Research Article

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# VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF GLECAPREVIR AND PIBRENTASVIR BY USING ANALYTICAL QUALITY BY DESIGN (AQBD) METHOD

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**ABSTRACT**

The quantitative measurement of Glecaprevir and Pibrentasvir has been created using a simple, quick, precise, sensitive, and reproducible reverse-phase high-performance liquid chromatography (RP-HPLC) method. It is more difficult to analyse varying amounts of pharmaceutical active medicinal ingredients in dosage forms without interferences. Therefore, the objective of the current work is to estimate Glecaprevir and Pibrentasvir simultaneously by adopting an Analytical Quality by Design (AQbD), a rotatable central composite-based technique using RP-HPLC-based method development and validation. Glecaprevir and Pibrentasvir were separated by chromatography using a Kinetex RP-18 Column (100x4.6mm, 2.6µ) column and a mobile phase made up of Acetonitrile: 0.1% tri fluoro acetic acid in a ratio of 26.464:73.536 v/v. The flow rate was 1.0 ml/min, and a photodiode array detector operating at room temperature was used to detect absorption at 236 nm. ICH criteria have been used to validate the offered techniques' linearity, accuracy, precision, and other attributes. The degradation study's findings showed that the medications deteriorated in high-stress situations. The chemical and pharmaceutical sectors might easily implement this unique AQbD-based analytical technique for routine analysis without any regulatory constraints.

**Keywords:** RP-HPLC, Analytical quality by design (AQbD), Central Composite Design, Glecaprevir and Pibrentasvir

## INTRODUCTION

For most people with chronic hepatitis C, Glecaprevir-Pibrentasvir offers a powerful treatment alternative, including an 8-week option for those who have never received treatment. It is the first pangenotypic NS3/4A protease inhibitor-NS5A inhibitor combination to be approved. Glecaprevir-Pibrentasvir is a fixed-dose prescription drug used to treat adults and children three years of age and older with chronic (lasting for a very long time) hepatitis C virus (hep C): Infections of Genotypes (GT) 1, 2, 3, 4, 5, or 6 that are not cirrhotic or that are cirrhotic but have compensated; Infections of Genotypes (GT) 1 that were previously treated with a regimen that includes either an NS3/4A protease inhibitor or a hepatitis C NS5A inhibitor, but not both [1].

The NS3/4A protease inhibitor Glecaprevir stops the cleavage of the HCV polyprotein. A common HCV genotype one variant with mutations (at Q80, R155, and D168) that confer resistance to older-generation HCV protease inhibitors shows significant in vitro activity (on the order of less than or equivalent to 5 nanomolar). The next-generation NS5A inhibitor Pibrentasvir (PIB, formerly ABT-530), which maintains strong antiviral activity against common HCV NS5A single-position variants that confer resistance to first-generation NS5A inhibitors such as Daclatasvir, Dedipasvir, and Ombitasvir is available now [2].

Due to its popularity, evaluating the Glecaprevir-Pibrentasvir combination is of utmost necessity. This study validated the Glecaprevir-Pibrentasvir by analytical quality by design (AQbD) method using Reverse Phase High-Performance Liquid Chromatography (RP-HPLC).

## MATERIALS AND METHODS

### Drugs, chemicals, solvents, instruments and software

Glecaprevir and Pibrentasvir were generously gifted from the Shree Icons laboratories in Vijayawada, India. HPLC grade tri fluoro acetic acid (TFA) was purchased from Thermo Fisher Scientific (Maharashtra, India). HPLC grade acetonitrile from Rankem Fine Chemicals Limited (New Delhi, India).

The High-performance liquid chromatographic (HPLC) system utilised for the whole analysis was Waters E 2695 HPLC (Wilmslow, England), united with a double solvent manager with a photodiode array detector (PDA) along with an autosampler. Unichrome ultrasonic baths have been used to solubilise and degas the sample and solvents. The pH of the mobile phase was adjusted with an Eutech Digital pH Meter (Maharashtra, India).

Waters HPLC system unified with Empower 2.0 software for data management. AQbD was developed using Design-Expert® trail version 13 (Stat-Ease Inc., Minneapolis-USA).

### Determination of Working Wavelength ($\lambda_{max}$)

In the simultaneous estimation of two drugs, isosbestic wavelength was used. The isosbestic point is the wavelength where the molar absorptivity is the same for two interconvertible substances. So, this wavelength was used in simultaneous estimation to estimate two drugs accurately.

The wavelength of maximum absorption of the solution of the drugs in a mixture of Acetonitrile and 0.1% TFA (30:70) was

scanned using a PDA Detector within the wavelength region of 200–400 nm against Acetonitrile and 0.1% TFA (30:70) as blank. The absorption curve shows an isosbestic point at 236 nm. Thus, 236 nm was selected as the detector wavelength for the HPLC chromatographic method.

**Chromatographic conditions**

Several trials were carried out during the selection of chromatographic conditions, and the best trial was selected for the optimised method.

**Preparation of standard solution**

Accurately weigh and transfer 40 mg of Pibrentasvir and 100 mg of Glecaprevir working standard into a 100 ml clean, dry volumetric flask; add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further, pipette 5 ml of the above stock solutions into a 50 ml volumetric flask and dilute up to the mark with diluent. (40 ppm of Pibrentasvir, 100 ppm of Glecaprevir)

**Sample Solution Preparation:** Accurately weigh and transfer 242 mg of Glecaprevir and Pibrentasvir sample into a 100 mL clean, dry volumetric flask; add diluent and sonicate it up to 30 minutes to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further, pipette 5 ml of the above solution into a 50 ml volumetric flask and make to the mark with diluents. (100 ppm of Glecaprevir and 40 ppm of Pibrentasvir)

**Final optimised chromatographic conditions in RP-HPLC using AQbD**

The separation and determination of two drugs have been achieved with the help of rotatable central composite-based AQbD [4] using the Kinetex RP-18 Column (100x4.6 mm, 2.6 µ) column. The mobile phase comprises HPLC grade acetonitrile: 0.1% TFA in a ratio of (26.464:73.536) v/v. The optimised chromatographic conditions were validated according to the ICH Q2R1 guidelines for specificity, linearity, accuracy, precision, LOD and LOQ and ICH Q2B for degradation studies [3,5].

**Chromatographic condition**

Use suitable High-Performance Liquid Chromatographic equipped with a PDA detector.
Column: Kinetex RP-18 Column (100x4.6mm, 2.6µ)
Mobile phase ratio: Acetonitrile: 0.1%TFA (26.464:73.536)
Detection wavelength: 236 nm
Flow rate: 1 ml/min
Injection volume: 20 µl
Run time: 6 minutes

**Preparation of Diluent:** Methanol as diluent.

**METHOD VALIDATION**

**Specificity**

The specificity of an analytical method is the ability to measure specifically the analyte of interest without interference from blank and known impurities. For this purpose, blank chromatogram, standard chromatogram and sample chromatogram were recorded. The chromatogram of blank shows no response at the retention times of drugs, which confirms the response of drugs was specific.

The formula for Assay:

$$\%\ Assay = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{Average\ weight}{Label\ Claim} * \frac{P}{100} * 100$$

**Linearity**

Preparation of stock solution:
Accurately weigh and transfer 40 mg of Pibrentasvir and 100 mg of Glecaprevir working standard into a 100 ml clean, dry volumetric flask; add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Preparation of Level – I (25 ppm of Glecaprevir, 10 ppm of Pibrentasvir):
1.25 ml of the above stock solutions have been taken in different 50 ml of volumetric flasks and diluted up to the mark with diluent.

Preparation of Level – II (50 ppm of Glecaprevir, 20 ppm of Pibrentasvir):
2.5 ml of the above stock solutions were taken in different 50 ml of volumetric flasks, diluted up to the mark with diluent.

Preparation of Level – III (75 ppm of Glecaprevir, 30 ppm of Pibrentasvir):
3.75 ml of the above stock solutions have been taken in different 50 ml of volumetric flasks, diluted up to the mark with diluent

Preparation of Level – IV (100 ppm of Glecaprevir, 40 ppm of Pibrentasvir)
5 ml of the above stock solutions have been taken in different 50 ml of volumetric flasks, diluted up to the mark with diluent**.**

Preparation of Level – V (125 ppm of Glecaprevir, 50 ppm of Pibrentasvir)
6.25 ml of the above stock solutions were taken in different 50 ml of volumetric flasks, diluted up to the mark with diluent**.**

Preparation of Level – VI (150 ppm of Glecaprevir, 60 ppm of Pibrentasvir)
7.5 ml of the above stock solutions have been taken in different 50 ml of volumetric flasks, diluted up to the mark with diluent**.**

*Procedure*
Inject each level into the chromatographic system and measure the peak area.
Plot a graph of peak area versus concentration (on the X-axis concentration and the Y-axis Peak area) and calculate the correlation coefficient.

**Accuracy**

For the preparation of 50% solution (Concerning target Assay concentration), accurately weigh and transfer 121 mg of Glecaprevir and Pibrentasvir sample into a 100 mL clean, dry volumetric flask; add diluent and sonicate it for up to 30 minutes to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further, pipette 5 ml of the above solution into a 50 ml volumetric flask and make to the mark with diluents. (50 ppm of Glecaprevir and 20 ppm of Pibrentasvir)

For the preparation of 100% solution (Concerning target Assay concentration)

Accurately weigh and transfer 242 mg of Glecaprevir and Pibrentasvir sample into a 100 mL clean, dry volumetric flask; add diluent and sonicate it for up to 30 minutes to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further, pipette 5 ml of the above solution into a 50 ml volumetric flask and make up the mark with diluents. (100 ppm of Glecaprevir and 40 ppm of Pibrentasvir)

For the preparation of 150% solution (Concerning target Assay concentration):
Accurately weigh and transfer 363 mg of Glecaprevir and Pibrentasvir sample into a 100 mL clean, dry volumetric flask; add diluent and sonicate it for up to 30 minutes to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further, pipette 5 ml of the above solution into a 50 ml volumetric flask and make to the mark with diluents. (150 ppm of Glecaprevir and 60 ppm of Pibrentasvir)

*Procedure*: Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

*Acceptance Criteria*: The % Recovery for each level should be between 98.0 to 102.0%.

**Precision**

Precision is the degree of repeatability of an analytical method under normal operation conditions. Precision is of 3 types
1. System precision
2. Method precision
3. Intermediate precision (a. Intra-day precision, b. Inter day precision)

System precision is checked using standard chemical substances to ensure the analytical system works appropriately. In this peak area, the % of drug of six determinations is measured, and % RSD should be calculated.

In method precision, a homogenous sample of a single batch should be analysed six times. This indicates whether a method is giving constant results for a single batch. In this, analyse the sample six times and calculate the % RSD.

The instrument's precision was checked by repeatedly injecting (n=6) solutions of 100 ppm of Glecaprevir and 40 ppm of Pibrentasvir).

*Acceptance Criteria*: The % RSD for the six replicate injection results absorbance should not exceed 2%.

**Limit of detection (LOD) and limit of quantification (LOQ)**

The limit of detection (LOD) and limit of quantification (LOQ) of the drug was calculated using the following equation as per International Conference Harmonization (ICH) guidelines.

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

LOD for Glecaprevir was found to be 0.3 µg/mL and LOQ for Glecaprevir was found to be 1 µg/ml, LOD for Pibrentasvir was found to be 0.12 µg/ml and LOQ for Pibrentasvir was found to be 0.40 µg/ml.

**Robustness**

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, and Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied from 0.9 ml/min to 1.1 ml/min.
Standard solutions of 100 ppm of Glecaprevir and 40 ppm of Pibrentasvir were prepared and analysed using the varied and method flow rates.

On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence, it indicates that the method is robust even with changes in the flow rate ±10%.

B. The variation of organic phase ratio from (23.814:76.186) to (29.1114:70.886).

A standard solution of 100 ppm of Glecaprevir and 40 ppm of Pibrentasvir was prepared and analysed using the varied mobile phase ratio.

**System suitability**

The tailing factor for the peaks due to Glecaprevir and Pibrentasvir in standard solution should not be more than 2.0.
The theoretical plates for the Glecaprevir and Pibrentasvir peaks in standard solution should not exceed 2000.
Resolution for the Glecaprevir and Pibrentasvir peaks in standard solution should not be less than 2. [6,7]

**Degradation Studies:**

*Stock solution preparation*: Accurately weigh and transfer 242 mg of Glecaprevir and Pibrentasvir sample into a 100 mL clean, dry volumetric flask; add diluent and sonicate it for up to 30 minutes to dissolve it completely and make volume up to the mark with the same solvent.

*Acid degradation*: Pipette 5 ml of the above solution into a 50 ml vacuum flask, followed by 1 ml of 1N HCl and leave it for 15 min. After 15 minutes, neutralised with 1 N NaOH and diluted to 50 ml with diluent. Filter the solution using 0.45-micron syringe filters and transfer it to the vial.

*Alkali degradation*: Pipette 5 ml of the above solution into a 50 ml vacuum flask, followed by 1 ml of 1N NaOH and leave it for 15 minutes. After 15 minutes, neutralised with 1 N HCl and diluted to 50 ml with diluent. Filter the solution using 0.45-micron syringe filters and transfer it to the vial.

*Thermal degradation*: Glecaprevir and Pibrentasvir sample was taken in a Petri dish and kept in a Hot air oven at 105 $^0$C for 3 hours. Then, the sample was taken and diluted with diluents injected into HPLC and analysed.

*Reduction degradation*: Pipette 5 ml of the above solution into a 50 ml vacuum flask; 1 ml of 10-cent Sodium bisulphate was added to the flask and left for 15 minutes. After 15 minutes, make the flask up to the mark, filter the solution using 0.45-micron syringe filters and transfer it to vials.

*Peroxide degradation*: Pipette 5 ml of the above solution into a 50 ml vacuum flask; 1 ml of 3 per cent w/v hydrogen peroxide was added to the flask and left for 15 minutes. After 15 minutes, make the flask up to the mark, filter the solution using 0.45-micron syringe filters and transfer it to vials.

*Photolytic degradation*: Glecaprevir and Pibrentasvir sample was placed in a photostability chamber for 3 hours. Then, the sample was taken and diluted with diluents injected into HPLC and analysed.

*Hydrolysis degradation*: Pipette 5 ml of the above solution into a 50 ml vacuum flask, 3 ml of HPLC water added to the flask, and leave it for 15 minutes. After 15 minutes, make the flask up to the mark, filter the solution using 0.45-micron syringe filters and transfer it to vials. [8,9]

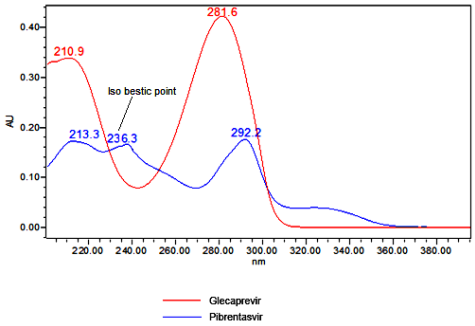


**Figure 1: PDA - Spectrum of Glecaprevir and Pibrentasvir**

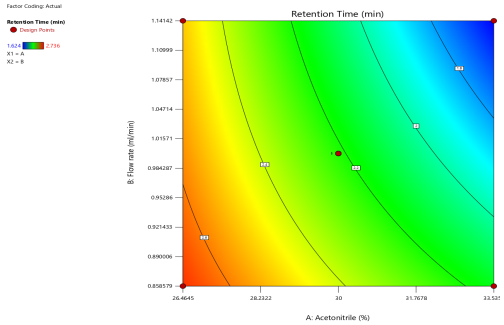


**Figure 2: Contour plots for retention time as a function of mobile phase and flow rate**

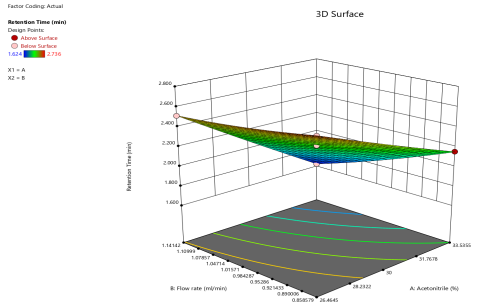


**Figure 3: Response surface for retention time as a function of mobile phase and flow rate**

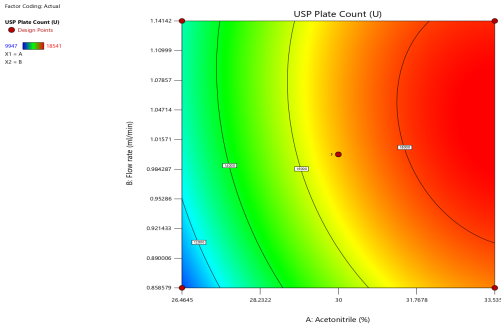


**Figure 4: Contour plots for plate count as a function of mobile phase and flow rate**

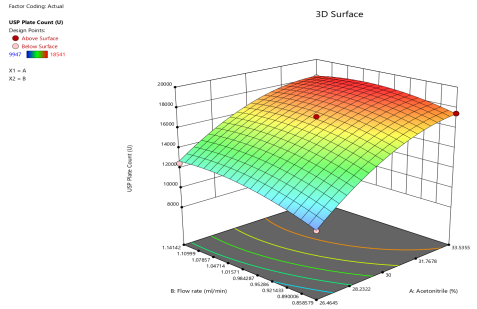


**Figure 5: Response surface for plate count as a function of mobile phase and flow rate**

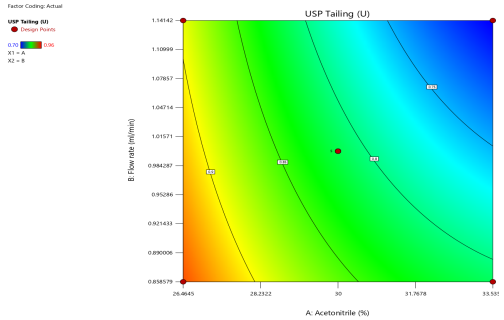


**Figure 6: Contour plots for tailing as a function of mobile phase and flow rate**

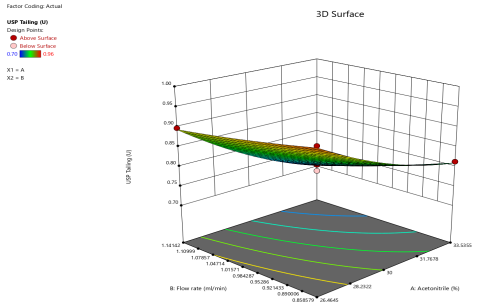


**Figure 7: Response surface for tailing as a function of mobile phase and flow rate**

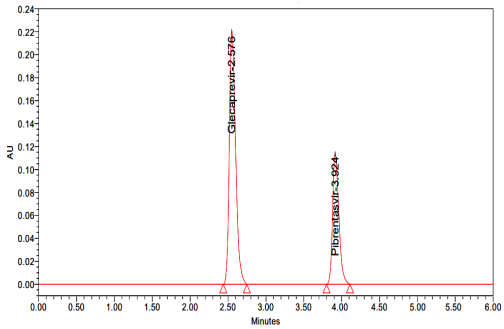


**Figure 8: Optimised chromatogram**

**Table 1: Optimised chromatographic conditions**

| Parameters | Observation |
|---|---|
| Instrument used | Waters HPLC with an autosampler and PDA detector. |
| Injection volume | 20 µl |
| Mobile Phase | Acetonitrile: 0.1% TFA (26.464:73.536) |
| Column | Kinetex RP-18 Column (100x4.6 mm, 2.6 µ) |
| Detection Wavelength | 236 nm |
| Flow Rate | 1 mL/min |
| Runtime | 6 minutes |
| Mode of separation | Isocratic mode |

**Table 2: Central composite design for screening of method parameters**

| Std | Run | Factor 1<br>A: Acetonitrile % | Factor 2<br>B: Flow rate ml/min | Response 1<br>Retention Time min | Response 2<br>USP Plate Count U | Response 3<br>USP Tailing U |
|---|---|---|---|---|---|---|
| 8 | 1 | 30 | 1.2 | 2.015 | 16628 | 0.76 |
| 4 | 2 | 33.5355 | 1.14142 | 1.624 | 18076 | 0.70 |
| 13 | 3 | 30 | 1 | 2.217 | 17152 | 0.83 |
| 7 | 4 | 30 | 0.8 | 2.504 | 14265 | 0.88 |
| 6 | 5 | 35 | 1 | 1.728 | 18541 | 0.74 |
| 12 | 6 | 30 | 1 | 2.217 | 16852 | 0.82 |
| 5 | 7 | 25 | 1 | 2.736 | 9947 | 0.96 |
| 11 | 8 | 30 | 1 | 2.216 | 16872 | 0.79 |
| 1 | 9 | 26.4645 | 0.858579 | 2.684 | 10516 | 0.95 |
| 9 | 10 | 30 | 1 | 2.216 | 16872 | 0.82 |
| 3 | 11 | 26.4645 | 1.14142 | 2.513 | 12505 | 0.90 |
| 2 | 12 | 33.5355 | 0.858579 | 2.156 | 17459 | 0.81 |
| 10 | 13 | 30 | 1 | 2.220 | 16852 | 0.82 |

**Table 3: Solutions found**

| Solutions found | | Glecaprevir and Pibrentasvir | | | | | |
|---|---|---|---|---|---|---|---|
| Number | Acetonitrile | Flow rate | Retention Time | USP Plate Count | USP Tailing | Desirability | |
| 1 | 26.464 | 1.000 | 2.579 | 12446.923 | 0.916 | 0.927 | Selected |

**Table 4: ANOVA for Quadratic model Response 1: Retention Time**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 1.29 | 5 | 0.2578 | 42757.28 | < 0.0001 | significant |
| A-Acetonitrile | 1.01 | 1 | 1.01 | 1.676E+05 | < 0.0001 | |
| B-Flow rate | 0.2428 | 1 | 0.2428 | 40273.06 | < 0.0001 | |
| AB | 0.0327 | 1 | 0.0327 | 5426.81 | < 0.0001 | |
| $A^2$ | 0.0004 | 1 | 0.0004 | 58.21 | 0.0001 | |
| $B^2$ | 0.0031 | 1 | 0.0031 | 507.17 | < 0.0001 | |
| **Residual** | 0.0000 | 7 | 6.029E-06 | | | |
| Lack of Fit | 0.0000 | 3 | 9.887E-06 | 3.15 | 0.1482 | not significant |
| Pure Error | 0.0000 | 4 | 3.136E-06 | | | |
| **Cor Total** | 1.29 | 12 | | | | |
| **Summary of the quadratic model** | | | | | | |
| **Std. Dev.** | 0.0025 | | **$R^2$** | 1.0000 | **Predicted $R^2$** | 0.9998 |
| **Mean** | 2.23 | | **Adjusted $R^2$** | 0.9999 | **Adequate Precision** | 664.6349 |

**Table 5: ANOVA for Quadratic model Response 2: USP Plate Count**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 9.700E+07 | 5 | 1.940E+07 | 573.20 | < 0.0001 | significant |
| A-Acetonitrile | 7.606E+07 | 1 | 7.606E+07 | 2247.51 | < 0.0001 | |
| B-Flow rate | 4.422E+06 | 1 | 4.422E+06 | 130.66 | < 0.0001 | |
| AB | 4.704E+05 | 1 | 4.704E+05 | 13.90 | 0.0074 | |
| $A^2$ | 1.343E+07 | 1 | 1.343E+07 | 396.88 | < 0.0001 | |
| $B^2$ | 4.323E+06 | 1 | 4.323E+06 | 127.74 | < 0.0001 | |
| **Residual** | 2.369E+05 | 7 | 33844.06 | | | |
| Lack of Fit | 1.692E+05 | 3 | 56409.48 | 3.33 | 0.1376 | not significant |
| Pure Error | 67680.00 | 4 | 16920.00 | | | |
| **Cor Total** | 9.723E+07 | 12 | | | | |
| **Summary of the quadratic model** | | | | | | |
| **Std. Dev.** | 183.9675 | | **$R^2$** | 0.9976 | **Predicted $R^2$** | 0.9865 |
| **Mean** | 15579.7532 | | **Adjusted $R^2$** | 0.9958 | **Adequate Precision** | 69.7825 |

**Table 6: ANOVA for Quadratic Model Response 3: USP Tailing**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 0.0696 | 5 | 0.0139 | 101.30 | < 0.0001 | significant |
| A-Acetonitrile | 0.0535 | 1 | 0.0535 | 389.35 | < 0.0001 | |
| B-Flow rate | 0.0127 | 1 | 0.0127 | 92.27 | < 0.0001 | |
| AB | 0.0009 | 1 | 0.0009 | 6.35 | 0.0398 | |
| $A^2$ | 0.0025 | 1 | 0.0025 | 18.43 | 0.0036 | |
| $B^2$ | 0.0001 | 1 | 0.0001 | 0.7584 | 0.4127 | |
| **Residual** | 0.0010 | 7 | 0.0001 | | | |
| Lack of Fit | 0.0001 | 3 | 0.0000 | 0.1267 | 0.9394 | not significant |
| Pure Error | 0.0009 | 4 | 0.0002 | | | |
| **Cor Total** | 0.0706 | 12 | | | | |
| **Summary of the quadratic model** | | | | | | |
| **Std. Dev.** | 0.0117 | | **$R^2$** | 0.9864 | **Predicted $R^2$** | 0.9721 |
| **Mean** | 0.8290 | | **Adjusted $R^2$** | 0.9766 | **Adequate Precision** | 33.5546 |

**Table 7: Assay of Glecaprevir and Pibrentasvir**

| Brand | Drug | Avg sample area (n=2) | Std. Conc. (µg/ml) | Sample Conc. (µg/ml) | Label amount (mg) | Std purity | Amount found (µg/ml) | % assay |
|---|---|---|---|---|---|---|---|---|
| - | Glecaprevir | 3848467 | 100 | 100 | 100 | 99.8 | 100.28 | 100.3 |
| | Pibrentasvir | 1189830 | 40 | 40 | 40 | 99.7 | 39.85 | 99.6 |

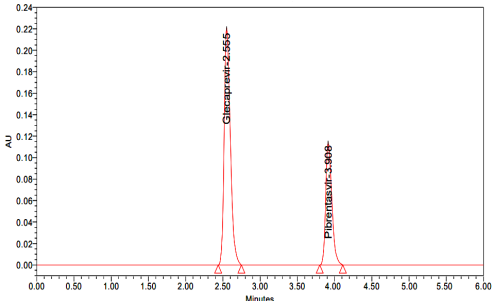


**Figure 9: Chromatogram of Assay-1**

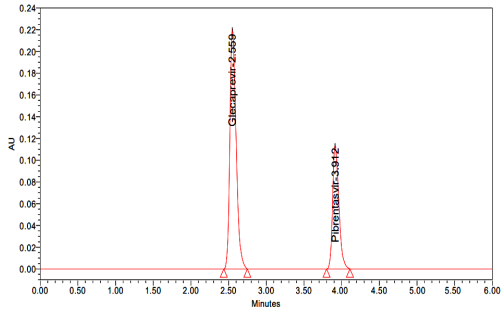


**Figure 10: Chromatogram of Assay-2**

**Table 8: Results of linearity for Glecaprevir and Pibrentasvir**

| S.NO | Glecaprevir | | Pibrentasvir | |
|---|---|---|---|---|
| | Conc.(µg/ml) | Peak area | Conc.(µg/ml) | Peak area |
| 1 | 25.00 | 969383 | 10.00 | 282624 |
| 2 | 50.00 | 1958638 | 20.00 | 597832 |
| 3 | 75.00 | 2924572 | 30.00 | 922173 |
| 4 | 100.00 | 3841582 | 40.00 | 1194620 |
| 5 | 125.00 | 4750920 | 50.00 | 1467913 |
| 6 | 150.00 | 5649679 | 60.00 | 1777425 |
| **Regression equation** | y = 37707.22x +42640.39 | | y =29641.58x+ 2550.89 | |
| **Slope** | 37707.22 | | 29641.58 | |
| **Intercept** | 42640.39 | | 2550.89 | |
| **$R^2$** | 0.99982 | | 0.99969 | |

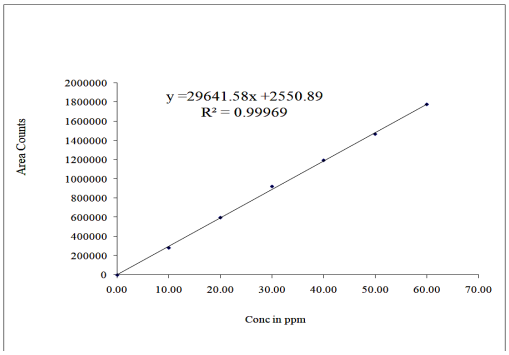


**Figure 11: Calibration curve for Pibrentasvir**

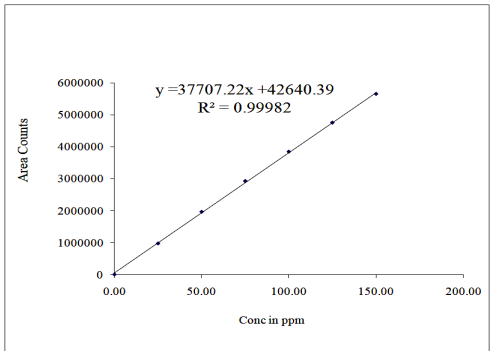


**Figure 12: Calibration curve for Glecaprevir**

**Table 9: Accuracy results of Glecaprevir by RP-HPLC method**

| % Concentration (at specification Level) | Area | Amount Added (mg) | Amount Found (mg) | % Recovery | Mean Recovery | %RSD |
|---|---|---|---|---|---|---|
| 50% | 1922882 | 50 | 50.1 | 100.2 | 100.4 | 0.71 |
| | 1940987 | 50 | 50.57 | 101.1 | | |
| | 1913964 | 50 | 49.87 | 99.7 | | |
| 100% | 3853619 | 100 | 100.41 | 100.4 | 100.1 | 0.32 |
| | 3829776 | 100 | 99.79 | 99.8 | | |
| | 3836913 | 100 | 99.97 | 100.0 | | |
| 150% | 5709614 | 150 | 148.77 | 99.2 | 99.7 | 0.57 |
| | 5773265 | 150 | 150.43 | 100.3 | | |
| | 5727741 | 150 | 149.24 | 99.5 | | |

**Table 10: The Accuracy results for Pibrentasvir by RP-HPLC method**

| % Concentration (at specification Level) | Area | Amount Added (mg) | Amount Found (mg) | % Recovery | Mean Recovery | %RSD |
|---|---|---|---|---|---|---|
| 50% | 599127 | 20 | 20.07 | 100.4 | 99.7 | 0.54 |
| | 593281 | 20 | 19.87 | 99.4 | | |
| | 594108 | 20 | 19.90 | 99.5 | | |
| 100% | 1189264 | 40 | 39.83 | 99.6 | 99.8 | 0.25 |
| | 1192791 | 40 | 39.95 | 99.9 | | |
| | 1195326 | 40 | 40.03 | 100.1 | | |
| 150% | 1788465 | 60 | 59.9 | 99.8 | 99.7 | 0.43 |
| | 1790870 | 60 | 59.98 | 100.0 | | |
| | 1776553 | 60 | 59.50 | 99.2 | | |

**Table 11: Method Precision for Glecaprevir and Pibrentasvir by RP-HPLC method**

| S. No. | Area for Glecaprevir | Area for Pibrentasvir |
|---|---|---|
| 1 | 3825123 | 1198276 |
| 2 | 3861249 | 1185840 |
| 3 | 3835631 | 1196354 |
| 4 | 3848103 | 1192136 |
| 5 | 3872867 | 1178209 |
| 6 | 3854147 | 1189823 |
| **Average** | 3849520 | 1190106 |
| **Standard Deviation** | 17286.913 | 7344.723 |
| **%RSD** | 0.45 | 0.62 |

**Table 12: Intermediate Precision (Day variation) for Glecaprevir and Pibrentasvir by RP-HPLC method**

| S. No. | Area for Glecaprevir | Area for Pibrentasvir |
|---|---|---|
| 1 | 3846721 | 1188072 |
| 2 | 3803216 | 1193217 |
| 3 | 3846241 | 1190328 |
| 4 | 3867548 | 1186219 |
| 5 | 3858452 | 1194382 |
| 6 | 3850543 | 1199201 |
| **Average** | 3845454 | 1191903 |
| **Standard Deviation** | 22222.799 | 4703.145 |
| **%RSD** | 0.58 | 0.39 |

**Table 13: System precision table of Glecaprevir and Pibrentasvir**

| S. No | Concentration Glecaprevir (µg/ml) | Area of Glecaprevir | Concentration of Pibrentasvir (µg/ml) | Area of Pibrentasvir |
|---|---|---|---|---|
| 1. | 100 | 3842651 | 40 | 1197235 |
| 2. | 100 | 3826772 | 40 | 1191208 |
| 3. | 100 | 3838974 | 40 | 1188335 |
| 4. | 100 | 3844609 | 40 | 1199276 |
| 5. | 100 | 3827564 | 40 | 1194523 |
| 6. | 100 | 3846812 | 40 | 1195281 |
| Mean | 3837897 | | 1194310 | |
| S.D | 8704.31 | | 3988.18 | |
| %RSD | 0.23 | | 0.33 | |

**Table 14: Sensitivity parameters (LOD and LOQ) by RP-HPLC**

| Name of drug | LOD(µg/ml) | s/n | LOQ(µg/ml) | s/n |
|---|---|---|---|---|
| Glecaprevir | 0.30 | 3 | 1.00 | 10 |
| Pibrentasvir | 0.12 | 3 | 0.40 | 10 |

**Table 15: Robustness results of Glecaprevir by RP-HPLC**

| Parameter | Glecaprevir | | | | | |
|---|---|---|---|---|---|---|
| | Condition | Retention time (minutes) | Peak area | Tailing | Plate count | % RSD |
| Flow rate Change (mL/min) | Less flow (0.9 ml) | 2.713 | 3561578 | 0.95 | 12535 | 0.36 |
| | Actual (1.0 ml) | 2.576 | 3842651 | 0.90 | 12452 | 0.23 |
| | More flow (1.1 ml) | 2.438 | 3951487 | 0.85 | 12411 | 0.50 |
| Organic Phase change | Less Org (23.814:76.186) | 2.872 | 3653640 | 0.98 | 12597 | 0.41 |
| | Actual (26.464:73.536) | 2.577 | 3826772 | 0.91 | 12447 | 0.23 |
| | More Org (29.1114:70.886) | 2.285 | 4054324 | 0.87 | 12364 | 0.36 |

**Table 16: Robustness results of Pibrentasvir by RP-HPLC**

| Parameter | Pibrentasvir | | | | | | |
|---|---|---|---|---|---|---|---|
| | Condition | Retention time(minutes) | Peak area | Resolution | Tailing | Plate count | % RSD |
| Flow rate Change (mL/min) | Less flow (0.9 ml) | 4.098 | 1021754 | 5.15 | 0.92 | 19876 | 0.41 |
| | Actual (1.0 ml) | 3.924 | 1197235 | 4.87 | 0.85 | 19819 | 0.33 |
| | More flow (1.1 ml) | 3.756 | 1328923 | 4.39 | 0.81 | 19773 | 0.85 |
| Organic Phase change | Less Org (23.814:76.186) | 4.222 | 998274 | 4.93 | 0.95 | 19941 | 0.10 |
| | Actual (26.464:73.536) | 3.921 | 1191208 | 4.84 | 0.87 | 19812 | 0.33 |
| | More Org (29.1114:70.886) | 3.843 | 1489263 | 5.96 | 0.84 | 19682 | 0.47 |

**Table 17: System suitability parameters for Glecaprevir and Pibrentasvir**

| Parameter | Glecaprevir | Pibrentasvir |
|---|---|---|
| Retention time | 2.576 | 3.924 |
| Plate count | 12452 | 19819 |
| Tailing factor | 0.90 | 0.87 |
| Resolution | ---- | 4.87 |
| % RSD | 0.23 | 0.33 |

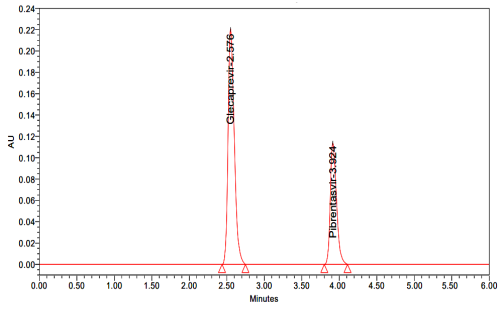


**Figure 13: Chromatogram of standard**

**Table 18: Forced Degradation results for Glecaprevir and Pibrentasvir**

| Results: % Degradation results | Glecaprevir | | | | | Pibrentasvir | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Area | % Assay | % Deg | Purity Angle | Purity Threshold | Area | % Assay | % Deg | Purity Angle | Purity Threshold |
| **Control** | 3835475 | 100 | 0 | 2.134 | 10.828 | 1194478 | 100 | 0 | 0.987 | 8.723 |
| **Acid** | 3358219 | 87.5 | 12.5 | 2.124 | 10.854 | 1065931 | 89.3 | 10.7 | 0.925 | 8.751 |
| **Alkali** | 3402440 | 88.7 | 11.3 | 2.131 | 10.831 | 1056178 | 88.4 | 11.6 | 0.947 | 8.709 |
| **Peroxide** | 3268711 | 85.2 | 14.8 | 2.108 | 10.827 | 1026525 | 86.0 | 14.0 | 0.939 | 8.798 |
| **Reduction** | 3732232 | 97.3 | 2.7 | 2.141 | 10.852 | 1152477 | 96.5 | 3.5 | 0.909 | 8.716 |
| **Thermal** | 3754823 | 97.9 | 2.1 | 2.152 | 10.809 | 1164373 | 97.5 | 2.5 | 0.918 | 8.793 |
| **Photolytic** | 3793047 | 98.9 | 1.1 | 2.175 | 10.834 | 1174017 | 98.3 | 1.7 | 0.944 | 8.758 |
| **Hydrolysis** | 3439588 | 89.7 | 10.3 | 2.119 | 10.808 | 1081754 | 90.6 | 9.4 | 0.952 | 8.711 |

## RESULTS

**Determination of Working Wavelength ($\lambda_{max}$):** Figure 1

**Selection of chromatographic conditions:** Acetonitrile and 0.1% TFA (26.464:73.536) were used as the mobile phase for chromatographic separation, with a 1 mL/min flow rate. The column was kept at a temperature of 30 °C, and detection was done at a wavelength of 236 nm (Table 1).

The **Model F-value** of 42757.28 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. **P-values** less than 0.0500 indicate model terms are significant. In this case, A, B, AB, $A^2$, and $B^2$ are

significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The **Lack of Fit F-value** of 3.15 implies the Lack of Fit is not significant relative to the pure error. There is a 14.82% chance that a Lack of Fit F-value this large could occur due to noise. A non-significant lack of fit is good -- we want the model to fit. The Predicted $R^2$ of 0.9998 is in reasonable agreement with the Adjusted $R^2$ of 0.9999; i.e. the difference is less than 0.2. Adequate Precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Your ratio of 664.635 indicates an adequate signal. This model can be used to navigate the design space (Table 4).

**3D and Contour graph for the retention time:** The relationship between the flow rate and percentage of the mobile phase was examined using contour and three-dimensional graphs at a constant temperature of 30 °C. According to the graph, better retention time can be obtained at lower flow rates and lower levels of the mobile phase (Figures 2 and 3).

The **Model F-value** of 573.20 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. **P-values** less than 0.0500 indicate model terms are significant. In this case, A, B, AB, $A^2$, and $B^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The **Lack of Fit F-value** of 3.33 implies the Lack of Fit is not significant relative to the pure error. There is a 13.76% chance that a Lack of Fit F-value this large could occur due to noise. A non-significant lack of fit is good -- we want the model to fit. The Predicted $R^2$ of 0.9865 is in reasonable agreement with the Adjusted $R^2$ of 0.9958; i.e. the difference is less than 0.2. Adequate Precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Your ratio of 69.783 indicates an adequate signal. This model can be used to navigate the design space (Table 5).

**3D and Contour graph for the plate count:** The relationship between the flow rate and percentage of the mobile phase was examined using contour and three-dimensional graphs at a constant temperature of 30 °C. From the graph, a lower flow rate and lower level of the mobile phase can give a higher number of theoretical plates (Figure 4 and 5).

The **Model F-value** of 101.30 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. **P-values** less than 0.0500 indicate model terms are significant. A, B, AB, and $A^2$ are significant model terms in this case. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The **Lack of Fit F-value** of 0.13 implies the Lack of Fit is not significant relative to the pure error. There is a 93.94% chance that a Lack of Fit F-value this large could occur due to noise. A non-significant lack of fit is good -- we want the model to fit. The Predicted $R^2$ of 0.9721 is in reasonable agreement with the Adjusted $R^2$ of 0.9766; i.e. the difference is less than 0.2. Adequate Precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Your ratio of 33.555 indicates an adequate signal. This model can be used to navigate the design space (Table 6).

**3D and Contour graph for the tailing:** The relationship between the flow rate and percentage of the mobile phase was examined using contour and three-dimensional graphs at a constant temperature of 30 °C. From the graph, a lower flow rate and lower level of the mobile phase can give a lesser tailing (Figure 6 and 7).

**Analytical method validation (HPLC):** The method was validated for its linearity, accuracy, precision, and specificity. Method validation was carried out as per ICH guidelines.

**Specificity:** Retention times of Glecaprevir and Pibrentasvir were 2.576 minutes and 3.924 minutes, respectively. We did not find interfering peaks in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific. (Figure 8)

**Assay:** (Table 7, Figure 9 and 10)

**Linearity:** (Table 8, Figures 11 and 12)

**Accuracy:** (Tables 9 and 10)

Three levels of Accuracy samples were prepared by the standard addition method. Triplicate injections were given for each level of accuracy, and mean % Recovery was obtained as 100.1% and 99.7% for

**Precision**
**Repeatability**
**Acceptance Criteria:** The % RSD for the area of six standard injection results should not be more than 2%**.** (Table 11)

**Intermediate precision (Day_ Day Precision):** (Table 12)

**Acceptance Criteria:** The % RSD for the area of six standard injection results should not be more than 2%.

**System Precision:** Six injections were given from a single volumetric flask of working standard solution, and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.23% and 0.33%, respectively, for Glecaprevir and Pibrentasvir. As the limit of Precision was less than "2", the system precision was passed in this method. (Table 13)

**Limit of detection (LOD) and limit of quantification (µg/ml)** (Table 14)

**Robustness:** (Tables 15 and 16)

**System suitability:** All the system suitability parameters were within the range and satisfactory as per ICH guidelines. (Table 17, Figure 13)

**Acceptance Criteria:** According to ICH guidelines, the plate count should be more than 2000, the tailing factor should be less than 2, and the resolution must be more than 2. All the system-suitable parameters were passed and were within the limits.

**Degradation studies:** Forced degradation studies of Glecaprevir and Pibrentasvir in various conditions such as acidic, basic, peroxide, reduction, thermal, photolytic, and hydrolytic were observed. The Glecaprevir and Pibrentasvir were stable under reduction, thermal, photolytic and hydrolytic conditions. The drug showed significant degradation in the acidic (12.5 and 10.7%), alkali (11.3 and 11.6%) and peroxide (14.8 and 14.0%) conditions for Glecaprevir and Pibrentasvir, respectively (Table 18).

## DISCUSSION

The mobile phase used was chromatographic separation using acetonitrile and 0.1% TFA (26.464:73.536) at a flow rate of 1 mL/min and a pH of 2.25. At 236 nm, detection was conducted while the column temperature was kept at ambient. Glecaprevir and Pibrentasvir had respective retention times of 2.462 and 3.771 minutes.

The design expert programme used the numerical optimisation method to assess the model's accuracy. The design expert recommended chromatographic settings of pH 2.25, ambient temperature, and mobile phase ratio of 26.464:73.536 with desirability of 0.927.

Under these circumstances, the model projected that the Glecaprevir retention time would be 2.462 minutes, whereas the Pibrentasvir retention time would be 3.771 minutes. The plate counts were 10541 and 19845, the tailing was 0.95 and 0.86, and the resolution was 4.14. The HPLC system was operated and maintained under the same experimental conditions.

The AQbD ANOVA results showed that all responses fell within the 95% confidence interval statistical limit, indicating a high degree of agreement between the observed and projected data. The optimised method was validated using ICH guidelines. The results showed that the method developed is linear, accurate, precise, robust, repeatable, and reproducible. Forced degradation studies of Glecaprevir and Pibrentasvir in various conditions such as acidic, basic, peroxide, reduction, thermal, photolytic, and hydrolytic were observed. The Glecaprevir and Pibrentasvir were stable under reduction, thermal, photolytic and hydrolytic conditions. The drug showed significant degradation in the acidic (12.5 and 10.7%), alkali (11.3 and 11.6%) and peroxide (14.8 and 14.0%) conditions for Glecaprevir and Pibrentasvir, respectively. The assay results also confirmed the percentage purity of 100.3% and 99.6 % for Glecaprevir and Pibrentasvir, respectively. Therefore, it is clear that more than any other stress state, the acid, alkali, and peroxide conditions significantly affect the stability of Glecaprevir and Pibrentasvir.

## CONCLUSION

Using RP-HPLC and design expert software, AQbD created a straightforward, accurate, and reliable analytical approach for simultaneous quantifying Glecaprevir and Pibrestasvir. Thirteen trials based on a central composite design were conducted. The responses from the trails were once more fed into Design Expert, where analytical parameters such as tailing, theoretical plates, and resolution were examined. The optimal approach was chosen based on the results of AQbD and verified in accordance with ICH guidelines. Additional tests on forced degradation were conducted, confirming the stability. The discovered method can be used for bulk and formulation estimation in the pharmaceutical industry.

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