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A NOVEL STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ANTI-VIRAL CLASS OF ELBASVIR AND GRAZOPREVIR IN BULK AND PHARMACEUTICAL DOSAGE FORM

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Keywords:

Elbasvir, Grazoprevir, Method development, RP-HPLC, Validation, Degradation

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ABSTRACT: Objective: To develop accurate, precise stability indicating a method for simultaneous estimation of Elbasvir and Grazoprevir in bulk and pharmaceutical dosage form. **Materials and Methods:** Simple, rapid, precise, sensitive and reproducible validated stability-indicating Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method for the quantitative analysis of Elbasvir and Grazoprevir in the pharmaceutical dosage form. Chromatographic separation was carried out on waters Alliance-2695, by using Luna C18 (150 mm × 4.6 mm, 5 µm) column and the mobile phase containing OPA buffer (0.1%) and acetonitrile in the ratio of 50:50 v/v. The flow rate was 1.0 ml/min; detection was carried out at 258 nm using a photodiode array detector at ambient temperature. **Results:** The number of theoretical plates and tailing factor for Elbasvir and Grazoprevir were obtained to be NLT 2000 and should not more than 2 respectively. The linearity of the method was excellent over the concentration range 1.53-22.95 µg/ml and 3.05-45.75 µg/ml for Elbasvir and Grazoprevir respectively. The correlation coefficient was 0.999%. The relative standard deviation of peak areas of all measurements was less than 2.0. The proposed method was validated according to ICH guidelines. **Conclusion:** The method was found to be a simple, economical, suitable, precise, accurate and robust method for quantitative analysis of Elbasvir and Grazoprevir in combination and its stability.

INTRODUCTION: Major issue concerned to global health found worldwide nowadays is hepatitis C. More than 150 million people worldwide are infected with the hepatitis C virus (HCV), the leading cause of liver disease and liver transplantations [1], about 3 million deaths occur worldwide each year due to hepatitis C virus (HCV) related cases.

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According to the centers for disease control and prevention estimates of the people infected, Grazoprevir (an NS3/4 protease inhibitor) and Elbasvir (an NS5A inhibitor) are being developed by Merck [2]. The combination is being studied as a once-daily, single-tablet regimen, with or without ribavirin. The two drugs are active against multiple genotypes of hepatitis C [3, 4].

Elbasvir is a drug approved by the FDA for the treatment of hepatitis C. It was developed by Merck and completed phase III trials, used in combination with the NS3/4A protease inhibitor Grazoprevir [5, 6]. The IUPAC name for Elbasvir is Dimethyl N, N'- ([(6S) -6H -indolo [1, 2-c] [1, 3]

benzoxazine - 3, 10 - diyl] bis {1H – imidazole -5,2 – diyl - (2S) – pyrrolidine - 2,1 – diyl[(2S)-1-oxo -3 – methyl butane-1,2- diyl]}) bis carbamate **Fig. 1**. It has a molecular formula of $C_{49}H_{55}N_9O_7$ and a molecular weight of 882.015 g/mol [7]. Grazoprevir is a second generation hepatitis C virus protease inhibitor acting at the NS3/4A protease targets. The IUPAC name for Grazoprevir is (1R, 18R, 20R, 24S,27S)-N-{(1R,2S)-1-[(cyclo propyl sulfonyl) carbamoyl] - 2 – vinyl cyclo propyl} -7 – methoxy – 24 - (2-methyl -2- propanyl)- 22, 25 –dioxo - 2, 21 – di oxa tetra azapenta cyclo [24.2.1.03, 12.05, 10.0, 18, 20] nonacosa-3, 5, 7, 9, 11- pentaene -27-carboxamide **Fig. 2**. It has a molecular formula of $C_{38}H_{50}N_6O_9S$ and a molecular weight of 766.901 g/mol [8].

The goal of this study was to develop and validate a novel RP-HPLC method for simultaneous analysis of Elbasvir and Grazoprevir in its bulk and pharmaceutical formulations by using simple C18 column. To develop an effective method for the analysis of the drugs, preliminary tests were performed to select adequate and optimum conditions. Parameters such as detection wave-length, ideal mobile phase, and its combination, optimum pH and concentration of the standard solution were studied and optimized.

Literature survey revealed few RP-HPLC methods for quantification of Elbasvir and Grazoprevir in bulk and pharmaceutical dosage form. The present study aims to develop and validate the novel, simple, sensitive, selective, rapid and reproducible analytical method for quantitative determination of Elbasvir and Grazoprevir in bulk and pharmaceutical dosage form by RP-HPLC method [6, 7, 8, 9].

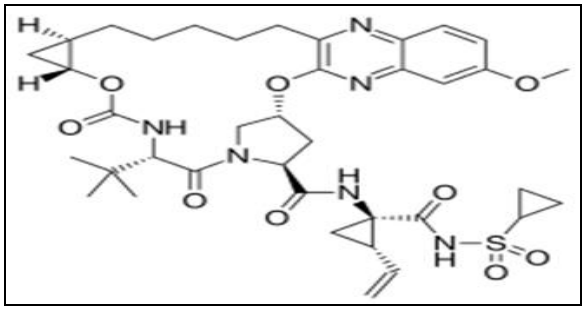

FIG. 1: STRUCTURE OF ELBASVIR

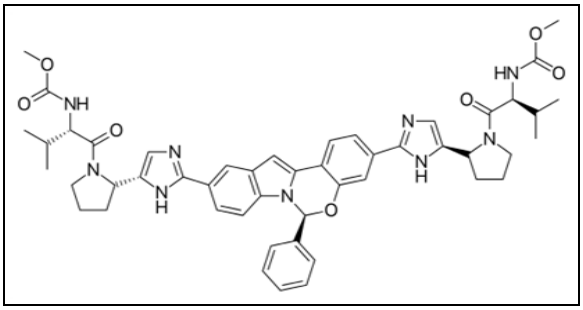

FIG. 2: STRUCTURE OF GRAZOPREVIR

MATERIALS AND METHODS:

Reagents and Chemicals: Water (HPLC grade) was used which is procured from Rankem and acetonitrile (HPLC grade), orthophosphoric acid (AR grade), sodium hydroxide (pure), hydrogen peroxide (pure) were procured from Merck Limited, 0.45 µm nylon filter was from Zodiac Life Sciences.

Instrumentation: The HPLC system consisted of Waters Alliance 2695 separations module, integrated with autosampler and equipped with waters 2998 photodiode array detector. The output of signal was monitored and integrated using waters empower 2 software.

Chromatographic Conditions: Luna phenyl-hexyl C_{18} column (250 × 4.6 mm, 5 µm) was used for separation. The mobile phase containing (0.1%) OPA and acetonitrile in the ratio of 50:50 v/v was used at a flow rate of 1.0 ml/min with detection at wavelength 258 nm. The injection volume was 10µl, and the analysis was performed at ambient temperature.

Preparation of Standard Solutions: Accurately weighed and transferred about 15.3 mg of Elbasvir working standard and 30.5 mg of Grazoprevir working standard into a 10 ml volumetric flask. Added 7 ml of diluent, sonicated to dissolve and diluted to the mark with diluent. Diluted 1 ml of the above solution to 10 ml with the diluent; further, dilute 1 ml of the above solution to 10 ml with the diluent.

Preparation of Sample Solution: Weigh 10 tablets and **an** average weight of tablet were determined. Weigh accurately about equivalent weight of 55.2 mg of sample powder into a 10 ml volumetric flask. Add 7 ml of diluent, sonicate to dissolve and dilute to volume with diluent. Dilute 1ml of the above solution to 10 ml with the diluent, further dilute 1 ml of the above solution to 10 ml

with the diluent filter through a 0.45µ nylon syringe filter.

Mobile Phase Preparation: 50 ml of 0.1% OPA was mixed with 50 ml of acetonitrile, and the mixture was used as mobile phase, and the same is used as diluent throughout the method development procedure.

Analytical Method Validation: The method was validated for accuracy, precision, linearity, limit of detection, limit of quantification and robustness as per ICH guidelines.

Linearity: The linearity of the method was determined at six levels of concentration ranging from 1.53-22.95 µg/ml of Elbasvir and 3.05-45.75 µg/ml of Grazoprevir respectively. The regression coefficient r^2 was found to be 0.999 for both the drugs.

Accuracy: Method accuracy was performed by adding known amounts of Elbasvir and Grazoprevir to the placebo solution and then comparing the added concentration with the standard concentration. Three levels of solutions were made which comprising of 50%, 100% and 150% of the nominal analytical concentration. Each level was made in triplicate **Table 1**.

The percentage of recovery in each case was calculated. The percentage recovery was found to be between 98% and 102% with the limit prescribed in ICH guidelines.

Precision: The precision for the quantification of Elbasvir and Grazoprevir by RP- HPLC is verified by repeatability (system precision and method precision).

System Precision: The system precision was performed by injecting six replicate injections of the standard solution into the chromatography. The % RSD for the peak of interest was calculated and found less than 1%.

Method Precision: The method precision performed by preparing six replicate sample preparations as per the testing procedure and injected into the chromatography. The percentage w/w of Elbasvir and Grazoprevir calculated from six replicates and % RSD.

Limit of Detection (LOD) and Limit of Quantification (LOQ): The limit of detection (LOD) and limit of quantification (LOQ) were calculated the formulae:

$$LOD = 3.3(SD) / S$$
$$LOQ = 10 (SD) / S$$

Where SD = Standard deviation of response (peak area) and S = Average of the slope of the calibration curve.

Robustness: Robustness of the method was determined by making slight changes in the composition of mobile phase ± 5%, flow rate by ± 0.2 ml. It was observed that there were no marked changes in the retention time and area of the chromatograms and the % RSD was less than 1%, which demonstrated that the developed RP-HPLC method was robust.

Forced Degradation Studies: The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing is carried out to elucidate the inherent stability characteristics of the active substance. This work aimed to perform the stress degradation studies on the Elbasvir and Grazoprevir by using the proposed method.

The degradation of a sample was prepared by the transfer the individual tablet powder was equivalent to the weight of each tablet was transferred into the 100ml flask, and it was treated under the acidic, alkaline, thermal, oxidizing and photolytic conditions. When degradation was complete, the solution was left to equilibrate to the room temp and dilute with mobile phase to furnish the solutions of a concentration equivalent to required concentrations. The specific degradative conditions are described below.

Acid Degradation Study: The acid degradation was done by the sample was treated with 3 ml of 1N hydrochloric acid and kept for 10 h at 60 ºC. After 10 h the solution was neutralized with 3 ml of 1N sodium hydroxide, made the volume up to the mark with mobile phase and analyzed using RP-HPLC.

Alkaline Degradation: The Alkaline degradation was done by the sample was treated with 3 ml of 1N sodium hydroxide and kept the sample for 10 h.

After 10 h solution was neutralized to add 3 ml of 1N hydrochloric acid, made the volume up to the mark with irrelevant media and analyzed using RP-HPLC.

Oxidative Degradation: The oxidative degradation was done by the sample was mixed with 3 ml of 30% v/v aqueous hydrogen peroxide solution and kept for 10 h. After 10 h made the volume up to the mark with mobile phase and analyzed using RP-HPLC.

Photolytic Degradation: The photolytic degradation was done by exposing drug content under the UV light for 15 min to 7 days.

Thermal Degradation: The thermal degradation is performed by exposing the solid drug at 80 °C for 15 min to 60 min and at 220 °C for 2-5 min. All of the above solutions were injected into the chromatographic system for the analysis of the drugs.

RESULTS AND DISCUSSION: Several trails have been performed, and the above mentioned optimized conditions were chosen which showed good resolution between Elbasvir and Grazoprevir is 13.86, indicates the good separation of both the compounds. The retention time of Elbasvir and Grazoprevir were 2.753 and 4.935 min, respectively. System suitability tests were carried out on freshly prepared standard solutions, and the parameters are summarized in **Table 1**.

TABLE 1: SYSTEM SUITABILITY AND VALIDATION PARAMETERS

Parameters	Elbasvir	Grazoprevir
Theoretical plates	2412	8927
Resolution	-	13.86
Tailing factor	1.45	1.21
Retention Time (min)	2.7153	4.935
Linearity range (µg/ml)	1.53-22.95	3.05-45.75
Correlation coefficient	0.999	0.9992
LOD (µg/ml)	0.3825	0.7625
LOQ (µg/ml)	0.765	1.525

* LOD-Limit of detection, LOQ-Limit of quantification

The values obtained demonstrated that the suitability of the system for analysis of these drugs in combined dosage form. Typical chromatogram showing separation of Elbasvir and Grazoprevir is given in **Fig. 3**. The calibration curves of Elbasvir and Grazoprevir were constructed and was found to be linear with a correlation coefficient of 0.999 and 0.999 which shows that good correlation exists between the area of the peak and the concentration **Fig. 4** and **5**. This method is validated for its intra-day and inter-day precision. The results obtained were in the acceptable limit **Table 1**. Robustness of the method was studied by changing the chromatographic conditions slightly, and the results were presented in **Table 2**.

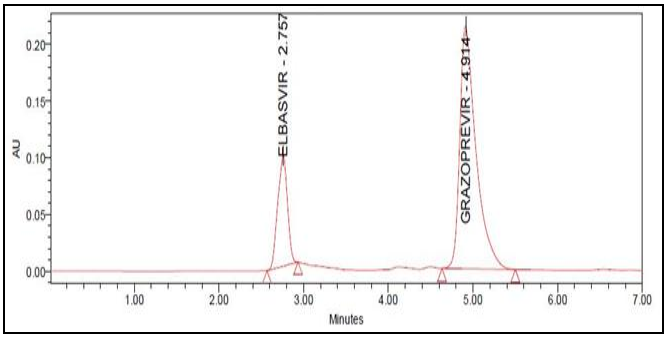

FIG. 3: TYPICAL CHROMATOGRAM SHOWING SEPARATION OF ELBASVIR AND GRAZOPREVIR IN COMBINED DOSAGE FORMULATION

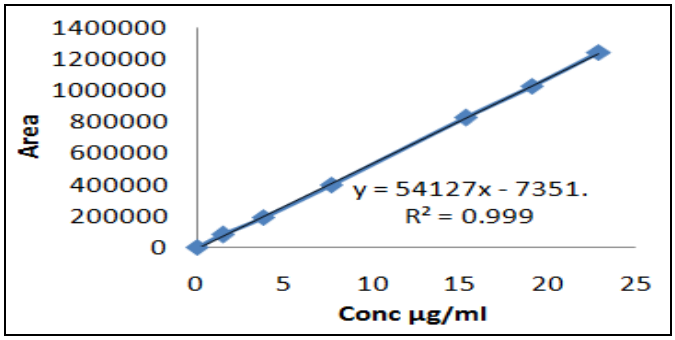

FIG. 4: CALIBRATION CURVE OF ELBASVIR

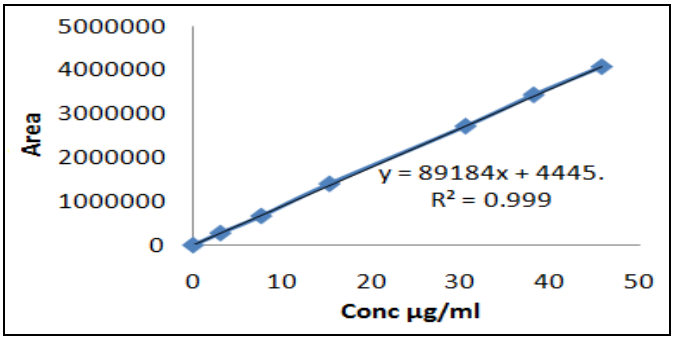

FIG. 5: CALIBRATION CURVE GRAZOPREVIR

TABLE 2: ROBUSTNESS DATA

Parameter	Actual	Changed	Elbasvir	Grazoprevir
			% RSD*	
Mobile phase	50:50 v/v	55:45	0.27	0.31
		45:55	0.35	0.44
Flow rate (ml/min)	1 ml/min	1.2	0.42	0.35
		0.8	0.45	0.47

TABLE 3: RESULTS OF THE RECOVERY STUDIES

Drug	Recovery level	Amount added (µg/ml)	Amount recovered* (µg/ml)	%Recovery ± SD*	%RSD*
Elbasvir	50%	8.08	8.14	100.7 ± 0.11	0.470
	100%	15.48	15.62	99.9 ± 0.04	0.300
	150%	24.2	24.24	100.0 ± 0.02	0.420
Grazoprevir	50%	100	100.09	100.09 ± 0.03	0.320
	100%	200	199.72	99.86 ± 0.19	0.570
	150%	300	300.86	100.28 ± 0.36	0.250

Detection limit for Elbasvir and Grazoprevir was 0.3825µg/ml, and 0.7625 µg/ml and quantitation limit were 0.765 µg/ml and 1.525 µg/ml, which suggest that a microgram quantity of both the compounds can be estimated accurately. The developed RP-HPLC method in the present study was used to quantify Elbasvir and Grazoprevir in the combined dosage form, and the results of the assay were comparable with the corresponding labeled amounts **Table 4**. High recovery values and no additional peaks, in the chromatogram, indicate that the proposed procedure is free from the interference of the commonly used excipients in the formulation. Degradation studies were performed for Elbasvir and Grazoprevir by exposing the drugs to different stress conditions where the results are given in the above **Table 5**.

TABLE 4: RESULTS OF ANALYSIS OF FORMULATION

Drug	Amount mg/tablet		% RSD	% Drug estimated
	Label claim	Amount found ± SD		
Elbasvir	50 mg	50.3 ± 0.15	0.378	100.7
Grazoprevir	100 mg	99.9 ± 0.17	0.63	99.9

*Average of six determinations, SD-Standard deviation

TABLE 5: FORCED DEGRADATION STUDY RESULTS FOR ELBASVIR AND GRAZOPREVIR

Stress condition	Elbasvir			Grazoprevir		
	Area	% Assay	% Degraded	Area	% Assay	% Degraded
Standard	786175	100.3	00	2774705	100.2	00
Acid	584395	75.3	24.7	2133836	77.6	22.4
Base	575476	77.6	22.4	2128676	76.4	23.6
Peroxide	575229	78.3	21.7	2148702	73.8	26.2
Thermal	586781	74.2	25.8	2274121	78.2	21.8
UV	581884	71.3	28.7	2144438	75.3	24.7
Water	575119	72.7	27.3	2774705	100.2	00

CONCLUSION: Very few specific and analytical methods are available according to the literature of Elbasvir and Grazoprevir the developed method gave adequate resolution between the compounds with a short analysis time. Validation was carried out according to ICH guidelines, and all the parameters were found to be within the acceptance range.

The method developed was accurate, reproducible, repeatable, linear, precise, and reliable. The results indicated the suitability of the method to study the stability of Elbasvir and Grazoprevir under various forced degradation conditions such as acid, base, dry heat, oxidation, and photolytic degradation. Based on the degradation studies even though the drugs are exposed to extreme conditions, they are stable. The method separates the drugs from their degradation products; it shows the method can be employed for analysis of stability for their tablet dosage form. These results show the method could find practical application as a quality control tool for analysis of the drug in its Tablet dosage forms in quality control laboratories.

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CONFLICT OF INTEREST: Authors declare no conflict of interest.

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