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Simultaneous Assay of Two Antiviral Agents, Pibrentasvir and Glecaprevir, Using Stability Indicating RP-HPLC Method in Bulk and Tablets

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ABSTRACT

The aim of the current investigation was to develop and validate a rapid and simple stability indicating RP-HPLC method for simultaneous determination of two antiviral agents - pibrentasvir and glecaprevir in bulk drug and tablet dosage forms. The chromatographic separation and analysis was done using Sunsil C18 column (250 mm × 4.6 mm; 5 µm particle size) with mobile phase mixture of potassium dihydrogen phosphage (0.1 M; pH 4.0) and methanol in a ratio 55:45 (v/v). The chromatographic data was acquired using photodiode array detector monitored at 226 nm. The method was validated for linearity, sensitivity, selectivity, specificity, precision, accuracy and robustness. The method had shown good linearity over the concentration range of 5-80 µg/ml (R2 - 0.9997) for pibrentasvir and 12.5-200 µg/ml (R2 - 0.9998) for glecaprevir. All validation parameters data recorded were in acceptable limits. Degradation products, produced after subjecting pibrentasvir and glecaprevir to ICH prescribed stress conditions, did not interfere with the assay of selected drug combination. Therefore, the assay could be considered as stability-indicating. The method has been applied for assay of pibrentasvir and glecaprevir in their tablet forms with satisfactory accuracy and precision.

Keywords: Pibrentasvir, Glecaprevir, Anti-viral agents, RP-HPLC, Analysis

INTRODUCTION

Pibrentasvir is a antiviral agent and 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] pyrro 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 (Figure 1). Pibrentasvir serve as nonstructural protease 5A inhibitor. Nonstructural protease 5A enzyme is required for hepatitis C viral RNA replication and viron assembly [1,2].

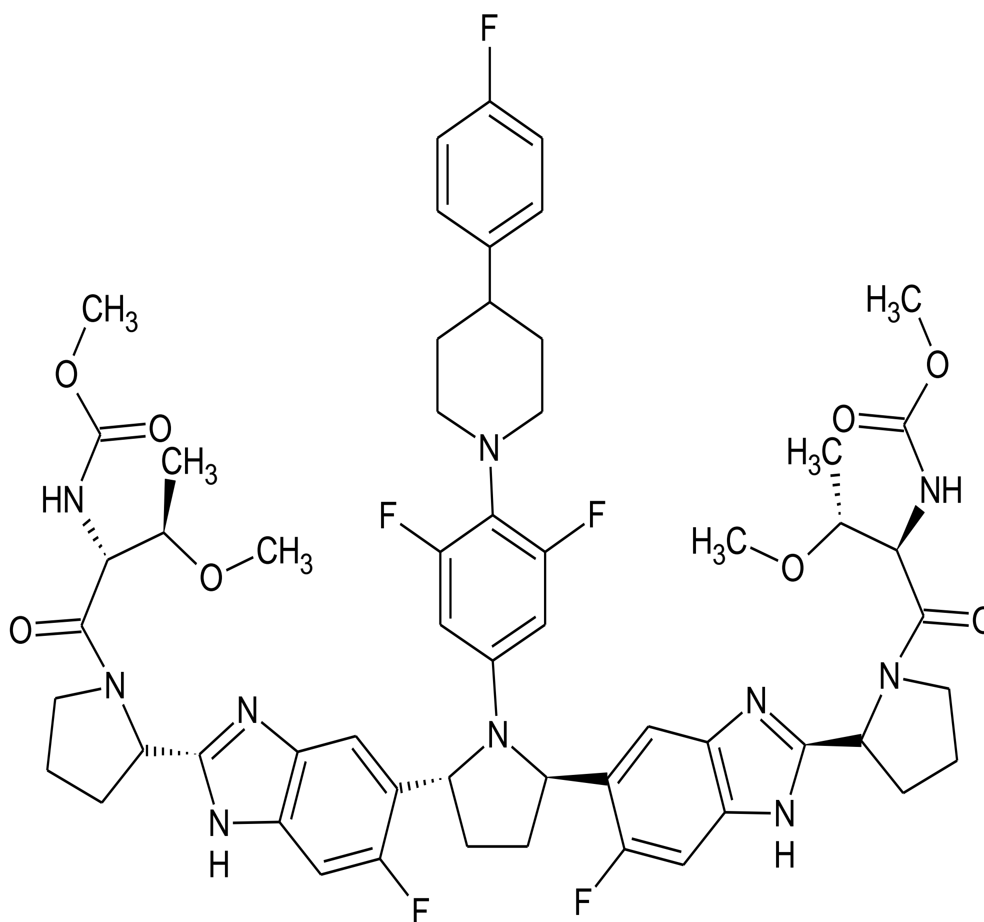

Figure 1: Pibrentasvir chemical structure.

Glecaprevir is also an antiviral agent, chemically described as (3aR,7S,10S,12R,21E,24aR)-7-tert-Butyl-N-{(1R,2R)-2-(difluoromethyl)-1-[(1-methylcyclopropane-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 (Figure 2). Glecaprevir acts nonstructural protease 3/4A protease inhibitor. These two enzymes are essential for hepatitis C viral RNA replication and viron assembly [3,4].

The combination of pibrentasvir and glecaprevir was accepted by Food drug and Administration (FDA) in 2017 August. This combination is prescribed to treat adult patients with chronic hepatitis C virus genotypes I to VI with/without mild cirrhosis. This is also used to treat patients infected with hepatitis C virus genotype 1 who had treatment with NS5A inhibitor alone or NS3/4A protease inhibitor alone, and not both in the past [5,6].

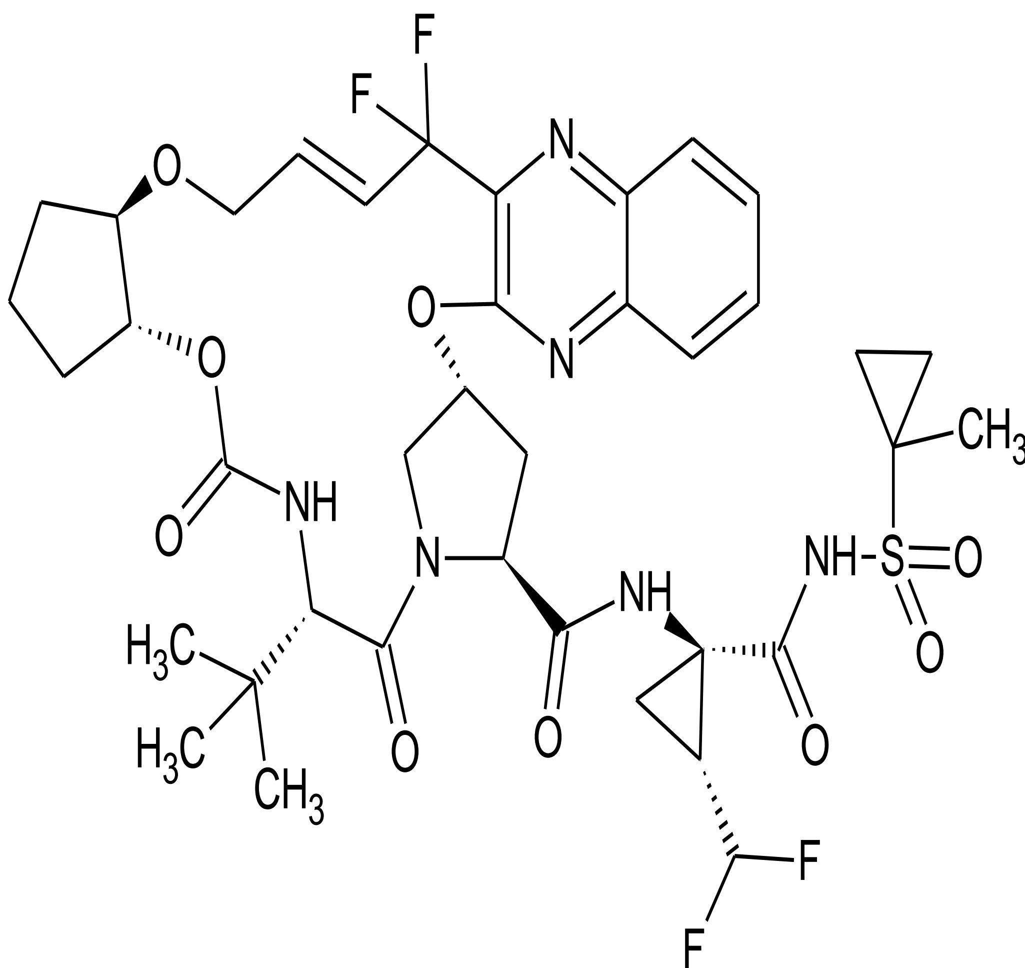

Figure 2: Glecaprevir chemical structure.

Simultaneous quantification of pibrentasvir and glecaprevir is not official in any major pharmacopoeias. So far only one HPLC [7] and one UPLC [8] methods have been reported for the simultaneous assay of pibrentasvir and glecaprevir. Till now no stability indicating RP-HPLC method was reported. In this present work, we are concerned with the development and validation of a stability indicating RP-HPLC methods using 0.1M KH_2PO_4: Methanol (55:45) as mobile phase and Sunsil C18 analytical column.

MATERIALS AND METHODS

Instrumentation

Pibrentasvir and glecaprevir are separated and analyzed on HPLC instrument (Waters 2695 alliance) equipped with a column heater, autosampler injector and quaternary pump. The detector used was Waters 2998 photodiode array detector and the stationary phase was SUNSIL C18 column (250 × 4.6 mm i.d, 5 µm particle sizes).

Materials

Reference standards

Pibrentasvir and glecaprevir reference standards were procured as gift samples from Lara Drugs Private Limited (Telangana, India).

Tablet formulation

Maviret® tablets (AbbVie Limited, Berkshire, UK) labeled to contain 100 mg glecaprevir and 40 mg pibrentasvir.

Chemicals and solvents

Chemicals and solvents used are analytical grade and HPLC grade, respectively. Orthophosphoric acid, potassium dihydrogen phosphate, hydrogen peroxide, hydrochloric acid and sodium hydroxide are obtained from SD. Fine Chemicals Ltd., Mumbai, India. Methanol is obtained from Merck India Ltd, Mumbai, India.

Stock solution of pibrentasvir (400 µg/ml) and glecaprevir (1000 µg/ml)

Prepared by accurately weighing 40 mg of pibrentasvir and 100 mg of glecaprevir in 100 ml volumetric flask and dissolving in mobile phase (0.1M KH_2PO_4: methanol, 55:45 v/v).

Working solutions of pibrentasvir and glecaprevir

Working solutions with concentration range of 5-80 (µg/ml) pibrentasvir and 12.5-200 (µg/ml) glecaprevir were prepared through apt dilution of stock solution with mobile phase(0.1M KH_2PO_4: methanol, 55:45 v/v).

Chromatographic conditions for the assay

Simultaneous RP-HPLC analysis of pibrentasvir and glecaprevir was carried out at a wavelength of 226 nm using 0.1M KH_2PO_4 and methanol in the ratio of 55:45 (v/v) as mobile phase set at a flow rate of 1.0 ml/min. 10 µL sample was injected into column for analysis and column temperature was 25°C. Filtered (with 0.45 µm pore size membrane filter) and degassed (sonicated for 20 min in sonicator) mobile phase was used during analysis.

General assay procedure

Working solutions having concentration 5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, 60 µg/ml, 70 and 80 µg/ml pibrentasvir, and 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml, 125 µg/ml, 150 µg/ml, 175 µg/ml and 200 µg/ml glecaprevir was injected (10 µl) into the HPLC system. Chromatography of pibrentasvir and glecaprevir was performed with photodiode array detector at 226 nm. Pibrentasvir and glecaprevir peak areas were recorded. Calibration curves for pibrentasvir and glecaprevir were plotted separately with drug concentration vs. respective peak area. Alternatively regression equation was derived.

Tablet sample assay

Ten Maviret® tablets were homogenized and accurately weighed. Amount of tablet powder equivalent to 100 mg of glecaprevir and 40 mg of pibrentasvir was transferred into 100 ml volumetric flask. 50 ml of mobile phase (0.1M KH_2PO_4: Methanol, 55:45 v/v) was added and the solution was sonicated for 20 min. The solution was filtered and the appropriate solvent (mobile phase) was added till the volume to prepare sample stock solution (pibrentasvir - 400 µg/ml and glecaprevir - 1000 µg/ml). Working sample solution (pibrentasvir - 40 µg/ml and glecaprevir - 100 µg/ml) for analysis was prepared in the mobile phase. This working solution was analyzed with the developed method. The content of glecaprevir and pibrentasvir in tablets was calculated either using respective calibration curve or regression equation.

Forced degradation studies

Forced degradation studies were executed using tablet sample solution (pibrentasvir-400 µg/ml and glecaprevir-1000 µg/ml) following ICH prescribed conditions [9]. 0.1 N HCl, 0.1 N NaOH, 30% hydrogen peroxide and Milli Q water was used for acid, base, oxidative and neutral degradation. 10 ml of each of 0.1N HCl, 0.1N NaoH, 30% hydrogen peroxide and Milli Q water were added into 10 ml tablet sample solution of all four sets in 100 ml volumetric flasks. All the resultant solutions were sonicated at room temperature for 30 min. After the specified degradation time, mixture was neutralized with sufficient volume of 0.1N NaOH (in acid degradation) and 0.1N HCl (in base degradation).

The resulting solutions were filtered and contents of all the four volumetric flasks were made up to mark with mobile phase. 10 ml of the above prepared solutions were diluted with mobile phase to 100 ml to get a working solution (pibrentasvir-40 µg/ml and glecaprevir-100 µg/ml) for analysis by the proposed method. Thermal degradation was conducted by taking tablet powder equivalent to 100 mg of glecaprevir and 40 mg of pibrentasvir and heated in oven at 105°C for 30 min. Photo degradation was conducted by taking tablet powder equivalent to 100 mg of glecaprevir and 40 mg of pibrentasvir and placed in direct sunlight for 24 hr. After degradation, tablet sample solution preparation and analysis was done as described in section "Tablet sample assay".

RESULTS AND DISCUSSION

Optimization of method

The RP-HPLC procedure was optimized with an aim to develop stability indicating method for the simultaneous estimation of glecaprevir and pibrentasvir. To achieve optimum separation and resolution between the peaks of glecaprevir and pibrentasvir and their possible degradants, different parameters, i.e., mobile phase composition, flow rate, pH, and column type, were studied.

Hiber C18 (250 mm × 4.6 mm, 5 µm), Inertsil C18 (250 mm × 4.6 mm, 5 µm), YMC C18 (250 mm × 4.6 mm, 5 µm) and Sunsil C18 (150 mm × 4.6 mm, 5 µm) analytical columns were investigated. Acceptable separation and resolution was achieved on Sunsil C18 (150 mm × 4.6 mm, 5 µm) with column temperature 25°C. Mobile phase combinations, methanol with 0.1% orthophosphoric acid and methanol with 0.1M KH_2PO_4, were tried in different ratios, flow rate and pH. Appropriate separation with best resolution, symmetrical and sharp shapes for studied drugs was achieved with 0.1M KH_2PO_4: methanol (55:45, v/v) with pH 4.0 (adjusted with dilute orthophosphoric acid) and a flow rate of 1.0 ml/min.

The peak area response of the glecaprevir and pibrentasvir was good at 226 nm. Hence, the wavelength of 226 nm was used for detection and quantification. Using the above mentioned chromatographic conditions, the run time was 6 min, and the retention times of pibrentasvir and glecaprevir were 1.810 in and 3.649 min, respectively (Figure 3).

Method validation

The RP-HPLC method developed was validated by means of ICH guidelines [10]. Validation parameters included system suitability, selectively, linearity, LOD, LOQ, accuracy, precision, robustness and specificity.

System suitability

The optimized RP-HPLC method was evaluated for system suitability by injecting working solution (glecaprevir-100 µg/ml and pibrentasvir-40 µg/ml) 5 times. The following parameters are investigated: plate count, tailing factor, resolution, relative standard deviation of retention time and relative standard deviation of peak area. As shown in Table 1, the values are within the accepted limits.

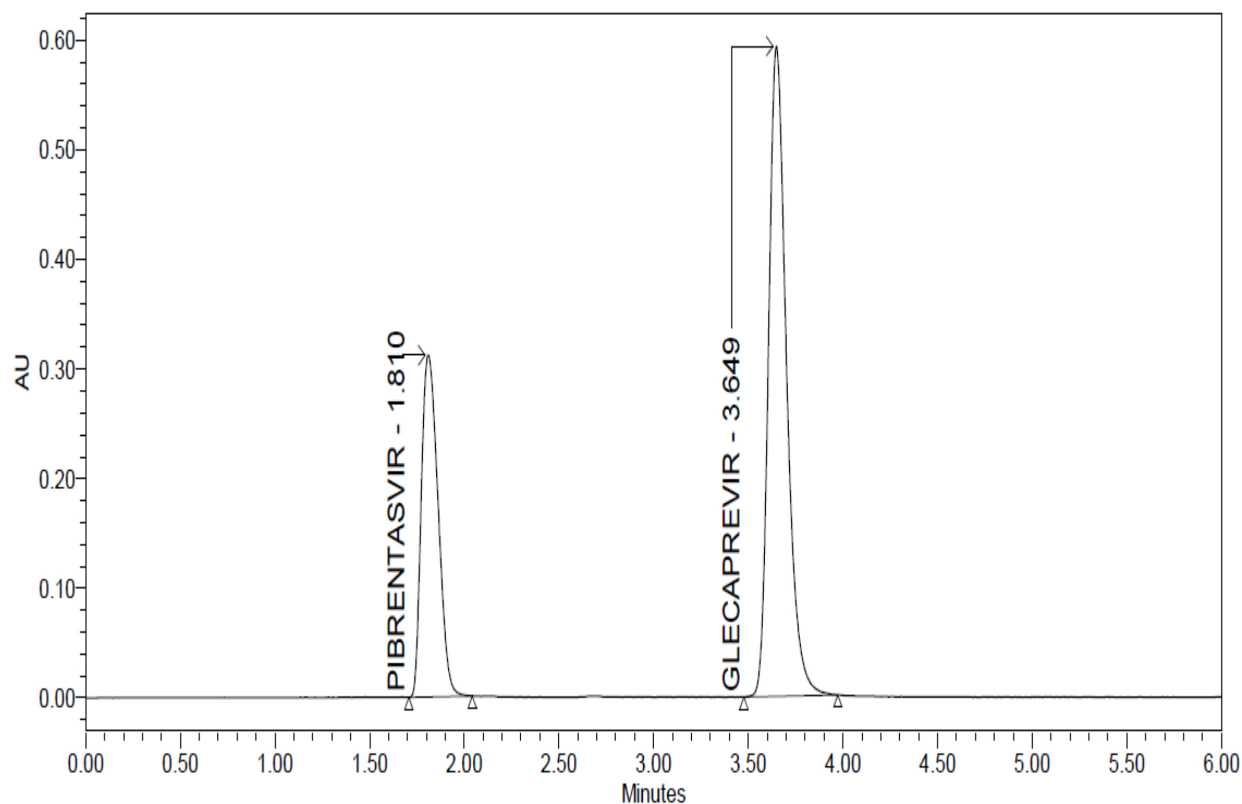

Figure 3: Chromatogram for separation of glecaprevir and pibrentasvir with optimized conditions

Table 1: System suitability test for the developed method.

Parameters	**Pibrentasvir**		**Glecaprevir**		**Recommended limit**
	Mean*	**RSD (%)**	**Mean***	**RSD (%)**	
Retention time	1.808	0.03	3.645	0.055	RSD ≤ 2
Peak area	1921529	0.205	3894481	0.342	RSD ≤ 2
Plate count	9902	0.706	7347	0.973	> 2000
Tailing factor	1.33	0.532	1.31	0.54	≤ 2
Resolution	-	-	10.784	1.087	> 2
Note: *Average of five determinations					

Selectivity

Selectivity was demonstrated by injecting working solution (glecaprevir-100 µg/ml and pibrentasvir-40 µg/ml), tablet sample solution (glecaprevir-100 µg/ml and pibrentasvir-40 µg/ml), mobile phase blank and placebo blank followed by comparing the chromatograms for any interference from excipients in placebo/tablet sample solution and components of mobile phase. Peaks are not observed in chromatogram of mobile phase blank (Figure 4) and placebo blank (Figure 4). The retention times of glecaprevir and pibrentasvir in chromatograms of working solution (Figure 4) and tablet sample solution (Figure 4) is same. The results proved the method's selectivity.

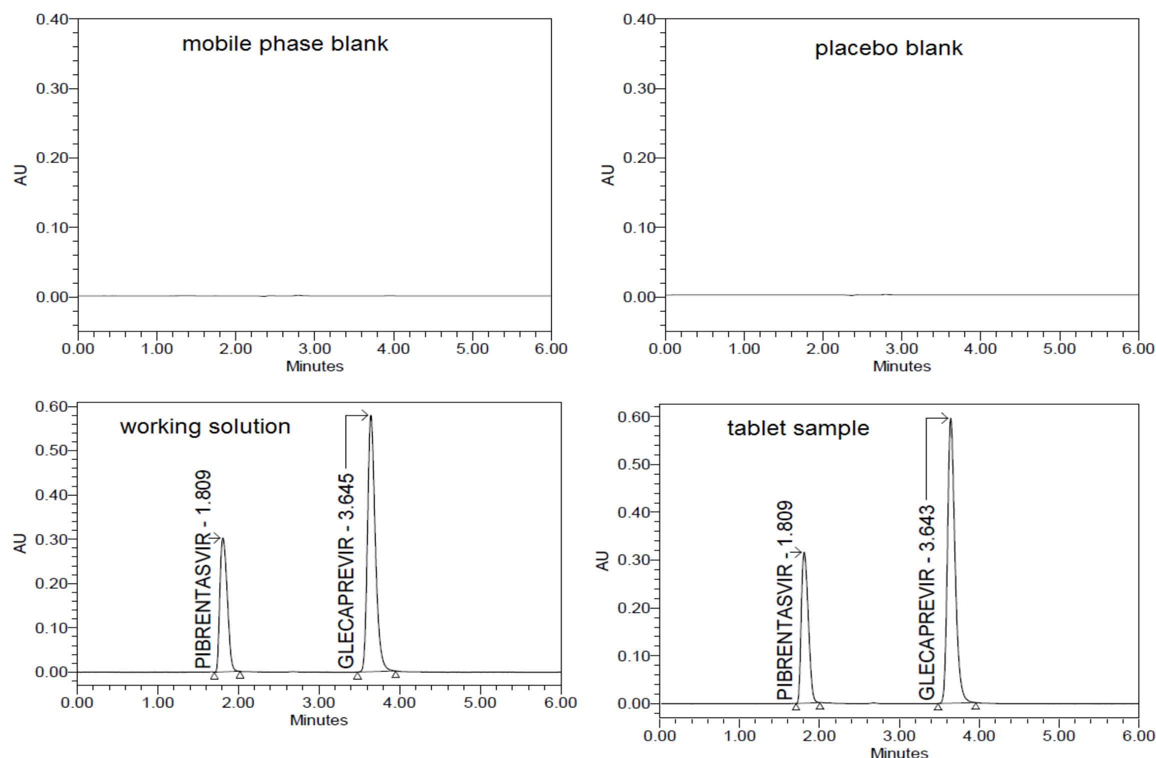

Figure 4: Chromatograms of method selectivity test.

Linearity and range

Calibration curve was found to be linear in range of 5-80 µg/ml for pibrentasvir and 12.5-200 µg/ml for glecaprevir. Y-intercept, Slope and regression coefficient (R^2) of regression analysis were calculated. The regression equation was:

For pibrentasvir - $y = 48093x + 1212$ ($R^2 = 0.9997$)

For glecaprevir - $y = 38923x + 1096$ ($R^2 = 0.9998$)

Regression coefficient was >0.999 and demonstrated a good degree of correlation and linearity of method.

Limits of detection (LOD) and quantitation (LOQ)

LOD and LOQ with 3:1 and 10:1 signal to noise ratio, respectively were determined for glecaprevir and pibrentasvir. Limit of detection was calculated as 0.154 µg/ml and 0.201 µg/ml for TP, pibrentasvir and glecaprevir, respectively whereas limit of quantitation was 0.512 µg/ml and 0.673 µg/ml, respectively.

Precision and accuracy

Injection samples containing pibrentasvir and glecaprevir with concentration 40 µg/ml and 40 µg/ml, respectively were analyzed 6 times by the developed method to test the precision and accuracy of the method. Percent relative standard deviations for pibrentasvir and glecaprevir peak area responses were found to be less than 0.5% which showed satisfactory precision of proposed method (Table 2) while percent assay for pibrentasvir and glecaprevir was found to 99.50% and 99.56%, respectively (Table 2) which proved the adequate accuracy of the method.

Tablet 2: Testing of precision and accuracy of the method.

Pibrentasvir			**Glecaprevir**		
Concentration (µg/ml)	**Peak area (mAU)**	**Assay (%)**	**Concentration (µg/ml)**	**Peak area (mAU)**	**Assay (%)**
40	1934072	99.85	100	3899056	99.62
40	1929918	99.63	100	3899951	99.64
40	1925637	99.41	100	3896264	99.55
40	1926042	99.43	100	3892937	99.46
40	1923179	99.29	100	3899175	99.62
40	1925256	99.39	100	3892411	99.45
Mean	1927351	99.5	**Mean**	3896632	99.56
RSD (%)	0.205	0.205	**RSD (%)**	0.085	0.085

Recovery studies

Recovery studies were carried out to further evaluate the method accuracy. Recovery studies were assessed via standard addition method. The preanalyzed tablet sample was spiked with additional 50%, 100% and 150% of pure drug and reanalyzed by developed method. Percent recoveries for pibrentasvir and glecaprevir were found in the range of 99.18-99.38% and 99.39-99.59%, respectively. The results at each level of pibrentasvir and glecaprevir are summarized in Table 3.

Table 3: Recovery of pibrentasvir and glecaprevir added to tablet sample.

Spiked level (%)	Amount of drug (mg)			Recovery (%)
	Tablet	**Added**	**Found***	
Pibrentasvir				
50	40	20	59.51	99.18
100	40	40	79.51	99.38
150	40	60	99.32	99.32
Glecaprevir				
50	100	50	149.08	99.39
100	100	100	199.03	99.52
150	100	150	248.83	99.53
Note: *Average of three determinations				

Stability studies

The summary of forced degradation study results for pibrentasvir and glecaprevir using the proposed method are given in Table 4. As in Figure 5, one degradation product was observed in all the applied stress conditions (acid, base, oxidative, thermal, photo and neutral). But the degradation product had no influence on peaks of pibrentasvir and glecaprevir. This confirmed the stability indicating nature of the proposed method for simultaneous quantification of pibrentasvir and glecaprevir in the presence of its degradants.

Table 4: Degradation of pibrentasvir and glecaprevir.

Variables	Pibrentasvir			Glecaprevir		
	Concentration (µg/ml)	**Recovered (%)**	**Degraded (%)**	**Concentration (µg/ml)**	**Recovered (%)**	**Degraded (%)**
0.1 N HCl	40	83.12	16.88	100	83.73	16.27
0.1N NaOH	40	87.88	12.12	100	86.95	13.05
30% H_2O_2	40	86.96	13.04	100	88.72	11.28

105°C	40	82.87	17.13	100	83.6	16.4
Sunlight	40	88.18	11.82	100	83.57	16.43
Water	40	88.52	11.48	100	88.43	11.57

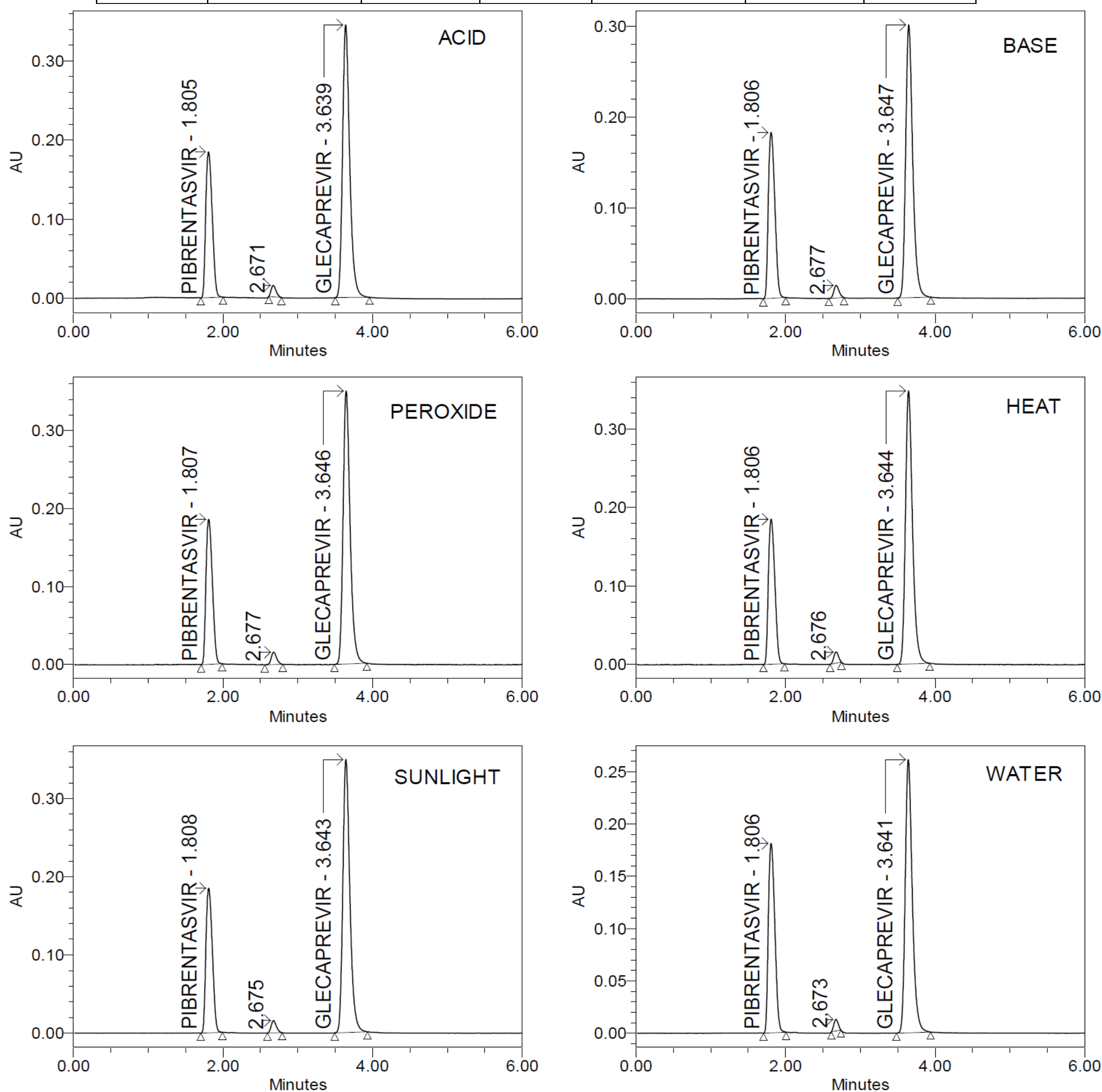

Figure 5: Chromatograms of stress degraded tablet sample.

Specificity

Specificity was assessed using photodiode array detection to make sure the homogeneity and to assess the purity of pibrentasvir and glecaprevir peaks under the applied stress conditions (acid, base, oxidative, thermal, photo and neutral). The peak purity values was less than peak threshold values in the applied stress conditions for pibrentasvir and glecaprevir peaks (Table 5). This confirmed the method's specificity.

Table 5: Homogeneity and purity of pibrentasvir and glecaprevir.

Condition	**Pibrentasvir**			**Glecaprevir**		
	Concentration	**Peak purity**	**Purity threshold**	**Concentration**	**Peak purity**	**Purity threshold**
	(µg/ml)			**(µg/ml)**		
0.1 N HCl	40	0.297	0.853	100	0.346	0.606
0.1 N NaOH	40	0.244	0.848	100	0.341	0.6
30% H_2O_2	40	0.298	0.849	100	0.35	0.601
105°C	40	0.215	0.847	100	0.336	0.6
Sunlight	40	0.275	0.852	100	0.341	0.605
Water	40	0.268	0.855	100	0.359	0.61

Robustness

The robustness of method was evaluated by assessing the effect of following deliberate variations in RP-HPLC conditions on the system suitability parameters:

Ratio of methanol in mobile phase (± 5%)

Flow rate of mobile phase (± 0.1 ml/min)

pH of mobile phase (± 0.2 units)

Column temperature (± 5°C)

Detection wavelength (± 2 nm)

The results are within the recommended limits, indicted the method robustness (Table 6).

Table 6: Method robustness data for pibrentasvir and glecaprevir.

Variation	Pibrentasvir			Glecaprevir		
	Plate count	Tailing factor	Resolution	Plate count	Tailing factor	Resolution
Ratio of methanol (+5%)	8903	1.30	-	7014	1.28	10.63
Ratio of methanol (-5%)	8056	1.31	-	7948	1.30	11.22
Flow rate (+0.1 ml/min)	7835	1.30	-	6637	1.27	10.43
Flow rate (-0.1 ml/min)	7833	1.30	-	6614	1.28	10.42
Temperature (+ 5°C)	9052	1.33	-	8089	1.34	11.25
Temperature (- 5°C)	9052	1.33	-	8089	1.34	11.25
pH (+ 0.2 units)	9922	1.32	-	7437	1.31	10.82
pH (- 0.2 units)	9903	1.32	-	7328	1.31	10.87
Wavelength (+ 2 nm)	9917	1.32	-	7288	1.32	10.79
Wavelength (- 2 nm)	9919	1.34	-	7402	1.31	10.85

Assay of pibrentasvir and glecaprevir in tablets

The developed and validated RP-HPLC method was applied for the assay of pibrentasvir and glecaprevir simultaneously in the tablet formulation. The tablet sample solution was prepared and injected into the HPLC system thrice. The percent recoveries and relative standard deviations of pibrentasvir and glecaprevir were calculated by using peak area response from the chromatograms. Acceptable mean percentage recoveries, 99.31% and 99.49% for pibrentasvir and glecaprevir, respectively were obtained. Relative standard deviations were 0.109% and 0.131% for pibrentasvir and glecaprevir, respectively. The values confirmed the accuracy and precision of the method for the estimation of pibrentasvir and glecaprevir simultaneously in the tablet formulation.

CONCLUSION

A rapid and simple stability indicating RP-HPLC method for the simultaneous assay of pibrentasvir and glecaprevir has been developed. The elution time for the selected analytes was short (6.0 min). The optimized chromatographic conditions provided excellent peak shapes with satisfactory system suitability parameters. The method was validated as stated by ICH guidelines and it was observed as linear, precise, accurate and robust. The developed method was applied productively for the assay of pibrentasvir and glecaprevir in their tablet forms. The adaptableness of the developed method to tablet formulations was proven by its satisfactory performance in terms of selectivity and recovery for pibrentasvir and glecaprevir in tablet samples. Therefore, the method may be utilized in quality control analysis of pibrentasvir and glecaprevir in bulk drugs or in the tablet forms. The stability indicating nature of the developed method was proved by determining pibrentasvir and glecaprevir in a single run with free of interference from stress degradation products.

REFERENCES

[1] Ng, V., et al. *In vitro* antiviral activity and resistance profile of the next-generation hepatitis C virus NS5A inhibitor pibrentasvir. *Antimicrob. Agents. Chemother,* **2017.** 61(5): e02558-16.

[2] Pibrentasvir. https://pubchem.ncbi.nlm.nih.gov/compound/Pibrentasvir#section=Top

[3] Glecaprevir. https://pubchem.ncbi.nlm.nih.gov/compound/Glecaprevir#section=Top

[4] Salam, KA., and Akimitsu, N., Hepatitis C Virus NS3 inhibitors: Current and future perspectives. *Biomed. Res. Int,* **2013.** 2013: 467869.

[5] Carrion, AF., and Martin, P., Glecaprevir + pibrentasvir for treatment of hepatitis C. *Expert. Opin. Pharmacother,* **2018.** 19: 413-419.

[6] T. Asselah, et al. Treatment of hepatitis C virus infection with direct-acting antiviral agents: 100% cure?, *Liver Int,* **2018.** 38: 7.

[7] Hemalatha, K., Simultaneous estimation of new analytical method development and validation of glecaprevir and pibrentasvir by high performance liquid chromatography. *Int. J. Med. Pharma. Sci,* **2018.** 3: 5.

[8] Sridevi, M., *European J. Biomed. Pharma. Sci,* **2018.** 5: 473.

[9] International Conference on Harmonization Q1B, Stability testing: Photostability Testing of New Drug Substances and Products. Proceeding of the International Conference on Harmonization, Geneva, **1996**.

[10] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonized Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology. ICH, Nov **2005**.