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Quantification of antiviral drug combination, glecaprevir and pibrentasvir, in bulk and tablet formulation by stability indicating RP-HPLC method

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Abstract
A sensitive, selective, reproducible and accurate stability indicating RP-HPLC method was developed for the estimation of glecaprevir and pibrentasvir in the presence of their stress degradation products. Degradation studies of *g*lecaprevir and pibrentasvir were carried out in acidic, alkaline, oxidative, thermal and photolytic stress conditions. Separation was performed on YMC C18 analytical column using 0.1M potassium dihydrogen phosphate: methanol: acetonitrile (40:35:25, *v/v/v*). Peaks at retention times 1.955 min and 3.189 min were recognized as *g*lecaprevir and pibrentasvir, respectively, and were quantified by measurements at wavelength 228 nm using photodiode array detector. The suggested method showed a linear range of 25–200 µg/ml with LOD and LOQ of 0.950 and 3.167 ng/ml for glecaprevir, and 10-80 µg/ml with LOD and LOQ of 0.273 and 0.910 µg/ml for pibrentasvir. The proposed method was applied successfully to the quantification of *g*lecaprevir and pibrentasvir in commercial tablet formulation. The proposed RP-HPLC method can be applied for routine analysis of glecaprevir and pibrentasvir in the presence of stress degradation products in the bulk as well as combined tablet formulation.

Keywords: Pibrentasvir, glecaprevir, antiviral, degradation, analysis

1. Introduction
Pibrentasvir and glecaprevir are antiviral agents [1]. Pibrentasvir is chemically known as methyl N-[(2S,3R)-1-[(2S)-2-[6-[(2R,5R)-1-[3,5-difluoro-4-[4-(4-fluorophenyl)piperidin-1-yl] phenyl]-5-[6-fluoro-2-[(2S)-1-[(2S,3R)-3-methoxy-2-(methoxycarbonylamino) butanoyl] pyro lidin-2-yl]-3H-benzimidazol-5-yl]pyrrolidin-2-yl]-5-fluoro-1H-benzimidazol-2-yl] pyrrolidin-1-yl]-3-methoxy-1-oxobutan-2-yl]carbamate (Fig. 1). Chemically, glecaprevir is described as (3aR, 7S, 10S, 12R, 21E, 24aR)-7-tert-Butyl-N-{(1R,2R)-2-(difluoromethyl)-1-[(1-methylcyclopro pane-1-sulfonyl)carbamoyl] cyclopropyl}-20, 20-difluoro-5, 8-dioxo-2, 3, 3a, 5, 6, 7, 8, 11, 12, 20, 23, 24 a-dodecahydro-1H, 10H-9, 12-methanocyclopenta [18, 19] [1, 10, 17, 3, 6] trioxadiazacyclonon adecino [11, 12-b] quinoxaline-10-carboxamide (Fig. 2).

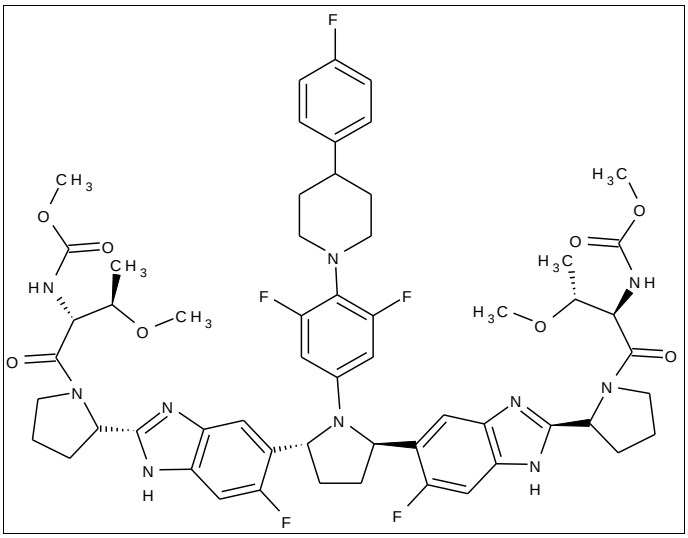


Fig 1: Chemical structure of pibrentasavir

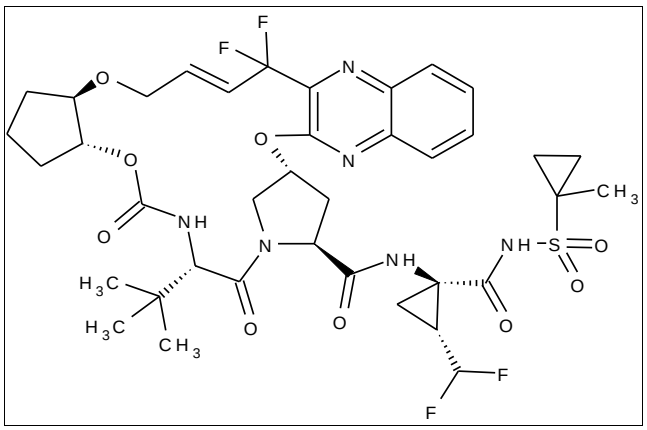


Fig 2: Chemical structure of glecaprevir

The pibrentasvir and glecaprevir combination was approved by Food Drug and Administration in 2017 August [2]. This combination is prescribed to treat patients (adults) with chronic hepatitis C virus genotypes 1 to 6 with no or with mild cirrhosis, patients with kidney disease, patients on dialysis, patients infected with hepatitis C virus genotype 1 who have been treated with an NS5A inhibitor or an NS3/4A protease inhibitor, but not both in the past [3-5]. The enzymes, nonstructural protease 3A (NS3A), 4A (NS4A) and 5A (NS5A) are involved in the viral RNA replication and viron assembly. Pibrentasvir acts as NS5A inhibitor whereas glecaprevir acts NS3/4A protease inhibitor. Thus, pibrentasvir and glecaprevir blocks the growth of hepatitis C virus in the patients by inhibiting its RNA replication and viron assembly [6].

The combination of these two drugs is not official in any pharmacopoeia. Hence, there is need to develop an analytical method that can quantify pibrentasvir and glecaprevir simultaneously. Only one HPLC [7] and one UPLC [8] methods have been previously reported for the simultaneous determination of pibrentasvir and glecaprevir. Yet to date simultaneous quantification of pibrentasvir and glecaprevir in bulk and tablet dosage forms using stability indicating RP-HPLC has not been reported. The present study describes a novel, simple, sensitive and rapid stability indicating RP-HPLC method for the simultaneous estimation of pibrentasvir and glecaprevir in bulk and in tablets using photodiode array detection with a run time of 7 min.

2. Experimental

2.1 Apparatus

HPLC apparatus used in the investigation is a Waters Alliance HPLC system (model 2965) equipped with 2998 photodiode array detector. Waters Empower2 software was used to process the chromatographic data. The chromatographic separation and analysis were carried out on an YMC C18 column (150 mm × 4.6 mm i.d., particle size 5 µm). Electronic balance model ELB 300 and Digisun pH meter were used for weighing samples and measuring pH, respectively.

2.2 Materials

Pibrentasvir and glecaprevir reference standard was procured from Lara Drugs Private Limited (Telangana, India) as gift samples. Maviret® tablets (AbbVie Limited, UK), labeled to contain 100 mg glecaprevir and 40 mg pibrentasvir per tablet was obtained from the local pharmacy market. HPLC grade acetonitrile and methanol were purchased from Merck Pvt Ltd (Mumbai, India). Analytical reagent-grade Potassium dihydrogen phosphate, orthophosphoric acid, hydrochloric acid, hydrogen peroxide and sodium hydroxide were obtained from Sd Fine Chemicals Ltd (Mumbai, India). Distilled water purified by a Millipore Milli-Q apparatus (Millipore, France) was used right through the investigation.

2.3 Preparation of mobile phase

Mobile phase consisted of 0.1M potassium dihydrogen orthophosphate, methanol and acetonitrile (40:35:25, *v/v/v*). The pH adjusted to 4.5 with dilute Ortho phosphoric acid solution. Prior to use, mobile phase was filtered via 0.45 µm membrane filter and sonicated for 10 min.

2.4 Preparation of standard solutions

An accurately weighed quantity Pibrentasvir (40 mg) and glecaprevir (100 mg) reference standard material was transferred to a 100 ml volumetric flask, and dissolved in 100 ml mobile phase. This stock solution (pibrentasvir - 400 µg/ml and glecaprevir - 1000 µg/ml) was further diluted aptly with mobile phase to produce a working standard solution of 40 µg/ml pibrentasvir and 1000 µg/ml glecaprevir.

2.5 Preparation of tablet sample solution

For tablet sample solution preparation, 20 tablets were weighed and the average weight was determined. Tablets were crushed to a fine powder. The quantity of the powder equivalent to 100 mg of glecaprevir and 40 mg of pibrentasvir was transferred to 100 ml volumetric flasks. A 30 ml of mobile phase was added. The contents of the flask were sonicated for 20 min and the volume was diluted to 100 ml with mobile phase. This solution ((pibrentasvir - 400 µg/ml and glecaprevir - 1000 µg/ml) was diluted appropriately with mobile phase to give a concentration 40 µg/ml pibrentasvir and 100 µg/ml glecaprevir. This solution was filtered through a 0.45 µm membrane filter.

2.6 Chromatography conditions

- Mobile Phase: 0.1M KH_2PO_4: methanol: acetonitrile (40:35:25,*v/v/v*)
- pH of mobile phase: 4.5
- Mode of elution: Isocratic mode
- Flow rate: 1.0 ml/min
- Column: YMC C18 analytical column (150 mm × 4.6 mm i.d., particle size 5 µm)
- Column temperature: 25˚C
- Volume of injection: 10 µl
- Detection wavelength: 228 nm

2.7 Preparation of stress degradation samples

Stress degradation was performed following ICH guidelines [9].

2.7.1 Acid and alkali hydrolysis

A quantity of tablet powder equivalent to 100 mg of glecaprevir and 40 mg of pibrentasvir was transferred to a 100 ml volumetric flask and mixed with 10 ml of 0.1 N hydrochloric acid (for acid hydrolysis) or 0.1 N sodium hydroxide (for alkali hydrolysis). The prepared solutions were subjected to sonication at room temperature for 30 min. The samples were neutralized with an amount of acid (in alkali hydrolysis) or base (in acid hydrolysis) equivalent to that of the earlier added. The volume of the flask was diluted to the mark with mobile phase.

2.7.2 Oxidation
Tablet powder (equivalent to 100 mg of glecaprevir and 40 mg of pibrentasvir) was transferred to a 100 ml volumetric flask. 10 ml of 30% hydrogen peroxide solution was added to the flask and the mixture was sonicated at room temperature for 30 min. The volume of the flask was diluted to the mark with mobile phase.

2.7.3 Thermal and photolytic degradation
Thermal and photolytic degradation samples were prepared using tablet powder (equivalent to 400 mg sofosbuvir and 100 mg velpatasvir). The tablet powder was exposed to 105ºC for 30 min in oven (for thermal degradation) or exposed to sun light for 24 h (or photolytic degradation). After the specified period of degradation, tablet powder was cooled and transferred to 100 ml volumetric flasks. 30 ml of mobile phase was added. The contents of the flask were sonicated for 20 min and the volume was diluted to 100 ml with mobile phase.

2.8 Calibration curve
Different concentrations of glecaprevir and pibrentasvir in the range of 25-200 µg/ml and 10-80 µg/ml, respectively were prepared in mobile phase from the stock standard solution (pibrentasvir - 400 µg/ml and glecaprevir - 1000 µg/ml). 10 µL was injected into HPLC system from each concentration. Separation and analysis was done using the described chromatographic conditions. The results were recorded as peak areas which collectively with the corresponding concentrations of the analytes were then used to plot the calibration curves and estimate the regression equations of each component.

2.9 Degradation study
The stress samples prepared in the section "preparation of stress degradation samples" were diluted aptly with mobile phase to get a concentration of 40 µg/ml pibrentasvir and 100 µg/ml glecaprevir for analysis. All the stress degraded samples were filtered via 0.45 µm membrane filter. 10 µl of degraded sample was injected into HPLC system. The peak areas of pibrentasvir and glecaprevir were recorded. Working standard solution at the same concentration level was used to calculate the percentage of drug remained in each degradation condition. The peak purity of pibrentasvir and glecaprevir was also determined for all degraded samples using photodiode array detector.

2.10 Analysis of tablet sample
10 µl of tablet sample solution (40 µg/ml pibrentasvir and 100 µg/ml glecaprevir) was injected into HPLC system and analyzed using the described chromatographic conditions. The concentrations of pibrentasvir and glecaprevir were determined from the corresponding calibration curves or from corresponding regression equations.

3. Results and Discussion
3.1 Method Development
Trials were done to develop a RP-HPLC method which was able to separate and quantify the pibrentasvir and glecaprevir in the presence of their stress degradants in short time with adequate sensitivity and selectivity. So as to achieve the good chromatographic separation of pibrentasvir, glecaprevir and their stress degradants, and to improve peaks symmetry, various parameters like choice of mobile phase, its composition, its flow rate and detection wavelength were considered during optimization of method.

During trials with different columns, it was observed that YMC C18 analytical column (150 mm × 4.6 mm i.d., particle size 5 µm) with temperature 25±2 ºC gave good results (good symmetric and sharp peaks). Hence the same column is used in the analysis. 0.1M KH_2PO_4, methanol and acetonitrile in different ratios, different flow rate and different pH were tried. Finally, 0.1M KH_2PO_4, methanol and acetonitrile in the ratio of 40:35:25 (*v/v/v*) with a flow rate of 1 ml/min and pH 4.5 provided less analysis time, good peak response, symmetric peaks and with best resolution. The sensitivity of the method was good at a wavelength of 228 nm. Therefore the same wavelength was selected as analytical wavelength. The chromatogram of pibrentasvir and glecaprevir after optimization is given in Fig. 3.

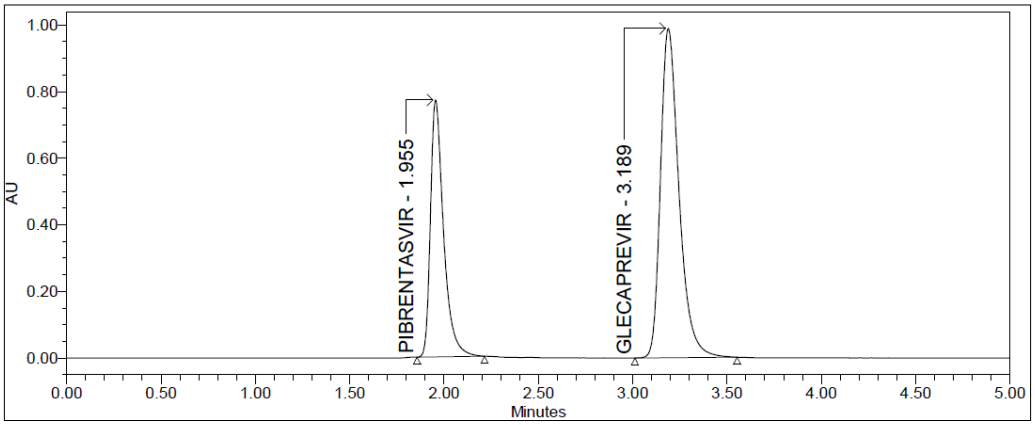


Fig 3: Chromatogram of pibrentasvir and glecaprevir

3.2 Method validation
Instructions as given by ICH guidelines for method validation were followed [10].

3.2.1 System suitability testing parameters
System suitability was assessed by calculating different parameters such as percent relative standard deviation of peak area response and retention time of drugs, plate count, tailing factor and resolution. For this, working standard solution at a concentration of 40 µg/ml pibrentasvir and 100 µg/ml glecaprevir was injected into the HPLC system five times. Results presented in Table 1 proved that the values are within the accepted limits indicating suitability of the system for good chromatographic separation and analysis.

Table 1: System suitability parameters of the developed method

Parameters	Pibrentasvir*	Glecaprevir*	Recommended limits
Retention time	1.197 (%RSD – 0.046)	3.220 (%RSD – 0.301)	RSD ≤2
Peak area	5832905 (%RSD – 0.213)	10665002 (%RSD - 0.150)	RSD ≤2
USP resolution	-	8.212	> 1.5
USP plate count	4084	5166	> 2000
USP tailing factor	1.452	1.308	≤ 2

* Values given are average of five determinations

3.2.2 Selectivity

The selectivity of the method was demonstrated by comparing the chromatogram of a placebo solution, a mobile phase blank, a tablet sample solution (40 µg/ml pibrentasvir and 100 µg/ml glecaprevir) with a drug standard solution (40 µg/ml pibrentasvir and 100 µg/ml glecaprevir). Chromatograms of placebo solution (Fig. 4a) and mobile phase blank (Fig. 4b) showed that there was no interference from common excipients and components of mobile phase. Chromatogram of tablet sample solution (Fig. 4c) proved that tablet excipients did not interfere as no additional interfering peaks were observed. These results proved the selectivity of the method.

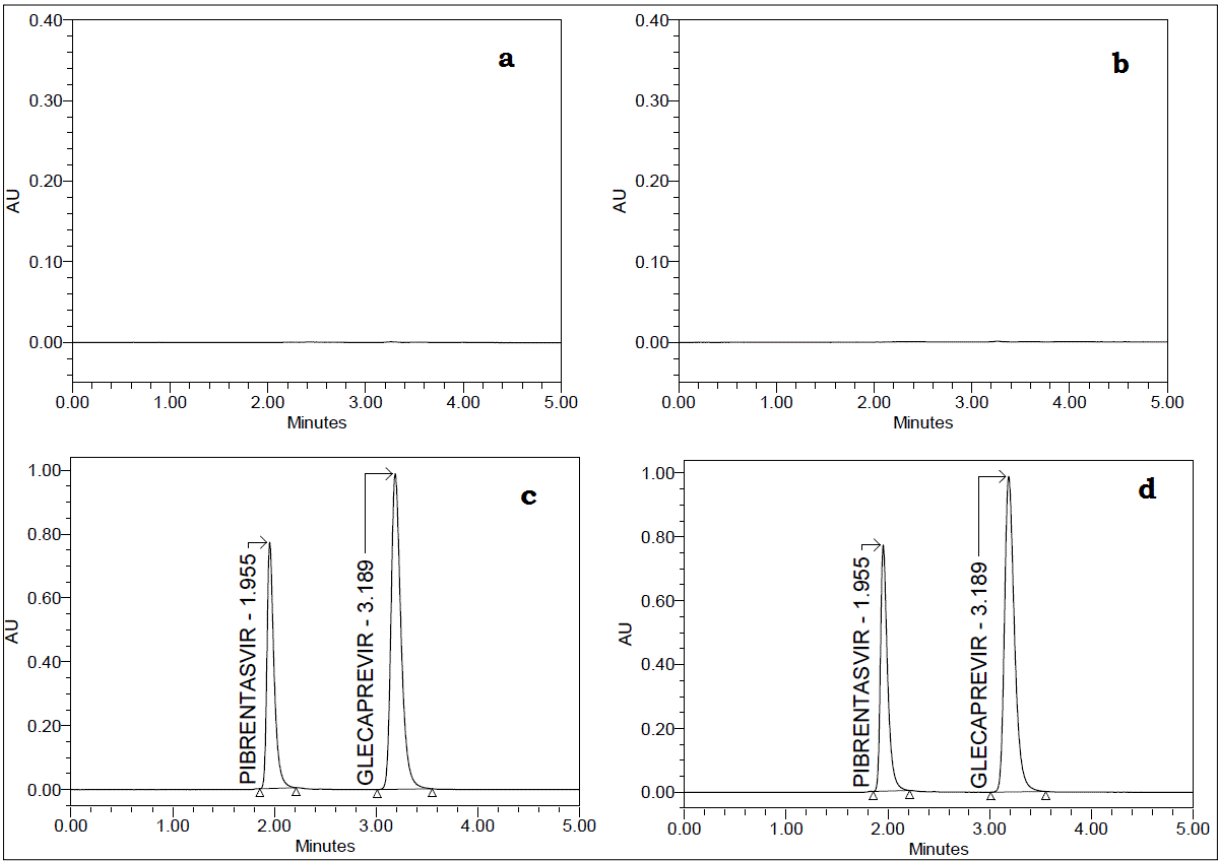


Fig 4: Chromatogram of (a) Placebo blank (b) Mobile phase blank (c) Tablet sample solution (d) Standard drug solution

3.2.3 Linearity

The peak area against the concentration of pibrentasvir and glecaprevir was linear in the range of 10–80 and 25–200 µg/ml, respectively. Linear regression data for the calibration curves are presented below.

PA = 14582 c + 0.052 (R^2 = 0.9996) for pibrentasvir

PA = 10665 c - 0.060 (R^2 = 0.9998) for glecaprevir

PA = peak area; c = concentration of drug (µg/ml); R^2 = Regression coefficient

Low values of intercept and slope, and good regression coefficient values confirming the excellent linearity of the method.

3.2.4 Limit of detection and limit of quantification

The limits of detection (LOD) and quantitation (LOQ) were calculated based on the signal-to-noise ratio of 3:1 and 10:1, respectively. LOD and LOQ of the method were found to be 0.273 µg/ml and 0.910 µg/ml as well as 0.950 µg/ml and 3.167 µg/ml for pibrentasvir and glecaprevir, respectively. Adequate sensitivity of the method was confirmed through the low values of LOD and LOQ.

3.2.5 Precision and accuracy

Method precision, expressed as relative standard deviation of peak area, was tested by analyzing working standard solution with concentration 40 µg/ml pibrentasvir and 100 µg/ml glecaprevir for five times. All results in Table 2 did not go beyond the acceptance criteria which were ≤ 2%. Method accuracy was tested similarly as precision and was calculated as percentage recovery. The mean recovery values of pibrentasvir and glecaprevir by the proposed method are ~ 100%, Table 2. These values of relative standard deviation and percent recoveries proved the precision and accuracy of the method, respectively.

Table 2: Method precision and accuracy results for analysis of pibrentasvir and glecaprevir

Sample no.	Pibrentasvir			Glecaprevir		
	Concentration of drug (µg/ml)	Peak area (mAU)	Recovery (%)	Concentration of drug (µg/ml)	Peak area (mAU)	Recovery (%)
1	40	5835296	99.74	100	10654081	99.70
2	40	5831450	99.68	100	10676200	99.90
3	40	5832983	99.70	100	10669336	99.84
4	40	5833889	99.72	100	10677620	99.92
5	40	5835905	99.75	100	10642274	99.59
6	40	5837504	99.78	100	10659774	99.75
Mean	40	5834505	99.73	100	10663214	99.78
RSD (%)	-	0.037	0.036	-	0.129	0.128

3.2.6 Recovery test

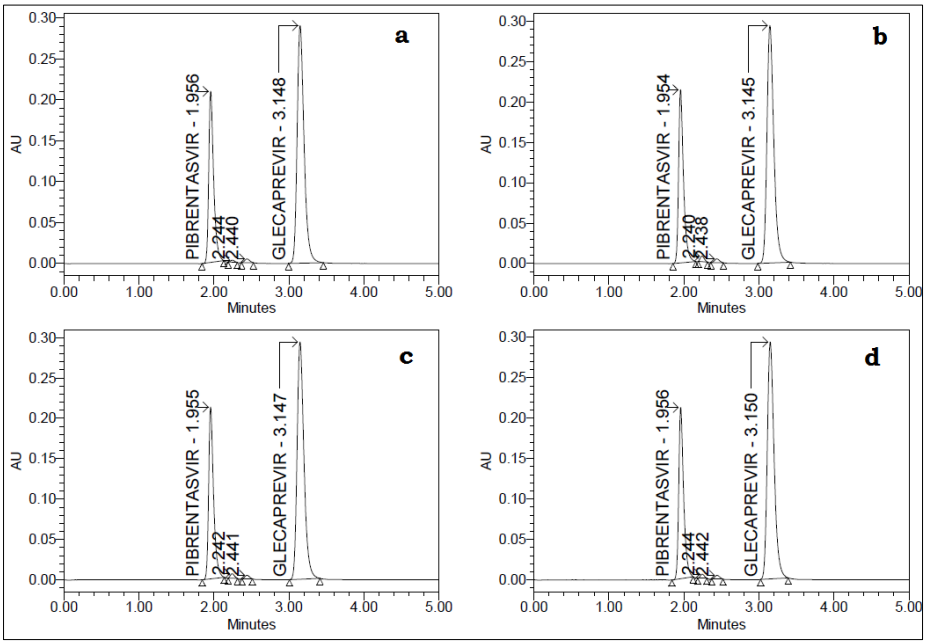
The excipients effect on the analysis was assessed by recovery studies using the standard addition samples. Standard addition samples were prepared by adding the pure pibrentasvir and glecaprevir into the solution of placebo blank. These standard addition samples have three different concentration levels (50%, 100% and 150%) for pibrentasvir and glecaprevir. The results of analysis, which correspond to recovery (%) were given in Table 3. The analysis of the samples was repeated three times for each concentration level. As it was shown in Table 3, the effect of excipients in the analysis of pibrentasvir and glecaprevir was not observed.

Table 3: Analysis results of pibrentasvir and glecaprevir in standard addition samples

Spiked level (%)	Concentration of drug (µg/ml)		Recovery (%)	Mean (%)	Concentration of drug (µg/ml)		Recovery (%)	Mean (%)
	added	found			added	found		
	Pibrentasvir				Glecaprevir			
50	20.00	19.92	99.61	99.67	50.00	49.91	99.82	99.83
	20.00	19.93	99.64		50.00	49.88	99.77	
	20.00	19.95	99.74		50.00	49.95	99.90	
100	40.00	39.92	99.81	99.73	100.00	99.60	99.60	99.65
	40.00	39.87	99.68		100.00	99.37	99.37	
	40.00	39.87	99.69		100.00	99.99	99.99	
150	60.00	59.79	99.65	99.64	150.00	149.02	99.35	99.62
	60.00	59.79	99.65		150.00	149.60	99.74	
	60.00	59.76	99.61		150.00	149.68	99.79	

3.2.7 Specificity

To establish specificity and stability indicating nature of the proposed method stress degradation studies were performed. For this tablet sample solution of pibrentasvir and glecaprevir was subjected to various stress degradation conditions like acid, alkali, oxidation, thermal and photolytic. The chromatograms of pibrentasvir and glecaprevir after stress degradation are shown in Fig. 5. In all the chromatograms, one peak of pibrentasvir, one peak of glecaprevir as well as two additional peaks of degradants was observed. Pibrentasvir and glecaprevir showed more degradation under thermal and alkali, respectively. Results of the stress degradation studies are given in Table 4. Peak purity study was performed on all degraded samples with a photo diode detector. In all the degraded samples, purity angle was lower than the purity threshold for pibrentasvir and glecaprevir Table 4. This indicated the peaks of pibrentasvir and glecaprevir passed the peak purity test and were pure in all the degraded samples.



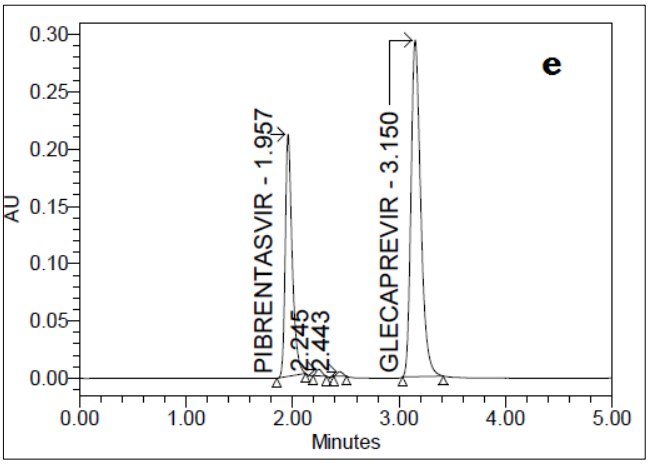


Fig 5: Chromatogram of (a) Acid degradation (b) alkali degradation (c) Peroxide degradation (d) Thermal degradation (e) Photo degradation

Table 4: Summary of stress degradation studies of pibrentasvir and glecaprevir

Drug	Type of degradation	Peak area (mAU)	Assay (%)	Degradation (%)	Purity Angle	Purity Threshold
Pibrentasvir	Acid	5141724	87.89	12.11	0.259	0.400
	Base	5018548	85.78	14.22	0.230	0.467
	Oxidative	5014749	85.72	14.28	0.194	0.303
	Heat	4838483	82.7	17.3	0.315	0.411
	Sunlight	4998965	85.45	14.55	0.233	0.472
Glecaprevir	Acid	9654480	90.34	9.66	0.622	0.841
	Base	9546179	89.33	10.67	0.625	0.899
	Oxidative	9741201	91.16	8.84	0.590	0.754
	Heat	9548476	89.35	10.65	0.593	0.790
	Sunlight	9558303	89.44	10.56	0.598	0.859

3.2.8 Robustness

The method robustness was studied by making small and deliberate variations in the chromatographic conditions such as altering the flow rate and column temperature. Robustness was studied with standard solution of concentration 40 µg/ml pibrentasvir and 100 µg/ml glecaprevir. The system suitability parameters were assessed in the varied conditions. The results are summarized in Table 5. From the values obtained, it was evident that the system suitability parameters in the varied conditions were within the acceptance limits. Hence, the method was robust.

Table 5: Robustness data for pibrentasvir and glecaprevir

Parameter	Investigated value	System suitability parameters		
		USP Plate Count	USP Tailing	USP resolution
Pibrentasvir				
Flow rate (ml/min)	1.0 - 0.1	3819	1.39	-
	1.0 + 0.1	4441	1.46	-
Temperature (°C)	25 - 2	3872	1.41	-
	25 + 2	4386	1.46	-
Glecaprevir				
Flow rate (ml/min)	1.0 - 0.1	4940	1.25	7.60
	1.0 + 0.1	5471	1.28	7.85
Temperature (°C)	25 - 2	4976	1.26	7.67
	25 + 2	5438	1.29	7.84

4. Conclusion

In this study, a sensitive, precise, accurate and selective stability indicating RP-HPLC method was established to quantify glecaprevir and pibrentasvir simultaneously. The lower analysis time (5 min) showing the rapidity of the method. The developed and validated method was applied with a good success for quantitation of glecaprevir and pibrentasavir in tablet dosage forms. Therefore, the proposed method can be used in the analysis of glecaprevir and pibrentasavir tablets in quality control laboratories. Forced degradation study demonstrated that stress degradants were well separated from glecaprevir and pibrentasvir. Hence confirming the proposed method as stability-indicating and can be employed for the estimation of glecaprevir and pibrentasvir in stability studies.

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