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Box-Behnken Design Assisted AQbD Approach for the Optimization and Quality Assessment of Ensitrelvir in Bulk and Dosage forms by RP-HPLC

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Ensitrelvir, is an oral SARS-CoV-2 3CL protease inhibitor that was approved in Japan to treat SARS-CoV-2 infections. This paper describes the AQbD approach and Box Behnken Design assisted development of a HPLC method and its validation for Ensitrelvir in bulk and dosage form. The three independent variables of the RP-HPLC method were flow rate, organic ratio in mobile phase and runtime and the responses retention time and tailing factor were taken as dependent variables. The study utilized a PLATSIL C18-EP column (4.6 x 250mm, 5μm) the chromatographic conditions were optimized using Acetonitrile: Triethylamine pH 4 (60:40) as the mobile phase, 1 mL/min as flow rate with a Rt of 9.609 min, at a λ max of 228 nm. The devised technique was found to be linear with a serial dilution of 20–100 μg/mL with (r2) of 0.991. The tailing factor (TF) and theoretical plates (N) were 1.28 and 4883 results indicated the system suitability test, respectively. The precision for Intraday and Interday were determined and % RSD was observed to be 1.6 and 0.9 %. The robustness values were below 2%. According to the chromatographic peak purity data no other coeluting peaks were found with the Ensitrelvir peak. The method could also be applied for the quantification of Ensitrelvir in dosage form and in its pure form.

Keywords: Analytical Quality by Design; Box-Behnken Design; Ensitrelvir; Optimization; RP-HPLC.

In preclinical trial studies, the oral SARS-CoV-2 3C-like protease inhibitor ensitrelvir fumaric acid has exhibited antiviral activity which was active against variants of SARS-CoV-2, including subvariants of Omicron.[1] Ensitrelvir has been approved since September 2023, it was approved for emergency use in Japan under Fast Track clinical trial conditions.[2] The Registry of Clinical Trials Japan has conducted a clinical trial 2/3, randomized double-blind, placebo-controlled clinical trial.[3] Treatment with ensitrelvir showed improvements in respiratory symptoms and in the multiple respiratory symptoms and slight feverishness was observed in phase 2b.[4] In phases

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2a and 2b it exhibited lower viral load compared to placebo. [5, 6]

The study was determined as per ICH Q8 for product development and ICH Q11 for manufacture and development of drug substance. [7, 8, 9] A well-understood product and process that reliably achieves its intended performance are the result of using QbD concepts. The information gathered throughout development can help construct a design space and choose appropriate process controls. The Analytical QbD (AQbD) refers to the use of these similar QbD concepts to the development of analytical procedures. [10]

No analytical methods for quantifying ensitrelvir had previously been published, according to a thorough assessment of the literature. [11 12] This is the first method reported in literature for method development and validation for Ensitrelvir in dosage form or in its pure form. Developing and refining the Ensitrelvir HPLC technique in a solid dosage form through a QbD approach is the aim of this undertaking. [13, 14]

MATERIALS AND METHODS

Materials

Ensitrelvir was procured as complementary sample from MSN Lab Pvt. Ltd., Hyderabad. KH_2PO_4 was purchased from Finar chemical LTD, Acetonitrile, Methanol and Water for HPLC were obtained from Standard solutions Ltd, HCl, H_2O_2, NaOH procured from Merck Pvt. Ltd. Mumbai.

Instruments

A HPLC WATERS, software: Empowered, 2487 UV detector, was utilized for the study.

Chromatographic conditions

The study utilized a PLATSIL C18-EP column (4.6 x 50 mm, 5µm) the chromatographic conditions were optimized using Acetonitrile: Triethylamine pH: 4 (60:40 mL) as the mobile phase, to adjust the mobile phase to pH 4, triethylamine (0.01%) was added. 1 mL/min as flow rate with a Rt of 9.609 min, at a ë max of 228 nm.

Preparation of reference standard solution

By precisely dissolving 25 mg of Ensitrelvir into 25 ml VF, the standard stock solution was prepared. After diluting the stock solution, a sub-stock was prepared. Dissolving the solution was done with a sonicator. Using diluent to dilute the stock solution, 0.6 ml of the aforementioned solution was taken in a 10 ml VF.

HPLC Method Development by Analytical QbD

Selection of quality target product profile

Finding the variables that influence the QTPP parameters is a crucial task for the QTPP. For the suggested HPLC approach, QTPP was found to be the retention time and tailing factor. [13, 14]

Determination of critical quality attributes

The CQAs selected for the study were runtime, flowrate and organic phase in mobile phase ratio considered to be independent variables and dependent variables (responses) were retention time and tailing factor.[14]

Factorial design

The various interaction effects and quadratic influences of run time, organic phase in mobile phase ratio and flow rate on the retention time and tailing factor were investigated using a central composite statistical screening strategy. A 3-factor, flow rate, organic ratio in mobile phase and run time at three different levels were used in the design process using Design Expert software (Version 12.0). Three essential components were selected and optimized utilizing the central composite experimental design once the QTPP and CQAs were defined. [10] Because multivariable interactions between variables and process parameters have been explored, preliminary analysis was conducted to identify the components. [11] Table 1 lists the independent variables that were chosen, including run time, the organic phase in mobile phase and flow rate. The tailing factor and retention time were considered as the factors. [12]

Analysing experimental findings and choosing the ideal procedure parameters

We evaluated these method variables using the BBD approach. The circumstances for the retention time and tailing factor have to be assessed in the first phase. This produced many chromatographic conditions for Ensitrelvir. Robust zones, wherein deliberate modifications to the procedure parameters have no effect on the quality, are found within the suggested acceptable limits. This technique ensures that validation testing can continue without concern for method failures in the future. Modifying the variable at different levels is necessary until the results fall within acceptable limits if the expected response from the modeling experiments is not attained. [15] The use of Design

Expert tools optimized ideal chromatographic conditions.

Risk assessment

The final optimized method was selected by taking into account factors like long-term functionality and dependability. The ICH Q8 and ICH Q9 guidelines' QbD principles have an impact on the risk-based methodology used to assess the method's robustness and ruggedness.[7]

Analytical method validation

Method validation is the process of confirming suitable analytical procedure for the purpose for which it is meant to be used. This can be confirmed using recorded evidence that provides a high level of certainty. The developed HPLC technique for Ensitrelvir estimation was evaluated in compliance with ICH Q2 (R1) requirements.[9]

Linearity

To examine the linearity of Ensitrelvir, five concentration ranges spanning from 10 to 50ìg/ml were considered. The standard curve was plotted. The r^2 value and the linear regression coefficient were calculated.

Precision

Repeatability was evaluated using measurements of six Ensitrelvir samples at 100ìg/ml. Three distinct Ensitrelvir various concentrations were tested, once within the day at a 2-hour gap and on separate days in order to compute the intraday and interday precision. Less than two was the allowed range for the % RSD.

Accuracy

Recovery trials from commercial formulations at three distinct concentrations—50%, 100%, and 150% of standard addition—were used to evaluate the method's accuracy. A recovery % was calculated for Esitrelvir. 98–102% of standard addition was the acceptable range for recovery % according to ICH recommendations.

LOD and LOQ

A drug's LOD and LOQ refer to the lowest concentration at which a drug can be consistently detected and separated from the background and, respectively. To calculate the LOD and LOQ in accordance with ICH guidelines, the following formula was utilized.

$$\sigma LOD = 3.3 \times \sigma/SD$$
$$\sigma LOQ = 10 \times \sigma/SD$$

Robustness and Intermediate precision Studies

By changing minor adjustments to the procedure such as changing the pump flow rate and composition of mobile phase, the robustness of the methodology was evaluated. The analysis of the robustness studies required substituting an analyst for an extraneous influencing component. The allowed limit for the peak area's estimated % RSD was below 2.

System suitability studies

Ensitrelvir was analysed six times in replicate to assess the system suitability. For standard solutions, the Rt, column efficiency, asymmetry factor and column efficiency were calculated.

Assay

Weigh out a powder to the exact quantity of 25 mg of Ensitrelvir and pour it into a 25 ml volumetric flask. Add diluent once the powder has completely dissolved, then sonicate for 15 minutes. After then, utilize the mobile phase to maintain the volume to desired level. The resulting solution can be filtered using 0.42ì Whatman filter paper. HPLC was used to analyze the solution under the same chromatographic conditions as linearity. Three separate assays' means were used for the calculation.

RESULTS AND DISCUSSION

Initially, we tried a mobile phase ratio of ACN and water 50:50 v/v the retention time was too lengthy. The next mobile phase tried was ACN and water in the ratio 80:20 v/v, no peak was observed. Finally, we used Acetonitrile to Triethylamine in water at a ratio of 60:40 v/v. By modifying the pH of the buffer, the peak's symmetry and shape were improved. The adjusted chromatographic conditions satisfied the specifications of the system suitability test. The ideal mobile phase was ACN to water 60:40 v/ v, with pH 4 adjusted with 0.01% triethylamine. Additional parameter optimization within the design space was conducted using the central composite design.

HPLC method development by QbD approach [14]

Quality target product profile

Retention time, theoretical plates, and peak asymmetry were the QTPP used in order to optimize the chromatographic conditions of HPLC.

Factorial design[17]

The Independent Variables (factors) and Dependent Variables (Responses) are shown in Table 1. Analysis of variance (ANOVA) test results are displayed in Table 2. Represents variables and their minimum and maximum levels are shown in Table 3, Represents Regression analysis for different responses and Fit Summary Table 4. Figures 2-6 represents the 2D contour plots for Retention time of Ensitrelvir, Figure 3 illustrates the 3D response surface plots for Retention time of Ensitrelvir, Figure 4 shows the 3D Surface for Tailing Factor of Ensitrelvir, Figure 5 illustrates the

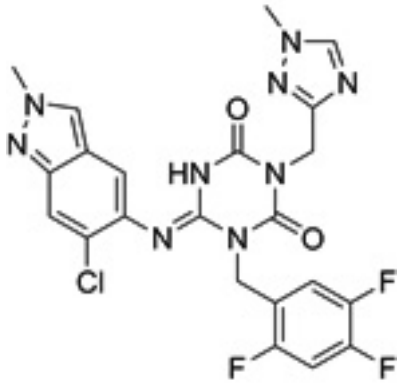

Fig. 1. Structure of Ensitrelvir

Table 1. Independent Variables (factors) and Dependent Variables (Responses)

Std	Run	Factor 1 A: Flow rate	Factor 2 B: Organic ratio in MP	Factor 3 C: run time	Response1 Retention time mins	Response2 tailing factor
6	1	1.00	55.00	14.00	9.5	1.27
4	2	1.00	60.00	14.50	9.6	1.28
12	3	0.90	60.00	15.00	9.4	1.6
10	4	0.90	60.00	14.00	9.2	1.6
15	5	0.90	55.00	14.50	8.5	1.6
2	6	1.00	50.00	14.50	8.2	1.6
1	7	0.80	50.00	14.50	8.6	1.6
9	8	0.90	50.00	14.00	8.6	1.6
17	9	0.90	55.00	14.50	8.5	1.6
3	10	0.80	60.00	14.50	8.3	1.6
7	11	0.80	55.00	15.00	8.7	1.6
16	12	0.90	55.00	14.50	8.5	1.6
5	13	0.80	55.00	14.00	8.7	1.6
13	14	0.90	55.00	14.50	8.5	1.6
8	15	1.00	55.00	15.00	8.3	1.6
14	16	0.90	55.00	14.50	8.5	1.6
11	17	0.90	50.00	15.00	8.6	1.6

Table 2. Analysis of variance (ANOVA) test results

Source	Sum of Squares	df	mean Square	F-Value	p-value Prob > F	
Mean vs Total	1291.96	1	1291.96	Suggested		
Linear vs Mean	1.12	3	0.37	2.59	0.0972	
2FI vs Linear	1.09	3	0.36	4.70	0.0269	
Quadratic vs 2FI	0.49	3	0.16	3.95	0.0611	Suggested
Cubic vs Quadratic	0.29	3	0.096	6.366E+007	< 0.0001	Aliased
Residual	0.000	4	0.000			
Total	1294.94	17	76.17			

2D contour plots for Tailing factor of Ensitrelvir, Figure 6 shows the Overlay plot for Ensitrelvir.

Design space

The 17-run quadric design model, the RSM, and the BBD were used. After comparing the flow rate, mobile phase ratio and run time to the two responses, retention time, and tailing factor, the findings of the suggested BBD experimental design were presented. The flow rate has impact on tailing factor, when flow rate increases tailing factor decreases, run time decreases tailing factor decreases. The impact on retention time, organic phase decreases retention time decreases, runtime decreases retention time decreases. The observed value for responses was computed using the HPLC peak for the designated organic phase ratio and buffer pH in order to get the percentage of prediction error. A comparison with the anticipated values followed subsequently.

Method validation

System suitability

The system suitability test was examined by taking number of characteristics, including the Rt, which was determined to be 9.609 mins, the number of theoretical plates, 4883, the peak asymmetry, 1.28, and the % RSD of

Table 3. Represents variables and their minimum and maximum levels

Factor	Name	Type	Low Actual	High Actual	Low Coded	High Coded	Mean	Std. Dev.
A	Flow rate	Numeric	0.80	1.00	-1.000	1.000	0.900	0.069
B	Organic mobile phase	Numeric	50.00	60.00	-1.000	1.000	55.000	3.430
C	runtime	Numeric	14.00	15.00	-1.000	1.000	14.500	0.343

Table 4. Regression analysis for different responses and fit summary

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Y1	Rt	min	17	Polynomial	8.20	9.60	8.72	0.42	1.17	None	No model chosen
Y2	tailing factor		17	Polynomial	1.27	1.60	1.56	0.10	1.26	None	2FI

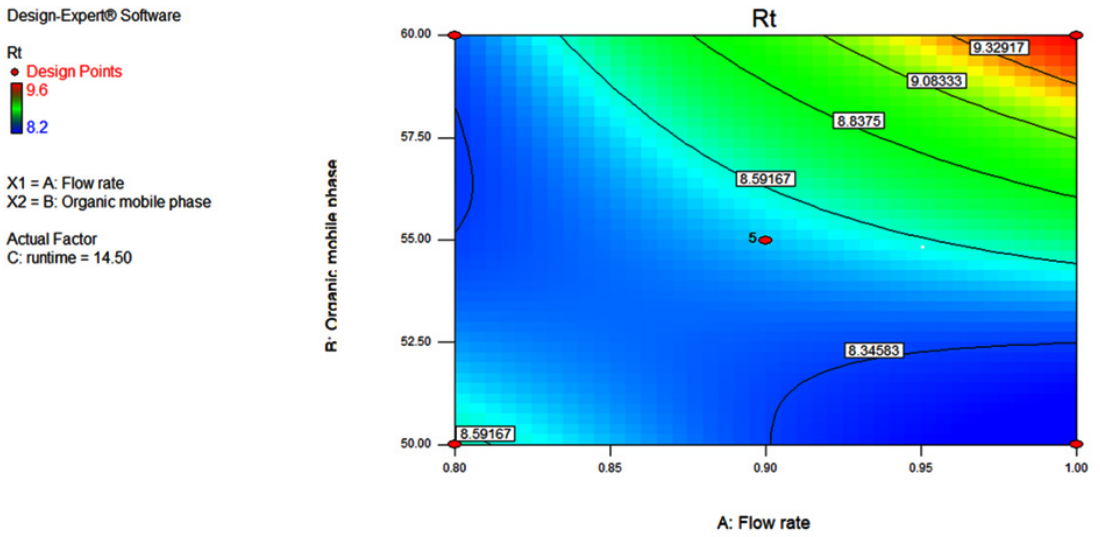

Fig. 2. 2D contour plots for Retention time of Ensitrelvir

six injections was 0.82. Table 5 represents the results of system suitability parameters. Figure 7 depicts blank Chromatogram, Figure 8 represents Standard Chromatogram, Figure 9 displays Sample Chromatogram.

Linearity

Within the linear range of 10–50ig/ml, the regression line for Ensitrelvir that developed was linear, as shown in Figure 2 and Table 6. The calibration curve's regression equation, y = 5038.6x + 3999.7, with a correlation coefficient of 0.9996, was often identified by plotting the graph of peak area vs concentration.

Precision

Based on six injections of the same range, the % RSD for reliability for Ensitrelvir was

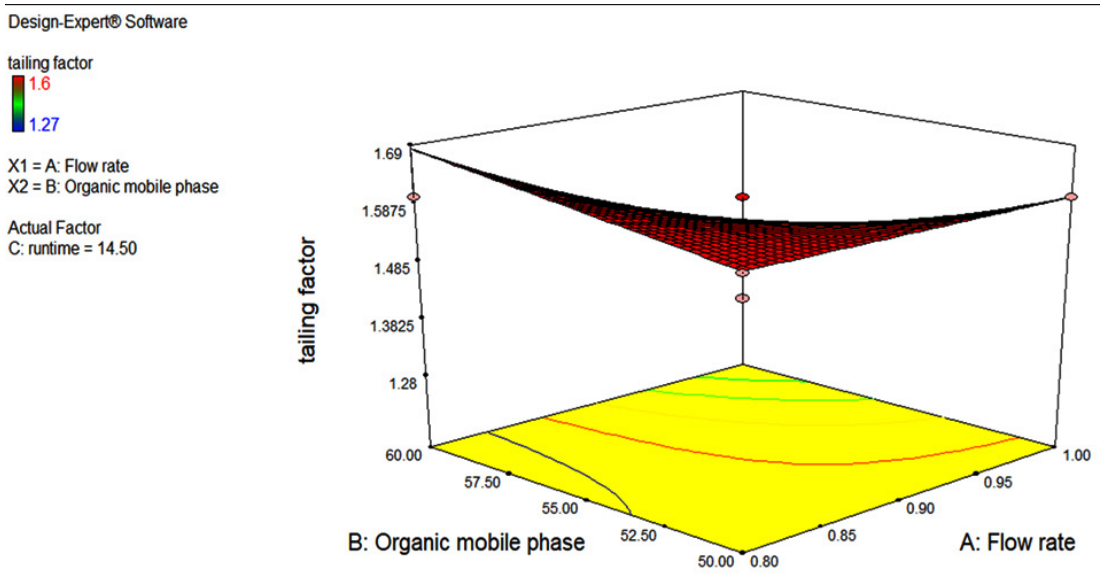

Fig. 3. The 3D response surface plots for Retention time of Ensitrelvir

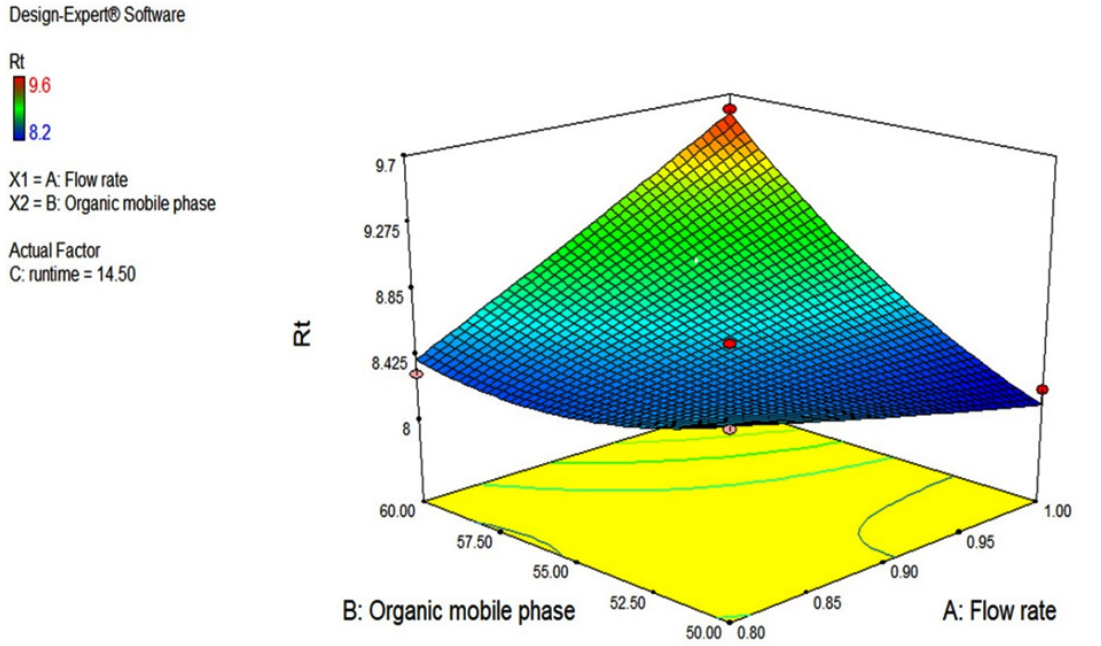

Fig. 4. 3D Surface for Tailing Factor of Ensitrelvir

observed to be 1.6 & 0.9. The developed method was considered to be precise if the % RSD value was below 2. The intraday and interday precisions values are displayed in Table 7.

Accuracy

Recovery study was used to ensure accuracy. Three different amounts of spiking were used to prepare sample solutions: 50%, 100%, and 150%. As per ICH Q2 (R1) requirements, the developed method was deemed accurate based on the recovery percentage of 98–102%. Table 8 displays the percentage recovery statistics that were acquired using the suggested HPLC procedure.

Robustness studies

In order to assess robustness, a minor but deliberate modification of intrinsic technique characteristics including the pH and mobile phase flow rate was investigated. The ruggedness was examined as an unrelated contributing factor by a different analyst. A percentage RSD of less than 2 was obtained for the peak area due to variations in the mobile phase pH, the flow rate and the analyst. Ensitrelvir's robustness results are shown in Tables 9 and 10.

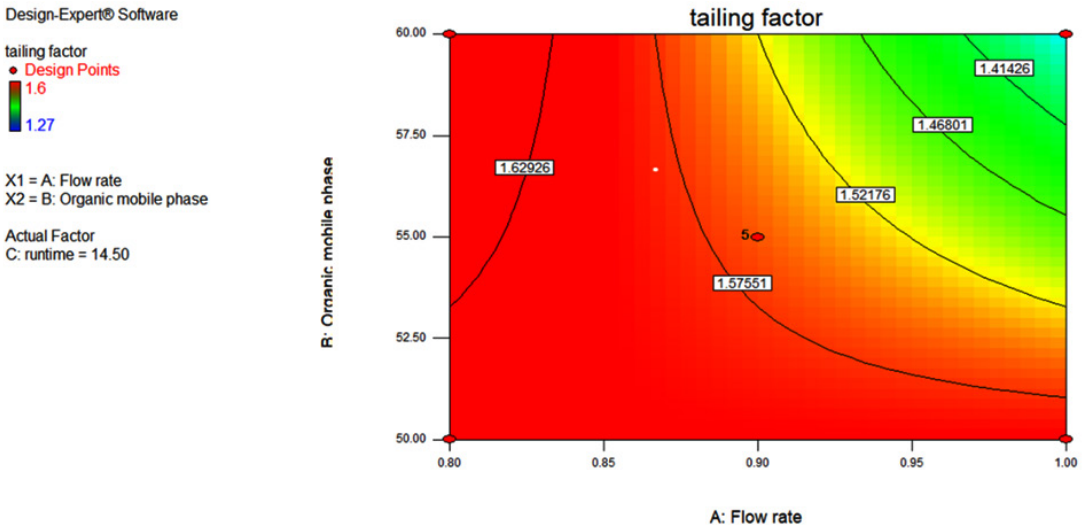

Fig. 5. 2D contour plots for Tailing factor of Ensitrelvir

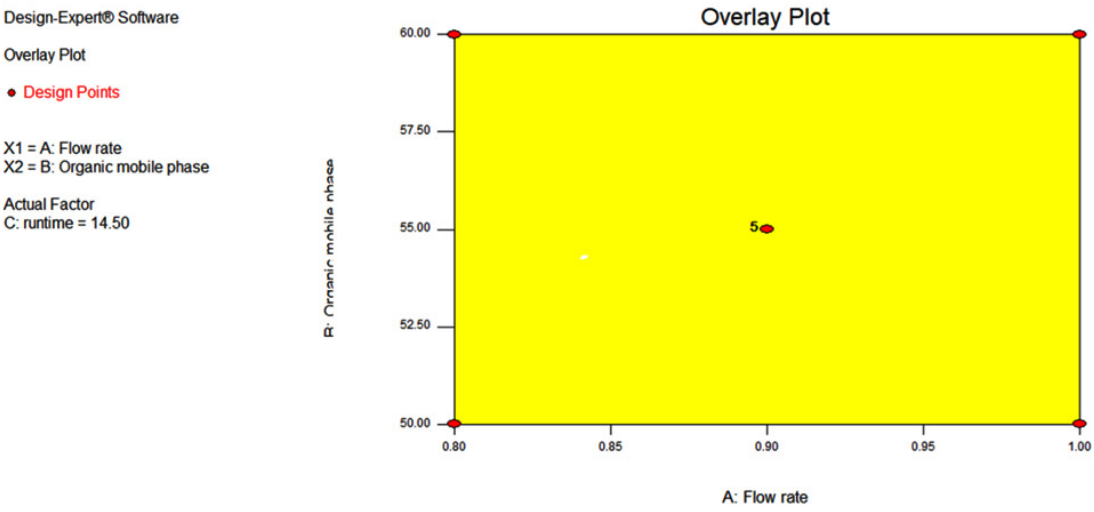

Fig. 6. Overlay plot for Ensitrelvir

LOD and LOQ

The LOD and LOQ for Ensitrelvir were determined to be 2.96ìg/ml and 9.97ìg/ml. The results for LOD & LOQ are displayed in Table 11.

Assay

Using tablets for the experiment, the optimized chromatogram for Ensitrelvir revealed a resolved peak at retention time 9.607 min. Ensitrelvir's label claim was found to have a drug

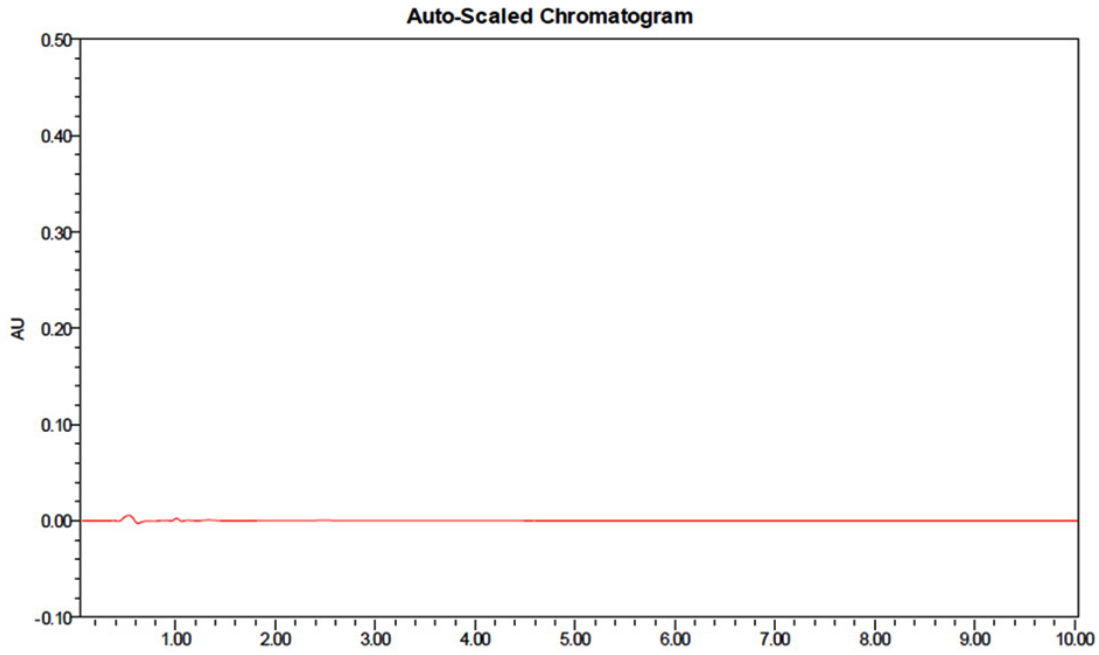

Fig. 7. System suitability Chromatogram for Ensitrelvir

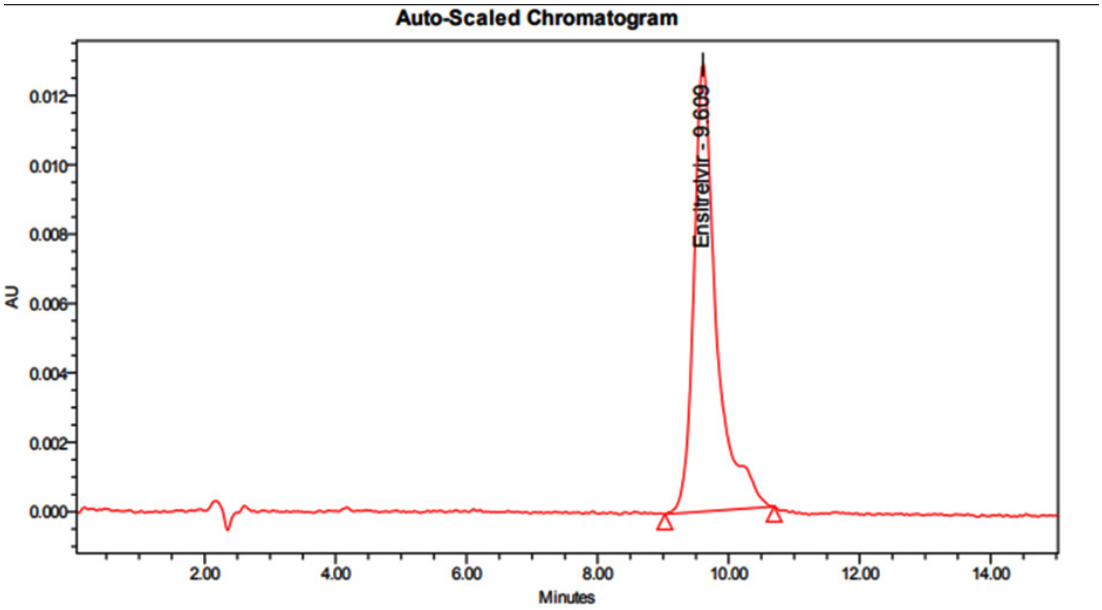

Fig. 8. Standard Chromatogram for Ensitrelvir

content assay percentage of 100.5. The assay result showed that, in the presence of excipients included in tablet powder, the method was found to be more precise and specific.

DISCUSSION

Using HPLC, an analytical QbD-driven method has been established for estimating ensitrelvir in solid dosage forms. The retention

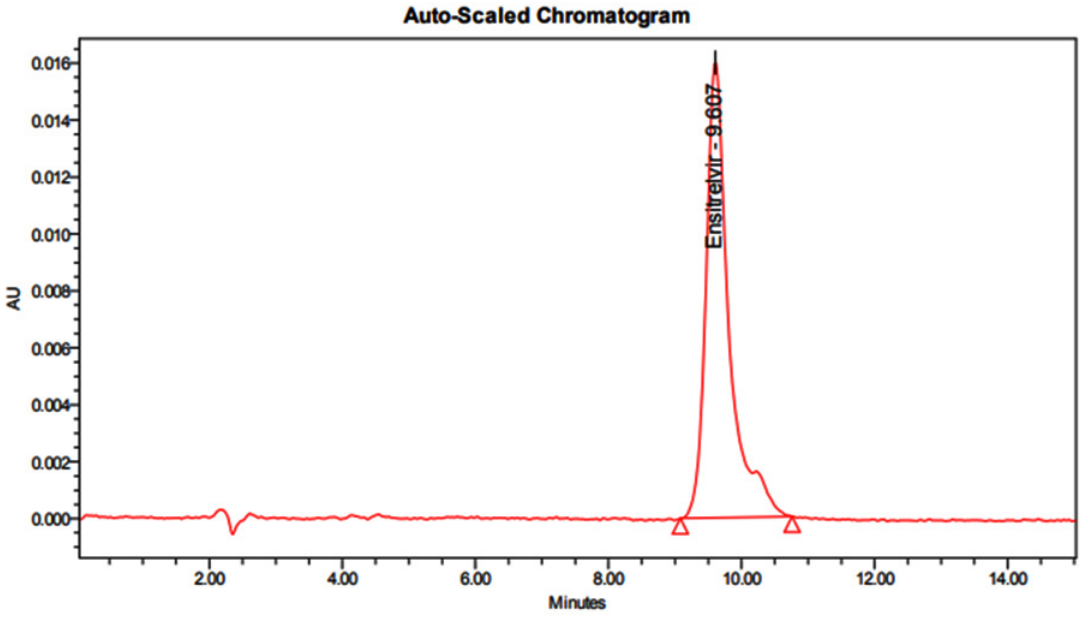

Fig. 9. Sample Chromatogram for Ensitrelvir

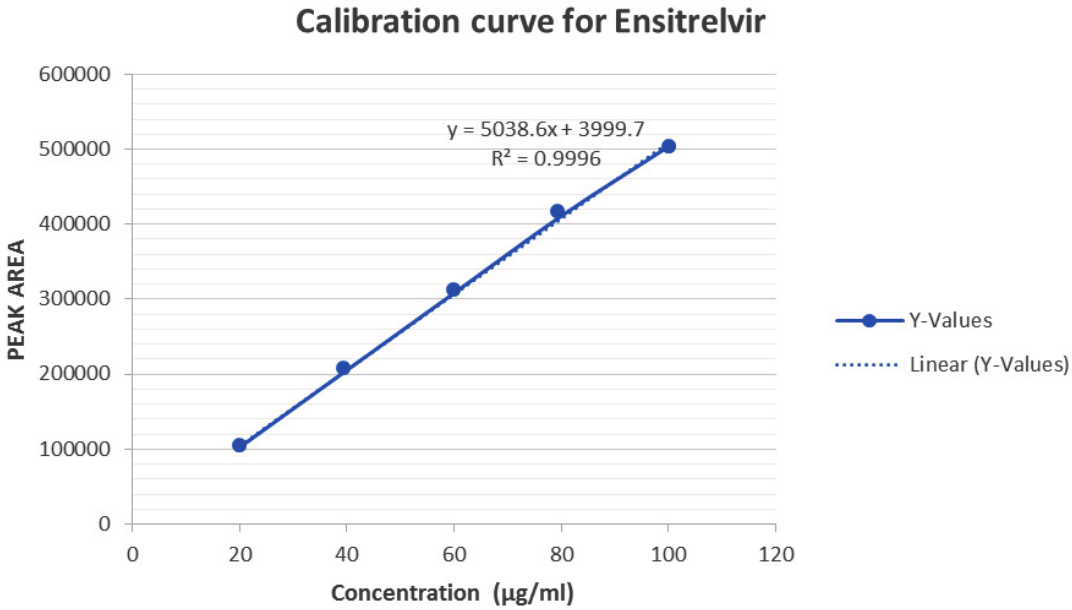

Fig. 10. Calibration graph for Ensitrelvir

time and tailing factor served as the analytical target product profile for the Ensitrelvir HPLC investigation. Three components have been recognized as CQAs that impact the measurement of quality attributes (ATP): run time, flow rate, and organic phase in mobile phase. Using Design Expert Software Version 12.0, the BBD was applied for three variables at three separate levels. Important factors influencing the analytical target profile were found through a risk assessment analysis. .[15, 16, 17] In order to retain control over variables related to column selection, instrument design, and injection volume, robustness study was assigned to govern several components of chromatographic separation, including flow rate and organic phase ratio of mobile phase. The study utilized a PLATSIL C18-EP column (4.6 x 250mm, 5µm) the chromatographic conditions were optimized using Acetonitrile: Triethylamine pH: 4 (60:40 mL) as the mobile phase, 1 mL/min as flow rate with a Rt of 9.609 min, at a ë max of 228 nm. The devised technique was observed to be with a linear range of 10–50ìg/ml with (r^2) of 0.991. The asymmetric factor (Tf) and theoretical plates (N) were 1.28 and 4883 results indicated the system suitability test. The precision for Intraday and interday were determined and found to be 1.6 and 0.9 % RSD. No other coeluting peaks were

Table 5. Results of system suitability parameters

No.	Name	RT (min)	Area (µV sec)	Height (µV)	USP tailing	USP plate count
1	Ensitrelvir	9.609	308314	12899	1.28	4883

Table 6. Area of different concentration of Ensitrelvir

No.	Ensitrelvir Concentration (µg/ml)	Area
1	20	102771
2	40	205542
3	60	308314
4	80	411085
5	100	503856

Table 7. Results of Precision for Ensitreivir

Injection	Area	Injection	Area
Injection-1	319439	Injection-1	309439
Injection-2	314607	Injection-2	304607
Injection-3	307106	Injection-3	307106
Injection-4	312764	Injection-4	312764
Injection-5	305953	Injection-5	305953
Injection-6	307962	Injection-6	307962
Average	311305.2	Average	307971.8
Standard Deviation	5227.715	Standard Deviation	2872.803
%RSD	1.6	%RSD	0.9

Table 8. Accuracy (recovery) data for Ensitrelvir

% Concentration (at specification Level)	Area*	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	99678.5	12.5	12.30	98.4	98.86
100%	12988	25	24.92	99.68	
150%	28451.9	37.5	36.95	98.5	

Table 9. Results for variation in flow for Ensitrelvir

No.	Flow Rate (ml/min)	System Suitability Results USP Plate Count	USP Tailing
1	0.8	4876	1.30
2	1	4883	1.28
3	1.2	4889	1.29

Table 10. Results for variation in mobile phase composition for Ensitrelvir

No.	Change in Organic Composition in the Mobile Phase	System Suitability Results USP Plate Count	USP Tailing
1	10 % less (54ml)	4880	1.30
2	*Actual (60ml)	4883	1.28
3	10 % more (66ml)	4891	1.29

Table 11. Results of LOD & LOQ

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio	Conc.
Ensitrelvir	83	246	2.96	0.1µg/ml
Ensitrelvir	83	828	9.97	0.38µg/ml

found with the Ensitrelvir peak, according to the chromatographic peak purity data. According to ICH specifications, the parameters for method validation were within the permissible range.

CONCLUSION

The current study offers a unique RP-HPLC method that was effectively developed and validated for estimation of Ensitrelvir. The use of AQbD with the Design expert Software significantly improved the method performance and robustness for successfully separating and estimating Ensitrelvir. The developed method was proven to be efficient for analysis of Ensitrelvir in bulk and tablet dosage forms. The technique minimizes the number of experimental runs and validation meet ICH Q2 R (1) requirements. The method could also be applied for the quantification of Ensitrelvir in dosage form and in its pure form.

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Conflict of Interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Author Contributions

Chandrasekar R: Conceptualization, Methodology, Writing – Original Draft; Sivagami B: Data Collection, Analysis, Writing – Review & Editing; Pavan Kumar V: Visualization, Supervision, Project Administration; Satheesh Kumar G: Funding Acquisition, Resources, Supervision

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