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A NEW VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF GRAZOPREVIR AND ELBASVIR IN TABLET DOSAGE FORMS

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Keywords:

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ABSTRACT: A combination of Grazoprevir and Elbasvir is used to treat Hepatitis C virus (HCV). A selective, accurate and precise RP-HPLC method was developed and validated for the simultaneous estimation of these drugs in combined tablet dosage forms. The drugs were resolved on a BDS C18 column using 0.1% orthophosphoric acid: Acetonitrile (45:55 v/v) as the mobile phase. The detection wavelength was 260 nm. The retention times obtained for Grazoprevir and Elbasvir were 2.400 & 3.018 min respectively. The linearity ranges were 25-150 & 12.5-75µg/ml respectively with Regression coefficients of 0.999. The % R.S.D. of precision studies was found to be 0.6 & 0.4 respectively. The accuracy of the proposed method was determined by recovery studies and the mean recovery was 99.14 & 100.34%, respectively. The method was also applicable for quantitative analyses of the marketed tablet formulations and in studying the stability of the drugs under acidic, alkaline, oxidation, thermal and UV conditions.

INTRODUCTION: Hepatitis C virus (HCV) infection is a significant public health concern. Globally, between 130-150 million people have chronic hepatitis C infection. Approximately 3.99 lakh people die each year due to Hepatitis C, mostly from cirrhosis and hepatocellular carcinoma [1, 2]. Hepatitis C is a liver disease caused by HCV. It is a blood-borne virus and most common modes of infection are through exposure to small quantities of blood [3]. Grazoprevir is a direct-acting antiviral medication used as part of combination therapy to treat chronic hepatitis C. It is a second-generation hepatitis C protease inhibitor acting at the NS3/4A protease targets [4].

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Grazoprevir is chemically known as (1R, 18R, 20R, 24S, 27S)- N- {(1R, 2S)-1- [(cyclopropylsulfonyl) carbamyl]- 2-vinyl(cyclopropyl)- 7-methoxy-24-(2-methyl-2-propanyl)-22,25-dioxo-2, 21-dioxa-4, 11, 23, 26-tetraazapentacyclo[24.2.1.03.12.05.1.0.0.18. 20] nonacosa-3,5,7,9,11-pentaene-27-carboxamide [5]. Elbasvir is first line therapy and classified Direct acting antiviral (DAA) and prevents viral replication in HCV genotype 1a, 1b and 4 of Hepatitis C. It is chemically known as 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-methylbutane-1, 2-diyl]})biscarbamate [6].

The literature survey shows that there are few methods for the determination of Grazoprevir and Elbasvir individually in tablet dosage form by using various analytical instruments like UV-Vis spectrophotometer [7], HPLC [8-11], RP-UPLC [12, 13] and LC-MS/MS [14].

So, the attempt has been made to develop a new validated stability-indicating RP-HPLC method for simultaneous estimation of Grazoprevir and Elbasvir in tablet dosage form as per International Conference on Harmonization (ICH) guidelines.

MATERIALS AND METHODS: The API gift samples of Grazoprevir & Elbasvir were provided by Spectrum Pharma Research solutions, Hyderabad. HPLC grade Acetonitrile, water and other chemicals obtained from the Rankem, Hyderabad. WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector and Autosampler integrated with Empower 2 Software. UV-VIS spectrophotometer T60 with special bandwidth of 2 mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Grazoprevir and Elbasvir solutions.

Preparation of Buffer: Accurately pipette 1.0 mL of OPA into clean & dried 1000 mL volumetric flask, add 900 mL of milli-Q water, stir well, Degas to sonicate and make up the volume with milli-Q water.

Preparation of Mobile Phase: It consisting of a mixture of buffer and Acetonitrile at ratio 45:55 v/v.

Preparation of Diluent: It is a mixture of Acetonitrile and milli-Q water at ratio 50:50 v/v.

Preparation of Standard Solution: Accurately weighed 10 mg of Grazoprevir (API) & 5 mg of Elbasvir (API) and transferred into a clean and dried 10 ml volumetric flask separately. Add 3/4th of diluents to both of these flasks, sonicate for 10 min and finally made up to the mark with diluent. The resultant concentrations are 1000 µg/ml of Grazoprevir and 500 µg/ml of Elbasvir.

Preparation of Standard Working Solutions (100% Solution): Pipette 1 ml from each stock solution and transferred into a clean and dried 10 ml volumetric flask and finally makeup to the mark with diluent. The resultant concentrations are 100 µg/ml of Grazoprevir and 50 µg/ml of Elbasvir.

Preparation of Sample Stock Solutions: 10 tablets are randomly selected, weighed and the average weight of each tablet is calculated, all tablets were grounded into a fine powder. The weight equivalent to 1 tablet was transferred into 100ml volumetric flask, add 60 ml diluent, sonicated for 25 min and finally makeup to the mark with diluent. All the content was passed through 0.45 µ filter paper. The resultant concentration 1000 µg/ml of Grazoprevir and 500 µg/ml of Elbasvir.

Preparation of Sample Working Solution (100% Solution): Pipette 1 ml of filtered sample stock solution, transfer it into 10 ml volumetric flask and makeup to the mark with diluent. The resultant concentrations were 100 µg/ml of Grazoprevir and 50 µg/ml of Elbasvir.

Optimized Chromatographic Method: The separation of Grazoprevir and Elbasvir was achieved on a BDS C_{18} column (150 × 4.6 mm; 5.6 µ) and eluting with a mobile phase consisting of a 45:55 v/v mixture of Buffer [0.1% orthophosphoric Acid] and Acetonitrile at a flow rate of 1.0 mL/min. The analytes were monitored at 260 nm. The injection volume was 10 µl. The total run time for elution of compound was 6 min.

Column	:	BDS C18; 150×4.6 mm; 5µ
Column temperature	:	30 °C
Flow rate	:	1 mL/min
Injection volume	:	10 µL
Detector wavelength	:	260 nm
Run time	:	6 min

Method Validation: The US Food and Drug Administration (FDA) and US Pharmacopeia (USP) both refer to ICH guidelines. The most widely applied validation characteristics are accuracy, precision, specificity, linearity, range, robustness, the limit of detection, limit of quantification, limit of detection and limit of quantification.

Accuracy: The accuracy of the method was evaluated by the standard addition method. The known amount of the reference standard was added to the known amount of standard solution at three different levels. The solutions were analyzed for mean recovery and % RSD. The studies were performed for both Grazoprevir & Elbasvir at three different levels 50%, 100%, and 150% solution. The 10 µL was injected into HPLC and % recovery and % RSD was noted as shown in **Table 1**.

TABLE 1: RECOVERY STUDIES OF GRAZOPRAVIR AND ELBASVIR

Drug	Level of spike solution	Amount present (mg/mL)	Amount added	Amount recovered	% Recovery	% RSD
Grazoprevir	50%	100	50	49.74	99.48	0.49
	50%	100	50	49.89	99.79	
	50%	100	50	50.30	100.60	
	100%	100	100	99.18	99.18	
	100%	100	100	99.61	99.61	
	100%	100	100	99.90	99.0	
	150%	100	150	148.86	99.24	
	150%	100	150	148.71	99.14	
	150%	100	150	148.63	99.09	
Elbasvir	50%	50	25	25.06	100.75	0.92
	50%	50	25	25.43	101.75	
	50%	50	25	25.0	100.04	
	100%	50	50	50.42	100.86	
	100%	50	50	49.52	99.05	
	100%	50	50	49.97	99.95	
	150%	50	75	74.54	99.39	
	150%	50	75	76.24	101.66	
	150%	50	75	75.113	100.15	

Precision: Precision is the degree of agreement among individual test results when an analytical method is used repeatedly to multiple sampling of a homogenous sample. The precision was determined as reproducibility precision and studied for method precision and inter-day precision by injecting 10 µL for six times and peak areas of replicated injections as shown in **Table 2**.

TABLE 2: METHOD PRECISION AND INTERDAY PRECISION STUDIES OF GRAZOPRAVIR AND ELBASVIR

S. no.	Injection	Method Precision		Interday Precision	
		Grazoprevir	Elbasvir	Grazoprevir	Elbasvir
1	Injection -1	2462393	1378315	2452398	1365279
2	Injection-2	2463923	1383171	2461777	1361090
3	Injection-3	2441051	1374590	2463436	1362001
4	Injection-4	2460796	1384792	2460552	1355833
5	Injection-5	2477760	1389189	2459586	1359189
6	Injection-6	2484486	1382350	2458105	1364374
	Average	2465068	1382068	2459309	1361294
	SD	15110.8	5087.8	3845.9	3468.8
	% RSD	0.6	0.4	0.2	0.3

System Suitability: It is the checking of a system to ensure system performance before or during the analysis of the unknown**.** It tests are an integral part of chromatographic method and are used to verify that the resolution & reproducibility of the system are adequate for the analysis to be performed. In this, plate count (N), tailing factor (T), resolution (Rs) and reproducibility (% RSD) are determined from replicate injection of standard. The acceptable limit of % RSD is less than 2% **Table 3**.

TABLE 3: SYSTEM SUITABILITY PARAMETERS

Drug	Retention time (min)	Area	USP Plate Count	USP Tailing
Grazoprevir	2.400	2460590	6411	1.06
Elbasvir	3.016	1385776	5138	1.45

Specificity: The ability of the method is to accurately measure the analyte response in the presence of all potential sample components. In this study, the method was evaluated by injecting 10µl of blank sample, placebo and standard solution into HPLC. As shown in **Fig. 1, 2,** and **3** respectively.

Linearity and Range: Linearity is the ability of the method to elicit test results that are directly or by a well-defined mathematical transformation to analyte concentration within a given range. The range is the interval between the upper and lower levels of analyte.

The linearity determined for Grazoprevir and Elbasvir concentration range of 25-150 µg/ml and 12.5-75 µg/ml respectively. As shown in **Table 4** and **Fig. 4** and **5**. The linearity of the method was evaluated by linear regression analysis.

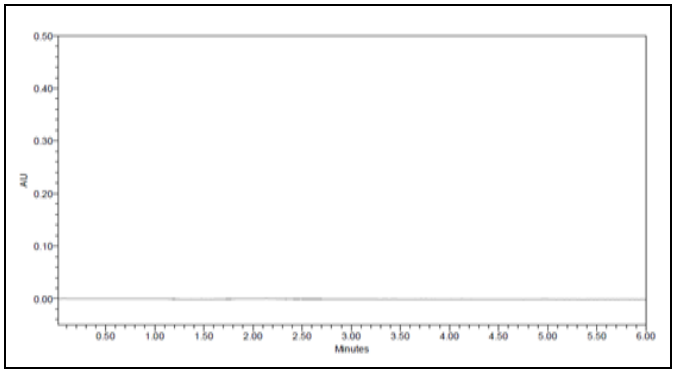

FIG. 1: TYPICAL CHROMATOGRAM OF BLANK

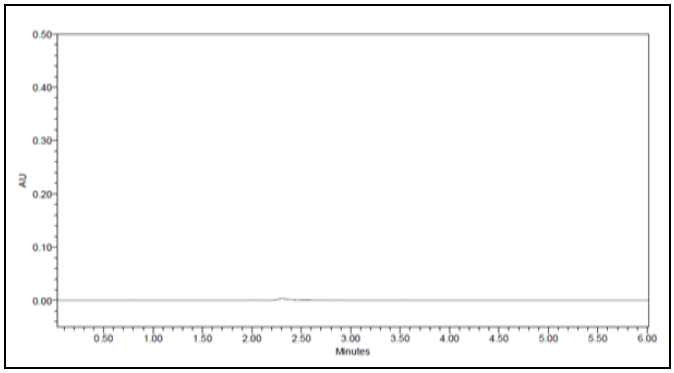

FIG. 2: TYPICAL CHROMATOGRAM OF PLACEBO

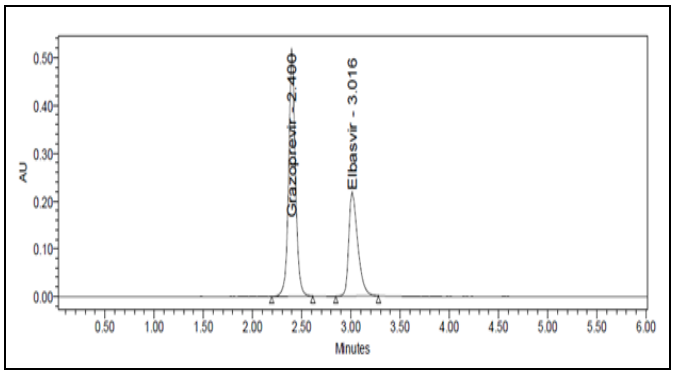

FIG. 3: STANDARD CHROMATOGRAM OF GRAZOPREVIR AND ELBASVIR

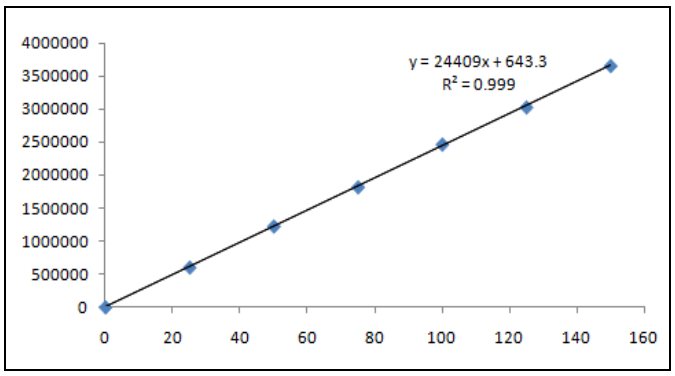

FIG. 4: LINEARITY CURVE OF GRAZOPREVIR

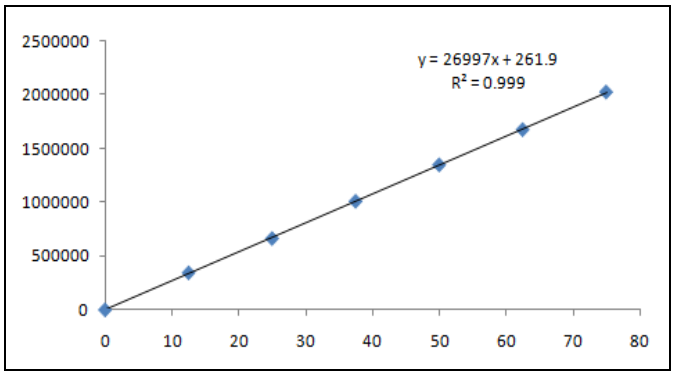

FIG. 5: LINEARITY CURVE OF ELBASVIR

TABLE 4: LINEARITY DATA OF GRAZOPREVIR AND ELBASVIR

S. no.	Grazoprevir		Elbasvir	
	Concentration (µg/mL)	Peak Area	Concentration (µg/mL)	Peak Area
1	25	604351	12.5	345798
2	50	1227773	25	665982
3	75	1821306	37.5	1012838
4	100	2470325	50	1352420
5	125	3033677	62.5	1680251
6	150	3661621	75	2031158
Correlation coefficient (R^2)		0.999	0.999	

Robustness: It is the capacity of a method to remain unaffected by small, deliberate variations in method parameters. It was indicated by changing the flow rate, mobile phase composition and temperature **Table 5**.

TABLE 5: ROBUSTNESS STUDIES OF GRAZOPREVIR AND ELBASVIR

Parameter	Change in parameter	Peak Area		SD		% RSD	
		Grazoprevir	Elbasvir	Grazoprevir	Elbasvir	Grazoprevir	Elbasvir
Flow rate	0.8mL/min	2836037	1589652	18450.7	3234.0	0.7	0.2
	1.2 mL/min	2317811	1307215	11409.0	7137.5	0.5	0.5
Mobile phase composition	2.4	2157911	1378579	13635.1	4094.0	0.6	0.3
	2.8	2122288	1336857	6824.4	10404.1	0.3	0.8
Temperature	25ºC	2602049	1458596	8733.8	4918.0	0.3	0.3
	35ºC	2142119	1365064	4259.4	3929.5	0.2	0.3

Limit of Detection (LOD) & Limit of Quantification (LOQ): LOD is the lowest concentrations of an analyte in a sample that can be detected. LOQ is the lowest concentration of an analyte in a sample that can be quantized. The LOD and LOQ of Grazoprevir and Elbasvir were determined from the standard deviation of the response and the slope **Table 6**.

TABLE 6: LOD AND LOQ OF GRAZOPREVIR AND ELBASVIR

Parameter	Grazoprevir	Elbasvir
LOD	0.03	0.07
LOQ	0.11	0.20

Assay Procedure: The assay performed by the marked product (Zepatier - 100mg/50mg of Grazoprevir & Elbasvir). The prepared sample and standard solution were injected into HPLC and peak areas were recorded. Finally, percentage amount of drugs was calculated. As shown in **Table 7**.

TABLE 7: ASSAY OF SAMPLE (TABLE DOSAGE FORM)

Drug	Label claim	Amount present (mg)	% Drug content
Grazoprevir	100	99.5	99.5
Elbasvir	50	49.3	98.6

Degradation Studies:

Oxidation: Pipette 1 ml of standard stock solution of Grazoprevir and Elbasvir into volumetric flask separately, add 1 ml of 20% hydrogen peroxide (H_2O_2), and these solutions were kept for 30 min at 60 ºC. The resultant solutions were diluted to obtain 500 µg/ml and 12.5 µg/ml solution, and 10 µl were injected into the system, and the chromatograms were recorded to assess the stability of the sample.

Acid Degradation Studies: Pipette 1ml of stock solution of Grazoprevir and Elbasvir into volumetric flask separately, add 1 ml of 2N Hydrochloric Acid and reflex for 30 min at 60 ºC. The resultant solutions were neutralized with 2N NaOH, diluted to obtain 100 µg/ml, and 50 µg/ml solution and 10 µl were injected into the system, and the chromatograms were recorded to assess the stability of the sample.

Alkali Degradation Studies: Pipette 1ml of stock solution of Grazoprevir and Elbasvir into volumetric flask separately, add 1 ml of 2N sodium hydroxide and reflex for 30 min at 60 ºC. The resultant solutions were neutralized with 2N HCl, diluted to obtain 100 µg/ml, and 50 µg/ml solution and 10 µl were injected into the system, and the chromatograms were recorded to assess the stability of the sample.

Dry Heat Degradation Studies: The standard drug solutions were placed into an oven at 105 ºC for 6hours. The resultant solutions were diluted to obtain 100 µg/ml of Grazoprevir, and 50 µg/ml of Elbasvir solution and 10µl were injected into the system, and the chromatograms were recorded to assess the stability of sample.

Photo Stability Studies: The photochemical stability of the drug was also studied by exposing the stock solutions to UV light by keeping the beaker in UV chamber for 7 days or 200-watt hours/m^2 in photo-stability chamber. The resultant solutions were diluted to obtain 100µg/ml of Grazoprevir, and 50 µg/ml of Elbasvir solution and 10 µl were injected into the system, and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies: Stress testing under neutral conditions was studied by refluxing the drug in water for 6 h at 60 ºC. The resultant solutions were diluted to obtain 100 µg/ml of Grazoprevir and 50 µg/ml of Elbasvir solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

TABLE 8: FORCED DEGRADATION STUDIES OF GRAZOPREVIR AND ELBASVIR

Forced Degradation				
Parameters		**% amount retained**	**Purity Angle**	**Purity threshold**
Grazoprevir	Acid	96.07	0.114	0.293
	Alkaline	96.27	0.106	0.291
	Oxidation	97.41	0.114	0.288
	Photo Stability	98.12	0.103	0.284
	Thermal	98.46	0.113	0.285
	Neutral	99.28	0.114	0.285
Elbasvir	Acid	97.51	0.131	0.339
	Alkaline	97.69	0.124	0.333
	Oxidation	98.10	0.128	0.329
	Photo Stability	98.34	0.114	0.323
	Thermal	99.27	0.112	0.325
	Neutral	99.35	0.115	0.318

RESULTS AND DISCUSSION: The proposed method was simple, precise and accurate for the simultaneous determination of Grazoprevir and Elbasvir in the combined tablet dosage form. The drugs were resolved on a BDS C18 column using 0.1% orthophosphoric acid buffer: Acetonitrile (45:55 v/v) as mobile phase, flow rate of 1ml/min and detection wavelength was 260 nm. The retention time for Grazoprevir and Elbasvir was found to be 2.400 and 3.016 min respectively.

The developed method was validated for accuracy, precision, linearity, robustness, LOD, and LOQ. The linearity of the method was determined by Regression analysis. A linear relationship was evaluated in the concentration range of 25-150 µg/mL of Grazoprevir and 12.5-75µg/mL of Elbasvir with correlation coefficient of 0.999 respectively. The system suitability studies and method precision were carried and %RSD was found to be less than 2%. The accuracy of the method was determined by recovery studies and mean recovery was observed to be 99.14% for Grazoprevir and 99.63% for Elbasvir. The LOD and LOQ were found to be 0.03 µg/mL & 0.11 µg/mL for Grazoprevir and 0.07 µg/mL & 0.20 µg/mL for Elbasvir. It indicates that the method was very sensitive. The robustness of the method was studied by deliberate changes in the flow rate, mobile phase composition, and temperature.

The %RSD was found to be not more than 2% and results indicate that the slight variations on the chromatographic conditions have negligible effect and confirmed that the method was highly robust. The proposed method was successfully applied to the assay of commercial formulation and showed 99.5% and 98.6% of Grazoprevir and Elbasvir respectively. The specificity of the developed method was evaluated by applying different stress conditions like acid, base, oxidation, thermal, photolytic and neutral to Grazoprevir and Elbasvir in combined dosage form.

The result obtained indicates that the purity angle was always less than the purity threshold, and it indicates the proposed method was stable.

CONCLUSION: The developed method was simple, precise, accurate and reliable for the simultaneous estimation of Grazoprevir and Elbasvir in combined dosage form and envisages the stability behavior of both the drugs as per ICH guidelines. The % RSD of all results is less than 2% that shows a high degree. Hence, the proposed method was simple, easy, cost-effective and can be used for routine analysis of Grazoprevir and Elbasvir combined dosage form.

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CONFLICTS OF INTEREST: The combination of Elbasvir and Grazoprevir used for the treatment of the chronic HCV genotype 1 and 4 in adults. Where Elbasvir is an NS5A inhibitor prevents HCV RNA replication and virion assembly and Grazoprevir is an HCV NS3/4A protease inhibitor that prevents cleavage of the polyprotein necessary for replication.

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