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Research Article

Bio-Analytical Method Development and Validation for Simultaneous Quantification of Glecaprevir and Pibrentasvir in Rat Plasma by Using RP-HPLC

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Abstract

A novel bio-anlytical method was developed for the simultaneous determination of Glecaprevir and Pibrentasvir in rat plasma by using RP-HPLC method. The chromatographic separation was performed on Xterra RP18, (150 mm × 4.6 mm and 3.5 µm) column using the mobile phase ACN: 0.1% formic acid (50:50 v/v). The internal standard used was Voxilaprevir. Glecaprevir, Pibrentasvir and Voxilaprevir peaks were detected at 2.5 min, 5.2 min and 6.3 min respectively. Linear response was obtained in the range of 0.15 µg/mL-2.25 µg/mL for Glecaprevir and 0.06 µg/mL-0.9 µg/mL for Pibrentasvir. All of the parameters must be validated like selectivity, accuracy, precision, linearity, lower limit of quantification, matrix effect, and recovery reached the acceptance criteria under the following of US FDA guidelines.

Keywords: Glecaprevir; Pibrentasvir; Voxilaprevir; Matrix effect; Recovery

Highlights

(RP-HPLC) Reverse PhaseHigh Performance Liquid Chromatography; (ACN) Acetonitrile; (C) Centigrade; (NS) Non Structural; (UV) Ultra Violet; (CV) Coefficent of Variation; (ISTD) Internal Standard; (LLOQ) Lower Limit of Quantitation; (LOQ) Limit of Quantitation; (HQC) High Quality Control; (MQC) Mid Quality Control; (LQC) Low Quality Control; (RS) Related Substances; (SD) Standard Deviation; (P and A) Precision and Accuracy.

Introduction

Infection with hepatitis C virus (HCV) genotype 3 is associated with higher rates of liver steatosis and achieving sustained virologic response quantifiably reverses its progression in those patients. GT3 has been shown to be an independent predictor of fibrosis progression and is associated with a higher incidence of hepatocellular carcinoma. Thus, effective HCV treatment options are critical for patients with HCV GT3 infection, particularly those with advanced liver disease and/or prior treatment experience.

Glecaprevir is an antiviral agent and Hepatitis C virus NS3/4A protease inhibitor which directly targets the the viral RNA replication (Figure 1). Glecaprevir is chemically known 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 ,5,6,7,8,11,12,20,23,24a-dodecahydro-1H,10H-9,12methanocyclopenta[2-8]trioxadiazacyclononadecino[11,12-b] quinoxaline-10¬carboxamide hydrate. Glecaprevir disrupts the intracellular processes of the viral life cycle through inhibiting the NS3/4A protease activity of cleaving downstream junctions of HCV polypeptide and proteolytic processing of mature structural proteins [1-12].

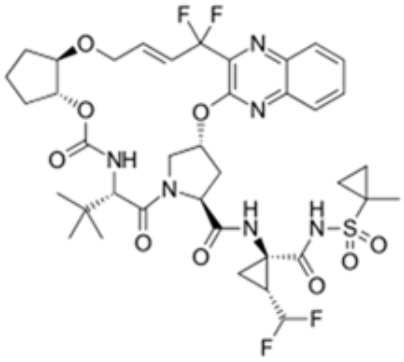

Figure 1: Structure of Glecaprevir.

Pibrentasvir is chemically dimethyl ((2S,2'S,3R,3'R)-((2S,2'S)-(((2R,5R)-1-(3,5-difluoro-4-(4-(4-fluorophenyl) piperidin-1-yl)phenyl)pyrrolidine-2,5-diyl)bis(6-fluoro-1H-benzo[d]imidazole-5,2-diyl))bis(pyrrolidine-2,1-diyl))bis(3-methoxy-1-oxobutane-1,2-diyl))dicarbamate2 (Figure 2). It is a direct acting antiviral agent and Hepatitis C virus (HCV) NS5A inhibitor that targets the viral RNA replication and viron assembly. NS5A is a phosphoprotein that plays an essential role in replication, assembly and maturation of infectious viral proteins. The combination of Glecaprevir and Pibrentasvir seems to be effective option for treatment regardless of which genotype they have, and

whether or not they have severe renal impairment or liver cirrhosis.

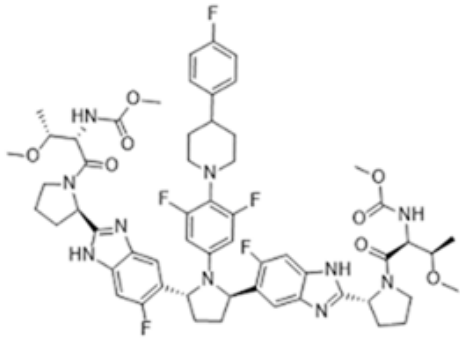

Figure 2: Structure of Pibrentasvir.

Literature survey revealed that HPLC3-12 methods were reported for simultaneous estimation of Glecaprevir and Pibrentasvir in pharmaceutical dosage forms but no method was developed for the estimation of these drugs in bio fluids by using RP-HPLC. In the present investigation, a specific RP-HPLC method was developed for simultaneous estimation of Glecaprevir and Pibrentasvir in rat plasma. This paper reports the novel, sensitive, rapid, precise and accurate method for the estimation of both Glecaprevir and Pibrentasvir in rat plasma by using HPLC

Materials and Methods

Pure Glecaprevir and Pibrentasvir samples were procured from Glenmark Pharmaceuticals, Mumbai. HPLC grade acetonitrile, formic acid and all other chemicals were obtained from Merck chemical division, Mumbai. HPLC grade water obtained from Milli-Q water purification system was used throughout the study.

Instrument

Chromatography was performed with Waters 2695 HPLC provided with high speed auto sampler, column oven, degasser and Waters 2998 PDA Detector to provide a compact and with class Empower-2 software.

Preparation of stock solutions

Buffer preparation: Transfer 1 mL formic acid into a 1000 mL volumetric flask and dilute to mark with water and mixed to preapare 0.1 M solution. The solution was filtered through 0.45 µ membrane filter paper.

Preparation of mobile phase

The mobile phase was composed of acetonitrile and 0.1% formic acid in the ratio 50:50 v/v and was used as diluent.

Preparation of glecaprevir and pibrentasvir stock solution

15 mg of Glecaprevir standard and 6 mg of Pibrentasvir standard was accurately weighed and transferred into a 100 mL volumetric flask and diluted to volume with mobile phase. Further dilute 1 mL of the above solution to 100 mL with diluent.

Preparation of internal standard stock solution

20 mg of Voxilaprevir standard was accurately weighed and transferred into a 100 mL volumetric flask and diluted to volume with diluent. Transfer 1 mL of the above solution to 100 mL volumetric flask and make upto volume with diluent.

Preparation of working standard solution

Transfer 5 mL of standard stock solution and 5 mL of ISTD stock solution into 50 mL volumetric flask and diluted to volume with diluent.

Preparation of linearity solution

Prepare the linearity solutions of concentration ranging from 0.06 µg/mL-0.9 µg/mL of Pibrentasvir and 0.15 µg/mL-2.25 µg/mL of Glecaprevir from the above working standard solution. Add 250 µL rat plasma. Centrifuge at 4000 rpm for 15 min–20 min. Collect the supernatant solution in HPLC vial and inject into the chromatograph.

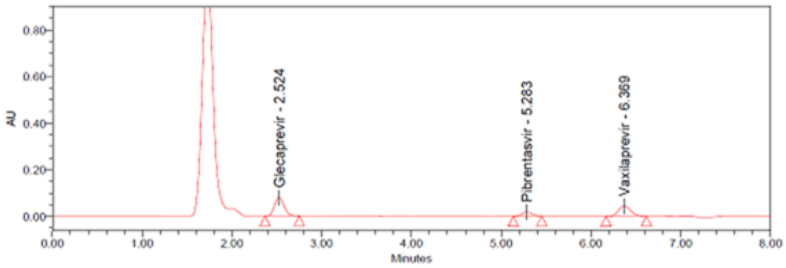

Figure 3: Optimized chromatogram.

Preparation of spiked plasma sample

Blood samples were collected into blank tubes and centrifuged for 5 minutes at approximately 4000 rpm at 4°C. The plasma samples (supernatant) were separted and stored at -20°C until analysis was completed. Drugs were extracted from the plasma samples with the method of protein precipitation. Concisely 250 µL of plasma sample were extracted in 4 mL of extraction solvent (acetonitrile:0.1% formic acid, 50:50, v/v) including the internal standard (Voxilaprevir at 200 µg/mL). The mixture was vortexed for 20 min and centrifuged at 4000 rpm, for 5 min at 4°C. After the centrifugation collect the supernatant layer and injected into chromatograph.

Results

Method development

The development of RP-HPLC method involves perform-

ing different trails using various proportions of mobile phases. Finally acetonitrile and 0.1% formic acid buffer in the ratio of 50:50, v/v was selected because it was found to be satisfied all peak parameters like theoretical plates, tailing factor, resolution etc. UV spectra of individual drugs were recorded at the wavelength range from 200 to 400 nm and the response for optimization was compared. The choice of wavelength 225 nm was considered satisfactory, permitting the detection of drugs with adequate sensitivity. Xterra RP18, (150 mm X 4.6 mm and 3.5 µm) column was optimised for better resolution of components. A suitable internal standard (ISTD) i.e. Voxilaprevir was used for controlling the variability in extraction recoveries. Preotein precipitation method was used for sample preparation. Flow rate was adjusted to 0.8 mL/min and the retention time for peaks of Glecaprevir, Pibrentasvir and Voxilaprevir was observed at 2.5 min, 5.2 min and 6.3 min respectively with a total run time of 10 minutes. The optimised chromatogram was depicted in Figure 3.

Validation

The final developed method was validated by checking for linearity, precision, accuracy, matrix effect, and stability studies.

System suitability

System suitability parameter was validated by injecting six consecutive injections of MQC concentration of the calibration curve for all analytes and 1000 µg/mL for IS. The % CV of retention times for Glecaprevir and Pibrentasvir and ISTD area was found to be 0.16, 0.97 and 0.98%. Acceptance limit for retention time deviation and area deviation 2% and 5%CV respectively were passed. The results were given in Table 1.

Table 1: System suitability results.

Sample Name	Glecaprevir		Pibrentasvir		Interna Standard	
	Analyte Area	Analyte RT(min)	Analyte Area	Analyte RT(min)	ISTD Area	ISTD RT (min)
MQC	328323	2.512	140069	5.288	306580	6.379
MQC	324814	2.512	140182	5.412	303097	6.53
MQC	320345	2.516	140073	5.412	307225	6.53
MQC	328712	2.516	140082	5.414	305873	6.534
MQC	324566	2.517	140052	5.416	302292	6.538
MQC	323610	2.523	140890	5.424	302394	6.543
Mean		2.516		5.3943		6.509
SD		0.00405		0.05228		0.06388
%CV		0.16		0.97		0.98

Auto sampler carryover

Carry over can be tested by injecting a sequence of un-extracted samples consisting of RS, HQC, RS, LLOQ and extracted samples containing standard blank, HQC, standard blank, LLOQ. Negligible % recovery was observed and tabulated in Table 2

Table 2: Auto sampler carryover.

Sample ID	Glecaprevir				Pibrentasvir			
	Peak Area	% Recovery	Peak Area	% Recovery	ed	ed	ed	ed
	Drug	ISTD	Drug	ISTD	Drug	ISTD	Drug	ISTD
Un Extracted Samples								
RS	0	0	N/A	N/A	0	0	N/A	N/A
HQC	608452	305416			211623	306512		
RS	0	0	0	0	0	0	0	0
LLOQ	202658	305416	N/A	N/A	7098	302699	N/A	N/A
Extracted Samples								
Std Blk	0	0	N/A	N/A	0	0	N/A	N/A
HQC	596841	304268	0	0	223682	304486	0	0
Std Blk	0	0			0	0		
LLOQ	192563	304785	N/A	N/A	7122	306984	N/A	N/A

Specificity and screening of biological matrix

The specificity of HPLC method was established by screening the standards blanks of different lots of rat plasma. Six different lots of plasma were required and these were found to be free of significant interferences at the retention time of all analytes. Inorder to meet the acceptane criteria the % interference of drug standard in extracted LLOQ samples should be ≤ 20.00% and ≤ 5.00% of the area of the IS in the extracted LLOQ samples. The results were satisfied and summarized in Table 3.

Table 3: Specificity and screening of biological matrix.

S. No.	Sample ID	Glecaprevir				Pibrentasvir			
		Area		% Interference		Area		% Interference	
		Drug	ISTD	Drug	ISTD	Drug	ISTD	Drug	ISTD
1	Std Blk 1	0	0	0	0	0	0	0	0

2	LLOQ 1	19991	306368	0	0	7380	306368	0	0
3	Std Blk 2	0	0	0	0	0	0	0	0
4	LLOQ 2	19754	306668	0	0	7551	306668	0	0
5	Std Blk 3	0	0	0	0	0	0	0	0
6	LLOQ 3	19991	301335	0	0	7204	301335	0	0
7	Std Blk 4	0	0	0	0	0	0	0	0
8	LLOQ 4	19832	302968	0	0	7139	302968	0	0
9	Std Blk 5	0	0	0	0	0	0	0	0
10	LLOQ 5	19991	304679	0	0	7380	304679	0	0
11	Std Blk 6	0	0	0	0	0	0	0	0
12	LLOQ 6	19754	308984	0	0	7204	308984	0	0

Sensitivity

The sensitivity of the method was evaluated by analyzing 6 LLOQ at 1 µg/mL for Glecaprevir and Pibrentasvir respectively. The % CV was found to be 2.2 for Glecaprevir and 3.79 for Pibrentasvir and 96%-106% of nominal concentration which satisfied the acceptance criteria stated at least 67% of the sample should be within 80%-120% of nominal and precision should be <20 % CV. The results were represented in Table 4 Matrix effect.

Table 4: Sensitivity results.

SamLple Name	Glecaprevir		Pibrentasvir	
	CalConc. (µg/mL)	% of Nominal Conc.	Cal Conc. (µg/mL)	% of Nominal Conc.
LLOQ-1	0.072	96	0.029	96.66
LLOQ-2	0.074	98.66	0.031	103.33
LLOQ-3	0.076	101.33	0.031	103.33
LLOQ-4	0.075	100	0.032	106.66
LLOQ-5	0.073	97.33	0.03	100
LLOQ-6	0.076	101.33	0.032	106.66
Mean	0.0743		0.03083	
SD	0.00163		0.00117	
%CV	2.2		3.79	

Matrix effect can be evaluated by analyzing QC samples, each prepared using matrix from atleast 6 different sources. Precision (%CV) is 0.08% and 0.21% for Glecaprevir at HQC and LQC, respectively. Precision (%CV) is 1.88% and 4.96% for Pibrentasvir at HQC and LQC, respectively which satisfied the acceptance criteria i.e. the % CV should be less than 15%. The results were given in Table 5.

Table 5: Sensitivity results.

S.No.	Plasma Lot No.	Glecaprevir		Pibentasvir	
		HQC	LQC	HQC	LQC
1	Lot 1	2.252	0.752	0.91	0.31
		2.254	0.754	0.95	0.33
		2.253	0.756	0.94	0.34
2	Lot 2	2.257	0.753	0.96	0.36
		2.254	0.754	0.92	0.35
		2.255	0.755	0.95	0.32
3	Lot 3	2.256	0.757	0.93	0.34
		2.257	0.753	0.96	0.35
		2.253	0.752	0.91	0.33
4	Lot 4	2.254	0.754	0.94	0.35
		2.252	0.756	0.96	0.34
		2.256	0.753	0.95	0.36
5	Lot 5	2.253	0.754	0.93	0.33
		2.257	0.757	0.92	0.32
		2.255	0.756	0.94	0.31

6	Lot 6	2.254	0.754	0.92	0.35
		2.256	0.755	0.91	0.36
			0.753	0.94	0.32
Nominal Conc.(µg/mL)		2.254	0.756	0.92	0.35
Nominal Conc. Range (µg/mL)		(2.252-2.257)	(0.752-0.757)	(0.91-0.96)	(0.31-0.36)
n		18	18	18	18
Mean		2.2547	0.7543	0.9356	0.3372
SD		0.00174	0.00157	0.01756	0.01674
%CV		0.08	0.21	1.88	4.96
% Mean Accuracy		100.40%	99.70%	99.50%	100.30%
No. of QC Failed		0	0	0	0

Linearity

Linearity curve was generated with the stanadard solutions in triplicate form which are showing linear response over the concentration range of 0.15 µg/mL-2.25 µg/mL of Glecaprevir and 0.06-0.9 µg/mL of Pibrentasvir. Samples were quantified using the ratio of peak area of analyte to that of ISTD. The response graph was plotted by peak area ratios Vs plasma concentrations. The correlation coefficient was found to be 0.999. The results were shown in Table 6 and linearity graph was represented in Figures 4 and 5.

Table 6: Results for linearity

Glecaprevir			Pibrentasvir		
Final conc. in µg/mL	**Area**	**Area response ratio**	**Final conc. in µg/mL**	**Area**	**Area response ratio**
0	0	0	0	0	0
0.15	57105	0.1327	0.06	11971	0.0304
0.375	98950	0.2623	0.15	21146	0.0561
0.75	199453	0.5406	0.3	45208	0.1225
1.5	403659	1.032	0.6	91579	0.2341
1.875	508362	1.2529	0.75	115802	0.2854
2.25	588402	1.9478	0.9	137432	0.4589

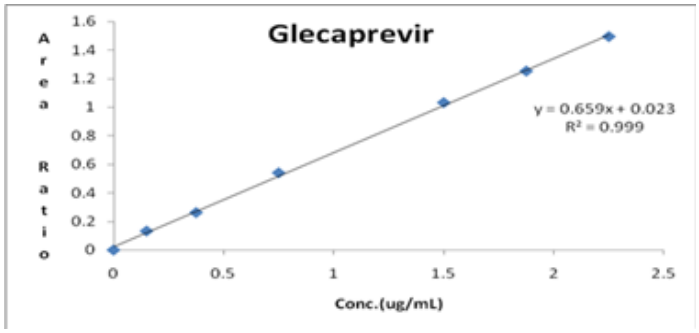

Figure 4: Calibration plot of Glecaprevir.

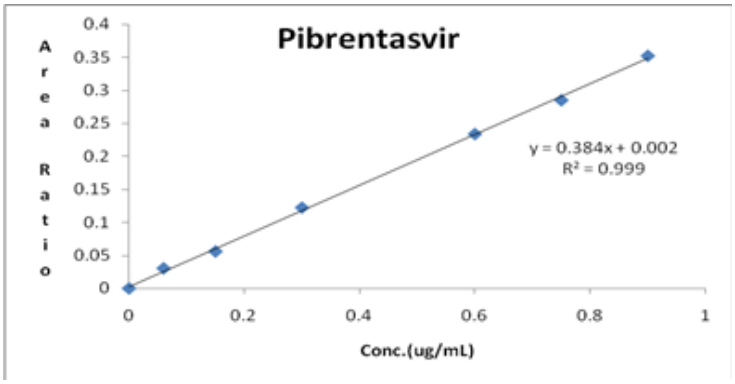

Figure 5: Calibration plot of Pibrentasvir

Precision and accuracy

The intra assay precision and accuracy was estimated by analyzing six replicates containing Glecaprevir and Pibrentasvir at four different QC levels. The inter assay precision was determined by analyzing the four levels QC samples on four different runs at each QC levels for Glecaprevir and Pibrentasvir were spiked combined with plasma sample and injected into HPLC. The percentage mean accuracy for all quality control samples with four replicates of three different batches for Glecaprevir and Pibrentasvir were within acceptance limits i.e 85%-115%. The results were tabulated in Tables 7 and 8

Table 7: Precision and accuracy results of Glecaprevir

Quality control sample	Spiked concentration (µg/ml)	Mean (µg/ml)	SD	Accuracy (%)	RSD (%)
Intra-day					
LLOQ	0.15	0.152	0.00232	101.33	1.52632
LQC	0.75	0.735	0.01171	98	1.5932
MQC	1.52	1.55	0.01652	101.97	1.06581
HQC	2	2.022	0.0261	101.10	1.2908
Intra-day					
LLOQ	0.15	0.153	0.00204	102	1.33137
LQC	0.75	0.759	0.02407	101.20	3.17128
MQC	1.52	1.49	0.03521	98.03	2.36309
HQC	2	1.987	0.01527	99.35	0.7685

Table 8: Precision and accuracy results of Pibrentasvir

Quality control sample	**Spiked concentration (µg/ml)**	**Mean (µg/ml)**	**SD**	**Accuracy (%)**	**RSD (%)**
Intra-day					
LLOQ	0.06	0.061	0.00216	101.67	3.54098
LQC	0.32	0.325	0.02483	101.563	7.64
MQC	0.62	0.623	0.0216	100.48	3.46709
HQC	0.8	0.78	0.02317	97.50	2.97051
Intra-day					
LLOQ	0.06	0.059	0.00205	98.33	3.47458
LQC	0.32	0.326	0.01295	101.875	3.97239
MQC	0.62	0.626	0.01985	100.97	3.17093
HQC	0.8	0.83	0.02522	103.75	3.03855

Recovery of analyte

The percentage mean recoveries were determined by measuring the responses of the quality control samples spiked into plasma against respective aqueous quality control samples at LQC, MQC and HQC levels. Three samples at each level were analyzed after extraction of each individual drug in separate solvent and % nominal concentration of the sample was calculated. Analyte recovery from a sample matrix (extraction efficiency) is a comparison of analytical response from an amount of analyte added to that determined from sample matrix.

Experiments with spiked compounds resulted in recoveries of analyte 85.1%-90.5% and for ISTD 84.25% which satisfied acceptance criteria. The recovery results were presented in Tables 9 and 10.

Table 9: Recovery of analyte of Glecaprevir

Sample	**Glecaprevir**		**Pibrentasvir**	
	Peak Area		**Peak Area**	
	Extracted	**Un Extracted**	**Extracted**	**Un Extracted**
HQC	593946	621697	217292	217444
	590813	620312	217384	217391
	595294	614930	213689	219056
	599317	626312	215792	218383
	597319	624441	211992	216200
	598980	625691	217458	219056
Mean	595944.83	622230.5	215601.17	217921.67
%CV	0.55	0.68	1.06	0.51

%Mean Recovery	95.15	92.14	95.4	91.1
MQC	325609	422749	144208	149603
	338362	422812	142936	149558
	321962	424665	140859	149558
	328091	423649	143072	140840
	321679	418600	147561	140320
	326003	424013	147886	149558
Mean	326951	422748	144420.33	146572.83
%CV	1.87	0.51	1.92	3.17
%Mean Recovery	95.2	91.5	95.6	93.5
LQC	194776	210729	70280	70787
	193956	211523	70056	70731
	193744	210830	70816	70150
	195745	209668	70495	70060
	195898	210318	70411	71868
	191613	209717	70260	78976
Mean	194288.67	210464.17	70386.33	72095.33
%CV	0.81	0.34	0.37	4.76
%Mean Recovery	95.3	93.2	95.20	92.30

Table 10: Internal standard results of Voxilaprevir

S. No.	Un Extracted Area	Extracted Area
1	307921	301678
2	306998	305389
3	306998	305389
4	301422	305996
5	303518	301839
6	307921	304985
n	6	6
Mean	307463	304212.67
SD	2333.325	1728.892
% CV	0.57	0.48
%Mean Recovery	90.30%	5.3

Ruggedness on precision and accuracy

Ruggedness was performed by different analysts using different columns and different instrument. The % CV for Glecaprevir and Pibrentasvir was found to be 1.22%-2.95% which meets acceptance criteria less than 15%. The results were tabulated in Tables 11 and 12

Table 11: Ruggedness on precision and accuracy results of Glecaprevir

Quality control sample	Concentration (µg/ml)	Mean concentration (µg/ml)	Mean SD	Mean % CV	Mean Accuracy (%)
Different Columns					
LLOQ	0.15	0.151	0.00187	1.24	100.67
LQC	0.75	0.76	0.0019	0.25	101.33
MQC	1.52	1.51	0.018	1.19	99.34
HQC	2	2.03	0.051	2.51	101.50
Different Analysts					
LLOQ	0.15	0.15	0.002	1.33	1.33333
LQC	0.75	0.74	0.019	2.57	2.57
MQC	1.52	1.54	0.017	1.10	1.10
HQC	2	2.04	0.0024	0.12	0.12

Table 12: Ruggedness on precision and accuracy of results of Pibrentasvir

Quality control sample	Concentration (µg/ml)	Mean concentration (µg/ml)	Mean SD	Mean % CV	Mean Accuracy (%)
Different Columns					
LLOQ	0.06	0.062	0.00184	2.96	103.333
LQC	0.32	0.33	0.03261	9.88	103.125
MQC	0.62	0.63	0.01987	3.15	101.613
HQC	0.8	0.82	0.02531	3.09	102.5
Different Analysts					
LLOQ	0.06	0.059	0.00156	2.65	98.3333

LQC	0.32	0.31	0.02418	7.80	96.875
MQC	0.62	0.618	0.01326	2.15	99.6774
HQC	0.8	0.81	0.03489	4.31	101.25

Ruggedness on reinjection reproducibility

Whole reinjection reproducibility was evaluated by reinjecting accepted Precision and Accuracy samples which were stored at 2°C-8°C for a period of 38 hours 32 minutes. The % mean accuracy for Glecaprevir was ranged from 97.2%-97.6% and for Pibrentasvir it was 97.4-97.8 which was within the limits 80%-120%. The results were presented in Tables 13 and 14.

Table 13: Ruggedness on reinjection reproducibility results of Glecaprevir

ed	HQC	MQC	LQC	LLOQ
P& A ID	Nominal Concentration (µg/mL)			
	2.253	1.52	0.74	0.073
	Nominal Concentration Range (µg/mL)			
	(2.252-2.216)	(1.52-1.56)	(0.72-0.76)	(0.072-0.076)
	Calculated Concentration (µg/mL)			
P & A 01	2.252	1.52	0.72	0.072
	2.256	1.54	0.76	0.076
	2.254	1.56	0.74	0.075
	2.255	1.15	0.73	0.073
	2.253	1.53	0.75	0.074
	2.254	1.54	0.74	0.073
n	6	6	6	6
Mean	2.25	1.47	0.74	0.07
SD	0.00141	0.15895	0.01414	0.00147
% CV	0.06	10.79	1.91	1.99
% Mean Accuracy	97.60%	97.50%	97.80%	97.40%

Table 14: Ruggedness on reinjection reproducibility results of Pibrentasvir

	HQC	MQC	LQC	LLOQ
P& A ID	Nominal Concentration (µg/mL)			
	0.92	0.63	0.32	0.032
	Nominal Concentration Range (µg/mL)			
	(0.92-0.98)	(0.62-0.68)	(0.32-0.38)	(0.032-0.038)
	Calculated Concentration (µg/mL)			
P & A 01	0.92	0.62	0.32	0.032
	0.96	0.65	0.35	0.035
	0.94	0.67	0.37	0.036
	0.93	0.63	0.33	0.034
	0.95	0.64	0.36	0.035
	0.97	0.62	0.34	0.037
n	6	6	6	6
Mean	0.95	0.64	0.35	0.03
SD	0.01871	0.01941	0.01871	0.00172
% CV	1.98	3.04	5.42	4.94
% Mean Accuracy	97.50%	97.50%	97.20%	97.60%

Stability on day zero

The stability of the analytes in rat plasma was evaluated by analysis six replicates of quality control samples at low and high concentration levels at room temperature over 24 h(day zero). The measured concentrations were compared with that of freshly prepared and processed samples. The % CV and mean accuracy for Glecaprevir and Pibrentasvir

were found to be 0.08% and 1.96%. The results were given in Table 15

Table 15: Stability on day zero

QC ID	Glecaprevir		Pibrentasvir	
	HQC	LQC	HQC	LQC
Nominal Conc.(µg/mL	2.253	0.755	0.95	0.34
Nominal Conc. range(µg/mL	(2.253-2.258)	(0.753-0.758)	(0.93-0.98)	(0.33-0.38)
Replicate No.	Calculated Concentration (µg/mL)		Calculated Concentration (µg/mL)	
1	2.253	0.755	0.93	0.33
2	2.255	0.753	0.95	0.36
3	2.257	0.754	0.96	0.37
4	2.256	0.756	0.94	0.35
5	2.254	0.758	0.98	0.34
6	2.258	0.757	0.97	0.33
n	6	6	6	6
Mean	2.26	0.76	0.96	0.35
SD	0.0019	0.00187	0.01871	0.01633
%CV	0.08	0.25	1.96	4.71
% Mean Accuracy	97.20%	97.60%	97.80%	97.50%

Long batch variation

The % CV and mean accuracy for Glecaprevir and Pibrentasvir were found to be 1.52%, 97.2%and 3.59%, 98.6% hence it passed the Long batch

Long term stability at -28°C

The stability of the analytes in rat plasma was evaluated by analysis six replicates of quality control samples at low and high concentration levels at -28°C temperature. Long term stability at -28°C stability was carried out by using six replicates of HQC and LQC level of samples. The long term stability of at -28°C±5°C was carried out by storing samples for 37 days. The results obtained are compared with those obtained by freshly prepared samples. The % CV and mean accuracy for Glecaprevir and Pibrentasvir were found to be 0.08%, 97.2% and 2.0%, 97.5% which is within the acceptance limit of 90.00 – 110.00%. The results are summarized in the Tables 16-18.

Table 16: Long batch Results of Glecaprevir

QC ID	Glecaprevir				Pibrentasvir			
	HQC	MQC	LQC	LL QC	HQC	MQC	LQC	LL QC
Mean	2.2548	1.531	0.7528	0.0736	0.9416	0.6566	0.3433	0.0338
SD	0.00263	0.0183	0.00194	0.00196	0.0278	0.02732	0.0216	0.00194
%CV	0.117	1.197	0.257	2.66	2.95	4.161	6.291	5.736
% Mean Accuracy	97.30%	97.20%	97.50%	97.40%	98.80%	98.60%	98.40%	98.80%

Table 17: Long Term at -280C

QC ID	Glecaprevir				Pibrentasvir			
	HQC	MQC	LQC	LL QC	HQC	MQC	LQC	LL QC
Mean	2.25	2.26	0.75	0.76	0.94	0.96	0.34	0.36
SD	0.00187	0.00187	0.00172	0.002	0.01871	0.01871	0.1871	0.01871
%CV	0.08	0.08	0.23	0.26	2	1.96	5.58	5.27
% Mean Accuracy	97.20%	96.30%	96.70%	97.60%	97.50%	97.80%	97.60%	97.50%

Table 18: Long Term at -800C

QC ID	Glecaprevir				Pibrentasvir			
	HQC	MQC	LQC	LL QC	HQC	MQC	LQC	LL QC
Mean	2.25	2.25	0.75	0.76	0.93	0.96	0.34	0.35
SD	0.00138	0.00264	0.00105	0.00187	0.01414	0.01871	0.01871	0.01049
%CV	0.06	0.12	0.14	0.25	1.52	1.96	5.58	3.04
% Mean Accuracy	97.30%	97.20%	97.50%	97.80%	97.60%	97.50%	97.20%	97.30%

Long term stability at -80°C

Long term stability of the spiked quality control samples was determined after stored at -80°C for 14 days. Stability was assessed by comparing them against the freshly spiked calibration standards. The % CV and mean accuracy for Glecaprevir and Pibrentasvir were found to be 0.06%, 97.3% and 1.52%, 97.6%. Acceptance Criteria is at least 67% QC samples should pass acceptance limit of 85%-115% and more than 50% at each QC level should fail. Results were summarized.

Discussion

The goal of present method was to develop a novel, simple and accurate bio-analytical method for simultaneous quantitation of Glecaprevir and Pibrentasvir in rat plasma by RP-HPLC. Voxilaprevir was used as internal standard for this estimation. Samples were extracted from rat plasma by using method of protein precipitation. All the analytes and internal standard were seperated on Xterra RP18, (150 mm X 4.6 mm and 3.5 µm) column using the mobilephase ACN:0.1%formic acid (50:50). The peak responses of Glecaprevir, Pibrentasvir and Voxilaprevir were observed at 2.5 min, 5.2 min and 6.3 min respectively. Detection of analysis was performed at 225 nm. The method was validated for all the parameter such as system suitability specificity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, dilution integrity, ruggedness and stability as per USFDA guidelines. The established method was found to be linear in the range of 0.15 µg/mL-2.25 µg/mL of Glecaprevir and 0.06 µg/mL-0.9 µg/mL of Pibrentasvir. The results of specificity demonstrated that there is no remarkable interference with the retention of analytes. % CV and accuracy values for all quality control samples were in between 0.11-3.05 for Glecaprevir and 2.17-6.26 for Pibrentasvir. The great extraction recovery for Glecaprevir at HQC, MQC, LQC was observed to be 93.78% and for Pibrentasvir it was 93.85% and 90.35% for internal standard. The stability of analytes was studied for HQC, LQC and MQC leves at zero hours, long batch LT at -28°C and LT at -80°C and the results were found to be greater than 95% which was within the acceptance criteria.

Conclusion

The current validated bio-analytical RP-HPLC method for simultaneous estimation of Glecaprevir and Pibrentasvir offers good accuracy and significant advantages in terms of sensitivity, selectivity and sample preparation. The single step protein precipitation, the short runtime of 10 min and isocratic elution makes the method economical and suitable for analysis of a large number of samples. The method is validated as per the requirements of US FDA guidelines. It can be concluded that the selected method was suitable for routine commercial and bioequivalence studies of Glecaprevir and Pibrentasvir samples.

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Authors' contribution

The authors have read and approved the manuscript

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