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**R E S E A R C H A R T I C L E**

# Development of new simultaneous RP-HPLC method for the estimation of Glecapravir and Pibrentasvir in tablet dosage form

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**A B S T R A C T**

The aim present research work to development and validation of RP-HPLC method for the simultaneous estimation of Glecapravir and Piberentasvir. Chromatographic separation was evaluated by Xterra C18 column (250 X 4.6 mm, 5 µm) using the mobile phase consisting of 50% Water: 50% Acetonitrile and the mobile phase was pumped at a flow rate of 1.0 mL/min and detection was done by UV detector at 255 nm. The retention time of Glecapravir and Piberentasvir were found to be 2.205 min and 4.996 min. The linearity was obtained in the range of 100-500µg/ml for Glecapravir and 40-200 µg/ml for Piberentasvir with correlation coefficient was 0.999. The proposed method was found to be simple, accurate, precise, robust and cost effective. It can be applied for routine quality control analysis for simultaneous estimation of Glecapravir and Piberentasvir in pharmaceutical dosage forms.

**Keywords:** Glecapravir, Piberentasvir, RP-HPLC, Mobile phase, Acetonitrile, Retention time

**A R T I C L E I N F O**

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**CONTENTS**



## 1. Introduction

Glecaprevir is a direct acting antiviral agent and Hepatitis C virus (HCV) NS3/4A protease inhibitor that targets the the viral RNA replication. In combination with Pibrentasvir, glecaprevir is a useful therapy for patients who experienced therapeutic failure from other NS3/4A protease inhibitors. It demonstrates a high genetic barrier against resistance mutations of the virus. In cell cultures, the emergence of amino acid substitutions at NS3 resistance-associated positions A156 or D/Q168 in HCV genotype 1a, 2a or 3a replicons led to reduced susceptibility to glecaprevir. The combinations of amino acid substitutions at NS3 position Y65H and D/Q168 also results in greater reductions in

glecaprevir susceptibility, and NS3 Q80R in genotype 3a patients also leads to glecaprevir resistance[5].

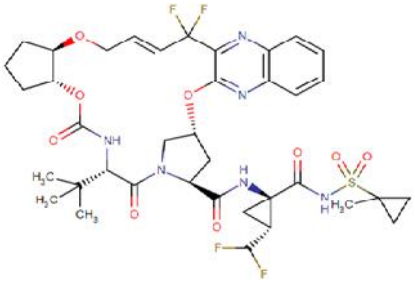

**Fig 1:** Chemical structure of Glecaprevir

Pibrentasvir is a direct acting antiviral agent and Hepatitis C virus (HCV) NS5A inhibitor that targets the the viral RNA replication and viron assembly. In combination with Glecaprevir, pibrentastiv is a useful therapy for patients who experienced therapeutic failure from other NS5A inhibitors. In cell cultures, the emergence of amino acid substitutions at known NS5A inhibitor resistance-associated positions in HCV genotype 1a, 2a or 3a replicons led to reduced susceptibility and resistance to pibrentasvir. Individual NS5A amino acid substitutions that reduced susceptibility to pibrentasvir include M28G or Q30D in a genotype 1a replicon and P32-deletion in a genotype 1b replicon[5].

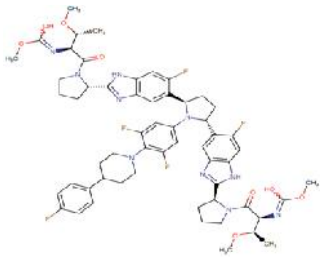

**Fig 2:** Chemical structure of Pibrentasvir

Glecapravir and Pibrentasvir are existing drugs. Literature reveals different methods for their analysis in their formulations6. But our present plan is to develop a new, simple, precise& accurate method for its analysis in formulation after a detailed study a new RP-HPLC method was decided to be developed and validated.

## 2. Materials and Methods

**Instruments used:** The following instruments are used to determination of Glecapravir and Piberentasvir.

**Table 1:** List of Instruments

| S. No | Instrument | Model |
|---|---|---|
| 1 | HPLC | WATERS, software: Empower, 2695 separation module.2487 UV detector |
| 2 | UV/VIS spectrophotometer | LABINDIA UV 3000$^+$ |
| 3 | pH meter | Adwa – AD 1020 |
| 4 | Weighing machine | Afcoset ER-200A |

**Chemicals used:**

The following chemicals are used to determination of Glecapravir and Piberentasvir.

**Table 2:** List of chemicals

| S. No | Chemical | Brand |
|---|---|---|
| 1 | Glecaprevir | Supplied by Pharmatrain |
| 2 | Pibrentasvir | Supplied by Pharmatrain |
| 3 | $KH_2PO_4$ | FINAR chemical LTD |
| 4 | Water and Methanol for HPLC | Standard solutions Ltd |
| 5 | Acetonitrile for HPLC | Standard solutions Ltd |
| 6 | WaterHPLC | MERCK |
| 7 | Ortho phosphoric acid | MERCK |

**HPLC Method Development**

**Mobile Phase Optimization:**

Initially the mobile phase tried was methanol: Ortho phosphoric acid buffer and Methanol: phosphate buffer, Acetonitrile: methanol with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to 0.1% OPA: Methanol in proportion 30: 70 v/v respectively.

**Wave length selection:**

UV spectrum of 10µg/ml Glecaprevir and 10 µg/ml Pibrentasvir in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 251 nm. At this wavelength both the drugs show good absorbance.

**Optimization of Column:**

The method was performed with various columns like C18 column Phenomenex column, YMC, and Inertsil ODS column. Xterra C18 column (4.6 x 150mm, 5µ) was found to be ideal as it gave good peak shape and resolution at 1 ml/min flow.

**Optimized Chromatographic Conditions:**

Instrument used **:**Waters HPLC with auto sampler and PDA detector.
Temperature **:** Ambient (25° C)
Mode of separation **:** Isocratic mode
Column **:** Xterra C18 column (4.6 x 150mm, 5µm)
Buffer **:** 0.1% OPA
Mobile phase **:** 30% buffer 70% Methanol
Flow rate **:** 1ml per min
Wavelength **:** 251 nm
Injection volume **:** 20 µl
Run time **:** 15 min.

**Preparation of mobile phase:**

Accurately measured 300 ml (30%) of above buffer and 700 ml of Methanol HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

**Standard Solution Preparation:**

Accurately weigh and transfer 25 mg of Glecaprevir and 10 mg of Pibrentasvir working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 3 ml

of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

**Sample Solution Preparation:** Accurately weigh and transfer equivalent to 25 mg of Glecaprevir and 10 mg of Pibrentasvir working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

**System Suitability:**

System suitability defined as integral part of many analytical procedures. The measured system suitability parameters like theoretical plates, tailing factor and resolution. Tailing factor for the peaks due to Glecaprevir and Pibrentasvir in Standard solution should not be more than 2.0 Theoretical plates for the Glecaprevir and Pibrentasvir peaks in Standard solution should not be less than 2000.Resolution for the Glecaprevir and Pibrentasvir peaks in standard solution should not be less than 2.

**Method Validation**

The developed method was statically validated according to ICH guidelines Q2(R1). The validation parameters like specificity, linearity, accuracy, precision, LOD & LOQ and robustness[10,11].

**Specificity:**

For Specificity Blank and Standard are injected into system. there is no any inteferece of any peak in blank with the retiontime of the analytical peaks.

**Linearity:**

The linearity was determined for Glecaprevir and Pibrentasvir five different concentrations were analyzed and calibration curve was constructed by plotting mean response factor against the respective concentration. The method was evaluated by determination of the correlation coefficient and intercept value. Linearity concentrations are made from in the range of 100-500µg/ml for Glecaprevir and 40-200 µg/ml for Pibrentasvir.

**Precision:**

The standard solution was injected for six times into the within the day and between the days and measured the area for all six Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

**Accuracy:**

Percentage mean recovery was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels such as 50%, 100% and 150% to the pre analysed sample formulation. From the amount of drug found, amount of drug recovered and percentage recovery were calculated which sense to conformation that the proposed method was accurate.

**LOD & LOQ:**

The sensitivity of the proposed method for measurement of Glecaprevir and Pibrentasvir were estimated in terms of Limit of Detection (LOD) and Limit of Quantification (LOQ).The LOD and LOQ were calculated by using the slope and SD of response (intercept).The mean slope value and SD of response were obtained after plotting six calibration curves.

**Robustness:**

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

**The flow rate was varied at 0.9 ml/min to 1.1ml/min:** Standard solution 300 ppm of Glecaprevir & 120 ppm of Pibrentasvir was prepared and analysed using the varied flow rates along with method flow rate. 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 by change in the flow rate ±10%.

**The Organic composition in the Mobile phase was varied from ±10%:** Standard solution 300 ppm of Glecaprevir & 120 ppm of Pibrentasvir was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10

**Degradation Studies:**

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Glecaprevir and Pibrentasvir using the proposed method. The standard solutions were subjected stress conditions like acid, base, thermal, photolytic and oxidative conditions. After some period of time observe the amount of drug degraded in selected stress conditions.

## 3. Results and discussion

**System suitability:**

The specificity of this method was determined by complete separation of Glecaprevir and Pibrentasvir. The tailing factor was less than 2% and resolution was satisfactory. The peaks obtained for sharp and have clear baseline separation. The system suitability parameters are given in Table 3 and fig 3.

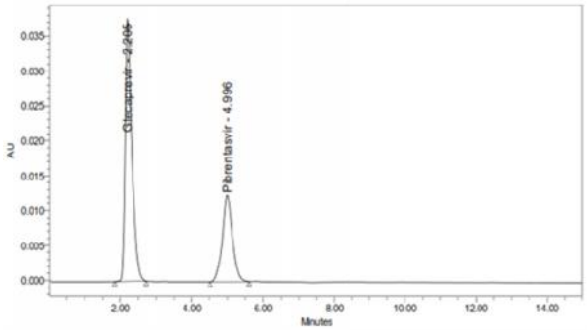


**Fig 3:** Chromatogram for system suitability

**Assay:**

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown in table 4.

Linearity:

The linearity range was found to lie from 100µg/ml to 500µg/ml of Glecaprevir, 40µg/ml to 200µg/ml of Pibrentasvir and chromatograms are shown in table 5 and fig 4 & 5.

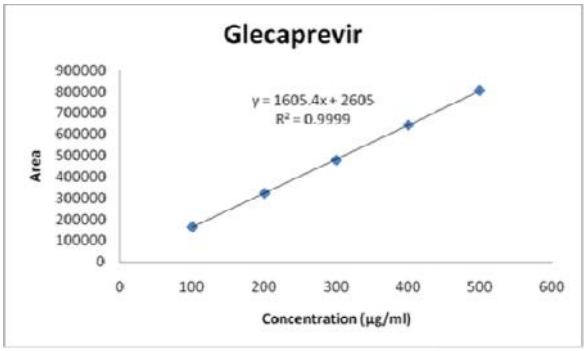


**Fig 4:**Calibration graph for Glecaprevir

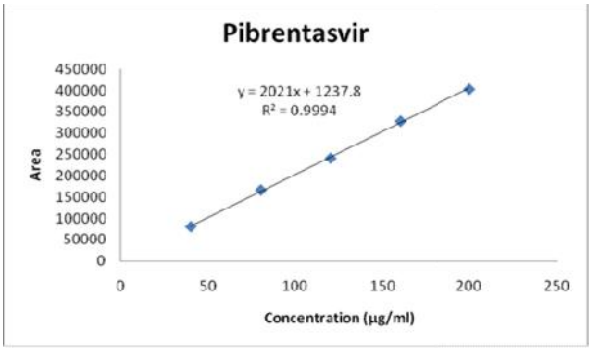


**Fig 5:** Calibration graph for Pibrentasvir

**Precision:** Precision of the method was carried out for both sample solutions as described under experimental work. The corresponding chromatograms and results are given in table 6.

**Accuracy:**

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated. The results were reported in table 7 & 8.

**Robustness:**

The standard and samples of Glecaprevir and Pibrentasvir were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. The data was given in table 10 & 11.

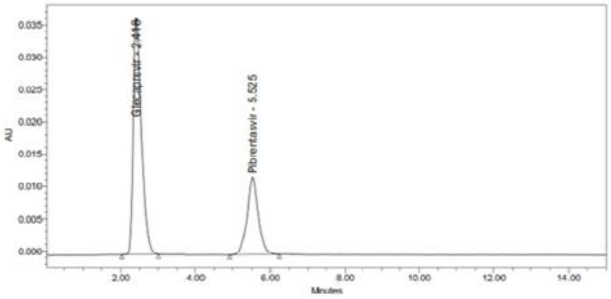


**Fig 6:**Chromatogram showing less flow

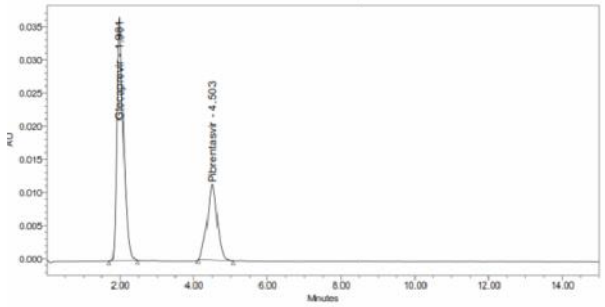


**Fig 7:** Chromatogram showing more flow

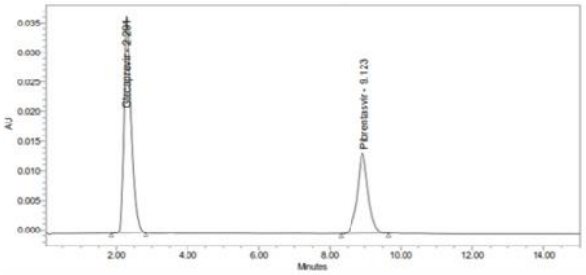


**Fig 8:**Chromatogram showing less organic composition

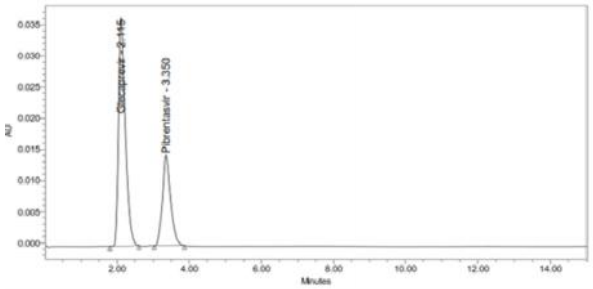


**Fig 9:**Chromatogram showing more organic composition

**Table 3:** Results of system suitability parameters

| S.No | Name | RT (min) | Area (µV sec) | Height (µV) | USP resolution | USP tailing | USP plate count |
|---|---|---|---|---|---|---|---|
| 1 | Glecaprevir | 2.205 | 478222 | 36550 | 6.76 | 1.58 | 3677.56 |
| 2 | Pibrentasvir | 4.996 | 239609 | 12483 | | 1.04 | 4683.62 |

**Table 2:**Results of Assay

| Drug | Label Claim (mg) | % Assay |
|---|---|---|
| Glecaprevir | 100 | 100.83 |
| Pibrentasvir | 40 | 100.23 |

**Table 3:** Area of different concentration of Glecaprevir and Pibrentasvir

| S. No | Glecaprevir | | Pibrentasvir | |
|---|---|---|---|---|
| | Concentration (µg/ml) | Area | Concentration (µg/ml) | Area |
| 1 | 100 | 165076 | 40 | 80057 |
| 2 | 200 | 323694 | 80 | 166200 |
| 3 | 300 | 480198 | 120 | 241067 |
| 4 | 400 | 645116 | 160 | 328200 |
| 5 | 500 | 807077 | 200 | 403253 |
| Slope (m) | 1605.4 | | 2021 | |
| Intercept (c) | 2605 | | 1237.8 | |
| Correlation coefficient ($R^2$) | 0.999 | | 0.999 | |

**Table 4:** Results for intraday and inter day precision

| Injection | Intraday precision | | Intermediate precision | |
|---|---|---|---|---|
| | Peak area of Glecaprevir | Peak area of Pibrentasvir | Peak area of Glecaprevir | Peak area of Pibrentasvir |
| Injection-1 | 483912 | 242261 | 482579 | 241793 |
| Injection-2 | 479899 | 241331 | 489171 | 241873 |
| Injection-3 | 487806 | 244327 | 482292 | 241291 |
| Injection-4 | 486352 | 243371 | 483377 | 241423 |
| Injection-5 | 482426 | 242500 | 483324 | 241328 |
| Injection-6 | 484893 | 241079 | 480775 | 242453 |
| Average | 484214.7 | 242478.2 | 483586.3 | 241693.5 |
| Std Dev | 2822.8 | 1227.7 | 2894.6 | 444.5 |
| %RSD | 0.6 | 0.5 | 0.6 | 0.2 |

**Table 7:** Accuracy (recovery) data for Glecaprevir

| %Concentration (at specification Level) | Area | Amount Added (mg) | Amount Found (mg) | % Recovery | Mean Recovery |
|---|---|---|---|---|---|
| 50% | 242024.3 | 12.5 | 12.57 | 100.54 | 100.40 |
| 100% | 484977.0 | 25 | 25.18 | 100.73 | |
| 150% | 721772.3 | 37.5 | 37.48 | 99.94 | |

*****Average of three determinations

**Table 8:**Accuracy (recovery) data for Pibrentasvir

| %Concentration (at specification Level) | Area | Amount Added (mg) | Amount Found (mg) | % Recovery | Mean Recovery |
|---|---|---|---|---|---|
| 50% | 120660.7 | 5 | 5.01 | 100.23 | 100.25 |
| 100% | 241976.0 | 10 | 10.05 | 100.50 | |
| 150% | 361205.0 | 15 | 15.00 | 100.02 | |

*Average of three determinations

**Table 9:** Results for LOD & LOQ

| Parameter | Drug name | Baseline noise(µV) | Signal obtained (µV) | S/N ratio |
|---|---|---|---|---|
| LOD | Glecaprevir | 58 | 173 | 2.98 |
| | Pibrentasvir | 58 | 174 | 3.00 |
| LOQ | Glecaprevir | 58 | 580 | 10.00 |
| | Pibrentasvir | 58 | 579 | 9.98 |

**Table 10:** Results for variation in flow for Glecaprevir and Pibrentasvir

| S. No | Flow Rate (ml/min) | System Suitability Results | | | | |
|---|---|---|---|---|---|---|
| | | Glecaprevir | | Pibrentasvir | | USP Resolution |
| | | USP Plate Count | USP Tailing | USP Plate Count | USP Tailing | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 0.9 | 3672.96 | 1.59 | 4701.86 | 1.04 | 6.84 |
| 2 | 1.0 | 3678.77 | 1.57 | 4652.35 | 1.04 | 6.71 |
| 3 | 1.1 | 3574.36 | 1.46 | 4388.51 | 1.01 | 6.20 |

* Results for actual flow (1.0ml/min) have been considered from Assay standard.

**Table 11:** Results for variation in mobile phase composition for Glecaprevir and Pibrentasvir

| S. No | Variation in mobile phase | System Suitability Results | | | | |
|---|---|---|---|---|---|---|
| | | Glecaprevir | | Pibrentasvir | | USP Resolution |
| | | USP Plate Count | USP Tailing | USP Plate Count | USP Tailing | |
| 1 | 10% less | 3668.63 | 1.45 | 4446.54 | 0.83 | 12.51 |
| 2 | Actual * | 3678.77 | 1.57 | 4652.35 | 1.04 | 6.71 |
| 3 | 10% more | 3575.02 | 1.51 | 4051.10 | 1.20 | 3.23 |

* Results for actual Mobile phase composition have been consideredfrom Accuracy standard.

**Table 12:** Results for Stability of Glecaprevir andPibrentasvir

| Sample Name | Glecaprevir | | Pibrentasvir | |
|---|---|---|---|---|
| | Area | % Degraded | Area | % Degraded |
| Standard | 480497 | - | 240280 | - |
| Acid | 446911 | 6.99 | 230160 | 4.21 |
| Base | 453105 | 5.70 | 222491 | 7.40 |
| Peroxide | 427335 | 11.06 | 213084 | 11.32 |
| Thermal | 421312 | 12.32 | 207446 | 13.66 |

## 4. Conclusions

The estimation of Glecaprevir and Pibrentasvir was done by RP-HPLC. The assay of Glecaprevir and Pibrentasvir was performed with tablets and the % assay was found to be 100.83 and 100.23 which shows that the method is useful for routine analysis. The linearity was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The method show precision 0.6 and 0.5 for Glecaprevir and Pibrentasvir which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.6 and 0.2 for Glecaprevir and Pibrentasvir which shows that the method is repeatable when performed in different days also. The total recovery was found to be 100.40% and 100.25% for Glecaprevir and Pibrentasvir. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The robustness limit for mobile phase variation and flow rate variation are well within the limit, the % degradation results are in limits. Which shows that the method is having good system suitability and precision under given set of conditions.

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