A New Force Indicating RP-HPLC Method Development and Validation for the Simultaneous Estimation of Pibrentasvir and Glecaprevir in Bulk and its Tablet Dosage Form

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ABSTRACT

Background: A simple reversed phase high performance liquid chromatographic method was developed for the simultaneous estimation of the Pibrentasvir and Glecaprevir in bulk and its solid dosage form. Materials and Methods: The method was developed on water’s C18 column capacitate 250x4.6 mm, 5 µm particle size, wavelength was fixed at 226 nm with photo diode array detection. The mobile phase was consisted mixture of 0.5 mM Ortho phosphoric acid buffer: Acetonitrile in the ratio of 75:25 v/v, pH 4.3 was adjusted with hydrochloric acid and flow of mobile phase through the column was maintained 1mL/min. Results: The retention times of Pibrentasvir and Glecaprevir were found to be 3.04 and 4.45 min respectively. The method was statistically validated with concern of precision, linearity, range and robustness of method was found for Pibrentasvir and Glecaprevir. The above method was afforded excellent percentage recovery and purity. The method was sensitive, precise, accurate, and robust, showed good linearity with different concentrations. The method is used for the routine analysis of Pibrentasvir and Glecaprevir in its bulk and pharmaceutical dosage form. Key words: Pibrentasvir, Glecaprevir, RP-HPLC, Ortho phosphoric acid buffer. Correspondence

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INTRODUCTION

The combination of Glecaprevir and Pibrentasvir was highly efficacious and well tolerated in patients with HCV genotype 1 infection and prior failure of directly acting antiviral containing therapy; RBV+so administration did not improve efficacy. Although direct acting antiviral therapies for chronic HCV infection have demonstrated high rates of sustained virologic response, virologic failure may still occur, potentially leading to the emergence of viral resistance, which can decrease the effectiveness of subsequent treatment. Glecaprevir is a next-generation HCV NS3/4A protease inhibitor, and Pibrentasvir is an NS5A inhibitor. They have been co formulated Glecaprevir and Pibrentasvir in clinical trials for the treatment of all six major HCV genotypes. The treatment of HCV with glecaprevir/pibrentasvir leading to first global approval in the European Union and subsequent approval in the United States of America for chronic Hepatitis C Virus infection. The chemical structures of Glecaprevir and Pibrentasvir were showed in Figures 1 and 2.

MATERIALS AND METHODS

All the chemicals and reagents were of analytical grade. Water was double distilled and filtered with a membrane filter. Acetonitrile – Hig Performance Liquid Chromatography grade (Merck, India), hydrochloric acid and ortho phosphoric acid (SD fine chem, India) were used to prepare mobile phase. Pharmaceutical grade standard drugs viz., Pibrentasvir and Glecaprevir were kindly gifted by Ajanta Pharma Limited, Mumbai, India. The combined tablet formulation contains 40 mg of Pibrentasvir and Glecaprevir 100 mg of (Mavyret) purchased from local market of Kurnool.

Preparation of standard solution

10 mg of Pibrentasvir and Glecaprevir were weighed and transferred to individual 10ml volumetric flasks with small quantity of mobile phase. The solutions were sonicated for 10 min and volume was made with mobile phase to give concentration 1000 µg/mL. This solution was further diluted with the mobile phase to get final concentrations of 80 µg/mL and 200 µg/mL working standard solutions for Pibrentasvir and Glecaprevir respectively.

Preparation of sample solution

Twenty tablets were weighed and finely powdered. The average weight of tablets was determined. The powder equivalent to 10 mg of Pibrentasvir was weighed and transferred to a 10 mL volumetric flask. 10 mL of diluent was added to dissolve contents of tablets completely by using ultra sonicator for 10 min. The aliquot portion of the filtrate was further diluted to get final concentrations of 80 µg/mL of Pibrentasvir and 200 µg/mL of Glecaprevir. This solution was filtered through membrane filter. The 20 µL of this solution was injected in to High Performance Liquid Chromatographic system.

Chromatographic Conditions

The mobile phase was consisted of 0.5 mM Ortho Phosphoric acid buffer: Acetonitrile in the ratio of 75:25 v/v and pH 4.3 was adjusted with hydrochloric acid. The mobile was filtered through 0.45µ membrane.
The mobile phase was pumped at a flow rate of 1mL/min. The effluents were monitored at 225 nm with Photo diode Array detection. The 20 µL solution was injected into High performance liquid chromatographic system.

**Method validation**

The method validation was performed according to International Council for Harmonization guidelines. The following method validation parameters resembling specificity, precision, accuracy, linearity, robustness, limit of detection and limit of quantification.

**Specificity**

The specificity was studied by injecting the mobile phase (blank), standard and sample solution prepared as per the developed method and injected into the HPLC system and study the any interference with retention times of Pibrentasvir and Glecaprevir.

**System suitability parameters**

To make certain critical parameters were m et al l system suitable requirements conducted on all the days. The chromatogram was eluted and showed symmetrical peaks. The standard, sample and blank chromatograms showed in Figures 3, 4, 5 and results were tabulated as in [Table 1].
Precision
The precision was assessed through assay with respect to intermediate precision and intraday precision. The repeatability of the system was studied by injecting analyte with 6 replicate injections. The %RSD values varied from 0.47-0.03%. The results showed good intra-day precision. The results were tabulated in Table 2.

Linearity
The linearity of the method was obtained through calibration curve (peak area vs concentration). The pure solution was checked in the concentration range of 20-120 µg/ml for Pibrentasvir and 50-300 µg/ml for Glecaprevir. The calibration curve was shown linear over concentration range and R² values were found to be 0.999 for Pibrentasvir and 1 for Glecaprevir, that results indicated good linearity between peak area and concentration. The data of graphs were showed in Figures 6 and 7. Acceptance criteria: Correlation coefficient should be not less than 0.999.

Accuracy
The accuracy of the method was studied by spiking standard solution with analyzed sample solution at three concentrations levels 50%, 100%, and 150%. The results are tabulated in Table 3.

Table 1: System suitability results of Pibrentasvir & Glecaprevir.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameter</th>
<th>PNR</th>
<th>GPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Theoretical Plate Count</td>
<td>3985</td>
<td>6425</td>
</tr>
<tr>
<td>2</td>
<td>Peak Area</td>
<td>905774.0</td>
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<tr>
<td>3</td>
<td>Peak Height</td>
<td>2151554</td>
<td>240210</td>
</tr>
<tr>
<td>4</td>
<td>RT</td>
<td>3.048</td>
<td>4.457</td>
</tr>
<tr>
<td>5</td>
<td>Tailing</td>
<td>1.147</td>
<td>1.096</td>
</tr>
<tr>
<td>6</td>
<td>Resolution</td>
<td>-</td>
<td>7.97</td>
</tr>
<tr>
<td>7</td>
<td>S/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Mean</strong></td>
<td></td>
<td></td>
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</table>

Table 2: Precision results of Pibrentasvir & Glecaprevir.

<table>
<thead>
<tr>
<th>S.NO</th>
<th>PEAK AREA</th>
<th>%ASSAY</th>
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<tbody>
<tr>
<td>PNR</td>
<td>GPR</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9110742</td>
<td>100.40</td>
</tr>
<tr>
<td>2</td>
<td>9061995</td>
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<td>3</td>
<td>9054193</td>
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<td>4</td>
<td>9038029</td>
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</tr>
<tr>
<td>6</td>
<td>9058562</td>
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<tr>
<td>AVG</td>
<td>9052951.50</td>
<td>99.75</td>
</tr>
<tr>
<td>STDEV</td>
<td>37763.41</td>
<td>0.47</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.42</td>
<td>0.03</td>
</tr>
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</table>

Table 3: Accuracy results of PNR & GPR.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Peak Area</th>
<th>Amount added(µg)</th>
<th>Amount recovered (µg)</th>
<th>% of recovery</th>
<th>% mean recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PIBRENTASVIR</td>
<td>PNR</td>
<td>GPR</td>
<td>50%</td>
<td>4524143</td>
</tr>
<tr>
<td></td>
<td>GLECAPREVIR</td>
<td>50%</td>
<td>9055022</td>
<td>79.80</td>
<td>79.98</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>150%</td>
<td>13534934</td>
<td>119.70</td>
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<td></td>
<td></td>
<td></td>
<td>50%</td>
<td>4500718</td>
<td>39.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100%</td>
<td>9018173</td>
<td>79.80</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>150%</td>
<td>13593541</td>
<td>119.70</td>
</tr>
</tbody>
</table>

Table 4: Results of Limit of Detection & Limit of Quantification results.

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Parameter</th>
<th>PNR</th>
<th>GPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Slope</td>
<td>10886</td>
<td>10886</td>
</tr>
<tr>
<td>2</td>
<td>STDEV</td>
<td>0.47</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>LOD</td>
<td>0.12 µg/ml</td>
<td>0.008 µg/ml</td>
</tr>
<tr>
<td>4</td>
<td>LOQ</td>
<td>0.43 µg/ml</td>
<td>0.027 µg/ml</td>
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The recovery studies were performed under optimized conditions in replicate. The results were showed in [Table 3]. The accuracy should between 98%-102%. The % RSD value should not more than 2.0.

**Limit of detection and Limit of quantification**

The detection limit and quantification limit were determined through signal to noise ratio 3:1 and 10:1 ratio. The limit of detection and limit of quantification were estimated 0.12 µg/mL-0.43 µg/mL for Pibrentasvir and 0.08 µg/mL-0.027 µg/mL for Glecaprevir. The results were tabulated in [Table 4]. The chromatograms of LOD and LOQ were showed in Figure 8 and 9.

**Robustness**

The method was unaffected with deliberate changes with respected to flow rate (± 0.2 ml/min) from the normal conditions of the method. The mixture of both drugs containing solution analyzed by HPLC method as three independent samples.

**Effect of flow rate**

The effect of temperature was assessed with the variation of ±5.0°C from the normal conditions of the method. The mixture of both drugs containing solution analyzed by HPLC method as three independent samples.

**Effect of temperature**

The effect of wavelength was evaluated with the variation of ±2.0 nm from the normal conditions of the method. The mixture

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**Figure 6**: Linearity graph for Pibrentasvir.

**Figure 8**: Chromatogram for LOD.

**Figure 7**: Linearity graph for Glecaprevir.

**Figure 9**: Chromatogram for LOQ.
of both drugs containing solution analyzed by HPLC method as three independent samples.

**Assay of Pibrentasvir and Glecaprevir in Solid dosage form**

Twenty tablets of marketed formulation (Mavyret) were weighed and finely powdered. Accurately weighed 10 mg equivalent quantity of tablet powder of Pibrentasvir and transferred into 10 mL volumetric flask. The volume was made with diluents. The final concentration of Pibrentasvir was 80 μg/ml and Glecaprevir was 200 μg/mL. Results were tabulated in Table 6.

**Force degradation studies**

The force degradation studies were performed on the PNR and GPR. There was no interference of degradants and blank, the developed RP-HPLC method proves the capability of stability indicating method for the analysis of Pibrentasvir and Glecaprevir. Different stress indicating studies were conducted like acid (0.1 N HCl, refluxed for 3 H at 60°C), Base (0.1 N NaOH refluxed for 4H at 60°C), H₂O₂ (3% H₂O₂ Stored at room temperature for 5 H), Hydrolytic (80°C refluxed for 8 H) and UV light (near UV ≥200 for 10 days). The degradation conditions were optimized to obtain target degradation between 10 to 30% as per ICH guidelines. The results were summarized in Table 7. Figures 10-14 shows chromatograms of different stress degradation conditions.

**RESULTS AND DISCUSSION**

The optimization of reversed phase high performance liquid chromatographic method, different columns like (Waters C₁₈; 250×4.6 mm, 5 μm and Zorbax C18; 250×4.6 mm, 5 μm), organic solvents (methanol and acetonitrile), buffers (acetates, citrates and phosphates) at different pH (4 and 4.5) were tested. The method was optimized with water’s C₁₈ column capacitate (250X4.6 mm, 5 µm particle size), The mobile phase was composed with mixture of 0.5 mM Ortho phosphoric acid buffer: Acetonitrile in the ratio of 75:25 v/v, pH 4.3 was adjusted with hydrochloric acid and flow of mobile phase through the column was maintained 1mL/min. The column temperature was maintained at 30°C. At all these conditions got excellent peak area, theoretical plates, good resolution between the peaks, low retention times and low tailing factor for Pibrentasvir and Glecaprevir. A flow rate of mobile phase was 1 mL/min gave an optimal signal to noise ratio with a reasonable separation time. The re-
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The retention times of Pibrentasvir and Glecaprevir were found to be 3.04 and 4.45 min. Total run time of analysis was 7 min. The complete resolution of both chromatographic peaks were observed at 225 nm.

The validation of analytical method and resolution between peaks were ensured by system suitability parameter. Both peaks were eluted by forming symmetrical single peaks well separated as showed in chromatogram.

The precision of the analytical method was found to be reliable based on % Relative Standard Deviation (< 2%) corresponding to the peak areas and retention times and tabulated in 2. The % Relative Standard Deviation values were less than 2, thus the method was found to be precise for Pibrentasvir and Glecaprevir. Excellent linearity was obtained in the range of 20-120 μg/mL for Pibrentasvir and 50-300 μg/mL for Glecaprevir. The correlation coefficient \( (R^2) \) value greater than 0.999 (n=6) in all instances for both Pibrentasvir and Glecaprevir. The results of the calibration curve showed in Figures 7 and 8. The developed method was afforded to high recoveries of Pibrentasvir and Glecaprevir in tablet. The results of recovery studies showed in Table 3, indicated that the method was showed good accuracy. The detection limit and quantization limit of the method were found 0.12 and 0.43 μg/mL and 0.008 and

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**Figure 10:** Chromatogram for acid degradation.

**Figure 11:** Chromatogram for base degradation.

**Figure 12:** Chromatogram of peroxide degradation.

**Figure 13:** Chromatogram of hydrolytic degradation.
0.027 µg/mL for Pibrentasvir and Glecaprevir. Results were tabulated in Table 4. Deliberate changes were performed from the normal conditions and got the % assay values within the range for both Pibrentasvir and Glecaprevir and results were tabulated in Table 5. The assay method was performed to Pibrentasvir and Glecaprevir, % assay values are 99.75% for Pibrentasvir and 99.87% for Glecaprevir. The results were showed in Table 6. The developed method was routinely used for qualitative analysis of combined dosage form of Pibrentasvir and Glecaprevir in tablet and its bulk dosage form.

The forced degradation studies were performed with acid (0.1 N HCl, refluxed for 1 H at 60°C), base (0.1 N NaOH refluxed for 4H at 60°C), H2O2 (3% H2O2 Stored at room temperature for 2 H), water at 60°C and UV light (near UV ≥200 for 10 days). Analytes were degraded significantly. Both the drugs were found to be sensitive to acid, base, peroxide and UV-light effect where as they found to resistance to hydrolytic degradation.

**CONCLUSION**

The developed RP-HPLC method was simple, sensitive, specific, accurate and precise, stability indicating simultaneous estimation of PNR and GPR in tablet dosage form. The developed method showed excellent resolution between PNR and GPR. The method was effectively validated in terms of system suitability, precision, linearity, range, accuracy, LOD, LOQ and robustness and stress indicating studies in agreement with ICH guidelines. Hence the method is routinely used for estimation and quality control, stability indicating samples of combined market formula-

**ACKNOWLEDGEMENT**

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ABBREVIATIONS**

PNR: Pibrentasvir; GPR: Glecaprevir; mL: Milli liters; (µg) Microgram; LOD: Limit of Detection; LOQ: Limit of Quantification; HCl: Hydrochloric acid; NaOH: Sodium hydroxide; H2O2: Hydrogen peroxide; UV: Ultraviolet; µL: Microliters; mg: milligrams; nm: Nanometers; HCV: Hepatitis C Virus; NS5A: Non structural 5A Protein; NS3/4A: Non structural 3,4A Protein; Min: Minutes; RP-HPLC: Reversed phase-High performance liquid chromatography.

**REFERENCES**

PICTORIAL ABSTRACT

The developed RP-HPLC method for the simultaneous estimation of Pibrentasvir and Glecaprevir showed good theoretical plates and low tailing factor. The method showed excellent linearity and $R^2$ were found to be 0.999 for PNR and 1 for GPR and also showed wider concentration range. The method showed good precision, accuracy and robustness, the values were within the limit as per ICH guidelines. The LOD and LOQ values were found to be 0.12-0.43 µg/mL for PNR and 0.008-0.027 µg/mL for GPR. The results were showed highly sensitivity of the method. The stress indicated studies were performed with different conditions of acid, alkaline, peroxide, hydrolytic and UV-light conditions, the results of the method were showed high stability of the method.

SUMMARY

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