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Stability-indicating high performance liquid chromatographic method for simultaneous assay of pibrentasvir and glecaprevir: Method development, validation and application to tablet dosage forms

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ABSTRACT: Pibrentasvir and glecaprevir combination therapy acts by inhibiting RNA replication and viron assembly in hepatitis C virus. The aim and objective of the present investigation is to develop and validate a stability indicating RP-HPLC method for simultaneous quantification of pibrentasvir and glecaprevir in bulk and tablets. Pibrentasvir and glecaprevir were separated and analyzed on Agilent Eclipse column (4.6 mm × 150 mm, 5 µm). The mobile phase consisted of 0.1% orthophosphoric acid and methanol (30:70, v/v), that was isocratically delivered at a flow rate of 1.0 mL/min. Retention times were 1.857 min for glecaprevir and 2.681 min for pibrentasvir. Good regression coefficients were obtained in range of 50-250 µg/mL for glecaprevir and 20–100 µg/mL for pibrentasvir. The results of validation parameters like selectivity, precision, accuracy and robustness are satisfactory. Tablet sample containing pibrentasvir and glecaprevir was subjected to degradation with 0.1N HCl, 0.1N NaOH, 30% hydrogen peroxide, thermal and photo conditions. The resulting degradants produced during the applied degradation conditions were well resolved from the peaks of pibrentasvir and glecaprevir. The utility of the proposed method was demonstrated by application to tablets containing pibrentasvir and glecaprevir combination. No interference from additives was observed. Therefore the method can be adapted in routine analysis of pibrentasvir and glecaprevir in quality control laboratories. The method can also be used for purity and degradation assessment of pibrentasvir and glecaprevir in tablets.

KEYWORDS: Antiviral drugs; pibrentasvir; glecaprevir; stability indicating; liquid chromatography.

1. INTRODUCTION

Hepatitis C virus is an RNA virus which causes progressive damage to the liver. As a result, liver cirrhosis and hepatocellular carcinoma might occur. Approximately 64 to 103 million people are infected chronically with hepatitis C virus [1]. As per WHO (World Health Organization), 350000 to 500000 people die each year because of this virus associated liver diseases. Though this virus was found worldwide, majorly affected regions included North Africa, Central Asia and East Asia [2, 3]. After better studying the properties of RNA, proteins and life cycle of hepatitis C virus, effective antiviral treatments were developed. The duration of therapy with antiviral and probability of response to antiviral depends on the number and genotype of RNA in hepatitis C virus [4].

The approval of pibrentasvir and glecaprevir combination was given by Food and Drug Administration in 2017 August [5]. This is used in healing the adult patients with chronic hepatitis C virus genotypes 1 to 6 (with no/mild cirrhosis), with kidney disease, those patients on dialysis, patients infected with hepatitis C virus genotype 1 who were already treated either with an NS5A inhibitor or an NS3/4A protease inhibitor, but not both in the past [6-8]. The enzymes, nonstructural protease 3A (NS3A), 4A (NS4A) and 5A (NS5A) are

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associated with viron assembly and viral RNA replication. Pibrentasvir enacts as NS5A inhibitor while glecaprevir enacts as NS3/4A protease inhibitor. Thus, pibrentasvir and glecaprevir blocks arrest the growth of hepatitis C virus through inhibition of its RNA replication and virion assembly [9]. The structure of pibrentasvir and glecaprevir are shown in Figure 1.

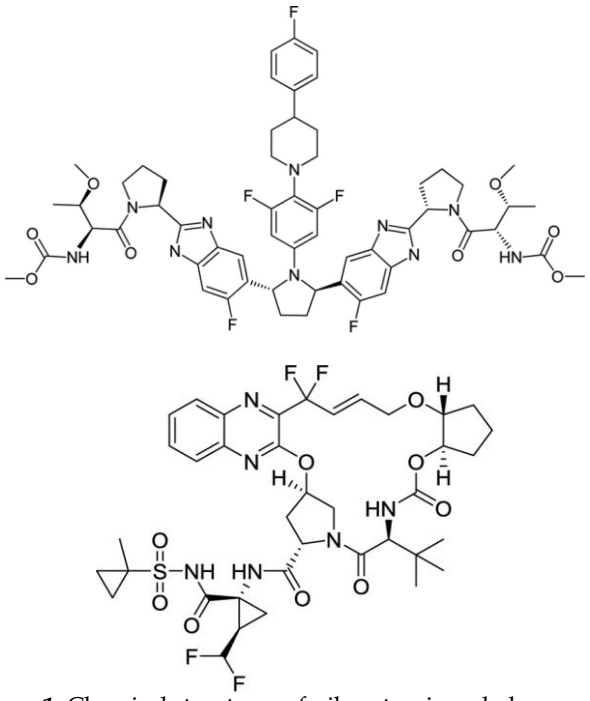

Figure 1. Chemical structures of pibrentasvir and glecaprevir.

In any pharmacopoeia, the combination of these two drugs is not included. Hence, there is a demand to establish an analytical method that can quantify pibrentasvir and glecaprevir simultaneously. Hitherto, only one HPLC [10] and one UPLC [11] methods are reported for the concurrent determination of pibrentasvir and glecaprevir. There is one method [12] established till date in simultaneous quantification of pibrentasvir and glecaprevir in bulk and tablet dosage forms using stability indicating RP-HPLC. The current study describes a novel, simple, precise and rapid stability indicating RP-HPLC method with UV detection for the simultaneous estimation of pibrentasvir and glecaprevir in bulk and in tablets with a run time of 10 min and less retention time.

2. RESULTS

2.1. Optimization of HPLC conditions

Three different columns were involved in performance investigations. They include Inertsil ODS C18 (4.6 mm × 150 mm, 5µm), Symmetry C18 (4.6 mm × 150 mm, 5µm) and Agilent Eclipse C18 column (4.6 mm × 150 mm, 5µm). The results revealed that the last column, Agilent Eclipse C18 column (4.6 mm × 150 mm, 5µm), was the most appropriate because it produced symmetrical peaks of glecaprevir and pibrentasvir with better resolution. The UV detector response of glecaprevir and pibrentasvir was recorded. The suitable detection wavelength was fixed at 244 nm. At 244 nm, both drugs showed good sensitivity with sensible response.

The effect of mobile phase composition, pH and flow rate on the resolution, retention time and peak symmetry of selected drugs was tested. For this purpose, different compositions (methanol: water, methanol: phosphate buffer, water: acetonitrile, phosphate buffer: acetonitrile and orthophosphoric acid: methanol) with different pH (3.0, 3.5, 4.0, 4.5) and a flow rate (0.8 mL/min, 1.0 mL/min, 1.2 mL/min) were tested. Finally, 0.1% orthophosphoric acid combined with methanol in the ratio 30:70 (v/v), having flow rate 1.0 mL/min and pH 3.5 gave better resolution and sharp symmetric peaks with retention times of 1.857 min 2.681 min for glecaprevir and pibrentasvir, respectively Figure 2.

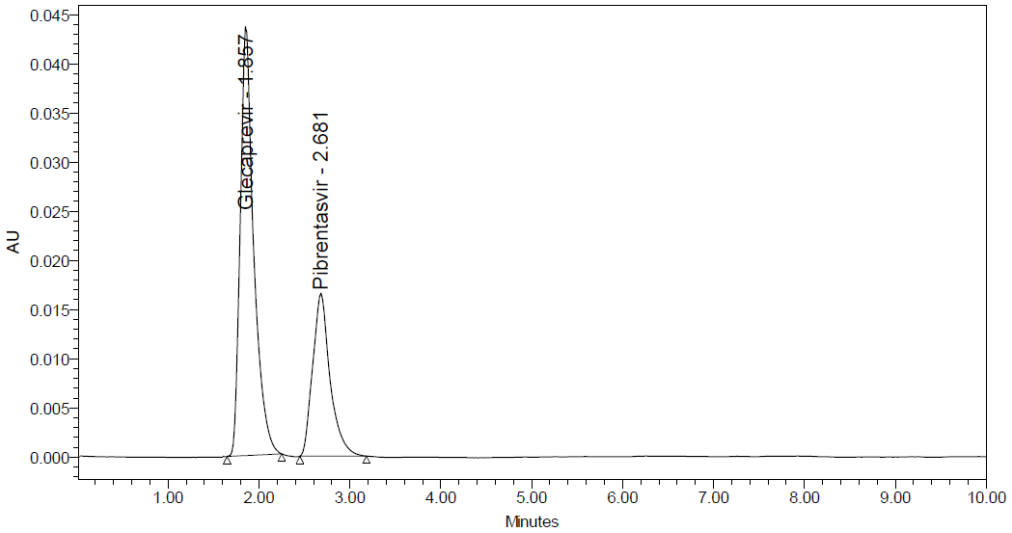

Figure 2. HPLC chromatogram of standard solution, containing glecaprevir and pibrentasvir with Agilent Eclipse column (4.6 x 150mm, 5µm) Mobile Phase: 0.1% orthophosphoric acid: methanol (30:70); flow rate 1mL/min; detection wavelength 244 nm; column temperature 25°C.

2.2. Method validation

International conference on harmonization guidelines was followed to validate the developed method [13-14].

2.2.1. System suitability

System suitability was done with respect to injection repeatability (relative standard deviation of retention time and peak area response), tailing factor, theoretical plate number and resolution for glecaprevir and pibrentasvir peaks using a standard solution (glecaprevir 150 µg/mL and pibrentasvir 60 µg/mL). Data summarised in Table1 shows satisfactory results for System suitability. The relative standard deviation of retention time and peak area response of 5 consecutive injections was observed as <1.0% for both drugs, indicating excellent injection repeatability. The tailing factor was found to be 1.463 (glecaprevir) and 1.287 (pibrentasvir), reflecting good peak symmetry. The theoretical plate number was found to be >4000 for both drugs which demonstrate satisfactory column efficiency. Finally, the resolution values of 3.183 show the good separation of glecaprevir and pibrentasvir peaks. The results assure the adequacy of the system for the simultaneous analysis of glecaprevir and pibrentasvir.

Table 1. System suitability data for the analysis of glecaprevir and pibrentasvir.

Parameter	Glecaprevir		Pibrentasvir	
	Value*	RSD	Value*	RSD
Retention time(min)	1.857	0.164	2.681	0.114
Tailing factor	1.463	0.395	1.287	0.449
Plate number	4738	0.547	6354	0.135
Resolution	-	-	3.183	0.181

*Average of five determinations.

2.2.2. Selectivity

Selectivity of method was assessed by checking that no interference peaks were found at the retention times of glecaprevir and pibrentasvir with mobile phase blank and tablet sample solutions. For this, chromatograms of solutions of standard (glecaprevir 150 µg/mL and pibrentasvir 60 µg/mL), tablet sample (glecaprevir 150 µg/mL and pibrentasvir 60 µg/mL) and mobile phase blanks are compared. The chromatograms of standard and tablet sample showed peaks for glecaprevir and pibrentasvir without any interfering peak Figure 3. In mobile phase blank chromatogram Figure 3, no peak was observed at the retention times of glecaprevir and pibrentasvir. Thus the method was proved selective.

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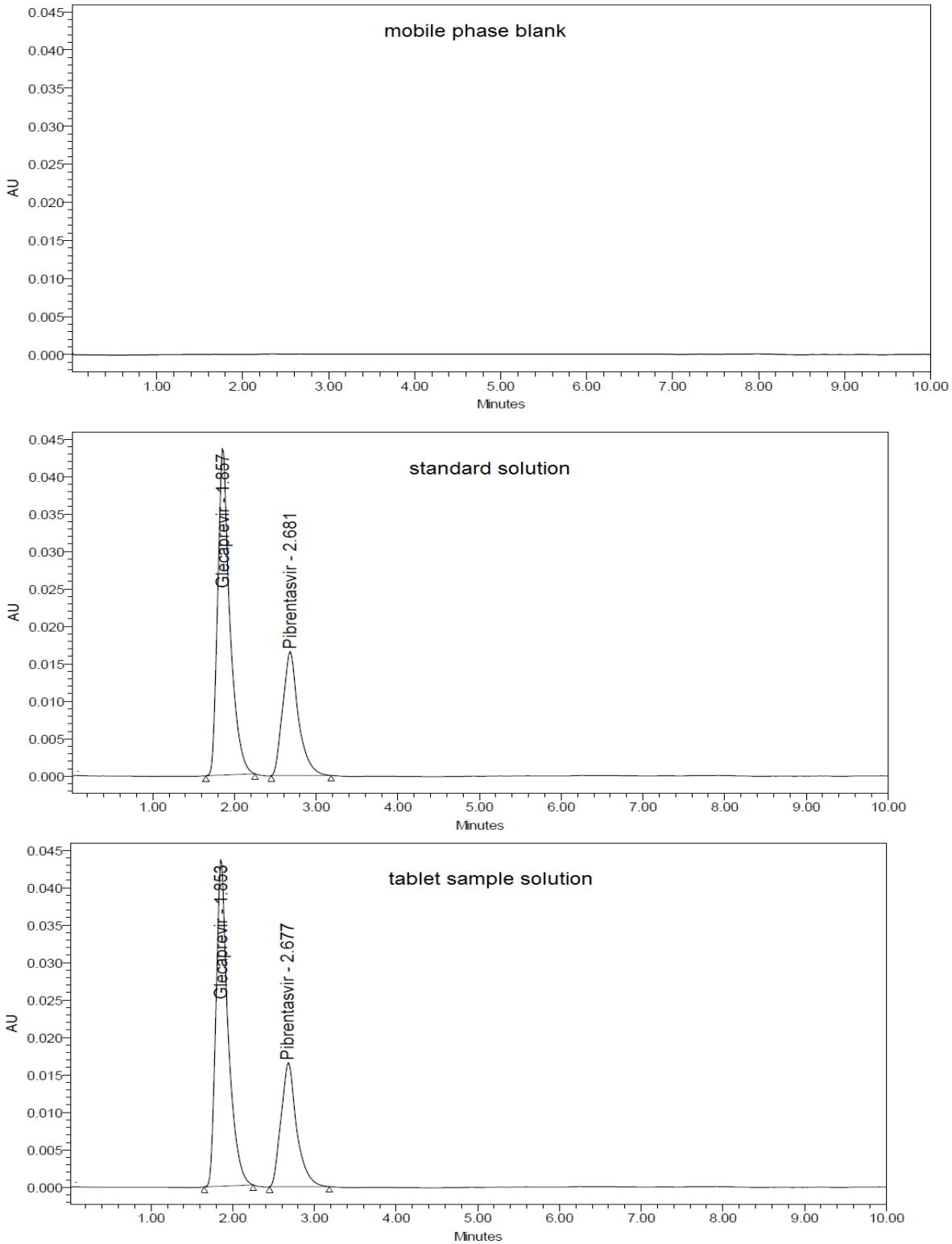

Figure 3. Selectivity study chromatograms.

2.2.3. Linearity

Calibration curves were linear over a glecaprevir concentration range of 50 to 250 µg/mL and pibrentasvir concentration range of 20 to 100 µg/mL. Linear regression of glecaprevir and pibrentasvir curves resulted in a linear fit of y = 3008 x – 6630 (R^2 = 0.9996) and y = 3586 x – 608.5 (R^2 = 0.9998), respectively. The results demonstrated good linearity of glecaprevir and pibrentasvir calibration curves.

2.2.4. Limit of detection and limit of quantitation

A signal to noise ratio of 3:1 and 10:1 is accepted to calculate the limit of detection (LOD) and limit of quantitation (LOQ), respectively. The limit of detection was calculated as 0.60 µg/mL for both drugs. The limit of quantitation was calculated as 1.95 µg/mL and 2.01 µg/mL for glecaprevir and pibrentasvir, respectively. Therefore, the method is sensible for glecaprevir and pibrentasvir analysis in tablet formulations. Chromatograms at LOD and LOQ level are shown in Figure 4.

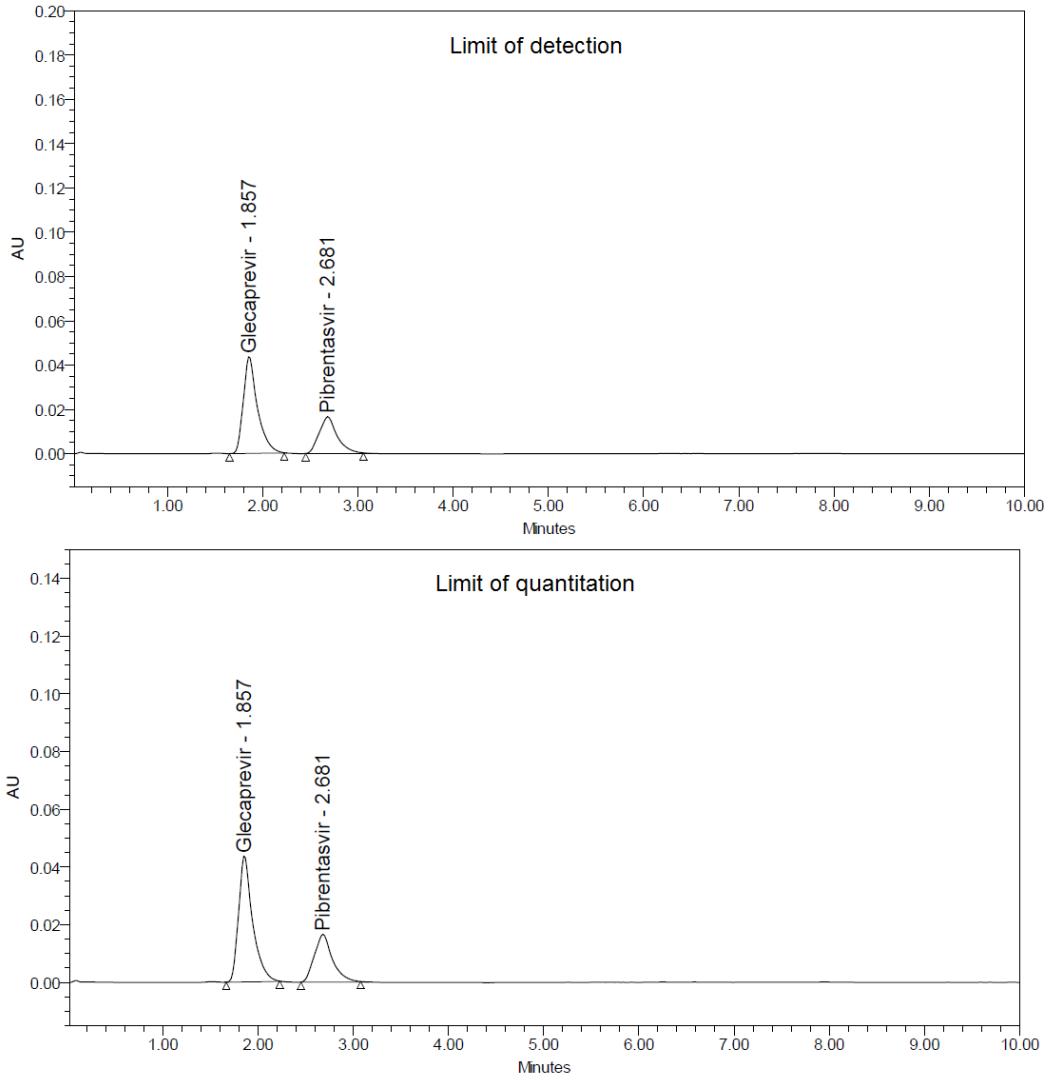

Figure 4. Chromatograms of glecaprevir and pibrentasvir at LOD and LOQ level.

2.2.5. Precision

System precision was tested using a standard solution with concentration 150 µg/mL and 60 µg/mL of glecaprevir and pibrentasvir, respectively. Data summarized in Table 2 revealed satisfactory values for system precision. The Relative standard deviation of peak area response was 0.203% for glecaprevir and 0.677% for pibrentasvir.

Method precision was assessed using tablet sample solution having concentration 150 µg/mL of glecaprevir and 60 µg/mL of pibrentasvir. Relative standard deviation of the percent assay of glecaprevir and

pibrentasvir were calculated. The data shown in Table 2 revealed satisfactory values for method precision. The relative standard deviation of the percent assay was 0.416% for glecaprevir and 0.473% for pibrentasvir.

Evaluation of intermediate precision/ruggedness of the method was performed using glecaprevir and pibrentasvir standard solution with concentration 150 µg/mL and 60 µg/mL, respectively on two different days. Peak area values for glecaprevir and pibrentasvir along with mean and percent relative standard deviation values are summarized in Table 2. Relative standard deviation of peak area response was less than 1% for glecaprevir and pibrentasvir indicating the acceptable values for intermediate precision/ruggedness of the method.

Table 2. Data of system, method, intermediate precision experiments.

Sample injection	**System precision**		**Method precision**	
	Peak area		**Percent assay**	
	Glecaprevir	**Pibrentasvir**	**Glecaprevir**	Pibrentasvir
1	448662	218753	99.00	100.52
2	446873	214829	99.79	99.67
3	446352	216426	99.32	100.10
4	447562	218452	99.53	99.90
5	447529	216468	100.00	100.18
6	446244	217567	98.99	99.15
Mean	447203.7	217082.5	99.44	99.92
%RSD	0.203	0.677	0.416	0.473
	Intermediate precision/Ruggedness			
	Glecaprevir peak area		**Pibrentasvir peak area**	
	Day 1	**Day 2**	**Day 1**	**Day 2**
1	448776	446582	218573	217584
2	445735	445154	218562	217685
3	447673	443565	214652	216452
4	448673	446352	215354	218574
5	445876	447545	216454	216854
6	448676	446585	216457	214578
Mean	447568	445964	216675	216955
%RSD	0.318	0.314	0.747	0.634

2.2.6. Accuracy

Accuracy was demonstrated in terms of recovery of known concentrations of glecaprevir and pibrentasvir spiked to preanalyzed tablet sample solution at three levels (50%, 100% and 150% of labeled claim). Calculated recoveries of glecaprevir and pibrentasvir at each level are shown in Table 3. The results suggest the acceptable accuracy of the developed method and non-interference of tablet excipients in the assay.

Table 3. Data of accuracy experiments.

Drug	**Level tested %**	**Labeled amount(mg)**	**Amount added (mg)**	**Amount Found(mg)**	**Recovery(%)**
Glecaprevir	50	100	50	151.05	100.70
	100	100	100	200.08	100.04
	150	100	150	251.12	100.45
Pibrentasvir	50	40	20	60.27	100.45
	100	40	40	80.40	100.50
	150	40	60	100.23	100.23

Table 4. Recovery, degradation and peak purity data of glecaprevir and pibrentasvir in applied stress conditions.

Drug	Condition	Peak area	% Recovery	% degraded	Purity angle	Purity Threshold
Glecaprevir	Undegraded	447408	100	-	-	-
	Acid	436572	97.57	2.43	0.589	1.619
	Base	428673	95.81	4.19	0.865	3.529
	Peroxide	439657	98.27	1.73	0.426	0.964
	Thermal	430876	96.30	3.70	0.379	1.146
	Photo	421862	94.29	5.71	0.786	2.744
Pibrentasvir	Undegraded	217707	100	-	-	-
	Acid	207853	95.47	4.53	0.167	0.354
	Base	196762	90.38	9.62	0.264	0.589
	Peroxide	206752	94.97	5.03	0.638	1.532
	Thermal	199672	91.72	8.28	0.397	0.733
	Photo	195534	89.92	10.18	0.369	0.846

2.2.7. Specificity

The peak area of glecaprevir and pibrentasvir in the stress degraded samples was less than the peak area of glecaprevir and pibrentasvir in standard solution indicating that the selected drugs undergoes partial degradation in all applied conditions. Peak area and % recoveries of glecaprevir and pibrentasvir remaining after applying degradation processes were calculated and summarized in Table 4. As per the results shown in Table 4, the glecaprevir and pibrentasvir are more susceptible towards photolysis condition. The chromatograms of degradation studies are shown in Figure 5. Chromatogram of tablet treated with 0.1N HCl shows one peak for unknown degradation product with retention time 5.256 min. Chromatograms of tablet treated with 0.1N NaOH and 30% hydrogen peroxide showing peaks for three unknown degradation products (retention time of degradants - 1.182 min, 4.406 min and 6.918 min in alkaline condition; 3.390 min, 5.256 min and 6.585 min in oxidative condition). Chromatogram of tablet undergone thermal and photo degradation shows peaks for 2 unknown degradation products (retention time of degradants – 3.685 min and 4.923 min in thermal degradation; 5.256 min and 7.260 min in photo degradation). The proposed method was able to detect and specifically quantitate glecaprevir and pibrentasvir peak in the presence of degradants. The results indicating that the proposed method can be used as a stability indicating method. In the applied forced degradation conditions, the purity of glecaprevir and pibrentasvir peaks were confirmed by the higher values of peak threshold value than peak purity angle acquired through the peak purity tool Table 4.

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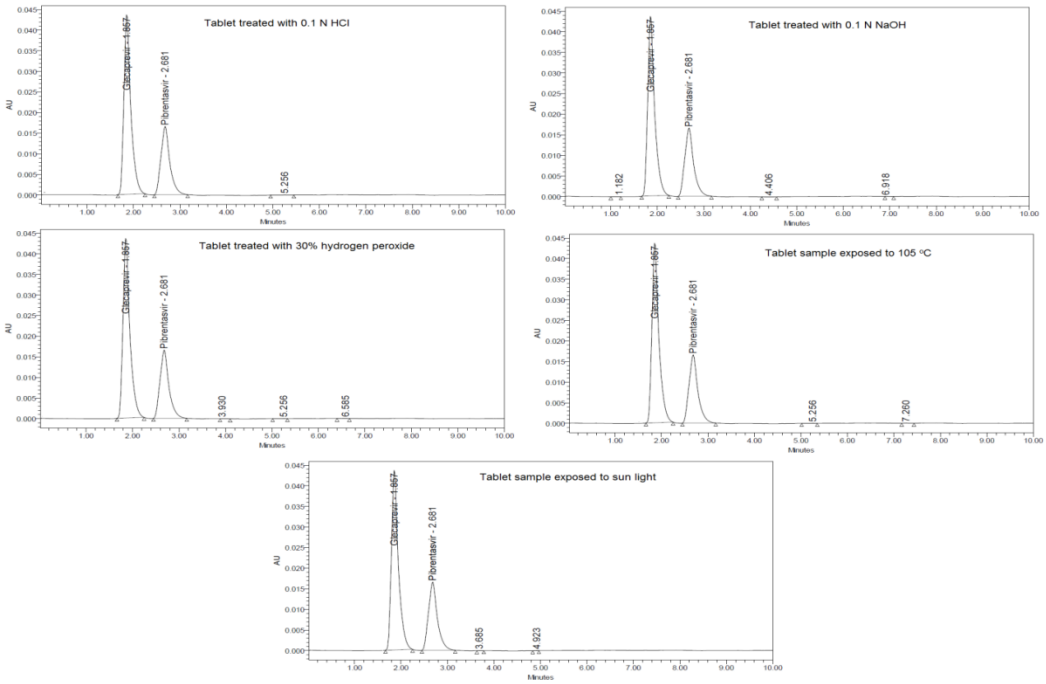

Figure 5. Chromatograms of degradation studies.

2.2.8. Robustness

During method robustness testing, deliberate change in the flow rate, mobile phase composition, pH of the mobile phase, detection wavelength and temperature variation was made to evaluate the impact on the system suitability parameters of the developed method. Results are presented in Table 5. No significant changes were observed in studied system suitability parameters when deliberate variations are made in the chromatographic conditions which mean that the proposed method is robust.

Table 5. Data of method robustness tests.

Chromatographic Condition	Value	Glecaperavir		Resolution	Pibrentasvir	
		Tailing factor	Plate count		Tailing factor	Plate count
Flow rate(mL/min)	0.9	1.463	4626.92	3.31	1.295	6132.29
	1.0	1.465	4725.92	3.18	1.293	6256.39
	1.1	1.466	4865.39	3.02	1.291	6352.29
Mobile phase cpmposition ratio (v/v)	25:75	1.467	4762.23	3.37	1.299	6214.27
	30:70	1.462	4725.92	3.18	1.292	6256.39
	35:65	1.464	4767.76	2.96	1.295	6232.23
p^H of mobile phase	3.3	1.469	4568.25	3.35	1.298	5895.25
	3.5	1.462	4725.92	3.18	1.294	6256.39
	3.7	1.460	4635.27	2.95	1.292	6145.95
Detection wave length(nm)	240	1.468	4635.25	3.18	1.293	6065.32
	244	1.460	4725.92	3.18	1.296	6256.39
	248	1.461	4762.35	3.18	1.297	6264.85
Column temperature(^{o}C)	23	1.466	4578.35	3.33	1.292	6145.35
	25	1.464	4725.92	3.18	1.295	6256.39
	27	1.465	4658.26	3.04	1.298	6758.15

2.3. Application of the method

The developed and validated assay method was applied to tablets containing glecaprevir and pibrentasvir. The mean concentration and recoveries for glecaprevir and pibrentasvir were found to be in good agreement with the nominal values Table 6. The method was also precise with relative standard deviation value <0.5%.

Table 6. Assay data of glecaprevir and pibrentasvir by the proposed method.

Glecaprevir			Pibrentasvir		
Labeled claim(mg)	Amount found(mg)	Recovery (%)	Labeled claim(mg)	Amount found(mg)	Recovery (%)
100	100.19	100.19	40	39.95	99.88
100	99.95	99.95	40	40.18	100.45
100	99.93	99.93	40	40.05	100.13
Mean	100.02	100.02	**Mean**	40.06	100.15
%RSD	0.145	0.145	**%RSD**	0.288	0.288

3. CONCLUSION

A stability indicating RP-HPLC method was developed for the determination of glecaprevir and pibrentasvir simultaneously. The developed method yielded satisfactory validation results concerning linearity, sensitivity, precision, accuracy and ruggedness. The present method for the assay of glecaprevir and pibrentasvir in tablets is specific and selective since the tablet excipients and degradants produced did not interfere with the peaks of selected drugs. The method can be adapted in quality control laboratories for the quantification of glecaprevir and pibrentasvir.

4. MATERIALS AND METHODS

4.1. Instrumentation

- HPLC system: Waters 2695 HPLC system provided with high speed auto sampler, column, oven, degasser and 2996 photodiode array detector.
- Software for data processing: Waters Empower 2 software.
- pH meter: Adwa AD 1020, Adwa Hungary Kft, Hungary
- Weighing balance: Afcoset ER-200A, Mumbai, India.

4.2. Materials

4.2.1. Reference standards

- Pibrentasvir: Pharmatrain, Hyderabad, India (procured as gift sample)
- Glecaprevir: Pharmatrain, Hyderabad, India (procured as gift sample)

4.2.2. Tablets

- Maviret ® tablets: manufactured by AbbVie Limited, Berkshire, UK. Labeled to contain 100 mg and 40 mg of glecaprevir and pibrentasvir, respectively.

4.2.3. Chemicals

- Analytical reagent grade orthophosphoric acid (Merck chemical division, Mumbai, India)
- Analytical reagent grade hydrochloric acid (SD Fine Chemicals Ltd, Chennai, India)
- Analytical reagent grade sodium hydroxide (SD Fine Chemicals Ltd, Chennai, India)
- Analytical reagent grade hydrogen peroxide (SD Fine Chemicals Ltd, Chennai, India)
- HPLC grade methanol (Merck chemical division, Mumbai, India)
- HPLC grade water (Milli-Q water purification system, Bangalore, India)

4.3. Chromatographic conditions

- Column : Agilent Eclipse column C18 (4.6 mm × 150 mm, 5 µm)
- Temperature : 25 °C
- Mobile phase : 30% 0.1% orthophosphoric acid : 70% methanol (degassed and filtered before use)
- pH of mobile phase : 3.5
- Detection wavelength : 244 nm

- Flow rate : 1 mL/min
- Injection volume : 20 µL
- Run time : 10 min

4.4. Standard solutions

Stock standard solution containing 1000 µg/mL and 400 µg/mL concentration of glecaprevir and pibrentasvir, respectively was prepared by dissolving appropriate quantities of drugs in the diluent (0.1% orthophosphoric acid: methanol in the ratio 30:70 v/v). Serial dilutions of the stock standard solution were made with diluent to get working standard solutions at concentrations of 50, 100, 150, 200 and 250 µg/mL of glecaprevir, and 20, 40, 60, 80 and 100 µg/mL of pibrentasvir.

4.5. Calibration curve

The standard solution (concentration: glecaprevir 50-250 µg/mL and pibrentasvir 20-100 µg/mL) was filtered through 0.44 micron syringe filters. The solutions were injected into the system with constant injection volume (20 µL). Calibration curve was plotted between peak area and respective concentration. The regression coefficient and slope were determined from the calibration curve.

4.6. Assay of selected drug combination in tablet

Five Maviret® tablets were crushed into fine powder. Powder equivalent to 100 mg glecaprevir and 40 mg pibrentasvir was accurately weighed and dissolved in 70 mL of diluent in a 100 mL volumetric flask. The content of the flask was sonicated for ten min to dissolve the drugs completely followed by dilution up to the mark with the same solvent. Pipette 1.0 mL of the prepared solutions into a 10 mL volumetric flask and dilute up to the mark with diluent (concentration: 100 µg/mL glecaprevir and 40 µg/mL pibrentasvir). 20 µL of the prepared tablet solution was injected (n=3) into the chromatographic system. Using the proposed method, measure the peak areas for glecaprevir and pibrentasvir and calculate their content in the tablet.

4.7. Forced degradation study

Forced degradation study was executed to analyze the method's stability indicating property, method's specificity and inherent stability characteristics of glecaprevir and pibrentasvir. Degradation was done by exposing the tablet solution (1500 µg/mL glecaprevir and 600 µg/mL pibrentasvir) to 5 stress conditions (acid, alkali, hydrogen peroxide, thermal and photo) [13]. Stressed samples were analyzed and the presence of related peaks and peak purity for glecaprevir and pibrentasvir was checked.

4.7.1. Hydrolytic degradation using 0.1N HCl

1.5 mL of tablet solution was transferred into a 10 mL volumetric flask and 3 mL of 0.1N HCl was added. The flask was refluxed at 60°C for 24 hours, neutralized with 0.1 N NaOH and make up to 10 mL with diluent. The solution was filtered with 0.44 micron syringe filters and injected into the system.

4.7.2. Hydrolytic degradation using 0.1N NaOH

Tablet solution (1.5 mL) was transferred into a 10 mL volumetric flask followed by the addition of 3 mL of 0.1N NaOH. The flask was refluxed at 60°C for 24 hours, neutralized with 0.1N HCl and diluted to 10 mL with diluent. The solution was filtered using 0.44 micron syringe filters and injected into the system.

4.7.3. Oxidative degradation using 30% hydrogen peroxide

1.5 mL of tablet solution and 1 mL of 30% hydrogen peroxide was added to a 10 mL volumetric flask. The volume was made up to the mark with diluent. The contents of the flask were kept at room temperature for 15 min. The solution was filtered using 0.44 micron syringe filters and injected into the system.

4.7.4. Thermal induced degradation

Tablet powder was taken in a petridish and kept in hot air oven set at 110°C for 3 h. Tablet powder equivalent to 100 mg glecaprevir and 40 mg pibrentasvir was transferred into a 100 mL volumetric flask. Add about 70 mL of diluent and sonicated for ten min and volume was made up to the mark with diluent. 1.5 mL of the above prepared solution was diluted to 10 mL with the diluent. The resulting solution was filtered using 0.44 micron syringe filters and injected into the system.

4.7.5. Photodegradation using sunlight

1.5 mL of tablet solution was transferred into a 10 mL volumetric flask and exposed to sunlight for 24 h. The solution was filtered using 0.45 micron syringe filters and injected into the system.

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Conflict of interest statement: The authors declare that no conflict of interest.

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