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A NEW METHOD WAS ESTABLISHED FOR SIMULTANEOUS ESTIMATION OF ELBASVIR AND GRAZOPREVIR BY RP-HPLC METHOD

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Abstract:

***Objective**: A simple, Accurate, precise method was developed for the simultaneous estimation of the Elbasvir and Grazoprevir in pharmaceutical dosage form.*

***Methods**: Chromatogram was run through symmetry C18, 250 x 4.6 mm, 5µm. Mobile phase containing 0.1% Ortho Phosphoric acid: Acetonitrile, (55:45, v/v) was pumped through column at a flow rate of 1 ml/min. Temperature was maintained at Ambient. Optimized wavelength for Elbasvir and Grazoprevir was 260 nm.*

***Results**: Retention time of Elbasvir and Grazoprevir were found to be 3.848 min and 2.313 min. The % purity of Elbasvir and Grazoprevir was found to be 100.4 % and 100.2 % respectively. The system suitability parameters for Elbasvir and Grazoprevir such as theoretical plates and tailing factor were found to be 3568.30 and 4836.12. The linearity study for Elbasvir and Grazoprevir was found in concentration range of 12.5 µg-75 µg and 25 µg-150 µg and correlation coefficient (r2) was found to be 0.999 and 0.999, % mean recovery was found to be 100.19 % and 100.84 %, %RSD for repeatability was 0.75 and 0.36 %. The precision study was precise, robust and repeatable. LOD value was 0.082 and 0.357, and LOQ value was 1.05 and 0.23 respectively*

***Conclusion**: The results of study showed that the proposed RP-HPLC method is a simple, accurate, precise, rugged, robust, fast and reproducible, which may be useful for the routine estimation of Elbasvir and Grazoprevir in pharmaceutical dosage form.*

***Keywords:** Elbasvir, Grazoprevir, RP-HPLC, Simultaneous estimation.*

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INTRODUCTION:

Elbasvir is a direct-acting antiviral medication used as part of combination therapy to treat chronic hepatitis C, an infectious liver disease caused by infection with hepatitis C virus (HCV).[1] HCV is a single-stranded RNA virus that is categorized into nine distinct genotypes, with genotype 1 being the most common in the United States, affecting 72% of all chronic HCV patients.Treatment options for chronic hepatitis C have advanced significantly since 2011, with the development of direct-acting antivirals (DAAs) such as elbasvir.[2] Elbasvir is an inhibitor of NS5A, a protein essential for viral replication and virion assembly. Elbasvir is an inhibitor of the HCV non-structural protein 5A. While the precise role of this protein is unknown, it is essential to viral replication and virion assembly.[3] Elbasvir maleate is a white to off-white, crystalline powder with a molecular weight of 882.035 g/mol. Chemical formula is $C_{49}H_{55}N_9O_7$. Elbasvir is practically insoluble in water (<0.1 mg/mL) and very slightly soluble in ethanol (0.2 mg/mL), but is very soluble in ethyl acetate and acetone.

Grazoprevir is a direct acting antiviral medication used as part of combination therapy to treat chronic Hepatitis C, an infectious liver disease caused by infection with Hepatitis C Virus (HCV).[4] HCV is a single-stranded RNA virus that is categorized into nine distinct genotypes, with genotype 1 being the most common in the United States, and affecting 72% of all chronic HCV patients.[5] Treatment options for chronic Hepatitis C have advanced significantly since 2011, with the development of Direct Acting Antivirals (DAAs) such as Grazoprevir. Grazoprevir is an inhibitor of NS3/4A, a serine protease enzyme, encoded by HCV genotypes 1 and 4.[6] Molecular weight is 776.093 g/mol. Chemical formula is $C_{38}H_{50}N_6O_9S$. Grazoprevir is soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide (DMF), which should be purged with an inert gas. The solubility of grazoprevir in these solvents is approximately 15, 25, and 30 mg/ml, respectively. Grazoprevir is sparingly soluble in aqueous buffers.

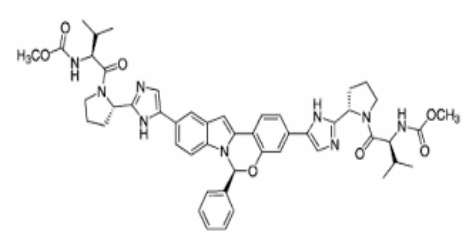

Figure 1: Structure of Elbasvir

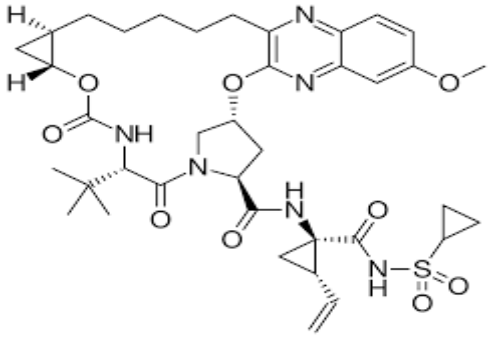

Figure 2: Structure of Grazoprevir

The literature survey revealed that There are Various analytical methods were carried out for the estimation of Elbasvir and Grazoprevir as a single or combined with other drugs in pharmaceutical dosages Literature survey reveals that the retention time for the simultaneous estimation of Elbasvir and Grazoprevir is more.[7-11] Hence the present study, we had made an attempt to develop simple, accurate, precise, less time consuming and with less retention time using RP-HPLC for the simultaneous estimation of Elbasvir and Grazoprevir in bulk and pharmaceutical dosage form by RP-HPLC. To validate the developed method in accordance with ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the drug in its dosage form.

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MATERIALS AND METHODS:

Chemicals and Reagents: Grazoprevir and Elbasvir were Purchased from market. NaH_2PO_4 was analytical grade supplied by Finerchem limited, Orthophosphoric acid (Merck), and Water and Methanol for HPLC (Lichrosolv (Merck).

Equipment and Chromatographic Conditions: The chromatography was performed on a Waters 2695 HPLC system, equipped with an auto sampler, UV detector and Empower 2 software. The experiments were carried out on the chromatographic system Agilent consisting of HPLC Pump, auto sampler and PDA detector. Empower software was used for data collection and analysis. The partial loop injection volume was 10µL. Chromatographic separations were performed on symmetry C18, 250 x 4.6 mm, 5m particle size column with UV detection at 260 nm. The flow rate was 1 mL/min and the column temperature was set at 30^0C.

METHOD DEVELOPMENT

Selection and preparation of mobile phase:

Several mobile phases containing orthophosphoric acid and acetonitrile in different ratios were tried by different columns, flowrates. Good peak symmetry, resolution and retention time was observed with mobile phase comprised of 0.1% Ortho Phosphoric acid: Acetonitrile, (55:45, v/v) premixed. Further sonication was done for 30 min and filtered.

Preparation of standard stock solution:

Accurately weighed 5 mg of Elbasvir & 10 mg of Grazoprevir standards were taken in a 10mL clean dry volumetric flask respectively and 5mL of diluent was added and sonicated for 30 minutes. The final volume is made upto the mark with diluents to get a concentration of 100 µg/mL of Grazoprevir and 50µg/mL of Elbasvir. From the above two stock solutions, 1mL was diluted to 10mL using diluent.

Preparation of Buffer (0.1%OPA):

Buffer was prepared by taking 1mL of Ortho phosphoric acid solution in a 1000mL of volumetric flask and about 100mL of milli-Q water was added. The final volume was made up to mark with milli-Q water.

Selection of wavelength:

Good response for both the drugs was detected from UV spectra as 260 nm.Hence detection was executed at 260 nm.

The developed chromatographic method was validated for system suitability, linearity accuracy, precision, ruggedness and robustness as per ICH guidelines.

System suitability parameters: To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 1.0 ml/min to equilibrate the column at ambient temperature. Chromatographic separation was achieved by injecting a volume of 10 µL of standard into symmetry C18, 250 x 4.6 mm, 5µm, the mobile phase of composition 0.1% Ortho Phosphoric acid: Acetonitrile, (55:45, v/v) was allowed to flow through the column at a flow rate of 1 ml per minute. Retention time, tailing factor and USP theoretical plate count of the developed method are shown in table 1.

Assay of pharmaceutical formulation: The proposed validated method was successfully applied to determine Grazoprevir and Elbasvir in their tablet dosage form. The result obtained for was comparable with the corresponding labeled amounts and they were shown in Table-2

RESULTS AND DISCUSSION

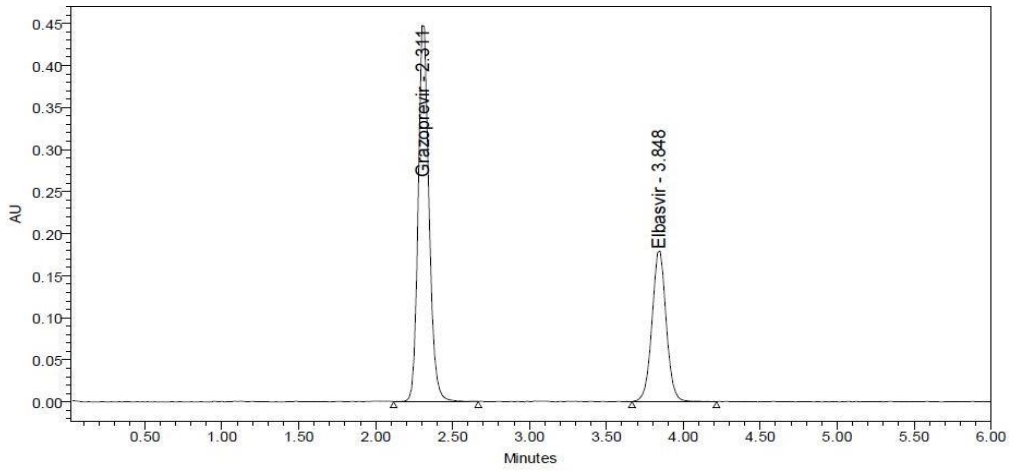

Figure 3: Standard chromatogram

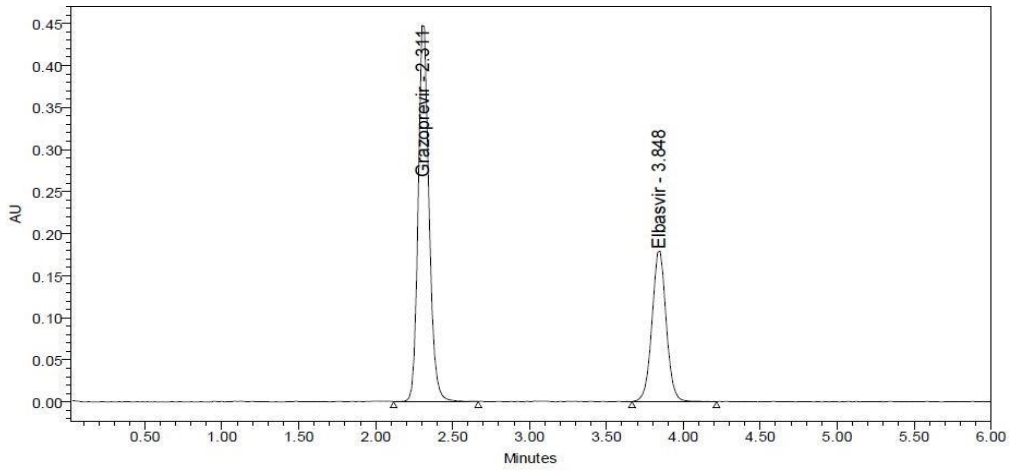

Figure 4: Sample chromatogram

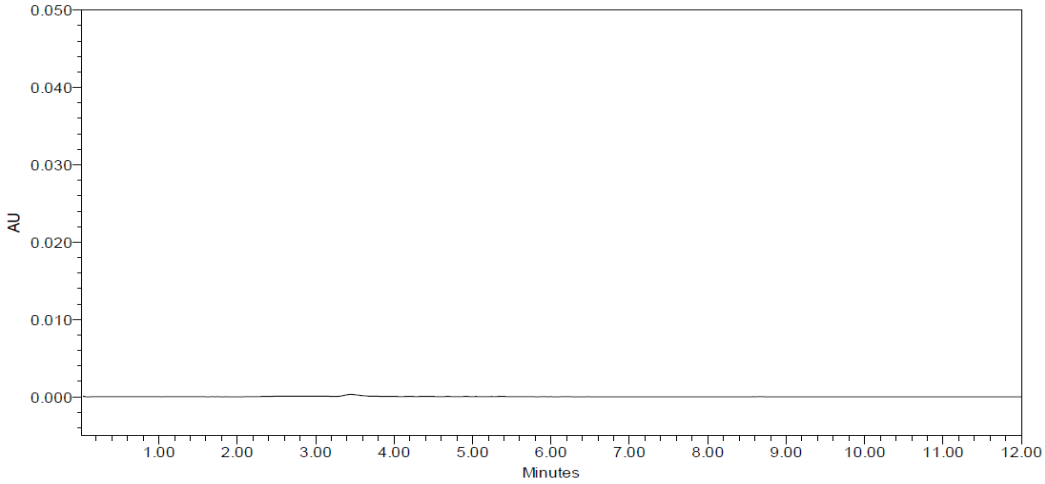

Figure 5: Blank chromatogram

Table 1: System suitability parameters

Parameters	Elbasvir	Grazoprevir
Retention time	3.848	2.313
USP Plate count	3568.306	4836.128
USP Tailing	1.4	1.6

Table 2: Assay results for Elbasvir and Grazoprevir

	Label Claim (mg)	% Assay
Elbasvir	5	100.2
Grazoprevir	10	100.4

Validation of Analytical method:

Linearity: For the estimation of accuracy and linearity eight solutions containing Elbasvir and Grazoprevir were prepared concentration range of 12.5–150 µg/mL in the mixture of acetonitrile and water (50:50 v/v). These solutions were further sonicated for 30 min and was stirred on the magnetic stirrer for 2 hrs. A calibration curve was plotted using peak area versus concentration. The results are shown in table 3.

Table 3: Linearity results of Grazoprevir and Elbasvir

Elbasvir		Grazoprevir	
Concentration of drug(µg/mL)	**Peak Area**	**Concentration of drug(µg/mL)**	**Peak Area**
12.5	286235	25	555581
25	563670	50	1115880
37.5	866584	75	1662593
50	1180129	100	2262199
62.5	1434876	125	2748549
75	1698233	150	3252133
Slope (m)	22881	Slope (m)	21841
Intercept (c)	3308	Intercept (c)	18686
Correlation coefficient	0.999	Correlation coefficient	0.999

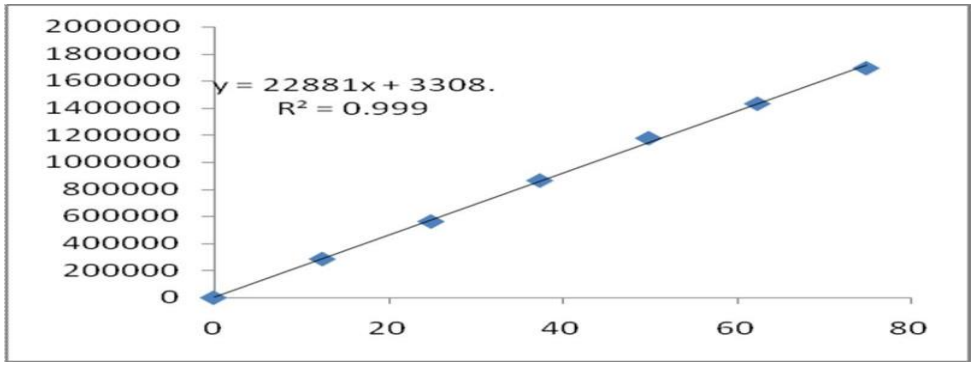

Figure 6: Linearity graph for Elbasvir

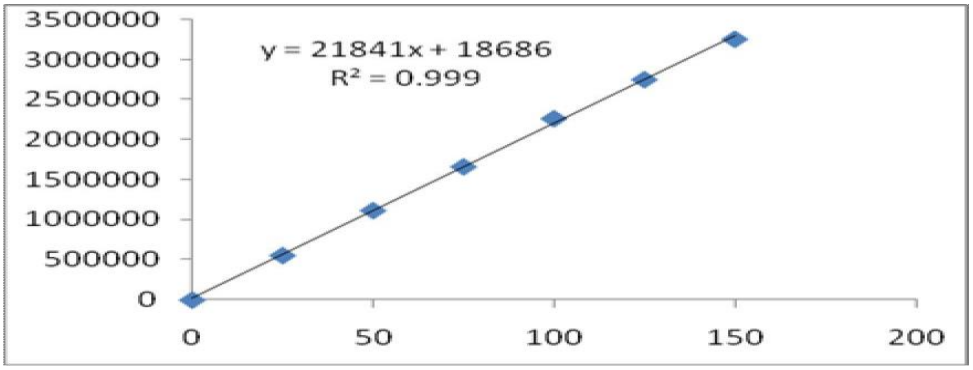

Figure 7: Linearity graph for Grazoprevir

Accuracy studies: Accuracy was estimated for analyte in samples at different concentration levels (50%, 100% and 150%) (n=3). The accuracy of Elbasvir and Grazoprevir is estimated by performing the recovery experiment at 50%, 100% and 150% of the label claim of the drug.The results are shown in table 4,5.

Table 4: Showing accuracy results for Elbasvir

Analyte Level	Analyte Peak Area	Nominal Concentration (µg/ml)	Actual Concentration (µg/ml)	Individual % Recovery	Mean % Recovery	% RSD
Level 1	1714244	25	24.7754	99.10	99.60	0.43
	1718715	25	24.97081	99.88		
	1718324	25	24.95372	99.81		
Level 2	2286191	50	49.77199	99.54	99.95	0.56
	2298411	50	50.30193	100.59		
	2288277	50	49.86587	99.73		
Level 3	2864873	75	75.06577	100.12	100.10	0.23
	2860294	75	74.86275	99.73		
	2867570	75	75.18451	100.23		

Table 5: Showing accuracy results for Grazoprevir

AnalyteLevel	Analyte Peak Area	Nominal Concentration (µg/ml)	Actual Concentration (µg/ml)	Individual % Recovery	Mean % Recovery	% RSD
Level 1	3283739	50	49.49192	98.98	99.28	0.29
	3289931	50	49.77542	99.55		
	3287308	50	49.65533	99.31		
Level 2	4406492	100	100.8977	100.90	100.00	0.82
	4371719	100	99.30557	99.31		
	4382254	100	99.78792	99.79		
Level 3	5461965	150	149.223	99.48	99.50	0.21
	5469805	150	149.5819	99.72		
	5455996	150	148.9497	99.30		

Precision Studies: Weight corresponding to 100 mg of Grazoprevir and 50 mg of Elbasvir were placed into a 100 mL volumetric flask and extracted with the mixture of acetonitrile and water (50:50 v/v) followed by sonication and filtration. From that stock solution, six solutions containing 50µg/mL of Elbasvir and 100µg/mL Grazoprevir were prepared. 10 µl of these were injected and the chromatograms were recorded. The peak areas were noted and the % RSD was calculated.The results are shown in table 6.

Table 6: Precision results for Elbasvir and Grazoprevir

Grazoprevir			Elbasvir		
Determination	**Area of Analyte**	**% Assay**	**Determination**	**Area of Analyte**	**% Assay**
100	2244319	99.11	50	1184782	99.00
100	2240188	98.84	50	1172172	98.91
100	2240190	99.48	50	1182476	99.82
100	2246117	100.29	50	1174120	99.07
100	2239860	99.23	50	1179670	99.56
100	2238559	99.51	50	1180246	99.61
Average	2241392	99.41	**Average**	1178896	99.51
SD	2931.2	0.45	**SD**	4941.8	0.42

Robustness: Robustness of the developed method was reviewed by small variations in the three important factors which influence dramatically chromatographic separation which include flow rate (mL/min, ± 1), temperature (ºC, ±50 C) and organic phase composition (±5 %). The results are shown in table 7.

Table 7: Robustness results for Elbasvir and Grazoprevir

Factors	**Elbasvir (RT minutes)**	**Grazoprevir (RT minutes)**
A. Flow rate (mL/min)		
0.7 mL	3.76	2.31
1.1 mL	3.77	2.32
B. Temperature (^{0}C)		
25 ^{0}C	3.78	2.32
35 ^{0}C	3.81	2.32
C. Organic phase composition		
Acetonitrile (35 %)	3.75	2.31
Acetonitrile (55 %)	3.77	2.32

LOD and LOQ: The lower detection limit and lower quantitation limit was necessarily determined and calculated from the signal-to-noise ratio using 100 µg/ml of Grazoprevir and 50 µg/mL of Elbasvir.10µL of these were injected and the chromatograms were recorded. The peak areas were observed.The sensitivity of RP-HPLC was determined from LOD and LOQ. Which were calculated from the calibration curve using the following equations as per ICH guidelines. The results are shown in table 8.

LOD = 3.3σ/S and
LOQ = 10 σ/S, where
σ= Standard deviation of y intercept of regression line,
S = Slope of the calibration curve

Table 8: LOD, LOQ of Elbasvir and Grazoprevir

Drug	**LOD**	**LOQ**
Elbasvir	0.081	1.04
Grazoprevir	0.357	0.21

Forced degradation study

The forced degradation study was carried out on Elbasvir and Grazoprevir in bulk and stress studies were carried out at a initial concentration of 1mg/mL. Both the drugs were exposed to acidic, alkaline, oxidative, thermal and photolytic conditions. The procedure followed for preparation of samples and degradation studies is represented as follows.

Acid degradation

1mg/mL mixture of Elbasvir and Grazoprevir in X1 Molarity of HCl was heated under reflux at X1 M, X2 0 C for X3 minutes. Three independent factors were studied at two levels (-1 and +1). The low level for (-1) for X1, X2 and X3 are 0.30 M, 25 0C and 45 min and high level for (+1) for X1, X2 and X3 are 0.80 M, 60 0C and 65 min. 23 factorial design was employed and 20 experiments were conducted since three variables are considered at two levels.

Alkaline degradation

1mg/mL mixture of Elbasvir and Grazoprevir in X1 molarity of Sodium hydroxide was exposed to heat under reflux at X2 0 C for X3 minutes. Three independent factors at two levels (-1 and +1) were studied. The low level for (-1) for X1, X2 and X3 are 0.30 M, 250 C and 45 minutes and high level for (+1) for X1, X2 and X3 are 0.80 M, 60 0 C and 65 minutes. Total of 20 experiments were conducted.

Oxidative degradation

1mg/mL mixture of Elbasvir and Grazoprevir was exposed to oxidative studies at room temperature by treating the drug to X1 % hydrogen peroxide in dark for 24hrs.

Two levels were chosen for X1 and X2 . The low level for (-1) for X1 and X2 are 5 % hydrogen peroxide and 5 min and high level for (+1) for X1 is 25% hydrogen peroxide and 20 minutes.

Thermal degradation

For the dry heat study, 100 mg of drug powder of Elbasvir and Grazoprevir placed in individual petri dishes was heated in hot air oven at 105 °C for 48 hrs.

Photo degradation

The photodegradation was performed by exposing 100 mg of drug powder, spread as a thin film in a covered petri plates and exposed to direct sunlight for 48 hrs.

Solutions of photo degraded and dry heat condition samples of drug powder were prepared by dissolving 10 mg of Grazoprevir and 5 mg of Elbasvir in diluents to produce 100 mL and sonicated for 10 minutes. The filtered solution volume was made upto the mark using diluent. Further dilutions were made to attain final concentration of 10 µg mL−1. To minimize the errors analyte, blank and control were analysed under same conditions.

The results are shown in table Table 9 and 10.

Table 9: Degradation results, purity angle and purity threshold of Elbasvir

Stress condition	Purity angle	Purity threshold	% Degradation
Acid degradation	1.319	1.714	14.23
Alkaline degradation	0.620	1.764	20.74
Oxidative degradation	0.417	1.819	16.55
Thermal degradation	1.416	1.531	13.21
Photodegradation	1.326	1.518	No degradation

Table 10: Degradation results, purity angle and purity threshold of Grazoprevir

Stress condition	Purity angle	Purity threshold	% Degradation
Acid degradation	0.358	0.520	9.51
Alkaline degradation	0.541	1.621	17.21
Oxidative degradation	1.582	1.714	17.50
Thermal degradation	0.481	0.671	18.11
Photodegradation	0.341	0.571	No degradation

CONCLUSION:

The Developed HPLC method was validated and it was found to be simple, precise, accurate and sensitive for the simultaneous estimation of Elbasvir and Grazoprevir in its pure form and in its pharmaceutical dosage forms. Hence, this method can easily and conveniently adopt for routine quality control analysis of Elbasvir and Grazoprevir in pure and its pharmaceutical dosage forms.

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