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A NEW ROBUST UPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ELBASVIR AND GRAZOPREVIR IN TABLET DOSAGE FORMS

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Abstract:
A novel, simple, accurate and rapid method has been developed and validated for the estimation of Elbasvir and Grazoprevir in bulk and in tablet dosage form. In RP-UPLC method, elution was achieved in isocratic mode using combination of 0.1% Formic acid in water and Acetonitrile in the ratio of 50:50 v/v using an Acquity UPLC BEH C18, 75 x 2.1 mm, 1.7 µm column. The flow rate was 0.3mL/min and detection was done at 264 nm. The method was validated for linearity, accuracy, precision, specificity and sensitivity. The peak retention times obtained for elbasvir and grazoprevir were 0.6 and 1.2 min respectively. The linearity range for elbasvir was found to be 5 to 30 µg/mL and for grazoprevir was 10 to 60 µg/mL, respectively. The forced degradation studies were also conducted on drug substances as per ICH norms and found that the developed method was stability indicating. ***Keywords: Elbasvir, Grazoprevir, RP-UPLC, Validation.***

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INTRODUCTION:

Elbasvir[1]:

Elbasvir is an anti-viral agent used as a combination therapy along with grazoprevir to treat chronic hepatitis C virus (HCV) genotypes 1 or 4 infection in affected adults. Elbasvir is a carbamate ester from imidazoles family which is a valine derivative with N-acylpyrrolidine ring. HCV is classified into nine diverse genotypes which is a single-stranded RNA virus. Elbasvir acts by inhibition of NS5A protein responsible for viral multiplication and assembly synthesis of virion. Its chemical structure is given below figure 1.

Figure 1: Chemical Structure of Elbasvir

Grazoprevir[2]:

Grazoprevir is an anti-viral agent used in combination with elbasvir (under the brand name Zepatier) for diagnosis of chronic HCV genotypes 1 or 4 infection in patients. It belongs to azamacrocycle class of compounds. It is a quinoxaline derivative with carbamate ester and an aromatic ether, cyclopropane derivative, a N-sulfonylcarboxamide. HCV is a RNA virus with single strand which is classified into nine different genotypes with genotype 1 as the utmost predominant one, affecting a total of 72% HCV patients. Its chemical structure is given below figure 2.

Figure 2: Chemical structure of Grazoprevir

A very few spectroscopic and liquid chromatographic procedures have been reported for the simultaneous estimation of Elbasvir and Grazoprevir. Therefore, there is need to develop a rapid, sensitive, robust and selective RP-UPLC method for simultaneous estimation of Elbasvir and Grazoprevir in bulk and tablet dosage form.

MATERIALS AND METHODS:

Chemicals and Solvents:

Reference standard samples of elbasvir and grazoprevir were procured from Clearsynth Laboratories Ltd, India. The commercial formulation "Zepatier" Tablets (50 mg Elbasvir + 100 mg Grazoprevir, Merck & Co. Inc.) was bought from the pharmacy store. Solvents like HPLC grade water and acetonitrile were bought from Merck, India. Formic acid was purchased from Sigma-Aldrich. Hydrochloric acid, hydrogen peroxide and sodium hydroxide were procured from S.D. Fine Chemicals, Hyderabad.

Instrumentation and Chromatographic Conditions:

Waters UPLC system equipped with photo diode array detector, auto sample injector and column Acquity UPLC BEH C18, 75 x 2.1 mm, 1.7µm respectively. The output signal was monitored and integrated using Waters Empower 2 software. Electronic balance was used for weighing the materials. Ultrasonicator was used for sonicating the solvents; Hot air oven was used for forced degradation studies. The mobile phase consisted of 0.1% Formic acid in water and Acetonitrile in 50:50 v/v ratio flowing through the column at a constant flow rate of 0.3 mL/min at ambient temperature with a sample injection volume of 2 µL. Detection of the analytes were carried out at a wavelength of 264 nm.

Preparation of Mobile Phase:

Mixture of 0.1% Formic acid in water and Acetonitrile in 50:50 v/v ratio filtered through 0.45µm filter under vacuum.

Diluent: The solution containing 50% acetonitrile in water was used as the diluent for preparing drug solutions.

Stock solution of the drugs: Independent stock solutions of the drugs were prepared by weighing 10 mg of elbasvir and 10 mg of grazoprevir into 10 mL volumetric flasks separately. To each volumetric flask, 7 mL of diluent was added and sonicated for 5 minutes to dissolve the drugs completely. Finally, the diluent was added up to the final volume in order to get 1.0 mg/mL concentration of the drugs.

Preparation of Working Standard Solutions: The working standard solution was prepared by taking 0.2 mL of elbasvir stock solution and 0.4 mL of grazoprevir stock solution into a 10 mL volumetric flask. The diluent was added up to the final volume in order to get the concentrations of 20 mcg/mL of elbasvir and 40 mcg/mL grazoprevir respectively.

RESULTS AND DISCUSSION:

Method development:

The method developed with the optimized wavelength as 264nm. In order to get the optimized RP-UPLC method various mobile phases and columns were used to get better resolution. Finally the analysis was performed by using 0.1% Formic acid in water and Acetonitrile in 50:50 v/v at a flow rate 0.3mL/min at an injection volume of 2µL and separation was carried out by using Acquity UPLC BEH C18, 75 x 2.1 mm, 1.7µm and final optimized chromatogram given in figure 3.

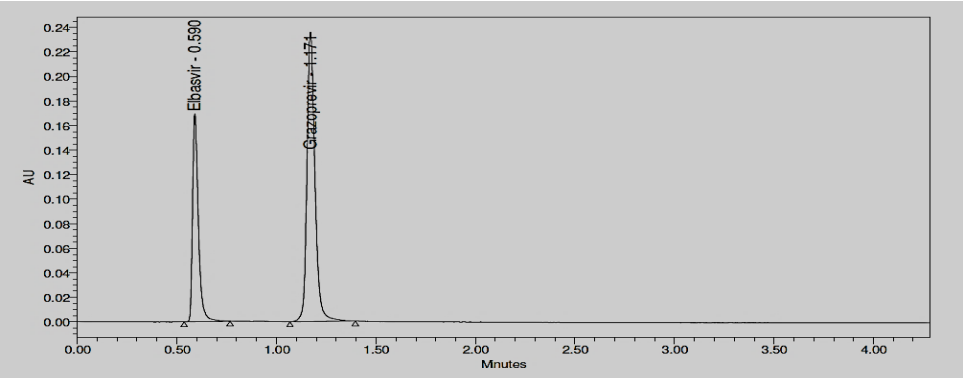

Figure 3: Final Optimized Chromatogram

Validation parameters as per ICH[3]:

System suitability:

System suitability experiment of the UPLC system was conducted to ensure that the analytical performance, equipment, and working standard solutions (WSS) to be analysed comprises of an integral system that can be assessed on the day of use for each validation run. System suitability was assessed by injecting six replicates of a freshly prepared WSS containing 20 mcg/mL of elbasvir and 40 mcg/mL of grazoprevir. The following parameters considered to assess the appropriateness of the system: peak area, retention time, USP peak tailing, USP plate count and %CV. The system suitability parameters are given in Table 1 by taking average values (n = 6).

Table 1: System suitability test parameter

Parameter	Elbasvir	Grazoprevir
Peak Area	442739	866594
Retention Time (min)	0.588	1.178
USP Plate count	2181	4614
USP Tailing	1.52	1.22
% CV	0.9	1.1
Resolution	9.6	

Specificity:

A study to establish the interferences of blank and placebo was conducted. Analysis was performed on solution placebo and formulation as per test method. Chromatograms of blank, placebo and formulation solution showed no peaks at the retention time.

Linearity:

The linearity of optimized analytical method was estimated by injecting three replicates of the mixed working standard solutions at six different concentration levels in the ranges of 5 to 30 mcg/mL for elbasvir and 10 to 60 mcg/mL for

grazoprevir respectively. The corresponding responses were found to be linear. The relevant results are summarized in Tables 2 and 3. The calibration curves were plotted by considering average analyte peak area (y-axis) over corresponding concentrations (x-axis) for elbasvir and grazoprevir individually and the regression equations were computed. The linear regression equation (y = mx + c) for elbasvir was found to be y = 22061x + 595.62. The R^2 correlation of coefficient was determined to be 0.9999 (Fig. 4). The linear regression equation (y = mx + c) for grazoprevir was found to be y = 21464x + 1034.3. The R^2 correlation of coefficient was determined to be 0.9997 (Fig. 5).

Table 2: Linearity data of Elbasvir and Grazoprevir

Elbasvir				Grazoprevir			
Conc. (mcg/mL)	**Area of Peak**	***Average Peak Area (n=3)**	**% RSD**	**Conc. (mcg/mL)**	**Area of Peak**	***Average Peak Area (n=3)**	**% RSD**
5	112392 112495 113062	112650	0.3	10	218395 218977 219188	218853	0.2
10	219200 218424 217576	218400	0.4	20	431611 434021 432711	432781	0.3
15	329127 330754 329762	329881	0.2	30	632152 632459 631240	631950	0.1
20	442745 444443 446389	444526	0.4	40	862859 867922 864017	864933	0.3
25	553471 553295 555442	554069	0.2	50	1075096 1080284 1075083	1076821	0.3
30	660132 660293 660939	660455	0.1	60	1286719 1289885 1288343	1288316	0.1

*= Average peak area of 3 replicate injection for each concentration

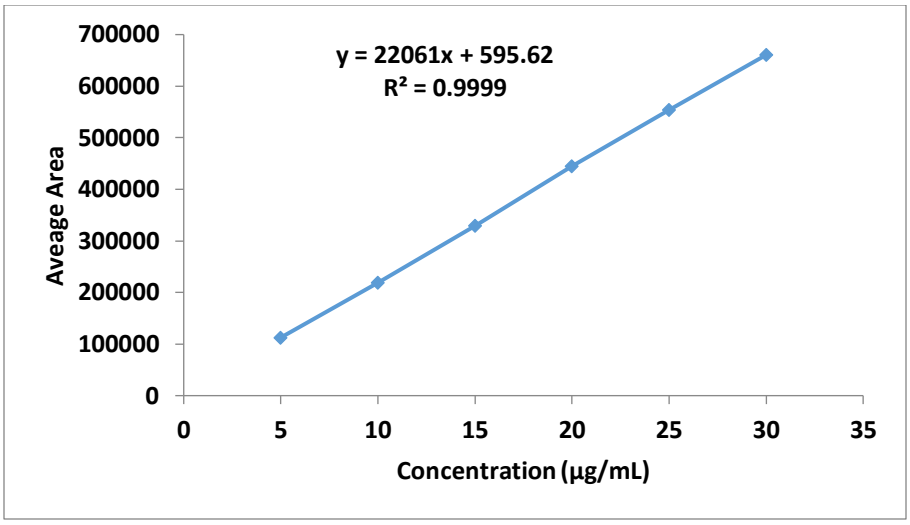

Figure 4: Standard calibration curve of Elbasvir

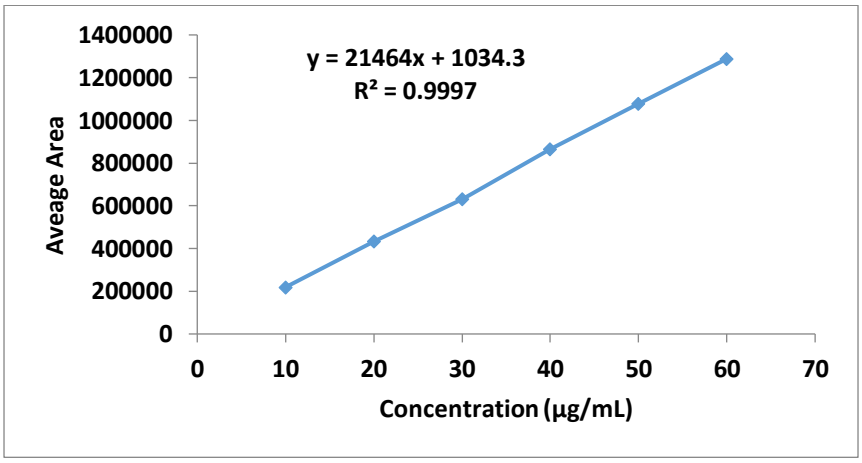

Figure 5: Standard calibration curve of Grazoprevir

Limit of Detection (LOD) and Limit of Quantification (LOQ):

A study to establish the LOD and LOQ for Elbasvir and Grazoprevir was conducted. Series of very dilute LOD and LOQ solutions were prepared as per the test method and injected triplicate into the UPLC system. The sensitivity results are shown in table no 3. Chromatograms of LOD and LOQ were given in figures no 6 to 7.

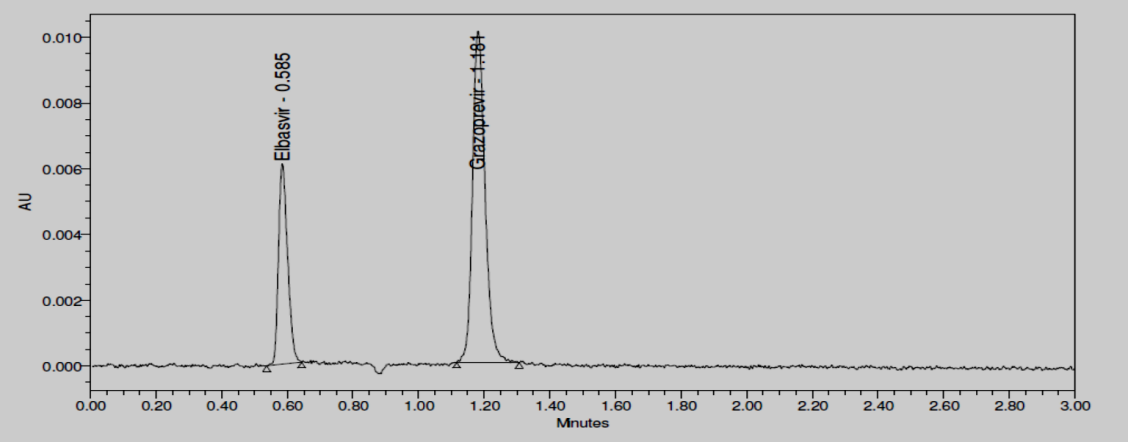

Figure 6: Chromatogram of LOD

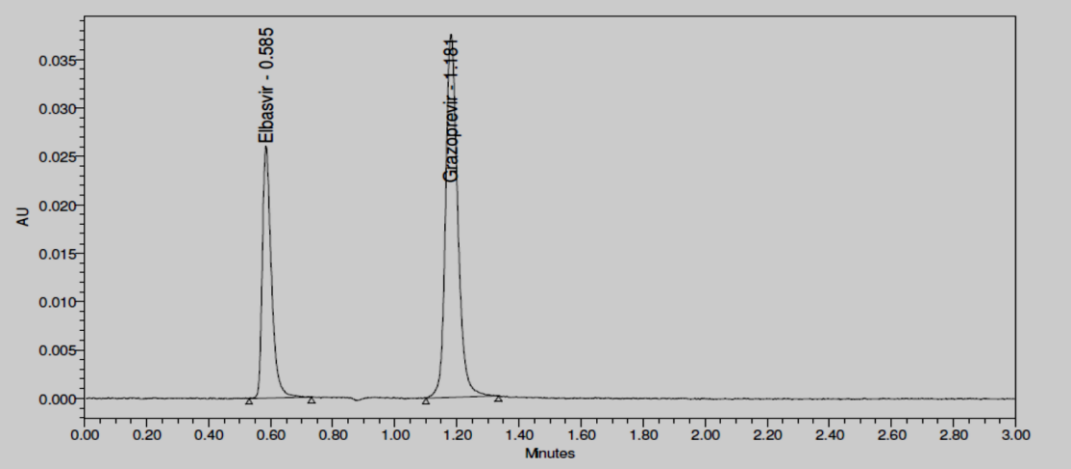

Figure 7: Chromatogram of LOQ

Table 3: Limit of Detection and Limit of Quantification data

Drug	LOD (mcg/mL)	LOQ (mcg/mL)
Elbasvir	0.050	0.150
Grazoprevir	0.030	0.080

Precision:

Repeatability (Intra-day precision) was assessed by injecting six independent drug samples and by computing the percent relative standard deviation. Intermediate precision for the analytical method was tested under the same experimental conditions by conducting same procedure on two consecutive days. The pre-defined concentration of elbasvir (20 mcg/mL) and grazoprevir (40 mcg/mL) analyte solutions were prepared. The percent CV obtained for precision experiment was below 2% which met the acceptance criteria. The repeatability and intermediate precision results of both the analytes are shown in Tables 4 and 5.

Table 4: Intra-day Precision data

S. No.	Elbasvir			Grazoprevir		
	*Area of Peak	Plate count	Peak Tailing	*Area of Peak	Plate count	Peak Tailing
1	1075536	5050	1.31	380735	4063	4063
2	1087510	5054	1.32	381267	3804	3804
3	1079258	4845	1.34	380705	3959	3959
4	1090527	4765	1.39	379293	3779	3779
5	1085000	5324	1.45	380442	3775	3775
6	1084453	5374	1.31	382317	3776	3776
Average (n=6)	1083714	-		380793	-	
SD	5473			993		
% RSD	0.5			0.3		

*= Average assay of 3 replicates injection at each time interval

Results showed lower %RSD values. This reveals that the method is quite precise

Table 5: Inter-day Precision data

Day	Elbasvir			Grazoprevir		
	Average Peak Area (n=6)	Plate count	Peak Tailing	Average Peak Area (n=6)	Plate count	Peak Tailing
Day 1	1072048	5087	1.33	364378	3841	1.20
Day 2	1084765	5123	1.27	361741	3902	1.27
Overall Average	1078406	-		363060	-	
SD	8993			1865		
% RSD	0.8			0.5		

Robustness

In some of the optimized chromatographic conditions, minor deliberate adjustments such as ratio of mobile phase, rate of flow (± 0.1 mL/min), and temperature of the column (± 2 °C) were made and examined for their effect on output with respect to the system conditions proposed. The results are summarized in Tables 6 to 8.

Table 6: Robustness data with change in flow rate

Flow rate (mL/min)		Drug	Retention time (min)	Asymmetry	Plate count	Resolution
Optimized	**Changed**					
0.3	**0.2**	Elbasvir	0.647	1.5	2293	10.1
		Grazoprevir	1.312	1.2	5013	
	0.4	Elbasvir	0.533	1.5	2087	9.7
		Grazoprevir	1.076	1.2	4599	

Table 7: Robustness data with change in mobile phase composition

Mobile Phase (Buffer-ACN v/v)		Drug	Retention time (min)	Asymmetry	Plate count	Resolution
Optimized	Changed					
50:50	45:55 v/v	Elbasvir	0.578	1.5	2132	8.5
		Grazoprevir	1.076	1.2	4494	
	55:45 v/v	Elbasvir	0.590	1.5	2219	11.5
		Grazoprevir	1.324	1.2	5073	

Table 8 Robustness data with change in column temperature

Column temperature (°C)		Drug	Retention time (min)	Asymmetry	Plate count	Resolution
Optimized	Changed					
30	28	Elbasvir	0.586	1.5	2174	10.6
		Grazoprevir	1.247	1.2	4934	
	32	Elbasvir	0.579	1.5	2134	9.2
		Grazoprevir	1.124	1.2	4638	

Accuracy:

The proposed UPLC method was evaluated for its accuracy by determining the recoveries of elbasvir and grazoprevir after adding pre-determined amounts to the sample solutions. Three solutions of sofosbuvir and velpatasvir at 50, 100 and 150% concentration levels were prepared. Three replicate injections were analyzed at all concentration levels and the chromatograms were recorded. The average percent recoveries were calculated from the peak areas. The mean percent recovery obtained was 98-102% for all the three concentration levels of both the drugs and found to be acceptable. The percent RSD were assessed and were found to be acceptable. The data is presented in Table 9.

Table 9: Accuracy data

Conc. Level	Elbasvir				Grazoprevir			
	Amount spiked (mcg/mL)	Recovery of Difference Amount (mcg/mL)	*Mean % Recovery	% RSD	Amount spiked (µg/mL)	Recovery of Difference Amount (mcg/mL)	*Mean % Recovery	% RSD
50%	10.000	9.962	99.9	0.35	20.000	20.251	100.6	0.63
		9.985				20.091		
		10.030				20.001		
100%	20.000	20.002	100.3	0.29	40.000	40.049	100.3	0.56
		20.103				39.916		
		20.105				40.352		
150%	30.000	30.188	100.2	0.70	60.000	59.664	99.6	0.17
		29.827				59.793		
		30.198				59.865		

*= Average results of 3 replicate injections

Forced Degradation Studies as per ICH[4,5]:

Elbasvir and Grazoprevir have shown degradation in acid induced degradation conditions only. The degraded peaks were not interfered with the peaks of the analytes of interest. It is clearly evident from the degradation studies, that the peaks of degradation products are not interfering with the peaks of the drugs and hence the developed stability indicating method is specific for the analysis of elbasvir and grazoprevir. The degradation results of Elbasvir and Grazoprevir in table 10.

Table 10: Degradation studies data

Nature of degradation	Stress conditions	Elbasvir		Grazoprevir	
		% Assay	% Degradation	% Assay	% Degradation
Acid	2N HCl refluxed at 60 ˚C for 30 min	95.78	4.22	96.35	3.65
Base	2N NaoH Refluxed at 60 ˚C for 30 min	98.14	1.86	98.10	1.90
Peroxide	20 % H_2O_2 at 60 ˚C for 30 min	98.54	1.46	98.05	1.95
Dry heat	Heat at 105 °C for 6 hrs	98.70	1.30	98.76	1.24
Photolytic	UV chamber at 200 Watts hours/m^2 for 7 days	98.21	1.79	99.66	0.34
Neutral	In water at 60 ˚C for 6 Hrs	99.15	0.85	99.06	0.94

CONCLUSION:

A new stability indicating RP-UPLC method was developed for the simultaneous estimation of Elbasvir and Grazoprevir in pharmaceutical dosage form and it was validated as per ICH guidelines. This method represents simple, economic, selective, and accurate with good precision and stability indicating analytical procedure for estimation of Elbasvir and Grazoprevir. Therefore, this method can be successfully applied to routine analytical purpose.

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