives of this study are as follows:

1. To develop an RP-HPLC method for the estimation of Lenacapavir with improved sensitivity and accuracy by utilizing hydrotropic agents.
2. To employ the Design of Experiment approach to systematically evaluate and optimize critical factors affecting the HPLC analysis, such as mobile phase composition, column temperature, flow rate, and injection volume.
3. To validate the optimized method in accordance with regulatory guidelines, ensuring its reliability and reproducibility for routine analysis.

The significance of this research lies in its potential to provide a more sensitive and efficient HPLC method for the estimation of Lenacapavir, thus contributing to the quality control of this important antiretroviral drug. Additionally, the utilization of Design of Experiment principles ensures a systematic and thorough exploration of the experimental space, leading to robust and transferable analytical methods.

The remainder of this paper will develop into the methodology, results, and discussion of the experimental findings. Furthermore, the validation and application of the optimized RP-HPLC method for the estimation of Lenacapavir in real pharmaceutical samples will be presented, along with a comparison to existing methods. Ultimately, this research aims to offer a valuable contribution to the field of analytical chemistry and pharmaceutical analysis.

Experimental:

Chemicals and reagents:

Lenacapavir (Sunlenca, **Manufacturer** – Gilead sciences pharmaceuticals LTD.), 2%Sodium benzoate and Methanol

METHOD DEVELOPMENT BY RP- HPLC[8-10]:

HPLC system (Shimadzu) with PDA detector was used. The software LC-Solution can be used and a Rheodyne injection with a 20µL loop was used for injection of the sample. Inertsil ODS $RPC_{18,}$ (4.6×150mm, 5µmparticle size) was used. The mobile phase was composed of 2% Sodium benzoate and methanol in the various ratios with flow rate of 0.8ml/min. HPLC system was operated at ambient temperature.

1. Preparation of Standard Stock Solution:

10mg of Lenacapavir standard was transferred into 10 ml volumetric flask and 7ml of diluent was added slowly and made up to the mark with diluent to obtain a concentration of 1000µg/ml.

2. Preparation of Working stock solution:

0.6ml of the standard stock solution was pipetted out and transferred into 10ml of volumetric flask and diluted up to the mark with diluent to obtain a concentration of 60µg/ml.

3. Preparation of Mobile phase:

2gm of Sodium benzoate is weighed and transferred into 100ml volumetric flask and dissolved slowly by adding HPLC grade water and made up to the volume with HPLC grade water to obtain 2%Sodium benzoate and methanol(50:50%v/v)as a mobile phase (Hydrotrope).It is also used as diluent.

4. Design of Experiment[5-7]: The standard drug sample of Lenacapavir was subjected to the design of experiment process. Box-Behnken response surface design was employed to identify the underlying facts of effects of factors and their interaction effects on selected method responses. A total of 17 runs were conducted.

Statistical analysis:

- By using ANOVA, the statistical calculations were processed for variables screening and optimization of the method.
- The statistical tools provide the numerical verification of variables and its effect on responses.

Method operable design region:

The different amalgamation and reciprocity of input factors produces the space referred as Design space. The establishment of design space was made by utilizing the contour graphs of Sigma tech software.

Method Verification[6,7]:

The optimized method conditions were proposed by the software in order to reach the desired method goals. The method was verified to check the predictability of the proposed model.

Table: 1 Box - Behnken design experimental runs

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	**A: Mobile Phase Ratio**	**B: Flow Rate**	**C: Buffer pH**	**Retention Time of Lenacapavir**	**Tailing factor**
				min	
1	30	0.8	3.5	5.2	0.8
2	40	0.8	3	4.6	0.9
3	40	0.9	3.5	4.4	1.2
4	30	0.9	4	4.3	1.1
5	30	1	3.5	4.8	1.2
6	50	0.9	4	4.5	1.1
7	40	0.9	3.5	4	1.2
8	50	1	3.5	4.7	1.3
9	40	0.9	3.5	4.4	1.2
10	50	0.8	3.5	4.9	1.1
11	40	1	3	4.2	1.1
12	40	1	4	4.5	1.2
13	40	0.8	4	4.8	1.2
14	50	0.9	3	4	0.9
15	40	0.9	3.5	4.5	1.2
16	40	0.9	3.5	4.5	1.2
17	30	0.9	3	4.3	1.1

METHOD VALIDATION[11-13]

1. Linearity:

From the above standard stock solution pipetted out 0.2, 0.4, 0.6, 0.8 and 1ml into a five 10ml volumetric flask and made up to the volume 10ml with diluent to get 2, 4, 6, 8 and 10µg/ml concentrated solutions of Lenacapavir was filtered and injected into HPLC system and peak area was measured. Plotted a graph between peak area and concentration. Correlation coefficient was determined by regression analysis.

2. Precision:

From the standard stock solution an aliquot of 0.6ml was added into a six 10ml volumetric flasks, made up to 10ml with diluent. Later it was filtered and six replicates were injected into HPLC system and measured the area for all six injections.

3. Accuracy:

Preparation of standard stock solution:

1000µg/ml of standard stock solution was prepared. Further pipetted out 0.6ml of Standard stock solution into 10ml volumetric flask and was diluted up to the mark with diluent.

Preparation of sample solution:

Accuracy solutions at 50% level:

5mg of sample is weighed and transferred into a 10ml volumetric flask added about 7ml of diluent and sonicated to dissolve it completely and made volume up to the mark with diluent. Further pipetted out0.6ml of above stock solution into a 10ml volumetric flask and made volume up to mark with diluent and injected sample into HPLC injector.

Accuracy solutions at 100% level:

10mg of sample is weighed and transferred into a 10ml volumetric flask added about 2ml of diluent and sonicated to dissolve it completely and made volume up to the mark with diluent. Further pipetted out 0.6ml of above stock solution into a 10ml volumetric flask and made volume up to mark with diluent and injected sample into HPLC injector.

Accuracy solutions at 150% level:

15mg of sample is weighed and transferred into a 10ml volumetric flask added about 2ml of diluent and sonicated to dissolve it completely and made volume up to the mark with diluent. Further pipetted out 0.6ml of above stock solution into a 10ml volumetric flask and made volume up to mark with diluent and injected sample into HPLC injector.

4. LOD and LOQ:

The limit of detection and limit of quantification was calculated based on the standard deviation of the response and slope of calibration curve.

5. Robustness:

It is the capacity of the method to remain unaffected by small deliberate variations like change in the flow rate and mobile phase composition was made to evaluate the impact on the method.

Flow rate variations:

60ppm of Lenacapavir was prepared and injected into HPLC system using the variation in flow rates along with method flow rate, i.e., 0.8ml/min, 1.0ml/min and 1.2ml

Variation in organic composition in mobile phase:

60ppm of Lenacapavir was prepared and injected into HPLC system using the varied organic composition in mobile phase along with method mobile phase composition i.e., 10% less, Actual and 10% more.

RETENTION TIME

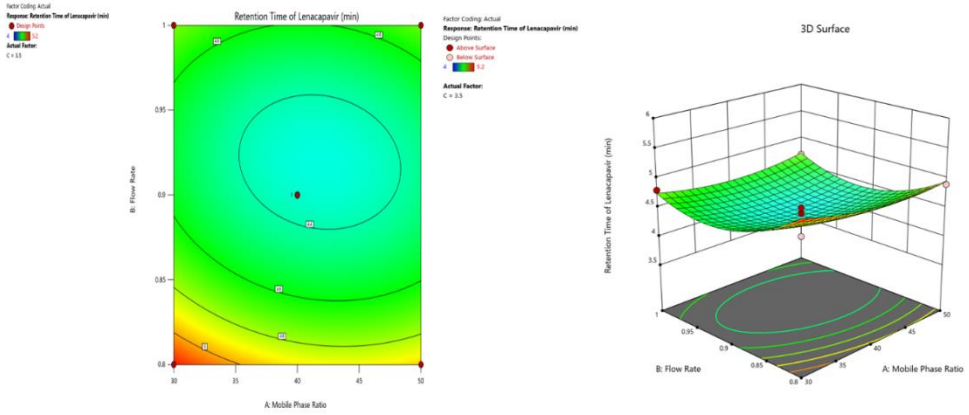

Figure 2: 3D RSM plots for retention time

TAILING FACTOR

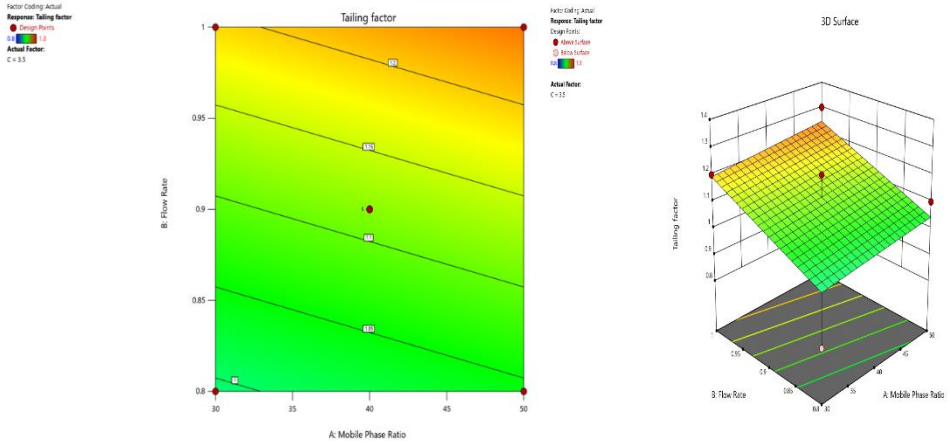

Figure 3: 3D RSM plots for Tailing factor

OPTIMIZATION

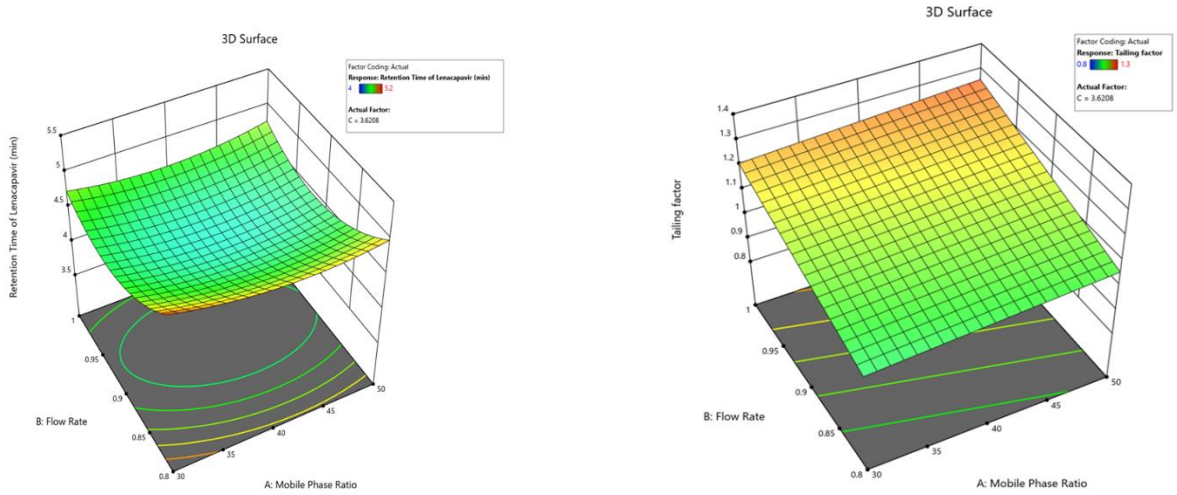

Figure 4: 3D RSM plots for optimized chromatogram

Optimization and development of RP-HPLC-PDA method using Box-Behnken design

- In the proposed investigation, 17 experimental runs were performed and analyzed for obtained results of retention time and tailing factors in accordance with the Box-Behnken design.
- Further investigation was performed using response surface methodology (RSM) to evaluate the relationship between the dependent responses and independent variables (Factors) using obtained data was reported in Table 1.
- The model was also validated by analysis of variance (ANOVA) using design expert software, and the results are as presented in Table 2. Based on value, a quadratic model was selected for responses such as retention time and tailing factor.
- The significant effects showed p value less than 0.05, while the low standard deviation (% C.V) and a high adjusted R-square value indicated a good relationship between the experimental data and those of the fitted model.
- The predicated R-square value was in acceptance concordance with the adjusted R-square value for all responses.
- The final equation in terms of actual components and factors which can be used to make predictions about the response for given levels of each factor,

Table: 2 ANOVA Table.

S.NO	Response	±S. D	Mean	%CV	R^2	Adjusted R^2	Predicated R^2	Adequate precision	P value
1	Retention time	0.1623	4.51	3.60	0.8824	0.7313	0.7013	9.7377	0.0039
2	Tailing factor	0.1091	1.12	9.76	0.4566	0.3312	0.0226	6.6143	0.0419

Coded Equations:

Retention time:

+2.20-0.3030A-0.4034B-0.1066C

Theoretical Plates:

+2109.60+10.12A-90.62B+114.50C-83.75AB+77.50AC-101.50BC-8.43A2-197.43B2-198.67C2

Tailing Factor:

+1.43+0.0200A-0.0162B+0.0463C

Positive values represent an effect that favours optimization while a negative value indicates an inverse relationship between the factors and responses. 3-D response surface plots revealed that effect of factors such as 2% Sodium benzoate and methanol (50:50% v/v) composition in mobile phase, Buffer pH and flow rate of HPLC system on the responses Retention time and Tailing factor.

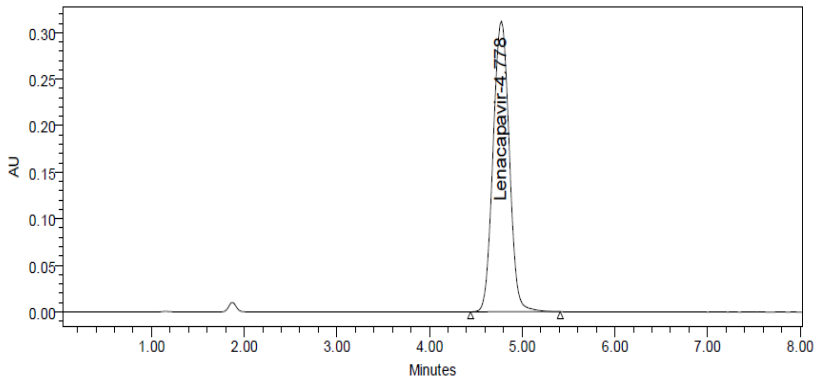

Figure 5: Optimized Chromatogram

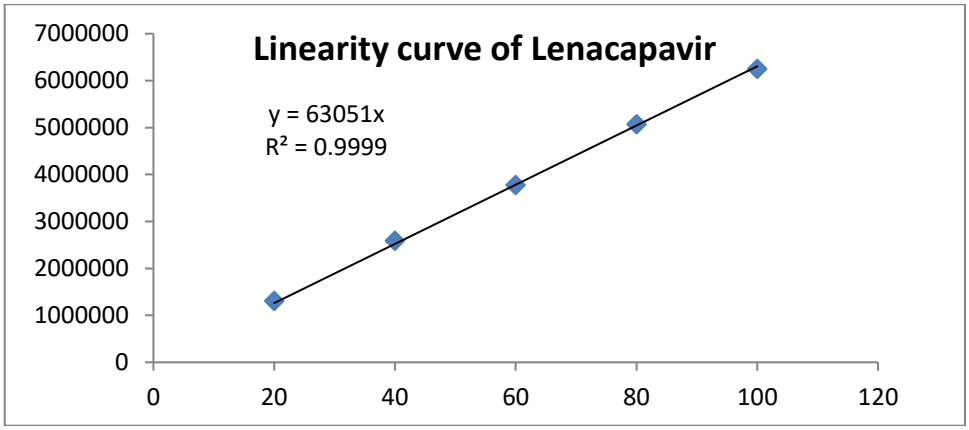

Figure 6: Linearity Curve of Lenacapavir

Name	Retention time	Peak area	Theoretical plates	Tailing Factor
Lenacapavir	4.778	3797331	3348	1.1

Observation:

The separation was good; peak shape was good, the retention of peak was good, resolution was good, tailing factor was less than 2, theoretical plates were more than 2000, and this trial was taken as optimized method.

METHOD VALIDATION:

Table: 3 Linearity studies of Lenacapavir

S. No	Linearity Level	Concentration	Area
1	I	20	1308152
2	II	40	2592905
3	III	60	3778327
4	IV	80	5070038
5	V	100	6249400
Correlation Coefficient			**0.999**

Acceptance criteria: Correlation coefficient should be not less than 0.999.

ACCURACY:

The accuracy is the method of closeness of the measured value to true value for the sample. Accuracy is usually determined by recovery studies.

Table 4: Accuracy results of Lenacapavir

Concentration (At specification Level)	Area	Amount Added(mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1907860	5	4.99	99.90	99.78
100%	3776045	10	9.88	98.86	
150%	5762457	15	15.08	100.58	

Acceptance criteria: The % recovery for each level should be between 98.0 to 102.0%.

PRECISION:

The precision studies were carried out by 6 replicate injections of Lenacapavir.

Table 5: Precision (Repeatability) results of Lenacapavir

Injection	Area
Injection-1	3794064
Injection-2	3800979
Injection-3	3800108
Injection-4	3801140
Injection-5	3814151
Injection-6	3814457
Average	**3804150.0**
Standard Deviation	**8287.8**
%RSD	**0.2**

Acceptance criteria: The % RSD for the area of six standard injections results should not be more than 2.

Table 6: Intermediate Precision results of Lenacapavir

Injection	Day1, Analyst 1	Day2, Analyst 2
Injection-1	3774765	3769004
Injection-2	3788388	3781898
Injection-3	3778269	3772903
Injection-4	3794002	3778043
Injection-5	3784010	3788995
Injection-6	3794707	3779928
Average	**3785690.2**	**3778461.8**
Standard Deviation	**8184.6**	**7006.1**
%RSD	**0.2**	**0.2**

Acceptance criteria:

The % RSD for the area of six standard injections results should not be more than 2.

ROBUSTNESS:

As part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method.

Table 7: Robustness results for Lenacapavir (Change in Flow rate)

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	3914	0.99
2	**1.0***	3347	1.1
3	1.2	2966	1.02

Table 8: Robustness results for Lenacapavir (Change in Organic Composition in the Mobile Phase)

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	3914	0.99
2	*Actual	3348	1.1
3	10% more	4361	1.08

ASSAY:

$$Assay\ \% = \frac{sample\ area}{Standard\ area} \times \frac{dilution\ sample}{dilution\ of\ standard} \times \frac{P}{100} \times \frac{Avg.wt}{Lc} \times 100 = \mathbf{99.87}$$

Where, Avg.wt = average weight of tablets
P= Percentage purity of working standard
LC= Label Claim of Lenacapavir mg/ml

Table 9: Assay

ASSAY	1065799	10	0.6	10	10	99	295.75	100	**99.87**
	3793522	10	10	10	0.6	100	25		

Discussion: The percentage assay of Lenacapavir was found to be 99.87%.

Table 10: Summary data of validation parameters

S.NO	VALIDATION PARAMETERS		ACCEPTANCE CRITERIA	RESULTS
1.	System suitability		%RSD for 5 replicate injections of standard solution NMT 2.0%	0.1
2.	Linearity		The correlation coefficient should be NLT 0.999	R^2=0.999
3.	Accuracy		The %Recovery at each level should be NLT 80.0% and NMT 120% of the amount added	%Recovery – 99.78
4.	Precision		The %RSD of peaks obtained from the 6 replicate injections should be NMT 2.0%	%RSD- 0.2
5.	LOD		-	0.0021µg/ml
6.	LOQ		-	0.0065µg/ml
7.	Robustness	Variation in flow rate(0.8ml/min-1.2ml/min)	%RSD should be NMT 2	%RSD 0.8ml/min – 0.00132 1.2ml/min – 0.00587
8.	Assay		-	99.87%

Conclusion

The proposed RP-HPLC method avoids the requirement for organic solvents by using a hydrotropic solution as the mobile phase, avoiding difficulties like volatility, pollution & cost. Furthermore, by using hydrotropic solvents as the mobile phase, the majority of the organic solvents in the analysis can be reduced. The Design of Experiment was carried out by using the Box-Behnken design & the assessment of independent variables. A rapid, novel, precise cost effective & robust RP-HPLC method for estimation of Lenacapavir in bulk and dosage form was developed. Hydrotropic solvent i.e., 2% sodium benzoate was found to be a more deliberate factor for method optimization, according to response surface plots. The use of DOE approach is a flexible strategy for reducing the no. of trial experimental runs required for a method to be created in a short period of time. The proposed method was found to be rapid, accurate, precise, specific, robust, rugged and economical.

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