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Method Development and Validation for Simultaneous Estimation of Grazoprevir and Elbasvir in Bulk Drug and Formulation by Using RP-HPLC

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Abstract
The current investigation described a sensitive, selective, precise and accurate RP-HPLC method with photodiode array detector for the simultaneous estimation of antiviral drugs, grazoprevir and elbasvir. The separation and analysis were done on Sunsil C18 analytical column (250 mm x 4.6 mm, 5 µ particle size). 0.1M NaH_2PO_4: methanol [60:40 *v/v*] in isocratic elution mode was used as mobile phase. The pH of the mobile was adjusted to 4.0 with orthophosphoric acid. The elution of grazoprevir and elbasvir was accomplished with a flow rate of 1.2 ml/min. Detection was performed with photodiode array detector set at a wavelength of 260 nm. The detector response was linear in the concentration of 25-75 µg/ml for elbasvir and 50-150 µg/ml for grazoprevir. The limit of detection and limit of quantitation values were found to be 0.137 µg/ml and 0. 574 µg/ml for elbasvir and 0.290 µg/ml and 0.968 µg/ml for grazoprevir, respectively. The method was validated following international conference on harmonization guidelines. The percentage recovery for grazoprevir and elbasvir were found to be in the range of 100.08%-100.45% and 99.60%-100.06%, respectively. The %RSD values are 0.130% and 0.161% for grazoprevir and elbasvir, respectively. The results of validation parameters were found in the acceptance range. The present investigation concluded that the RP-HPLC method with photodiode array detector method was selective for simultaneous estimation of elbasvir and grazoprevir in combined dosage form.

Keyword: Elbasvir , Grazoprevir , Method Development,RP-HPLC.

INTRODUCTION
Grazoprevir is a NS3/4A protease inhibitor used against different hepatitis C virus genotype variants [1]. Grazoprevir belongs to second generation hepatitis C virus protease inhibitor [2]. By inhibiting NS3/4A protease enzyme, grazoprevir stops the conversion of viral polyprotein into its functional proteins. Elbasvir is a NS5A protein inhibitor used in the treatment of hepatitis C viral infection [3]. NS5A is a protein important for replication of virus and assembly of virion. The combination of elbasvir with grazoprevir was approved by FDA in 2016 in the treatment of chronic Hepatitis C virus genotypes 1 and 4 [4].
The combination of elbasvir with grazoprevir is not listed official in any pharmacopoeia. Only few methods are found in the literature for the quantification of elbasvir and grazoprevir either individually or in combination. Haiyan et al., [5] established an ultra performance liquid chromatography with tandem mass spectrometry method for the quantification of elbasvir in rat plasma using deuterated elbasvir as internal standard. The separation and analysis was achieved with an UPLC BEH C18 column. The mobile phase consisted of acetonitrile–water (containing 5 mM ammonium acetate with 0.01% acetic acid, pH 4.5) at a flow rate of 0.3 ml/min for 3 min in gradient elution mode. This method was applied to the pharmacokinetics study of elbasvir in rats. Haritha et al., [6] described a liquid chromatography with tandem mass spectrometry method for estimation of grazoprevir and elbasvir simultaneously in human plasma. Agilent TC-C18 (4.6 x 75 mm, 3.5 µm, 80 Å) column as stationary phase and acetonitrile: 5 mM ammonium acetate (80:20 v/v) as mobile phase was used for the analysis. Akram [7] determinate elbasvir and grazoprevir in bulk and in its pharmaceutical dosage forms using an RP-HPLC method. The separation and analysis are performed using Ineertsil ODS column (4.6 ×2 50 mm, 5
µm). Acetonitrile and phosphate buffer (pH 3) in the ratio of 40:60 (v/v) with a flow rate of 1 ml\min was used.

The methods of Haiyan et al., [5] and Haritha et al., [6] were not applied to the quantification of elbasvir and grazoprevir in bulk and pharmaceutical dosage forms. Though the RP-HPLC method of Akram [7] was applied to pharmaceutical dosage forms, this method has disadvantages such as less sensitive, less precise increased retention time of drugs. The present study was aimed to develop a cost effective, sensitive and fully validated RP-HPLC method with photodiode array detection method for the simultaneous determination of elbasvir and grazoprevir in bulk and pharmaceutical dosage forms.

EXPERIMENTAL

Mobile phase

All the solvents and chemicals are used in the preparation of mobile phase were of HPLC grade and analytical grade, respectively. 0.1 M NaH2PO4 and methanol (Merck India Ltd., Mumbai) in the ratio of 60:40 (v/v) was used as mobile phase. NaH2PO4 solution (0.1M) was prepared by dissolving 12 g of NaH2PO4 (Sd. Fine Chemicals Ltd., Mumbai) in 300 ml of double distilled water in a 1000 ml volumetric flask and made up to the volume with the same solvent. pH of the mobile phase was corrected to 4.0 with dilute orthophoshoric acid (Sd. Fine Chemicals Ltd., Mumbai). Before use, the mobile phase was filtered through millipore membrane filter and degassed for 15 min.

Instrumentation and chromatographic conditions

Waters 2695 alliance with binary HPLC pump coupled with Waters 2998 PDA detector and Waters Empower2 software was used. Sunsil C18 analytical column (250 x 4.6 mm; 5 µm particle size) was used for separation and analysis of elbasvir and grazoprevir. The temperature of the column was maintained at 25±2oC. Isocratic elution with 1.2 ml/min as flow rate was used. The injection volume was 10 µl. The eluents were detected at 260 nm.

Standard solutions

Elbasvir and grazoprevir reference standard samples were obtained from Lara drugs pvt Ltd. (Hyderabad, Telangana, India). 50 mg of elbasvir and 100 mg of grazoprevir was dissolved in 100 ml of mobile phase in a 100 ml volumetric flask to prepare the stock standard solution. Working standard solutions in the concentrations 25, 37.5, 50, 62.5 and 75 µg/ml of elbasvir and 50, 75, 100, 125 and 150 µg/ml of grazoprevir was prepared from stock solution by aptly diluting the stock solution with the mobile phase.

Calibration curve

10 µl of each of the working standard solutions was injected automatically into the column (n=3) under the chromatographic conditions described. The chromatograms and the peak area response of selected drugs were recorded. The calibration curve was constructed by plotting the mean peak area vs concentration of analyte (µg/ml). The results of each drug were subjected to regression analysis to compute the regression equation and regression coefficients.

Assay of Elbasvir and Grazoprevir Content in Tablet Dosage Form:

Zepatier tablets (labeled to contain 50 mg of elbasvir and 100 mg of grazoprevir) are used. Ten tablets were weighed, powdered and an accurate weight of the powder corresponding to 50 mg of elbasvir and 100 mg of grazoprevir was transferred to a 100 ml volumetric flask. The analytes were extracted with 30 ml of mobile phase in an ultrasonic bath for 30 min. The resulting solution was diluted to volume with the mobile phase then filtered through a membrane filter (0.45 µm pore size). One ml of tablet sample solution prepared was diluted to 10 ml with mobile phase in a 10 ml volumetric flask. The resulting tablet sample solution contains 50 µg and 100 µg of elbasvir and grazoprevir, respectively. The solution thus prepared was filtered using membrane filter and then analyzed as described in the section “calibration curve”. The content of grazoprevir and elbasvir in the tablets

were obtained either the calibration curve or regression equation.

Results and Discussion

High Performance Liquid Chromatography Method Parameters Optimization

In order to achieve good resolution, better sensitivity, good symmetric peak shape for selected drugs several trails were conducted to optimize the chromatographic method parameters (analytical column, composition of the mobile phase, pH, flow rate and analytical wavelength). 0.1 M NaH2PO4 and methanol in different ratios and with different pH were tested. The best separation was obtained on Sunsil C18 (250 x 4.6 mm; 5 µm particle size) using a 0.1 M NaH2PO4 and methanol in the ratio of 60:40 (v/v) with pH 4.0 as mobile phase pumped with a flow rate of 1.2 ml/min. The column temperature was set at 25±2°C. The maximum response of grazoprevir and elbasvir together was detected at 260 nm and the same wavelength was chosen for the analysis. Using the above described conditions, the retention times for elbasvir and grazoprevir was observed to be 2.853 min and 3.882 min respectively (Figure 1). Total run time of analysis was 6 min.

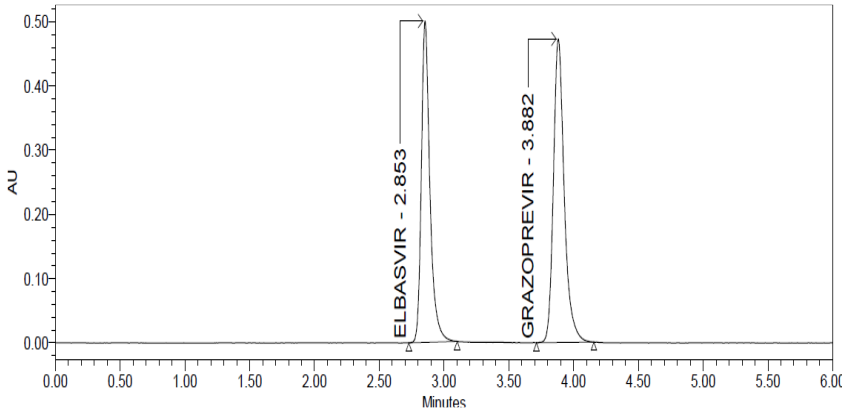

Figure 1: Chromatogram of elbasvir and grazoprevir by the developed method.

HPLC method validation

Method validation was done in accordance with ICH recommendation [8].

System suitability

Chromatographic parameters associated to the developed method must pass the system suitability limits before the analysis of sample. The relative standard deviation for peak area of drugs, relative standard deviation for retention time of drugs peak response, theoretical plates, resolution and tailing factor for elbasvir and grazoprevir peaks was evaluated using a working standard solution (50 and 100 µg/ml of elbasvir and grazoprevir, respectively). The results (Table 1) pledge the adequacy of the proposed method for routine analysis of grazoprevir and elbasvir simultaneously.

Table 1: System suitability results.

Parameters	Elbasvir	Grazoprevir	Recommended limits
Retention time	2.843 (%RSD – 0.435)	3.875 (%RSD – 0.344)	RSD ≤2
Peak area	2299022 (%RSD –	2778077 (%RSD –	RSD ≤2

	0.082)	1.008)	
USP resolution	-	7.524	> 1.5
USP plate count	9723	10709	> 2000
USP tailing factor	1.370	1.294	≤ 2

Selectivity

The selectivity study was assessed to verify the absence of interference by the components of mobile phase and tablet excipients. For this study, solutions of working standard (50 µg/ml-elbasvir; 100-µg/ml of grazoprevir), tablet sample (50 µg/ml- elbasvir; 100-µg/ml of grazoprevir), placebo blank (contains the tablet excipients and devoid of drugs) and mobile phase blank were injected into the chromatographic system. The chromatograms obtained are shown in Figure 2. The chromatogram confirmed the specificity of the method, because there were no peaks at the retention time of selected drugs in the chromatogram of mobile phase blank and placebo blank. The retention time of selected drug combination in the chromatograms of standard solution and tablet sample solution were almost same.

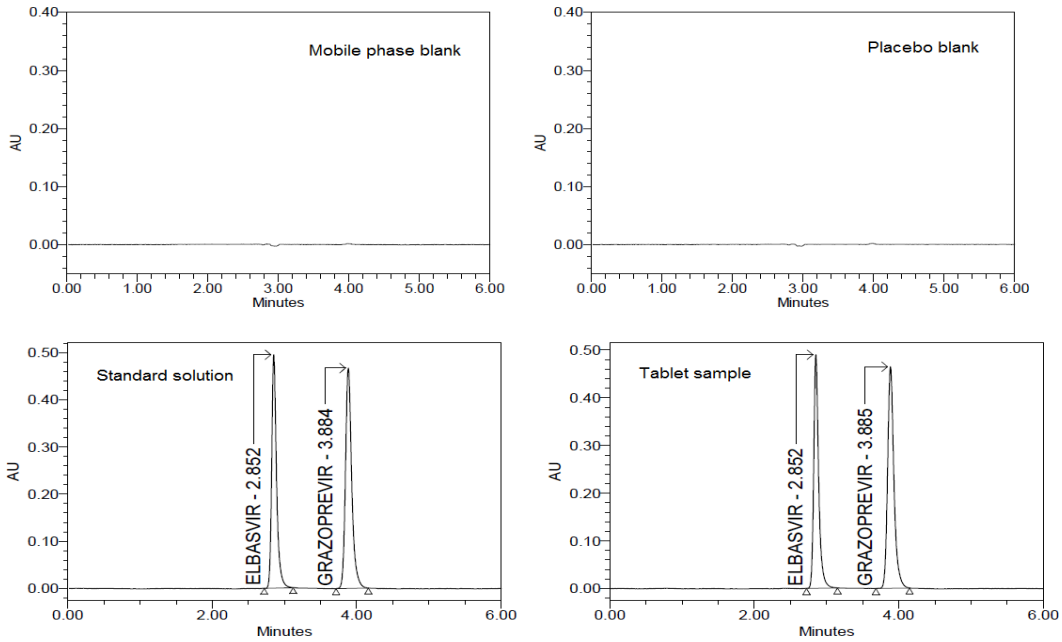

Figure 2: Chromatograms of selectivity studies.

Linearity and sensitivity (Limits of detection and quantification)

Table 2 presents the equation of the regression line, regression coefficient (R2), slope and intercept for each drug. Excellent linearity with good regression coefficient was found between the peak area and concentration. The linearity was found in the range of 25-75 µg/ml and 50-150 µg/ml for elbasvir and grazoprevir, respectively. The high R^2 value was indicative of good linearity.

The limit of detection (LOD) and limit of quantitation (LOQ) represents the sensitivity of the method. they were calculated based on the signal-to-noise ratio. LOD and LOQ were demonstrated by five injections of elbasvir and grazoprevir at concentrations of LOD and LOQ. The results presented in the Table 2 indicated the satisfactory sensitivity of the method for the assay of elbasvir and grazoprevir. The chromatograms of selected drug combination at LOD and LOQ levels are shown in Figure 3.

Table 2: Linearity and sensitivity results.

Drug	Regression equation (Y = m X + c)	Regression coefficient (R^2)	LOD (µg/ml)	LOQ (µg/ml)
Elbasvir	y = 45916x + 2322	0.9998	0.137	0.457
Grazoprevir	y = 27804x - 1757	0.9996	0.290	0.968

X = Concentration (µg/ml); Y=Area; m=slope; c=intercept.

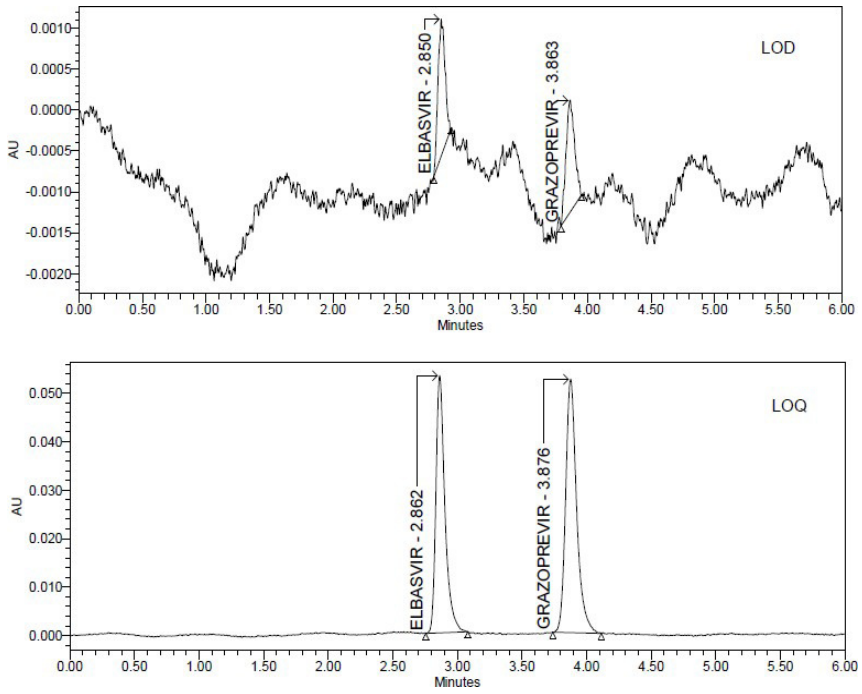

Figure 3: Chromatograms of elbasvir and grazoprevir at LOD and LOQ levels.

Precision

The precision of the method was checked by injecting elbasvir and grazoprevir standard solution 6 times at the 50 µg/ml and 100 µg/ml concentration level. The method precision was expressed as % RSD and found to be 0.161% and 0.130% for elbasvir and grazoprevir, respectively (Table 3). The low percent RSD values indicated the precision of the method.

Table 3: Method precision results.

Elbasvir			Grazoprevir		
Peak area			**Peak area**		
2299045	Mean	peak	2778843	Mean	peak
2292669	area:		2774658	area:	
2297777	2296191		2778971	2777029	
2299705			2779893		
2291795	%RSD:		2779769	%RSD:	
2299011	0.161		2771858	0.130	

Accuracy

A standard working solution containing elbasvir and grazoprevir, at concentration level 50 µg/ml and 100 µg/ml, respectively was prepared. The prepared standards were injected 6 times in the HPLC system as a test sample. From the respective peak area counts, the concentrations of elbasvir and grazoprevir were calculated using the detector responses. The accuracy represented in terms of percentage recovery is listed in Table 4. The good percent recovery values indicated the accuracy of the method.

Table 3: Method accuracy results.

Elbasvir			**Grazoprevir**		
Concentration taken (µg/ml)	**Recovery** (%)		**Concentration taken**(µg/ml)	**Recovery** (%)	
50	99.20	**Mean recovery**(%): 99.10	100	99.53	**Mean recovery**(%): 99.47
50	98.93		100	99.38	
50	99.15		100	99.53	
50	99.23		100	99.57	
50	98.89	**%RSD:** 0.150	100	99.56	**%RSD:** 0.120
50	99.20		100	99.28	

The accuracy of the proposed method was again established by recovery studies through standard addition method. For this, the preanalyzed sample solution was spiked with known concentration of elbasvir and grazoprevir at 3 diverse concentration levels (50%, 100% and 150%). The percentage recovery data presented in Table 5 show that the proposed method was accurate and the excipients present in tablets did not obstruct the assay of elbasvir and grazoprevir.

Table 5: Recovery study results.

Spiked level (%)	**Elbasvir**				**Grazoprevir**			
	Added (µg/ml)	**Found (µg/ml)**	**Recovery (%)**	**Mean (%)**	**Added (µg/ml)**	**Found (µg/ml)**	**Recovery (%)**	**Mean (%)**
	24.75	24.70	99.79		49.50	49.51	100.02	
50	24.75	24.62	99.49	99.66	49.50	49.60	100.19	100.08
	24.75	24.67	99.69		49.50	49.51	100.02	
	49.50	49.57	100.13		99.00	99.26	100.26	
100	49.50	49.41	99.81	100.01	99.00	99.56	100.57	100.42
	49.50	49.54	100.08		99.00	99.43	100.43	
	74.25	74.23	99.98		148.50	149.14	100.43	
150	74.25	74.28	100.04	100.06	148.50	149.24	100.50	100.45
	74.25	74.36	100.15		148.50	149.14	100.43	

Robustness

The method robustness was established at a concentration of 50 µg/ml (elbasavir) and 100 µg/ml (grazoprevir). To measure the method robustness, the chromatographic conditions were deliberately varied. The studied parameters were: column temperature (±2°C) and flow rate (±0.1). The system suitability parameters were determined to reveal the method robustness. The results shown in Table 6 indicated that the minute change in the chromatographic conditions did not notably affect the system suitability. Thus, the method is robust.

Table 5: Results of method robustness.

Parameter	Retention time	Peak area	USP plate count	USP Tailing	USP resolution
Elbasvir					
Flow rate – 1.1 ml/min	3.572	2841800	10645	1.44	-
Flow rate – 1.3 ml/min	2.384	1898190	9087	1.35	-
Column temperature-23°C	3.576	2855300	10744	1.39	-
Column temperature-27°C	2.387	1897586	9256	1.36	-
Grazoprevir					
Flow rate – 1.1 ml/min	4.815	3479251	11932	1.35	7.64
Flow rate – 1.3 ml/min	3.233	2272806	9903	1.24	7.14
Column temperature-23°C	4.822	3486149	11888	1.31	7.64
Column temperature-27°C	3.236	2303683	9810	1.25	7.14

CONCLUSION

The RP-HPLC photodiode array detector system with C18 reversed phase column (250 mm x 4.6 mm, 5 µm) was used in this investigation. NaH_2PO_4 (0.1M) and methanol in the ratio of 60:40 (*v*/*v*) with a flow rate of 1.2 ml/min was selected as the mobile phase. Analytical wavelength of 260 nm was used. The method validation was performed following the guidelines of the International Conference on Harmonization and the results of validation parameters were found to be within the acceptance criteria. The components of mobile phase and common tablet excipients did not interfere with the assay. Therefore, the present RP-HPLC method can be helpful for estimating the concentration of elbasvir and grazoprevir simultaneously in tablet dosage forms in quality control laboratories.

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