ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

Stability indicating RP-HPLC method for simultaneous determination of Grazoprevir and Elbasvir in bulk and tablet dosage form

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Article History:

Received on: 30.03.2018
Revised on: 17.07.2018
Accepted on: 22.07.2018

Keywords:

Grazoprevir,
Elbasvir,
Potassium dihydrogen,
Orthophosphate,
Acetonitrile,

ABSTRACT

A simple, rapid and sensitive isocratic RP-HPLC method was developed for the simultaneous estimation of Grazoprevir and Elbasvir in bulk and their Pharmaceutical dosage form using Waters C_{18} (250 x 4.6 mm x 5 µ particle size) analytical column in an isocratic mode with mobile phase comprising Acetonitrile: 0.25M Potassium dihydrogen orthophosphate buffer (pH 4.5) (55:45, v/v). The flow rate was 1.5 ml/ min and effluent was monitored at 235nm. The retention times were found to be 2.390 min for Grazoprevir and 4.603 min for Elbasvir. The assay exhibited a linear dynamic range of 100 - 300 µg/ml for Grazoprevir and 50-150 µg/ml for Elbasvir. The calibration curve was linear (r^2 = 0.9998 for Grazoprevir and r = 0.9999 for Elbasvir) over the entire linear range. Recovery was found to be 99.03% for Grazoprevir and 99.34 % for Elbasvir. The proposed method was statistically evaluated and validated as per ICH guidelines and can be applied for routine quality control analysis of Grazoprevir and Elbasvir in Pharmaceutical dosage form.

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ISSN: 0975-7538
DOI: https://doi.org/10.26452/ijrps.v9i4.1632

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INTRODUCTION

Hepatitis C is an infection caused by the hepatitis C virus (HCV) that attacks the liver and leads to inflammation. The World Health Organization estimates that about 3% of the world's population has been infected with HCV and that there are more than 170 million chronic carriers who are at risk of developing liver cirrhosis and/or liver cancer (Goossens, N 2015). For almost 25 years, Pegylated interferon and ribavirin have been the cornerstone of treatment for this disease until the revolutionary development of protease inhibitors. This class of direct-acting antiviral agents has led to all oral HCV treatment regimens that have changed the strategies of hepatitis C treatment (Sulejmani, N 2016).

Zepatier® is a novel combination of two new Food and Drug Administration (FDA) approved drugs Elbasvir (ELB) and Grazoprevir (GRA) (Food U 2016). It combines two direct-acting antiviral agents with distinct mechanisms of action that target HCV at multiple steps in the viral lifecycle. ELB (Figure 1(A)) is an inhibitor of HCV NS5A, which is essential for viral RNA replication and virion assembly. On the other hand, GRA (Figure 1 (B)) is an inhibitor of the HCV NS3/4 A protease which is necessary for the proteolytic cleavage of the HCV encoded polyprotein and is essential for viral replication (El Kassas, M *et al.,* 2016; Papudesu, C *et al.,* 2017).

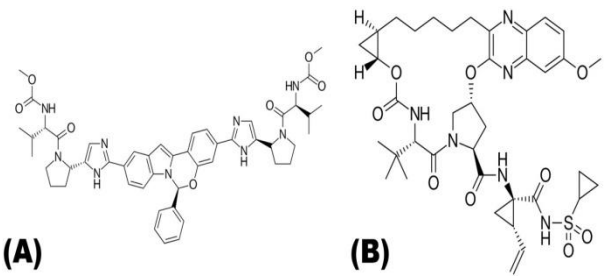

Figure 1: Chemical structures of (A) Elbasvir and (B) Grazoprevir

GRA is chemically(1R,18R,20R,24S,27S)-N-{(1R,2S)-1-[(Cyclopropylsulfonyl)carbamoyl]-2-vinylcyclopropyl}-7-methoxy-24-(2-methyl-2-propanyl)-22,25-dioxo-2,21-dioxa-4,11,23,26-tetraazapentacyclo [24.2.1.03,12.05,10.018,20] nonacosa-3,5,7,9,11-pentaene-27-carboxamide. GRA is a compound with molecular formula $C_{38}H_{50}N_6O_9S$ molecular weight of 766.903 gm/mol. Grazoprevir is very slightly soluble in water, soluble in ethanol and acetonitrile. ELB is chemically methyl N-[(2S)-1-[(2S)-2-[5-[(6S)-3-[2-[(2S)-1-[(2S)-2-(methoxycarbonylamino)-3-methylbutanoyl] pyrrolidin-2-yl] -1H-imidazol -5-yl]-6 –phenyl -6H-indolo [1,2- c] [1,3] benzoxazin-10-yl] -1H-imidazol -2-yl] pyrrolidin-1-yl] -3-methyl-1- oxobutan-2-yl] carbamate. ELB is a compound with molecular formula $C_{49}H_{55}N_9O_7$ and molecular weight of 882.035 gm/mol. It is sparingly soluble in ethanol and insoluble in water (Forns X *et al.,* 2015; Howe AY *et al.,* 2014; Sulejmani N *et al.,* 2016).

In literature, ELB was determined individually in rat plasma using ultra-performance liquid chromatography with tandem mass spectrometry (UPLC–MS-MS) method (Liu, H *et al.,* 2016). Moreover, few methods were described for simultaneous determination of GRA with ELB by spectrophotometry (Attia, K.A *et al.,* 2018) and HPLC (Pallapati, S *et al.,* 2017; Nallagundla, S *et al.,* 2017; Akram, N.M *et al.,* 2017). In this work, an HPLC analytical method has been developed and validated as per ICH guidelines (ICH, 2015) for the simultaneous estimation of GRA and ELB in their pharmaceutical dosage form.

Experimental

Chemicals

All reagents and solvents were of analytical and HPLC grade. GRA and ELB were kindly supplied by Merck, India. All other chemicals were commercial analytical reagent grade. In-house double distilled water was used for preparing solutions.

Instrumentation

The HPLC system consisted of a LC Waters (Waters, Milford, MA, USA) using a Water's C18 250 x 4.6 mm, 5µ column, a quaternary gradient system (600 Controller), in line degasser (Waters, model AF 24). The system was equipped with a photodiode array detector (Waters, 2998 model) and autosampler (Waters, model 717 plus). Data was processed using the Empower 2 software (Waters, Milford, MA, USA). The mobile phase was pumped at a flow rate of 1.5 mL min−1. Injection volume was 20 µL and the column temperature was 40°C. The detection wavelength (Isosbestic point) for GRA and ELB was 235 nm.

Preparation of Mobile Phase

450 mL (45%) of 0.25 M Potassium dihydrogen orthophosphate buffer pH 4.5 and 550 mL (55%) of ACN was mixed in a 1000 mL volumetric flask and filtered through a 0.45µ filter under vacuum filtration. The solution was kept for sonication and degassing in an ultrasonic water bath for 10 mins. The mobile phase was used as diluent.

Preparation of Primary GRA & ELB Standard Solution

100 mg of GRA and ELB were accurately weighed and transferred to individual 100 mL volumetric flask, diluent was added to dissolve and final volume was made up to the mark with the same to get the final concentration of 1 mg/mL (1000 µg/mL) of GRA and ELB.

Preparation of working standard solution of GRA and ELB

2 mL of GRA and 1 mL of ELB primary stock solution was diluted to 10 mL with diluent in 10 mL volumetric flask, diluent was added up to the mark to get the final concentrations of 200 µg/mL and 100 µg/mL of GRA and ELB respectively.

Assay of tablet dosage forms

Twenty tablets were accurately weighed and the average weight was determined. Tablets were ground and the tablet powder equivalent to 10 mg (91.052 mg) of ELB was weighed accurately and transferred to a 10 mL volumetric flask. 6 mL of mobile phase was added and sonicated for 10 mins. The final volume was made up to the mark with the same to get the sample stock solution of 1000 µg/mL. The resulting solution was filtered through the membrane filter of 0.45 µ. 1 mL of the above solution was transferred to a 10 mL volumetric flask and diluted up to the mark with mobile phase. 20 µL of the resulting solution was injected into the chromatograph. Peak area and RT were determined from chromatogram and the amount of GRA and ELB were calculated.

Table 1: Optimized chromatographic conditions for determination of GRA and ELB

S. No	Parameter	Description/Value
1.	Stationary Phase	Water's C18 (250X4.6X5)
2.	Mobile Phase	Acetonitrile : 0.25M Potassium dihydrogen orthophsophate buffer (pH 4.5) (55:45, v/v)
3.	Flow rate	1.5 ml/min
4.	Detection Wavelength (Isosbestic Point)	235 nm
5.	Detector	Photodiode array
6.	Injection	Autosampler -Waters, model 717 plus
7.	RTs	Grazoprevir: 2.390 min Elbasvir: 4.603 min
8.	Injection volume	20 µl
9.	Column Temperature	40°C
10.	Runtime	6 mins
11.	Diluent	Mobile Phase

Table 2: System suitability data of GRA and ELB

S. No.	Parameter*	GRA	ELB
1.	Theoretical Plate Count	4058	6412.75
2.	Average Peak Area	12549551.2	2163090.2
3.	Peak Height	2184745.667	243682
4.	RT	2.390	4.603
5.	Tailing	1.5	1.34
6.	Resolution	--	11.5
7.	S/N	3128	3482

* Average of 6 replicates

Table 3: Linearity data of GRA and ELB

S. No.	Linearity Level	Grazoprevir		Elbasvir	
		Concentration (µg/ml)	Peak Area	Concentration (µg/ml)	Peak Area
1.	50	100	8175171	50	1289368
2.	75	150	10632695	75	1781450
3.	100	200	12970191	100	2269583
4.	125	250	15555543	125	2756728
5.	150	300	17875994	150	3228628
Reg. Equation		y = 48649x + 3E+06		y = 19415x + 323632	
Slope		48649		19415	
Y-Intercept		3E+06		323632	
R^2		0.9998		0.9999	

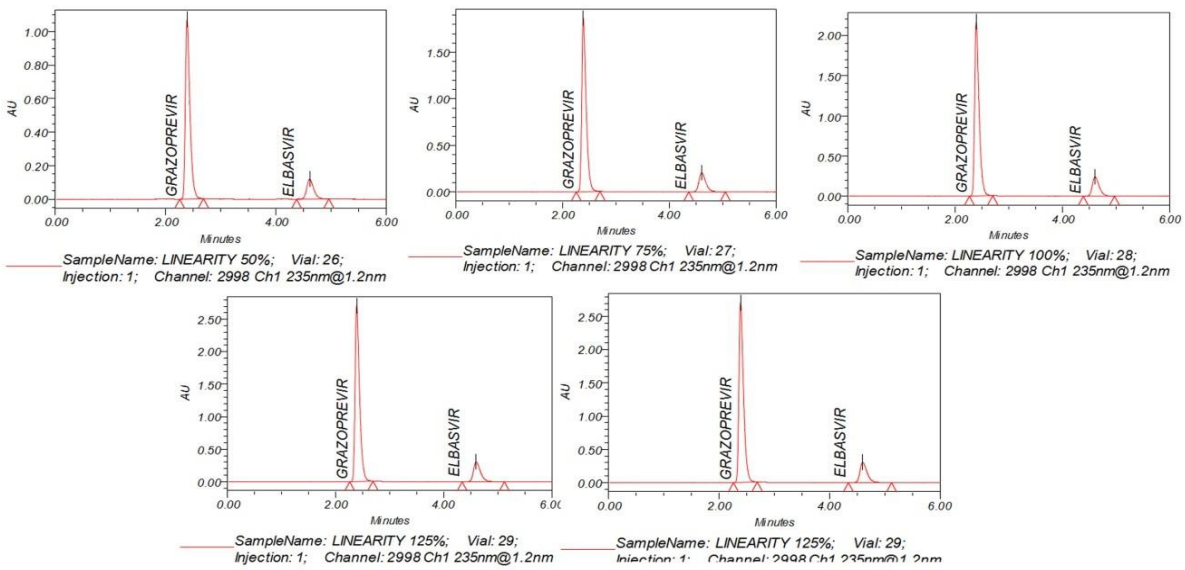

Figure 2: Linearity Chromatograms of GRA and ELB

Validation criteria

Selectivity

The selectivity is defined as the ability of the method to measure the analyte accurately and specifically in the presence of components present in the sample matrix, was determined by analysis of chromatograms of drug-free blank and formulation.

Linearity

Five-point standard curves for both compounds were constructed by drawing peak area versus GRA and ELB concentration at 100-300 µg/ml and 50-150 µg/ml respectively. The concentration ranges were selected based on optimized drug concentration. Calibration curves were generated using weighted linear regression analysis with a weighting factor of 1/x over the respective standard concentration range.

Accuracy

For the determination of the accuracy of the method, recovery study was carried out by analyzing the samples at three different concentrations at 50, 100 and 150%. The percentage of recoveries at three concentrations was calculated.

Precision

Repeatability of the method was checked by analyzing six replicate samples of GRA and ELB at 100% concentration. The %RSD was calculated in terms of % area. Intra-day and inter-day variations were studied to establish intermediate precision of the proposed method. Intraday precision was determined by analyzing six replicate samples of optimized concentration. The same procedure was followed for three different days to study inter-day variation (n = 18). The precision of the assay was evaluated by performing six independent assays of test samples of GRA and ELB. The %RSD of six results was calculated.

Sensitivity

The sensitivity of the method was proved by establishing the limit of detection (LOD) and limit of quantitation (LOQ) for GRA and ELB with a signal-to-noise ratio of 3:1 and 10:1, respectively. LOD and LOQ were determined by injecting a series of diluted solutions having known concentrations of drugs. The precision study was also carried out at the LOQ level by injecting six individual preparations of GRA and ELB at LOQ concentration and by calculating the %RSD for the areas of each peak. Accuracy at LOQ level was verified by injecting three individual preparations of GRA and ELB at LOQ level and by calculating % recoveries of each analyte.

Robustness

The robustness study was carried out to evaluate the influence of small but deliberate variation in the chromatographic conditions. The factors chosen for this study which were critical sources of variability in the operating procedures such as a temperature of the column (±5°C), mobile phase and flow rate (±0.2 mL/min) were identified. Resolution between GRA and ELB was evaluated in the deliberately altered experimental conditions.

Stress studies

Specificity is the ability of the method to measure the analyte in the presence of its potential degraded products. Specificity of the developed HPLC method for GRA and ELB was performed in the presence of degradation products. Stress studies were performed at concentration 100 µg mL−1 of both drug substance to indicate the stability indicating property and specificity of the proposed method.

For acid degradation condition, drug solution was treated with 2 ml of 0.5 N HCl and heated for 70°C for 8 H, cooled and added 2ml of 0.5 N NaOH to neutralize any excess acid present in the sample. 20 µl of the sample solution was injected into HPLC. For base degradation, drug solution was treated with 2 ml of 0.5 N NaOH and heated for 80°C for 8 H, cooled and added 2ml of 0.5 N HCl to neutralize any excess base present in the sample. 20 µl of the sample solution was injected into HPLC. For peroxide degradation, drug solution was treated with 1 ml of 3 % H_2O_2 and heated for 80°C for 8 H, cooled and 20 µl of the sample solution was injected into HPLC. For UV degradation, drug sample was exposed to UV light in a UV chamber at 256 nm for 25 H. 20 µl of the sample solution was injected into HPLC. For heat degradation, drug solution was refluxed at 80°C for 25 H. 20 µl of the sample solution was injected into HPLC.

RESULTS AND DISCUSSION

Method development

Estimation of GRA and ELB in tablet dosage form by RP- HPLC method was carried out using optimized chromatographic conditions. The typical chromatogram of standard and sample solution is given in figure 2 and 3 respectively. The peak area ratio of standard and sample solutions was calculated. The results of the analysis show that the amounts of drugs were in good agreement with the label claim of formulations. The mobile phase was optimized based on resolution, asymmetric factor and peak area obtained for both the analytes. The mobile phase composed of a mixture of Acetonitrile: 0.25M Potassium dihydrogen orthophosphate buffer (pH 4.5) (55:45, v/v) was found to be

satisfactory and gave two symmetric and well-resolved peaks for GRA and ELB. The summary of optimized chromatographic conditions was shown in table 1. The retention time of GRA and ELB was found to be 2.390 and 4.603 mins respectively. The total time of analysis was less than 6 minutes. Results of method validation showed excellent correlation response factor and concentration of drugs within the concentration range.

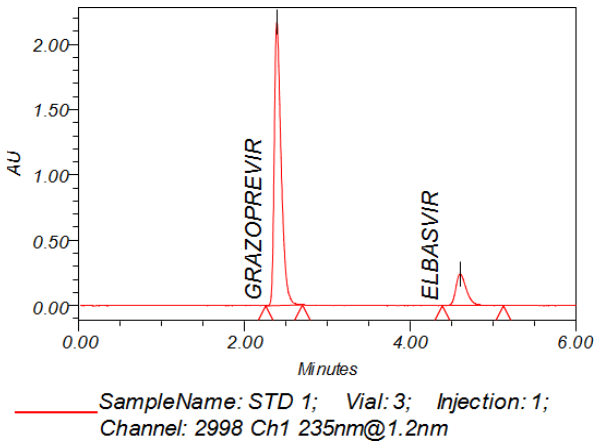

Figure 3: Representative chromatogram of Standard of GRA and ELB

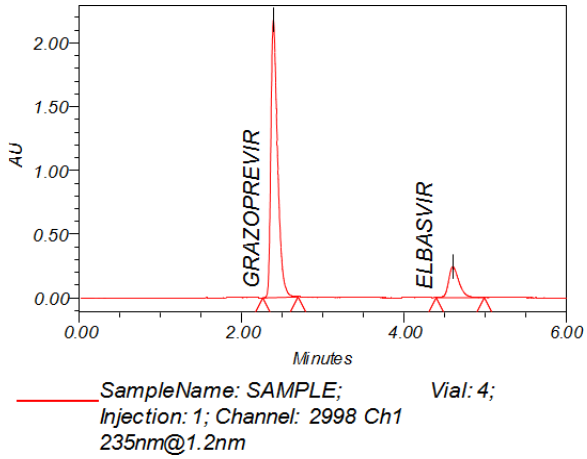

Figure 4: Representative chromatogram of Sample of GRA and ELB

Method validation

After method development, validation of the current test method for GRA and ELB was performed in accordance with ICH guidelines (ICH, 2015) which include accuracy, precision, specificity, linearity etc.

Specificity

The specificity of the HPLC method is illustrated in figure 2 and 3 where complete separation of GRA and ELB were noticed in the presence of tablet excipients. In addition, there was no any interference at the retention time of GRA and ELB in the chromatogram of the blank solution. In peak purity analysis with a photodiode detector, the purity angle was less than purity threshold for both the analytes. This shows that the peak of analytes was pure and excipients in the formulation did not interfere the analytes.

Linearity

Linearity was constructed with five concentration at the level of 50-150% (100, 150, 200, 250, 300 µg/ml of GRA and 50, 75, 100, 125, 150 µg/ml of ELB). The peak areas of the analytes were found to be linear in the studied concentration range the correlation coefficient was found to be 0.9998 for GRA and 0.9999 for ELB. The linearity data and curve was shown in table 3 and figure 5 & 6 respectively, and the Chromatograms of linearity was shown in Figure 2.

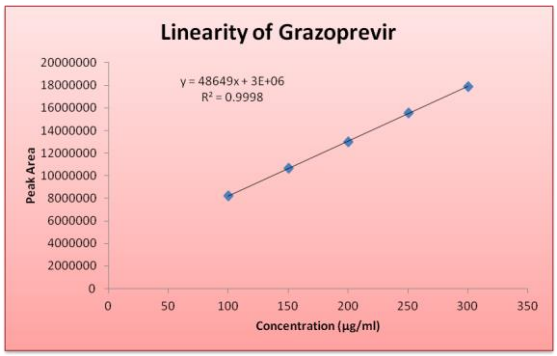

Figure 5: Linearity Plot of Grazoprevir

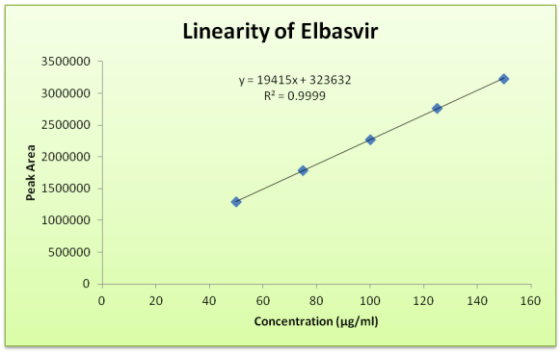

Figure 6: Linearity Plot of Elbasvir

Accuracy

The accuracy of the method was determined by the % recovery method at three concentration levels (50%, 100% and 150%) of the test solution. Six replicates were analysed for 50 % and 150 %, 3 replicates were tested for 100%. The mean recovery of GRA and ELB was found to be in between 99-100 %. Table 4 shows the results of accuracy.

Precision

Inter and Intra-day precision of the method was determined by performing precision for three times in the same day and followed by three consequent days. % RSD was calculated and found to be within the specified limits (<2%), which proves that the developed method was precise. Table 5 shows the precision results.

Table 4: Accuracy data of GRA and ELB

Level (%)	Sample Wt. (mg)	Mean Sample area	Amount added (µg/ml)	Amount found (µg/ml)	% Recovery	Mean % Recovery
Accuracy of Grazoprevir						
50	45.526	6202762.33	99.85	98.85	98.90	
100	91.052	12451735.33	199.91	198.12	99.11	99.03
150	36.578	18645930.83	299.86	297.16	99.10	
Accuracy of Elbasvir						
50	45.526	1075496	50.05	49.72	99.34	
100	91.052	2154725.33	100.10	99.61	99.51	99.34
150	36.578	32203395.33	150.16	148.88	99.15	

Table 5: Precision results of GRA and ELB

S. No	Peak Areas			
	Intraday precision		Interday precision	
	Grazoprevir	Elbasvir	Grazoprevir	Elbasvir
1	12490568	2139539	12514807	2176536
2	12521643	2149337	12523307	2154260
3	12372333	2129580	12536111	2172219
4	12372949	2157984	12529869	2153372
5	12381516	2146145	12553817	2175285
6	12424701	2169788	12604652	2160315
Average	12427285.00	2148728.83	12543760.50	2165331.17
STDEV	64779.27	14052.21	32609.96	10609.65
% RSD	0.52	0.65	0.26	0.49

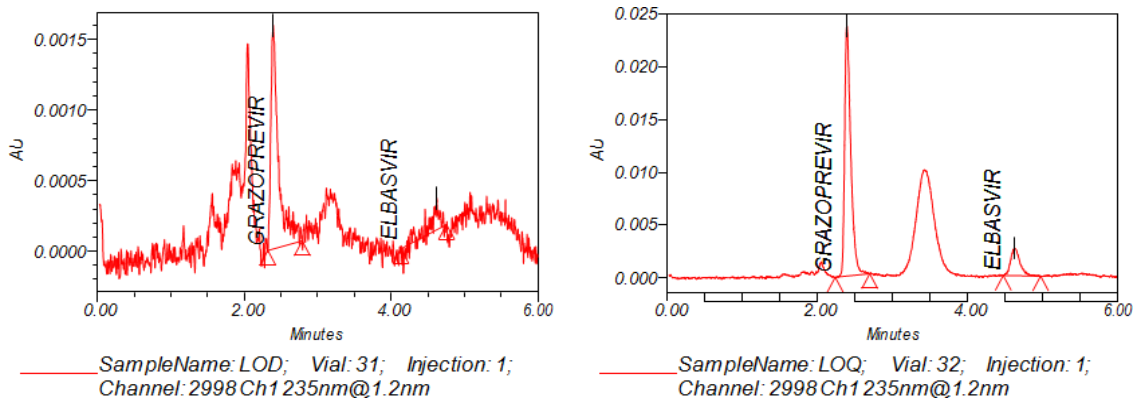

Figure 7: LOD and LOQ Chromatograms of GRA and ELB

Table 6: Robustness results of GRA and ELB

S. No	Parameter	Condition	Grazoprevir			Elbasvir		
			RT	Peak Area	% Assay	RT	Peak Area	% Assay
1		0.8 ml/min	3.281	12479835	99.44	6.195	2169233	100.28
2	Flow	1 ml/min	2.390	12514807	99.72	4.603	2176536	100.62
3		1.2 ml/min	1.922	12498176	99.59	3.872	2148792	99.34
4		25 °C	2.258	12656208	100.85	4.585	2189104	101.20
5	Temp	30 °C	2.390	12514807	99.72	4.603	2176536	100.62
6		35 °C	2.510	12581162	100.25	4.652	2166541	100.16
		Average	2.47	12540832.50	99.93	4.75	2171123.67	100.37

Sensitivity

LOD and LOQ were determined by using the standard deviation of response and slope of calibration curves. The LOD and LOQ for GRA of the proposed method were found to be 0.104 µg/ml and 0.347 µg/ml for ELB 0.0068 µg/ml and 0.029 µg/ml respectively. Figure 7 shows the chromatograms of LOD and LOQ.

Robustness

The robustness of the analytical method was evaluated by assaying the test solutions after slight but deliberate changes in the conditions like flow rate (± 0.1 ml/min) and the column temperature (± 2°C). System suitability data was found to be satisfactory during variations of the analytical conditions.

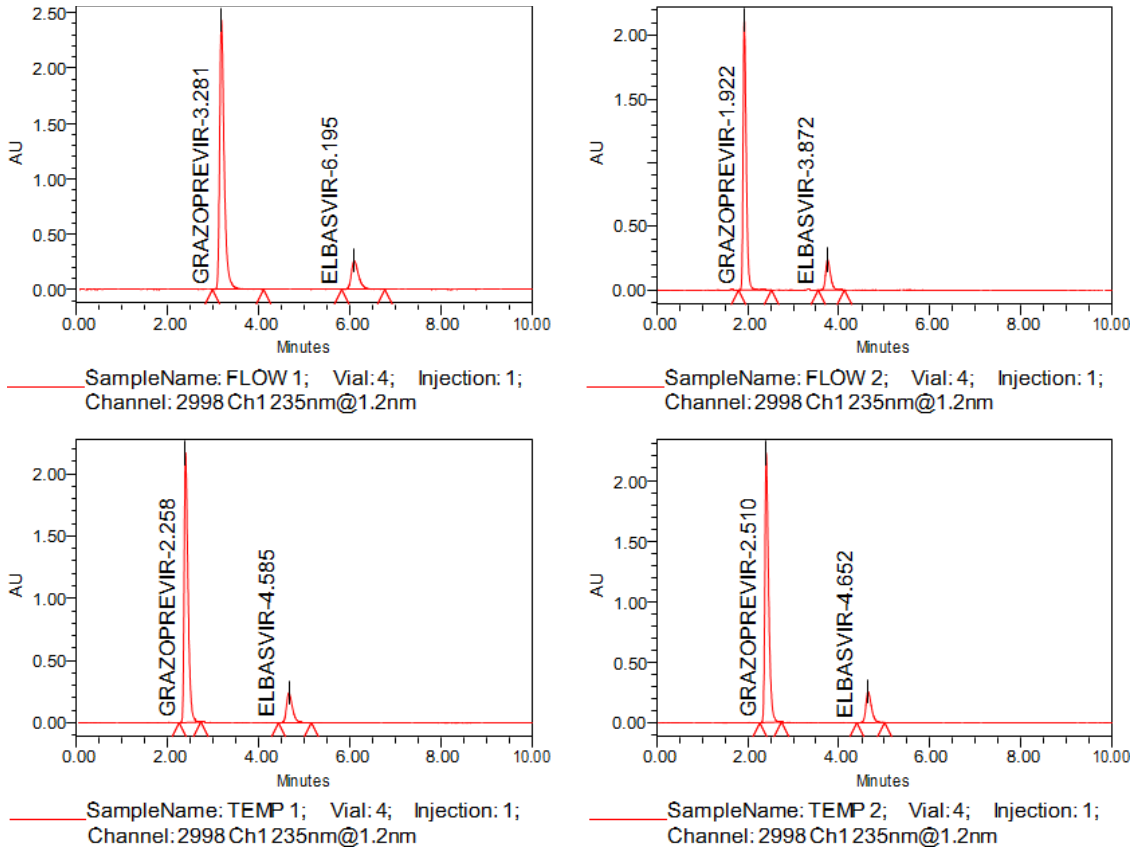

Figure 8: Robustness chromatograms of GRA and ELB

Table 7: Assay results of GRA and ELB

S. No	Sample Wt. (mg)	Grazoprevir		Elbasvir	
		Peak area	% Assay	Peak area	% Assay
1	91.052	12490568	99.53	2139539	98.91
2	91.052	12521643	99.78	2149337	99.36
3	91.052	12372333	98.59	2129580	98.45
4	91.052	12372949	98.59	2157984	99.76
5	91.052	12381516	98.66	2146145	99.22
6	91.052	12424701	99.01	2169788	100.31
	Average	12427285.00	99.03	2148728.83	99.34
	STDEV	64779.27	0.52	14052.21	0.65
	% RSD	0.52	0.52	0.65	0.65

System suitability results were also remained unaffected by slight changes in the analytical conditions. Table 6 shows the results and chromatograms were shown in figure 8.

Assay

The proposed method was applied to the tablets of GRA and ELB. The mean % assay was found to be 99.03 and 99.34% for GRA and ELB respectively. Results were given in table 7.

Stress studies

Stress studies were performed to evaluate the stability indicating an ability of the developed analytical method by exposing the sample solution to different the stress conditions viz., acid, base, peroxide, UV and heat. Assay studies were carried out for stress samples at 100µg/ml against a reference standard. The proposed analytical method can able to detect the analyte even in the presence of degraded products and thus confirms the stability indicating the power of the developed method. Results of stress studies were shown in table 8 and figure 9 shows chromatograms of Stress studies of GRA and ELB.

CONCLUSION

A simple, accurate and precise stability-indicating RP-HPLC analytical method was developed and validated for the simultaneous analysis of GRA and ELB in tablet formulations. Low LOD and LOQ of this method enable the detection and quantification of this impurity at low concentration. The method is very simple and specific as both peaks are well separated from one another and excipients peaks with a total runtime of 6 min, which

Table 8: Forced degradation results of GRA and ELB

S. No	Condition	Grazoprevir			Elbasvir		
		Peak Area	% Assay	% Degradation	Peak Area	% Assay	% Degradation
1.	Acid	11574907	92.23	7.77	1938839	89.63	10.37
2.	Base	11237957	89.55	10.45	1939460	89.66	10.34
3.	H_2O_2	12125314	96.62	3.38	2017386	93.26	6.74
4.	UV	11502149	91.65	8.35	2018717	93.33	6.67
5.	Heat	11065840	88.18	11.82	1956987	90.47	9.53

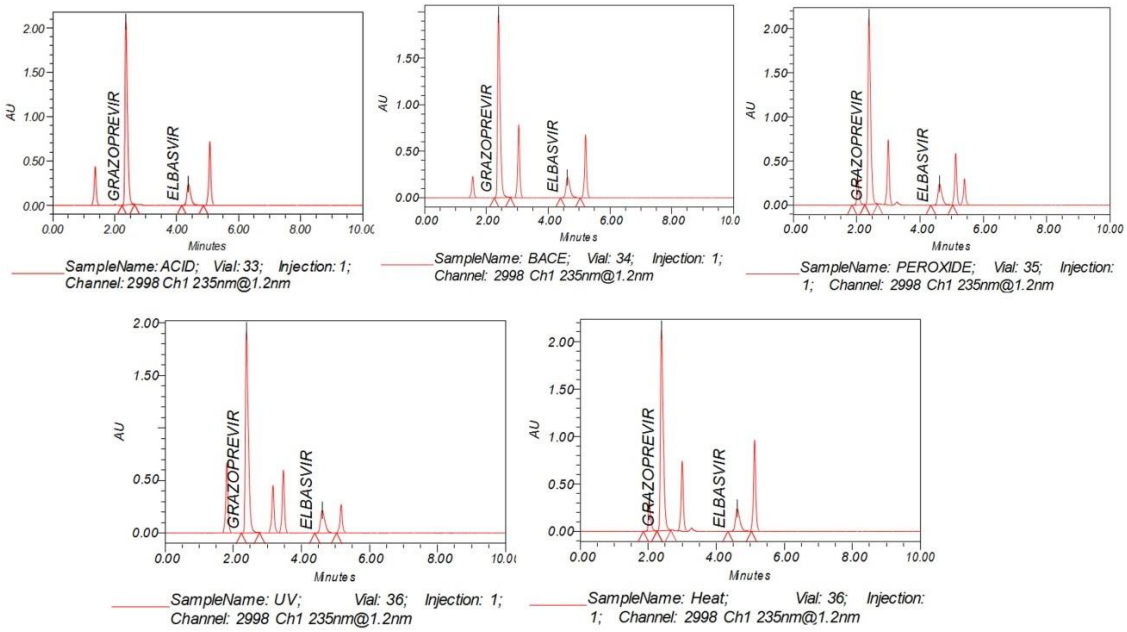

Figure 9: Degradation studies of GRA and ELB

makes it especially suitable for routine quality control analysis work.

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