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Research Article

Development and Validation of new RP-HPLC Method for the Simultaneous Estimation of Elbasvir and Grazoprevir in Combined Pharmaceutical Dosage Form

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ABSTRACT

A reliable and exact technique was formulated for concurrently determining Elbasvir and Grazoprevir in tablet dosage forms. Chromatogram was developed by running a sample through Zodiac C18 column (4.6 x 150 mm, 5 μm) with the mobile phase containing Orthophosphoric acid (0.1%) and Acetonitrile in the ratio 50:50 v/v. The solution was pumped through the column at a flow rate of 1 ml/min, while maintaining the column temperature at 30°C. The optimized wavelength selected was 260 nm. The retention times for Elbasvir and Grazoprevir were determined to be 2.32 min and 3.30 min, respectively. The percentage recovery was found to be 100.16% for Elbasvir and 99.49% for Grazoprevir. The LOD and LOQ values obtained from the regression equations for Elbasvir were 0.30 mg/ml and 0.92 mg/ml, and for Grazoprevir were 0.28 mg/ml and 0.86 mg/ml, respectively. The regression equation for Elbasvir was found to be y = 2282.5x + 2407.2, and for Grazoprevir, it was y = 2366.5x + 7740.4. In conclusion, the developed method proved to be simple and economical, demonstrating successful application for the simultaneous estimation of both Elbasvir and Grazoprevir in bulk and combined tablet formulations.

Keywords: Elbasvir; Grazoprevir; RPHPLC; Validation; Simultaneous estimation

INTRODUCTION

Elbasvir (Figure 1) is an inhibitor of the Hepatitis C Virus (HCV). Combining elbasvir with other drugs that target other points of the viral life cycle and with non-overlapping resistance profiles results in increased potency and an improved barrier to resistance. Elbasvir is currently approved for use in combination with grazoprevir (as the combination product Zapatier) for the treatment of chronic hepatitis C genotypes 1 and 4.[1–4]

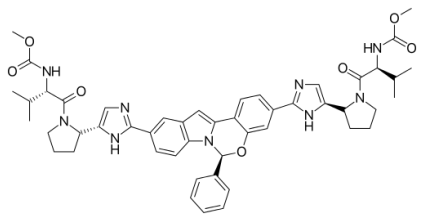

Fig. 1: Chemical structure of Elbasvir

Grazoprevir (Figure 2) is a second generation protease inhibitor approved for the treatment of hepatitis C virus (HCV) in combination with Elbasvir as the fixed-dose combination product Zepatier (FDA). Use of this medication is indicated, with or without ribavirin, for the treatment of adults with HCV genotypes 1a, 1b, or 4. NS3/4a protease is an integral part of viral replication as it is responsible for cleaving the long polypeptide produced following translation of the viral genome.

Literature

Many HPLC methods have been reported for the individual determination of Elbasvir[5] and Grazoprevir in pharmaceutical dosage forms and biological samples[6]. A few chromatographic and spectroscopic methods[7] have been reported for the simultaneous determination of Elbasvir and Gazoprevir[8–16] in combined dosage forms.

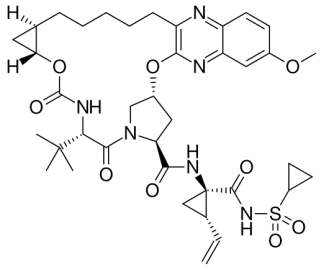

Fig. 2: Chemical structure of Grazoprevir

MATERIALS AND METHODS

Materials

The reference samples of Elbasvir and Grazoprevir were obtained from M/s. Swiss pharma private limited, Gujarat, India. The branded formulation (tablets) (Zepatier tablets containing 50 mg of Elbasvir and 100 mg of Grazoprevir) manufactured by M/s. Merck & Co., Inc. was procured from the United States of America. HPLC grade methanol, acetonitrile and analytical grade orthophosphoric acid were obtained from M/s. Rankem Chemicals Ltd, Mumbai, India. Milli-Q water dispensed through a 0.22 μ filter of the Milli-Q water purification system (Millipore, Merck KGaA, Darmstadt, Germany) was used throughout the study.

Instrumentation

The analytical method was performed by using the HPLC system Shimadzu (SPD-AT20) equipped with auto sampler, UV and PhotoDiode Array (PDA) detector, Rheodyne injector with 20 μl loop volume, analytical balance (Model AX200), pH analyser (Chemiline CL 180 based pH meter) and Toshcon Ultra Sonicator.

METHODS

Preparation of orthophosphoric acid solution (0.1%)

1 mL of Orthophosphoric acid (OPA) was transferred into a 1000 mL flask and 400 mL of Milli-Q water was added and mixed well. Then volume was made up to 1000 mL, sonicated for 5 min and then filtered through a 0.45 μ membrane filter.

Preparation of the mobile phase

A 50:50 v/v mixture of the above 0.1 % of OPA and acetonitrile was prepared and used as the mobile phase in the study.

The diluent

A 50:50 v/v mixture of water and acetonitrile was prepared and used as the diluent in the preparation of drug dilutions.

Preparation of mixed standard solution of Elbasvir and Grazoprevir and tablet solution

About 50 mg of Elbasvir and 100 mg of Grazoprevir were accurately weighed and transferred into a 50 mL clean dry volumetric flask containing 30 mL of the diluent. The solution was sonicated for 5 min and then volume was made up to the mark with a further quantity of the diluent to get a concentration of 1000 μg/mL of Elbasvir and 2000 μg/mL of Grazoprevir (Stock solution). A mixed working standard solution was prepared by further diluting the above stock solution to obtain a concentration of 100 μg/mL of Elbasvir and 200 μg/mL of Grazoprevir.

Twenty tablets from the commercial sample of 'Zepatier' were meticulously weighed and finely powdered. An accurately measured portion of the powdered sample, equivalent to the weight of one tablet (50 mg of Elbasvir and 100 mg of Grazoprevir), was transferred into a 50 mL volumetric flask containing 30 mL of the diluent. The flask contents were sonicated for approximately 10 minutes to ensure complete solubility of the drugs, and the volume was adjusted by adding more of the diluent. Subsequently, this mixture was filtered through a 0.45 μ membrane filter, and the resulting filtrate was employed for further analysis.

Method Development

Initially reverse phase liquid chromatography separation was tried to develop using various ratios of Methanol and Water, Water and Acetonitrile as mobile phases, in which the drug did not respond properly. The organic content of the mobile phase was also investigated to optimize the elution of the drug. To improve the tailing factor, the pH of mobile phase becomes an important factor. Thereafter, 0.1 % of OPA and Acetonitrile were taken in isocratic ratio 50:50 v/v and with a flow rate of 1.0 ml/min were employed. Zodiac C18 column (150 $\times$4.6 mm, 5m) was selected as the stationary phase to reduce the tailing of the peak. 260 nm was selected as the detection wavelength for PDA detector. The retention time was found to be 2.32min and 3.30min for Elbasvir and Grazoprevir respectively. The results are shown in Table 1 and Figure 3.

Table 1: Optimized chromatographic conditions for simultaneous estimation of in Elbasvir and Grazoprevir combined tablet dosage form

Column	:	Zodiac C18 column(150 mm x 4.6 mm, 5 μm)
Elution mode	:	Isocratic
Mobile phase	:	0.1%OPA:acetonitrile = 50:50 v/v
Column Temp	:	30^0 C
Wavelength	:	260 nm
Injection Volume	:	20 μL
Flow rate	:	1 mL/min
Run time	:	6 min

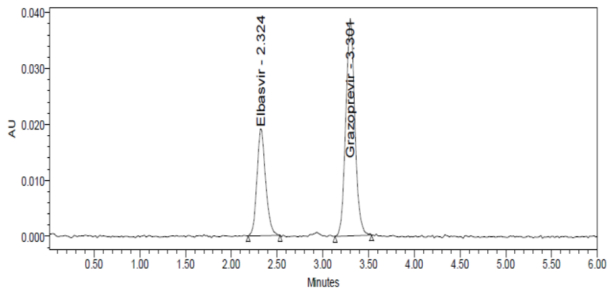

Fig. 3: Chromatogram of standard solution of Elbasvirand Grazoprevir

Method Validation

The method was validated by determining system suitability, linearity, precision, accuracy, specificity, ruggedness and robustness by analyzing Elbasvir and Grazoprevir The analytical method validation was carried out as per ICH method validation guidelines [17,18].

System Suitability

Before the validation runs, a system suitability test was conducted to assess chromatographic parameters such as retention time, number of theoretical plates, capacity factor, and asymmetry factor. The outcomes of these system suitability parameters are presented in Table 2.

Table 2: System suitability of Elbasvir and Grazoprevir

S. No.	Elbasvir			Grazoprevir		
	Area (AU)	USP Plate Count	USP tailing	Area (AU)	USP Plate Count	USP tailing
1	112377	2965	1.19	231971	5397	1.08
2	111882	2973	1.13	230048	5365	1.02
3	110239	2885	1.07	232135	5322	1.05
4	110188	2954	1.12	233299	5373	1.04
5	112502	2973	1.15	231612	5388	1.08
6	111925	2899	1.16	230739	5381	1.07
Mean	111519			231634		
Std. Dev.	1040.1			1136.1		
% RSD	**0.9**			**0.5**		

Linearity and Range

The linearity of Elbasvir and Grazoprevir were evaluated at six concentration levels by diluting the standard stock solution to give solutions of Elbasvir and Grazoprevir in the concentration range from 12.5-75 µg/mL µg/ml and 25-150 µg/mL . The regression analysis was carried out for the slope, intercept and correlation coefficient. The results were given in Tables 3 and 4 and Figures 4, 5 and 6.

Table 3: Linearity data of Elbasvir and Grazoprevir

Elbasvir		Grazoprevir	
Concentration (µg/mL)	Mean Peak area (n=3)	Concentration (µg/mL)	Mean Peak area (n=3)
12.5	30036	25	68895
25	61628	50	125996
37.5	88737	75	185768
50	114007	100	239012
62.5	143794	125	302970
75	175403	150	366192

Table 4: Regression Analysis of Calibration Curve

Parameters	Elbasvir	Grazoprevir
Slope (m)	2282.5	2366.5
Intercept (c)	2407.2	7740
Correlation coefficient (R^2)	0.9988	0.9993

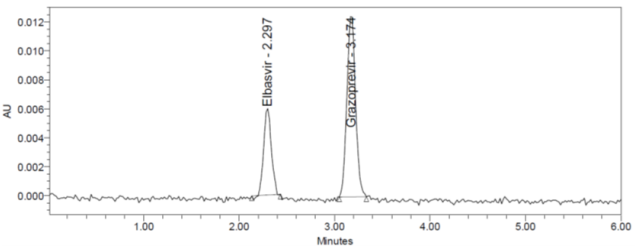

Fig. 4: Linearity Spectra of Elbasvir and Grazoprevirat 260 nm

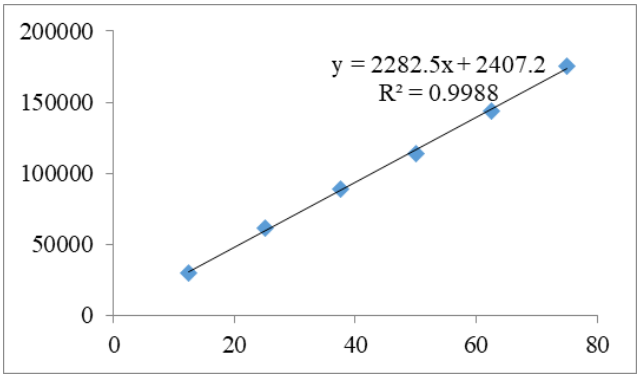

Fig. 5: Calibration curve for Elbasvir

Accuracy

To determine the accuracy of the method, the standard addition method was employed. A known quantity of the standard drug was added to a fixed amount of pre-analyzed standard solution at 50%, 100%, and 150% levels. These

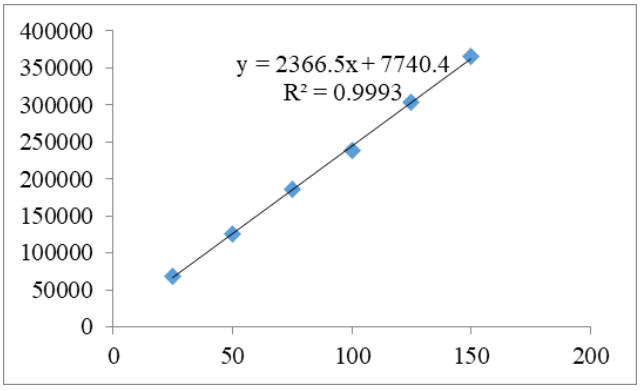

Fig. 6: Calibration curve for Grazoprevir

solutions were analyzed in triplicate using the proposed method, and the corresponding results are detailed in Table 5.

Table 5: Results of recovery experiments of Elbasvir and Grazoprevir

Preanalysed amount (μg/ml)		Spiked amount (μg/ml)		% recovered	
Elbas-vir	**Grazop-revir**	**Elbas-vir**	**Grazop-revir**	**Elbasvir**	**Grazo previr**
50	100	25	50	99.75	99.64
50	100	25	50	99.70	100.78
50	100	25	50	99.60	99.95
50	100	50	100	100.54	99.30
50	100	50	100	98.80	100.13
50	100	50	100	100.46	99.99
50	100	75	150	100.97	98.58
50	100	75	150	100.86	98.31
50	100	75	150	100.74	98.76
			MEAN	100.16	99.49
			SD	0.73	0.82
			%RSD	**0.73**	**0.82**

Precision

The assay's precision was examined for both repeatability and intermediate precision. Repeatability was derived from six replicate injections of freshly prepared test solution for Elbasvir and Grazoprevir in the equipment, with the chromatogram results documented in Table 6.

Intermediate Precision

Six replicate injections of the same dilution were analyzed on two different days by different analysts to assess precision variations. The % RSD for Elbasvir and Grazoprevir were found to be 0.9 and 0.8, respectively, well within the acceptable limit of ≤2. This suggests that the method is

Table 6: Results of repeatability of Elbasvir and Grazoprevir

S. No.	Elbasvir			Grazoprevir		
	Area	**USP Plate Count**	**USP Tailing**	**Area**	**USP Plate Count**	**USP Tailing**
1	110054	3319	1.18	232623	5679	1.11
2	111803	3320	1.16	232772	5523	1.07
3	111623	3216	1.22	232317	5517	1.08
4	110823	3125	1.18	234149	5606	1.09
5	112740	3039	1.17	234582	5260	1.08
6	110554	3073	1.19	233052	5454	1.06
MEAN	111266			233249		
SD	975.8			907.1		
% RSD	**0.9**			**0.4**		

reproducible on different days, indicating precision. Results are presented in Tables 7 and 8.

Table 7: Results of Intermediate Precision of Elbasvir

S. No.	Average area (n=6)	USP Plate Count	USP Tailing
Day 1	101580	2954	1.15
Day 2	101714	2942	1.14
Overall average	101647		
SD	887.1		
% RSD	**0.9**		

Table 8: Results of Intermediate Precision of Grazoprevir

S. No.	Average area (n=6)	USP Plate Count	USP Tailing
Day 1	223535	5365	1.04
Day 2	223733	5344	1.02
Overall average	223634		
SD	1809.0		
% RSD	**0.8**		

Robustness

For robustness assessment, slight changes in chromatographic conditions, including flow rate of the mobile phase, composition of the mobile phase, and column temperature, were made. The study revealed no significant changes in the chromatograms, indicating the robustness of the developed RP-HPLC method. Robustness study results are outlined in Tables 9 and 10.

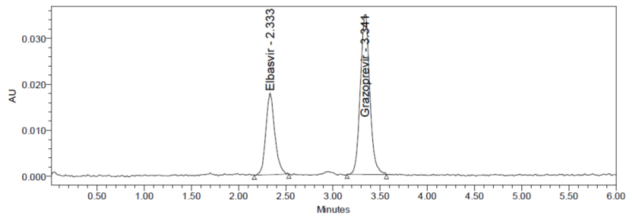

Fig. 7: Chromatogram showing separation of Elbasvir and Grazoprevir from tablet formulation

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

LOD and LOQ were determined following ICH guidelines. The values for Elbasvir were 0.30 and 0.92 μg/mL, while those for Grazoprevir were 0.28 and 0.86 μg/mL, respectively. The low LOD and LOQ values indicate the method's sensitivity.

Table 9: Robustness study for Elbasvir

Condition	Mean area	% assay	% difference
Optimised	796701	99.65	—–
Flow rate at 0.9 mL/min	792534	99.01	0.64
Flow rate at 1.1 mL/min	790125	99.85	0.20
Mobile phase: • Buffer-acetonitrile (55:45)	785981	100.06	0.41
• Buffer-acetonitrile (65:35)	745869	100.12	0.47
Column Temperature: • at 25^{0}C	789586	99.05	0.60
• at 35^{0}C	785241	99.67	0.02

Table 10: Robustness study for Grazoprevir

Condition	Mean area	% assay	% difference
Optimized	221969	99.45	—–
Flow rate at 0.9 mL/min	222845	100.35	0.9
Flow rate at 1.1 mL/min	222021	99.67	0.22
Mobile phase: • Buffer-acetonitrile (55:45)	223139	100.71	1.43
• Buffer-acetonitrile (45:55)	222685	100.09	0.64
Column Temperature: • at 25^{0}C	223413	101.01	1.56
• at 35^{0}C	222737	100.23	0.78

Analysis of Marketed Formulation by Developed Method

The validated RP-HPLC method was employed for the assay of a marketed tablet formulation containing 50 mg of Elbasvir and 100 mg of Grazoprevir. Three injections of prepared sample and standard solutions were made. The estimated values for the labeled claim of Elbasvir and Grazoprevir in Zepatier tablets were 99.38±0.5% and 99.19±0.66%, respectively. RSD values for Elbasvir and Grazoprevir are within the limit of ≤2. Results are depicted in Figure 7 and Table 11.

Table 11: Analysis of Tablet dosage form

S. No.	Drug Name	Labeled amount (mg)	Amount found (mg)	% recovery ± SD*
1	Elbasvir	50	49.30	98.59 ± 0.16
2	Grazoprevir	100	99.89	99.89 ± 0.68

* n=6 for each parameter

DISCUSSION

An Zodiac C18 column (4.6x150mm; 5μm) was selected as the stationary phase for separation of both drugs and detection was carried out at 260 nm. Initially, reverse phase liquid chromatography separation was attempted using various ratios of methanol and water and acetonitrile and water as the mobile phases, in which both the drugs were not eluted properly, and the resolution was also poor. Further, trials were also performed to optimize the organic content of mobile phase using 0.1% Orthophosphoric acid. The retention times were found to about 2.324 min and 3.301 min for Elbasvir and Grazoprevir respectively.

CONCLUSION

The findings and outcomes derived from this investigation, encompassing system suitability, linearity and range, accuracy, precision, and robustness, align comfortably with established criteria. Based on the experimental inquiries, one can infer that the suggested method is viable for the regular analysis of Elbasvir and Grazoprevir in their combined dosage form.

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