STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF GLECAPREVIR AND PIBRENTASVIR IN BULK FORM AND MARKETED PHARMACEUTICAL DOSAGE FORM BY USING RP-HPLC METHOD AS PER ICH GUIDELINES

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ABSTRACT

Objective: To develop and validate a simple, selective, precise, and accurate method for the simultaneous estimation of Glecaprevir and Pibrentasvir using reversed-phase high-performance liquid chromatography (RP-HPLC) technique in bulk and marketed formulation.

Method: The proposed method utilizes chromatographic conditions Symmetry C_{18}, 100A, 5µm, 250mm x 4.6mm i.d., mobile phase is consists of Methanol and Phosphate Buffer (pH-3.8-with Ortho Phosphoric Acid Solution) were taken in the ratio of 25:75 v/v, flow rate was maintained 1.0 ml/minute, column temperature was set at Ambient, detection wave length was 330 nm, and diluent was mobile phase.

Results: By injecting 6 times of the standard solution system suitability parameters were studied and results were found well under the acceptance criteria. The linearity study was performed by taking the concentration ranges from 60-140µg/ml and 24-56 µg/ml, and the R2 value was found to be 0.9996 & 0.9999 for Glecaprevir and Pibrentasvir respectively and the method precision (repeatability) and intermediate precision was found to be within the limits. The % recovery was found to be 100.02% and 100.05% for Glecaprevir and Pibrentasvir respectively. The Limit of detection and limit of quantitation for Glecaprevir and Pibrentasvir were found to be 0.09µg/ml and 0.29µg/ml & 0.34µg/ml and 0.923µg/ml respectively. Degradation study on Glecaprevir and Pibrentasvir was performed and concluded that the purity threshold was more than purity angle and within the acceptable range.

Conclusion: The developed RP-HPLC method for Glecaprevir and Pibrentasvir was found to be simple, precise, accurate, reproducible, and cost effective. Statistical analysis of the developed method conforms that the proposed method is an appropriate and it can be useful for the routine analysis. This method gives the basic idea to the researcher who is working in area such as product development and finish product testing.

Key Words: Glecaprevir and Pibrentasvir, RP-HPLC, Accuracy, Precision, ICH Guidelines.

INTRODUCTION

Glecaprevir is a Hepatitis C Virus NS3/4A Protease Inhibitor. The mechanism of action of glecaprevir[1] is as a HCV NS3/4A Protease Inhibitor, and P-Glycoprotein Inhibitor, and Breast Cancer Resistance Protein Inhibitor, and Organic Anion Transporting Polypeptide 1B1 Inhibitor, and Organic Anion Transporting Polypeptide 1B3 Inhibitor, and Cytochrome P450 3A Inhibitor, and Cytochrome P450 1A2 Inhibitor, and UGT1A1 Inhibitor. Glecaprevir[2] is a direct acting antiviral agent and Hepatitis C virus (HCV) NS3/4A protease inhibitor that targets 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 Label. The combinations of amino acid substitutions at NS3 position Y65H and D/Q168 also results in greater reductions in glecaprevir[3] susceptibility, and NS3 Q80R in genotype 3a patients also leads to glecaprevir resistance. The IUPAC Name of Glecaprevir is (1R,14E,18R,22R,26S,29S)-26-tert-butyl-N-[(1R,2R)-2-(difluoromethyl)-1-[(1-methylcyclopropyl) sulfonyl carbamoyl] cyclopropyl]-13,13-difluoro-24,27-dioxo-2,17,23-trioxa-4,11,25,28-tetrazapentacyclo [26.2.1.03,12.05,10.018,22] hentriaconta-3,5,7,9,11,14-hexaene-29-carboxamide. The Chemical Structure of Glecaprevir is as following

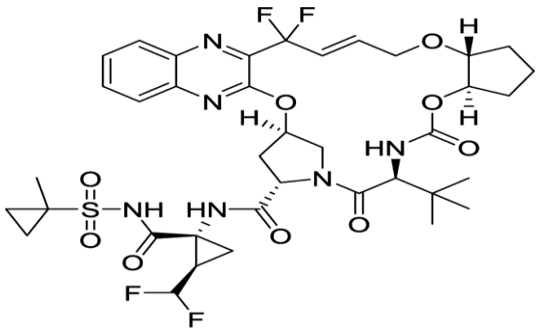

Fig-1: Chemical Structure of Glecaprevir

Pibrentasvir is a Hepatitis C Virus NS5A Inhibitor. The mechanism of action of Pibrentasvir[4] is as a P-Glycoprotein Inhibitor, and Breast Cancer Resistance Protein Inhibitor, and Organic Anion Transporting Polypeptide 1B1 Inhibitor, and Organic Anion Transporting Polypeptide 1B3

Inhibitor, and Cytochrome P450 3A Inhibitor, and Cytochrome P450 1A2 Inhibitor, and UGT1A1 Inhibitor. Pibrentasvir[5] is a direct acting antiviral agent and Hepatitis C virus (HCV) NS5A inhibitor that targets the viral RNA replication and viron assembly. In combination[7] 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[6]. These resistance-associated amino acid substitutions included Q30D/deletion, Y93D/H/N or H58D +Y93H in genotype 1a replicons, F28S + M31I or P29S + K30G in genotype 2a replicons, and Y93H in genotype 3a replicons. 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. The IUPAC Name of Pibrentasvir is Methyl N-[(2S,3R)-1-[(2S)-2-[6-[(2R,5R)-1-[3,5-difluoro-4-[4-(4-fluoro phenyl) piperidin-1-yl] phenyl]-5-[6-fluoro-2-[(2S)-1-[(2S, 3R)-3-methoxy-2-(methoxy carbonyl amino) butanoyl] pyrrolidin-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. The Chemical Structure of Pibrentasvir is follows

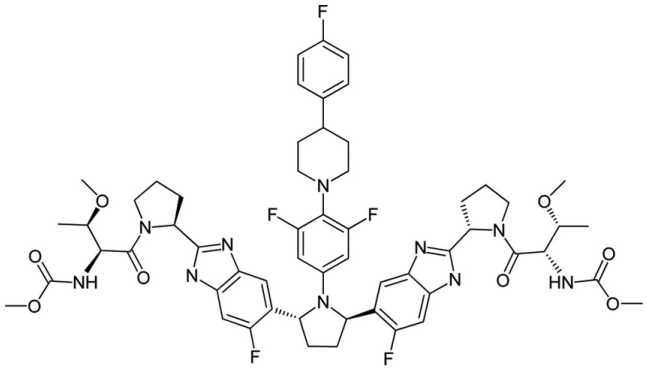

Fig-2: Chemical Structure of Pibrentasvir

MATERIALS AND METHODS

Table-1: Instruments used

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC** with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	**ELICO SL-159** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Thermal Oven
6.	Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.
7.	P^H Analyser **(ELICO)**
8.	Vacuum filtration kit **(BOROSIL)**

Table-2: Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
4.	Dipotassium hydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai

5.	Potassium dihydrogen orthophosphate	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
6.	Sodium hydroxide	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
7.	Hydrochloric acid	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
8.	Hydrogen Peroxide	99.9%	L.R.	Sd fine-Chem ltd; Mumbai

UV Analysis:

Preparation of Standard Stock Solution of Glecaprevir

Accurately weighed 10mg of Glecaprevir and it was transferred to clean and dry 100 ml of volumetric flask and dissolved in Methanol : buffer (25:75) and made-up the volume to 100 ml with same solvent system. The final solution contained 100 µg per ml of Glecaprevir solution.

Preparation of Standard Stock Solution of Pibrentasvir

Accurately weighed Pibrentasvir (10 mg) was transferred to 100 ml volumetric flask, dissolved in Acetonitrile: buffer (40:60) and made-up the volume to 100 ml with same solvent system. The final solution contained 100 µg per ml of Pibrentasvir solution.

Determination of Wavelength of Maximum Absorbance for of Glecaprevir

Standard of Glecaprevir solution (1ml) was transferred to separate 10 ml volumetric flask. The final volume was adjusted to 10 ml with the same mobile phase. The absorbance of the final resulted solution was scanned in the range 400 to 220 nm against mobile phase as blank.

Estimation of Maximum Wavelength for Pibrentasvir

First of all take 1ml of standard Pibrentasvir solution from the above standard solution (1 ml) was transferred to separate clean and dry of 10 ml volumetric flask. The final volume was adjusted to 10ml with same mobile phase (Solvent). The absorbance of the final resulted solution was scanned in the range 400 to 220 nm against solvent mixture as blank. The results are shown in following figure-5.2.

HPLC Method Development:

Selection of Wavelength

The λ_{max} of the two ingredients i.e. Glecaprevir and Pibrentasvir, were found to be 292 nm and 330 nm respectively in methanol as solvent system. As two drugs having almost near absorption max & at 330 nm Glecaprevir shows more intense as compare to Pibrentasvir at 292 nm, 342 nm has been chosen as common absorption maximum for HPLC analysis.

Preparation of Standard Solution of Glecaprevir

Weighed accurately 10mg of standard Glecaprevir and transferred into clean & dry 100 ml volumetric flask. Then 20 ml of mobile phase was added and sonicated to dissolve in 100ml of volumetric flask. The final volume was made up to the mark with same solvent. The final solution contained about 100 μg/ml of Glecaprevir.

Preparation of Standard Solution of Pibrentasvir

First 10 mg of Pibrentasvir was weighed accurately and transferred into clean & dry 100 ml volumetric flask. Then 20 ml of mobile phase was added and sonicated to dissolve it in mobile phase. The final volume was made up to the mark with same solvent. The final solution contained about 100 μg/ml of Pibrentasvir.

Initialization of the Instrument

The HPLC instrument was switched on. First the column was washed with the HPLC grade water for 45 minutes. After washing the column that the column is saturated with the mobile phase in 45 minutes. The mobile phase was run to find the peaks or identification of peaks. After 20 minutes the standard drug solution was prepared and injected in HPLC system.

Different Chromatographic Conditions used and their Optimizations

The various HPLC chromatographic conditions[8,10] are used to fin the optimum chromatographic condition for best elution of drugs in the mixture.

Preparation of Mobile Phase

The mobile phase can be prepared by taking Methanol: Phosphate Buffer and maintained pH-3.8 with diluted orthophosphoric acid (25:75% v/v). The resulted Mobile phase was filtered through 0.45 µm membrane filter and degassed under ultrasonic bath. The final obtained mobile phase[11] was pumped through the selected column and maintained at a flow rate of 1.0 ml/min.

Method Validation

The validation of an analytical method[12-18] confirms the characteristics of the method to fulfill the requirements of the application domain. The method was validated according to the ICH guidelines for specificity, linearity, precision, recovery, and stability.

System Suitability

A standard solution of Glecaprevir and Pibrentasvir working standard was prepared as per procedure and injected 6 times into the HPLC system. Then, the system suitability parameters were evaluated from standard chromatograms obtained. The % relative standard deviations (%RSD) of retention time, tailing factor, theoretical plates, and peak areas from six replicate injections were within range[19].

Specificity

Specificity[20] is the ability of a method to discriminate between the intended analyte(s) and other components in the sample, to check whether there is placebo, blank, impurity, and degradants interference at analyte concentration. Volume of 20µl of working placebo sample solution was injected, and the chromatogram was recorded.

Linearity

To demonstrate linearity[21] of the assay method, five standard solutions with concentrations of about 60-140 ppm and 24-56pp of Glecaprevir and Pibrentasvir was injected. Then, graphs were plotted between concentrations and peak area. Linearity plots were shown in Fig. 6 & 7.

Accuracy

Three concentrations of 80%, 100%, and 120% were injected in a triplicate manner then % recovery and % RSD were calculated and shown in Table 4 and 5.

Precision

Precision[22] was estimated by studying repeatability, intra- and interday tests by injecting 100 ppm & 40ppm concentrations of Glecaprevir and Pibrentasvir respectively. The results were calculated as standard deviation, relative standard deviation[23] and shown in Table 6.

Limit of detection (LOD)

LOD[24] is the lowest level of concentration of analyte in the sample that can be detected, though not necessarily quantitated. It can be calculated from the below formula.

$LOD = 3.3\ \sigma/S$

Where,

σ = Standard deviation of the response,

S = Slope of calibration curve.

Limit of quantitation (LOQ)

LOQ[25] is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied. It can be calculated from the below formula.

$LOQ = 10\ \sigma/S$

Where,

σ = Standard deviation of the response,

S = Slope of calibration curve.

Robustness

It is the capacity of the method to remain unaffected by small but deliberate variations in method parameters. The analysis was performed by slightly changing the temperature, mobile phase composition and flow rate. The results were calculated as % RSD and were given in Table 11 and 12.

RESULTS AND DISCUSSION

Method Development

Selection of Wavelength:

The λ_{max} of the two ingredients i.e. Glecaprevir and Pibrentasvir, were found to be 292 nm and 330 nm respectively in methanol as solvent system. As two drugs having almost near absorption max & at 330 nm Glecaprevir shows more intense as compare to Pibrentasvir at 292 nm, 342 nm has been chosen as common absorption maximum for HPLC analysis.

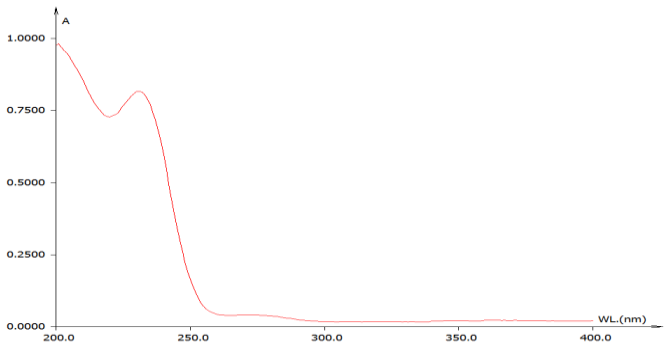

Fig-3: UV Spectrum of Glecaprevir (292 nm)

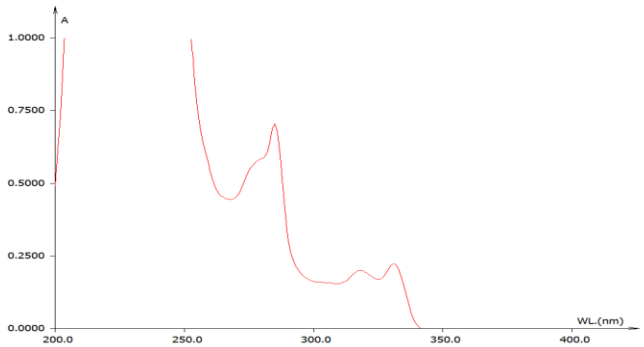

Fig-4: UV Spectrum of Pibrentasvir (342 nm)

Optimization of Chromatographic Method:

Table-3: Optimized Chromatographic Conditions

Mobile phase	**Methanol : Buffer pH-3.8 with OPA (25:75)**
Wavelength	330 nm
Flow rate	1.0 ml/ min.
Auto Sampler Temperature	Ambient
Injection Volume	20µl
Run time	10min.
Column	Symmetry C_{18}, 100A, 5µm, 250mmx4.6mm i.d.
Column Temperature	Ambient

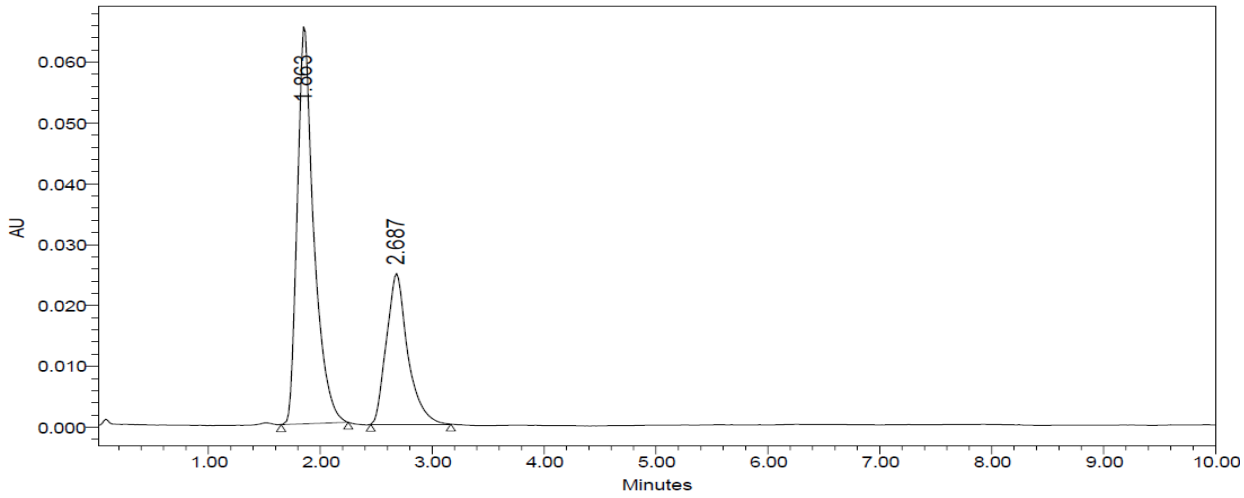

Fig-5: Optimized Chromatographic Condition

Final Result & Discussion: The selected and optimized mobile phase[26] was Methanol: Buffer pH-3.8 with OPA (25:75% v/v) and other conditions optimized were: flow rate (1.0 ml/minute), wavelength (330 nm), Run time was maintained at 10minutes. Here the peaks were separated and

showed better resolution, theoretical plates and has the better symmetry[27]. The proposed developed optimized chromatographic conditions were found to be appropriate for the quantitative estimation of the given drugs.

Validation of Analytical Method

The method was validated for its linearity range, accuracy, precision, and specificity. Method validation was carried out as per the ICH guidelines.

1. Accuracy:

To determine the accuracy of the projected technique, recovery studies[28] were distributed by adding totally different amounts (80%, 100%, and 120%) of pure drug of Ibrutinib were taken and side to the pre-analysed formulation of concentrations 100µg/ml & 40µg/ml of Glecaprevir and Pibrentasvir respectively. From that proportion recovery values were calculated.

Glecaprevir and Pibrentasvir

Recovery Study:

Table-4: Accuracy Results for Glecaprevir

Sample ID	Concentration (µg/ml)			% Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	80	80.092	602453	100.115	Mean= 100.177%
S_2 : 80 %	80	80.227	603457	100.283	S.D. = 0.091768
S_3 : 80 %	80	80.108	602574	100.135	% R.S.D.= 0.091605
S_1 : 100 %	100	99.915	749635	99.915	Mean= 99.887%
S_2 : 100 %	100	99.826	748969	99.826	S.D. = 0.05351947%
S_3 : 100 %	100	99.922	749687	99.922	R.S.D.= 0.05357965

S_7 : 120 %	120	119.879	897858	99.899	Mean= 100.016%
S_8 : 120 %	120	120.121	899654	100.100	S.D. = 0.104644
S_9 : 120 %	120	120.061	899213	100.050	% R.S.D.= 0.104627

Table-5: Accuracy Results for Pibrentasvir

Sample ID	Concentration (μg/ml)			% Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	32	32.018	36828	100.056	Mean= 100.152%
S_2 : 80 %	32	31.990	36796	99.968	S.D. = 0.247583
S_3 : 80 %	32	32.139	36967	100.434	% R.S.D.= 0.247206
S_4 : 100 %	40	39.932	45889	99.830	Mean= 99.907%
S_5 : 100 %	40	40.017	45986	100.042	S.D. = 0.11705269%
S_6 : 100 %	40	39.940	45898	99.850	R.S.D.= 0.11716126
S_7 : 120 %	48	48.051	55184	100.106	Mean= 100.092%
S_8 : 120 %	48	48.046	55179	100.095	S.D. = 0.015716%
S_9 : 120 %	48	48.036	55167	100.075	R.S.D.= 0.015702

Result & Discussion: The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

2. Precision

The precision of the analytical developed method was studied by analysis of multiple sampling of homogeneous (same) sample. The precision expressed as standard deviation[29] (SD) or relative standard deviation (%RSD). The precision[30] of the method can be analyzed by the intermediate precision. It includes the intra-day and inter-day variation[31-33].

2.1. Repeatability

The precision of each method was achieved separately from the peak areas obtained by actual estimation of 5 injections of fixed homogenous sample concentrations of Glecaprevir and Pibrentasvir. The % relative standard deviation[34] for the Glecaprevir and Pibrentasvir was calculated.

Table-6: Precision (Repeatability) Results of Glecaprevir and Pibrentasvir

Concentration of Glecaprevir and Pibrentasvir in ppm	Rt of Glecaprevir	Peak area of Glecaprevir	Rt of Pibrentasvir	Peak area of Pibrentasvir
100 +40	1.853	726358	2.677	41659
100 +40	1.857	728547	2.681	41847
100 +40	1.859	729568	2.683	41658
100 +40	1.861	724857	2.685	41369
100 +40	1.863	725894	2.687	41587
AVG		**727045**		**41624**
S.D.		**1949.72**		**172.122**
% RSD		**0.26817**		**0.413516**

Result & Discussion: The repeatability study which was conducted on the solution having the concentration of about 100 µg/ml for Glecaprevir and 40 µg/ml for Pibrentasvir (n =5) showed a RSD of 0.26817% for Glecaprevir and 0.413516% for Pibrentasvir respectively. It was concluded that the analytical technique showed good repeatability[35].

2.2. Intermediate Precision:

2.2.1. Intra-assay & inter-assay:

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations[36] for Glecaprevir & Pibrentasvir revealed that the proposed method is precise.

Table-7: Results of intra-assay & inter-assay

Conc. of Glecaprevir (API) (µg/ml)	Observed Conc. of Glecaprevir (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
80	79.879	0.8574	80.652	0.6895
100	99.861	0.9687	100.485	0.8457
120	120.05	0.7845	120.085	0.9652

Table-8: Data for Pibrentasvir intra-assay & inter-assay analysis

Conc. of Pibrentasvir (API) (µg/ml)	Observed Conc. of Pibrentasvir (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
32	32.584	0.4875	31.964	0.9687

40	40.153	0.6354	40.856	0.7489
48	48.048	0.8794	47.689	0.5478

Result and discussion:

The Intraday and interday related studies shows that the % RSD was found to be within limit i.e. (≤2%). So it is indicated that the developed is within the limits. Hence finally we concluded that the developed method was found to be precise.

3. Linearity and Range

Linearity range was found to be 0-140 µg/ml for Glecaprevir and 0-56 µg/ml for Pibrentasvir. The correlation coefficients[37] were found to be 0.999 & 0.999, the slopes were found to be 39036 & 60481 and intercept were found to be 34828 & 22371 for Glecaprevir and Pibrentasvir respectively.

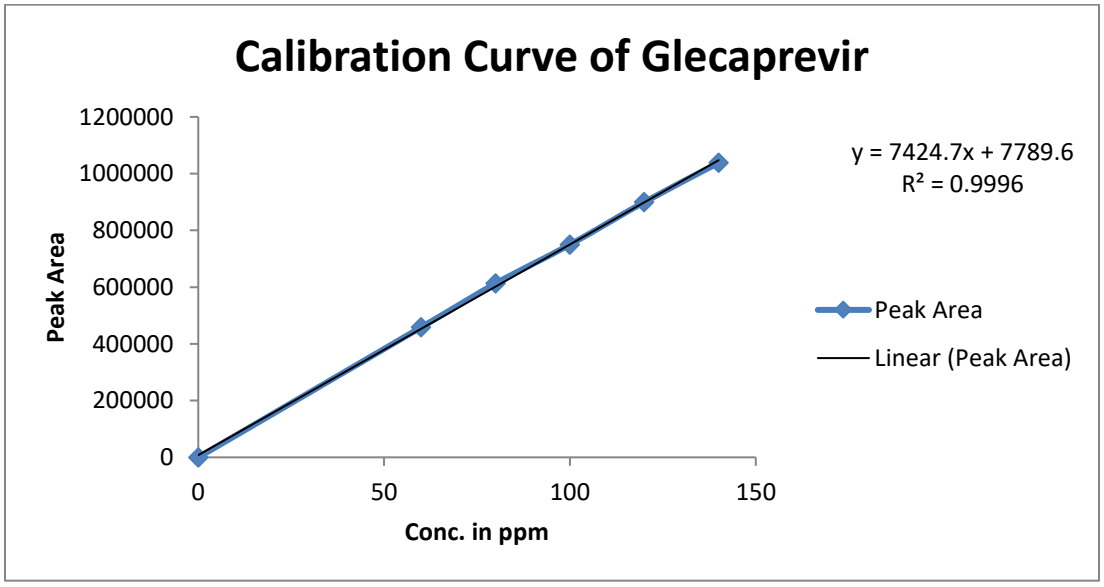

Fig-6: Standard curve for Glecaprevir

Table-9: Standard curve for Glecaprevir

CONC.(µg/ml)	MEAN AUC (n=5)
0	0
60	458636
80	612788
100	749854
120	899589
140	1038234

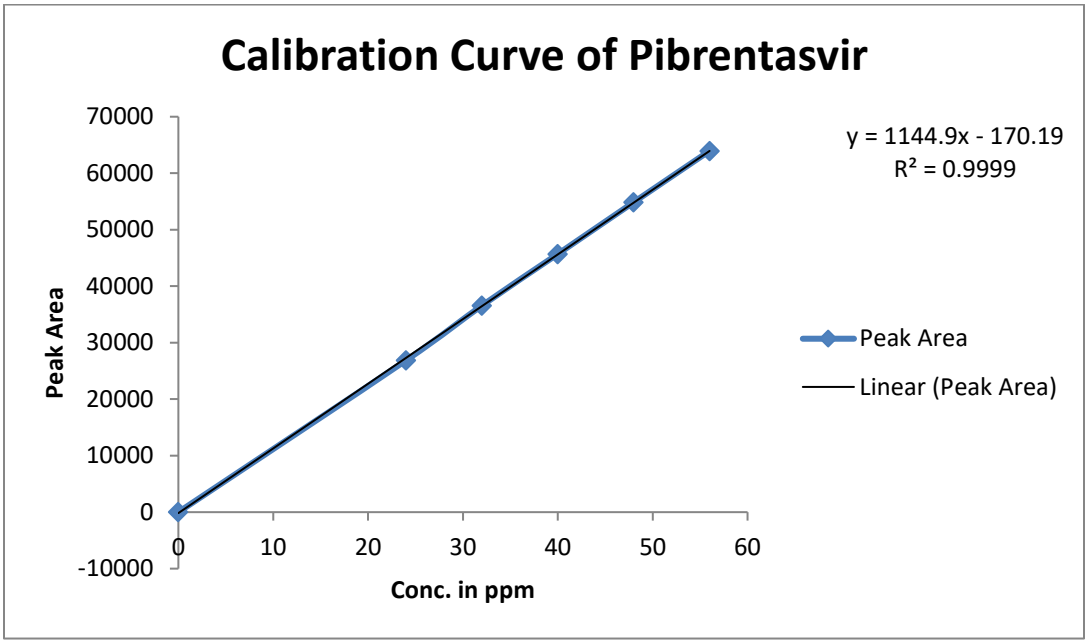

Fig-7: Standard curve for Pibrentasvir

Table-10: Standard Curve for Pibrentasvir

CONC.	AUC
0	0
24	26875
32	36574
40	45698
48	54865
56	63952

4. Method Robustness:

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & methanol content in mobile phase (±2%) studied to determine the robustness[38] of the method are also in favour of (Table-11, % RSD < 2%) the developed RP-HPLC method for the analysis of Glecaprevir (API).

Table-11: Result of Method Robustness Test

Change in Parameter	% RSD
Flow (1.1 ml/min)	1.058
Flow (0.9 ml/min)	0.674
Temperature (27^{0}C)	0.586
Temperature (23^{0}C)	0.613
Wavelength of Detection (332 nm)	0.386
Wavelength of detection (328 nm)	0.179

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & Methanol content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-12, % RSD <2%) the developed RP-HPLC method for the analysis of Pibrentasvir (API).

Table-12: Result of Method Robustness Test

Change in Parameter	% RSD
Flow (1.1 ml/min)	0.452
Flow (0.9 ml/min)	0.268
Temperature (27^{0}C)	0.365
Temperature (23^{0}C)	0.578
Wavelength of Detection (332 nm)	0.635
Wavelength of detection (328 nm)	0.429

5. Limit of detection (LOD) & Limit of quantification (LOQ):

The detection limit (LOD) and quantization limit (LOQ) may be expressed as:

$$L.O.D. = 3.3 (SD/S).$$

$$L.O.Q. = 10 (SD/S)$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Result & Discussion

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.09 & 0.29 µg/ml respectively for Glecaprevir.

The LOD was found to be 0.34 µg/ml and LOQ was found to be 0.923 µg/ml for Pibrentasvir which represents that sensitivity of the method is high.

6. System Suitability Parameter

It is an integral part of so many analytical procedures. The parameters are based on the idea that the equipment, electronics, analytical operations and the samples to be analyzed constitute as an integral system which can be examined. Finally system suitability[39] test parameters are established. The obtained data is shown in the following table-13.

Table-13: Data of System Suitability Parameter

S. No.	Parameter	Glecaprevir	Pibrentasvir
1	Retention time	1.861	2.862
2	Theoretical plates	5246	4857
3	Tailing factor	1.01	1.08
4	Area	726586	41578
5	Resolution	5.68	

The system suitability Parameters were found to be within the specified limits for the proposed method.

7. Assay of Glecaprevir & Pibrentasvir in Pharmaceutical Dosage Form:

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Finally, the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15

minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicate to degas the mobile phase (Solvent system). From this above stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system (Mobile phase).

The prepared solutions were injected in five replicates into the HPLC system and the observations were recorded.

A duplicate injection (Blank Solution) of the standard solution also injected into the HPLC system and the chromatograms and peak areas were recorded and calculated. The obtained data are shown in Table 14.

ASSAY:

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area

AS = Standard preparation Peak Area

WS = Working standard weight taken in mg

WT = Sample weight taken in mg

DS = Standard solution dilution

DT = Sample solution dilution

P = Working standard percentage purity

The assay[40] was performed as explained in the previous chapter (Above). The results which are obtained are following:

Table-14: Assay of Glecaprevir & Pibrentasvir Tablets

Brand name of Tablets	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
MAVYRET Tab	100/40	99.865 (±0.056)/ 39.864 (±0.035)	99.865 (±0.056) /99.754 (±0.789)

Results and Discussion:

The assay of MAVYRET Tablets containing Glecaprevir was found to be 99.865 (±0.056) and Pibrentasvir was found to be 39.864 (±0.035) and the % purity of the Glecaprevir & Pibrentasvir was found to be 99.865 (±0.056) /99.754 (±0.789) respectively.

Forced Degradation Studies

The APIs of Glecaprevir and Pibrentasvir was subjected to different stability conditions in various ways to observe the rate and extent of degradation occur in the course of storage after administration to body. This is one type of accelerated stability studies[41] that helps us determining the fate of the drug that is likely to happen after long time storage, within a very short time as compare to the real time or long term stability testing. The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation, photolytic degradation and oxidative degradation.

Results of Degradation Studies:

The results of the stress studies indicated the specificity of the method that has been developed. Glecaprevir and Pibrentasvir were stable only in photolytic stress conditions and little bit in thermal stress conditions. The results of forced degradation studies are given in the following Table-15 and 16.

Table-15: Results of forced degradation studies of Glecaprevir API

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	70.75	29.25	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	78.14	21.86	100.00
Thermal Degradation (50 ^{0}C)	24Hrs.	73.55	26.45	100.00
UV (254nm)	24Hrs.	77.39	22.61	100.00
3% Hydrogen peroxide	24Hrs.	68.52	31.48	100.00

Table-16: Results of forced degradation studies of Pibrentasvir API.

Stress Condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	81.35	18.65	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	83.26	16.74	100.00
Thermal Degradation (50 ^{0}C)	24Hrs.	87.32	12.68	100.00
UV (254nm)	24Hrs.	73.21	26.79	100.00
3% Hydrogen peroxide	24Hrs.	85.24	14.76	100.00

SUMMARY AND CONCLUSION

The developed HPLC method for the estimation of selected drugs such as Glecaprevir and Pibrentasvir is simple, rapid, accurate, precise, robust, and economical. The mobile phase and solvents are simple to prepare and economical, reliable, sensitive, and less time consuming. The sample recoveries were in good agreement with their respective label claims and they suggested non-interference of formulation excipients in the estimation and can be used in laboratories for the routine analysis of selected drugs. Since the system validation parameters of HPLC method used for estimation of selected drug in pure and have shown satisfactory, accurate and reproducible results (without any interference of excipients) as well, it is deduced that the simple and short proposed methods be most useful for analysis purpose. The present work concluded that stability indicating assay method by RP-HPLC was simple, accurate, precise, and specific and has no interference with the place and degradation products. Hence, these can be used for routine analysis of Glecaprevir and Pibrentasvir in bulk form and marketed pharmaceutical dosage forms.

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