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R E S E A R C H A R T I C L E

RP-HPLC Method Development and Validation for the Simultaneous Estimation of Elbasvir and Grazoprevir Fixed dosage form

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A B S T R A C T

The present research work focused on development and validation for the RP-HPLC method for the simultaneous estimation of Elbasvir and Grazoprevir in bulk and fixed dosage form. Chromatographic separation was achieved on by using Eclipse XDB -C18 (150x4.6mm, 5µm particle size) column and the flow rate was maintained at 1.0 ml/min. The injection volume was 20µl. Separation was carried on gradient mode. The mobile phase A consist of 0.01M Disodium hydrogen Orthophosphate in water and the pH 3.0 was adjusted with Dilute Ortho-phosphoric acid , mobile phase B consist of Acetonitrile. UV detector wavelength monitored at 215 nm and the run time was 15 min. The retention time was found to be 7.158 min for Elbasvir and 5.313 min for Grazoprevir. The linearity was obtained in the range of 300-900 µg/ml for Elbasvir and 600-1800µg/ml for Grazoprevir. The developed method was validated statically according to ICH guidelines. The proposed method was accurate, precise, reproducible and robust; it can be employed for routine quality control analysis of pharmaceutical formulations.

Keywords: RP-HPLC, Elbasvir, Grazoprevir, Mobile phase, Validation, Retention time

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

Elbasvir-grazoprevir (Zepatier) is an oral fixed-dose combination of an NS5A replication complex inhibitor (elbasvir), and a later -generation HCV NS3/4A protease inhibitor (grazoprevir). Elbasvir (formerly MK-8742) is a small-molecule inhibitor of nonstructural protein 5A and possesses in vitro activity against most major HCV genotypes and some viral variants resistant to earlier NS5A

inhibitors. Grazoprevir (formerly MK-5172) is a macrocyclic compound that reversibly binds to the HCV NS3/4A protease, an enzyme responsible for cleaving and processing the HCV-encoded polyprotein. It is distinct from earlier-generation protease inhibitors in its potent in vitro activity against a broader array of HCV genotypes, as well activity against some of the major resistance-associated variants (R155K and D168Y) resulting from failure with a first-generation protease inhibitors[1,2]. Elbasvir is a highly potent and selective NS5A inhibitor of the hepatitis C virus NS5A replication complex. It has only been investigated as a combination product with other complementary hepatitis C antiviral drugs such as grazoprevir and MK-3682, and it is unclear whether elbasvir would show robust antiviral activity if it was administered by itself[3].

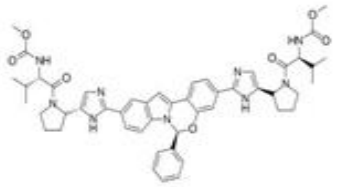

Fig 1: Chemical structure of Elbasvir

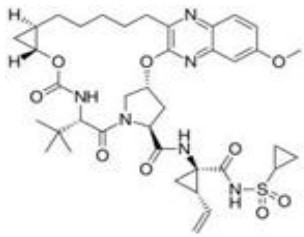

Fig 2: Chemical structure of Grazoprevir

Literature survey reveals that very few methods have been reported for the simultaneous estimation Elbasvir and Grazoprevir fixed dosage form by using RP-HPLC[4,5]. Then need to develop new method for the estimation Elbasvir and Grazoprevir. The method was developed to accordance with ICH guidelines Q $_2(R_1)$[8,9].

2. Materials and Methods

Chemicals and Reagents:

All the reagents were of analytical-reagent grade unless stated otherwise. Glass-distilled and deionized water (Nanopure, Barnstead, USA), HPLC-grade methanol, sodium hydroxide, hydrochloric acid, hydrogen peroxide and ortho-phosphoric acid (S.D.Fine chem, Mumbai, India) were used. Samples of active pharmaceutical ingredients as reference standards the active pharmaceutical ingredients were a kind of gift from M/s Mylan Laboratories India Pvt. Ltd, Hyderabad.

Instrument:

Waters Alliance 2695 separation module (Waters Corporation, Milford, USA) equipped with 2489 UV/visible detector or 2998 PDA detector with Empower 2 software was used for the analysis. The HPLC system was equipped with a column compartment with temperature control and an on-line degasser. The eluted compounds were monitored at 240 nm by using PDA detector. The column oven temperature was maintained at 30◦C. Data acquisition, analysis, and reporting were performed by Empower2 (Waters) chromatography software.

Preparation of mobile phase:

0.01M Disodium hydrogen Orthophosphate in water Adjust pH: 3.0 with Dilute Ortho-phosphoric acid as solvent-A and Acetonitrile as Solvent-B was used in gradient mode of separation. The resultant solution was thoroughly mixed and filtered through a poly-tetra-fluoro ethanol (PTFE) filter of 0.45 µm pore size using vacuum pump and degassed by sonication to expel the dissolved gases in solvent system.

Preparation of standard solution:

Transfer 1500 mg of Grazoprevir, and 750 mg of Elbasvir working standards into a 100 ml volumetric flask, dissolved and dilute with acetonitrile and water in the ratio of 40:60 (v/v) as diluent. 5 ml of the resulting solution is further diluted up to 50 ml in volumetric flask with diluents. The resulting solution contains 1500 µg/mL of Grazoprevir, and 750 µg/mL of Elbasvir as working standard solutions. The prepared stock solutions were stored at 4 0C and protected from light.

Preparation of Sample solution:

Twenty tablets of Zepatier®- containing 100 mg of Grazoprevir and 50 mg of Elbasvir were triturated in mortar and pestle to get uniform blend and free flowing powder. The contents were mixed properly to get a homogeneous powder. The resulting sample contents are measured a q uantity equivalent to 1500 mg of Grazoprevir, and 750 mg of Elbasvir working standards into a 100 ml volumetric flask, dissolve and dilute with acetonitrile and water in the ratio of 40 : 60 (v/v) as diluents and transferred in to a 100-mL volumetric flask, extracted in diluent by sonication, and filtered through Whatman no. 41 filter paper. The filtrate (5 mL) was quantitatively transferred to a 50-mL volumetric flask, and solution was diluted to volume with the diluents. The resulting solution contains 1500 µg/mL of Grazoprevir, and 750 µg/mL of Elbasvir as working test or sample solutions. The prepared stock solutions were stored at 4 0C and protected from light.

Optimized Chromatographic Conditions:

Chromatographic conditions as optimized above are shown below. These optimized conditions were followed for the determination of Grazoprevir and Elbasvir. The chromatograms of reference standard, sample and blank, and are shown in Figure

Chromatographic conditions:

Mobile phase	**:**0.01M Disodium hydrogen Orthophosphate in water Adjust pH: 3.0 with Dilute Ortho-phosphoric acid as solvent-A and Acetonitrile as Solvent-B
Elution mode	**:**Gradient elution
Column	**:**Eclipse XDB -C18 (150x4.6mm, 5µm particle size)
Flow rate	**:**1.0 ml/min
Injection volume	**:**20µl
Detector	**:**215 nm

wavelength

Column temperature	**:** Ambient
Diluent	**:**Acetonitrile and Water in the ratio of 40:60 (v/v)
Run time	**:** 15 min

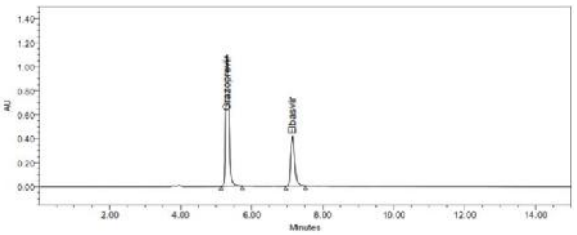

Fig 3: optimized Chromatogram for Elbasvir and Grazoprevir

3. Results and Discussion

The present study was aimed at developing a chromatographic method for separation and quantitative determination of Grazoprevir, and Elbasvir in fixed dosage form.

System suitability: The system suitability was assessed by six replicate analyses of the drugs at concentrations of resulting solution contains 1500 µg/mL of Grazoprevir, and 750 µg/mL of Elbasvir as working test or sample solutions. The acceptance criterion was ±2% for the RS2D for the peak area and retention times for all four analytes. The system suitability parameters with respect to theoretical plates, tailing factor, repeatability and resolution between Grazoprevir peak and peaks of the other two analytes were defined. The system was suitable for use if the tailing factors for Grazoprevir, Elbasvir and Elbasvir were <2.0 and the resolution was >2 and USP-theoretical plates were more than 2000. The tablet dosage form samples were analyzed under identical conditions. The quantities of Grazoprevir and Elbasvir were calculated from their respective peak areas. System suitability data is given in Table-1.

Specificity:

In specificity study, interference between drugs and tablet excipients were evaluated from the comparison of spectral purity obtained from the analysis for the standard solutions and sample solutions. The specificity of method will be demonstrated by the ability to analyze, Grazoprevir and Elbasvir as fixed dosage form in finished product sample matrix. The separate solution of blank and standard samples of three analytes were evaluated. There is no interference observed in the blank and standard solution. The results were shown in table 2.

Linearity:

Linearity of the method was evaluated at seven equispaced concentration levels by diluting the standard solutions to give solutions over the ranges 40–120% target concentration for both the analytes respectively. The calibration curves were constructed at seven concentrations between 600-1800 µg/ mL for Grazoprevir and 300-900 µg/ mL Elbasvir. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. The linearity data is reported in Table 3 and calibration graphs are shown in Figure-4.

Precision:

Precision was evaluated in terms of intra-day repeatability and inter-day reproducibility. The intra-day repeatability was investigated using six separate sample solutions prepared, as reported above; from the freshly reconstructed tablet formulations at 100% of the target level contains 1500 µg/mL of Grazoprevir and 750 µg/mL of the Elbasvir. Each solution was injected in six replicates and the peak areas obtained were used to calculate means and RSD% values. The inter-day reproducibility was, by preparing and analyzing in triplicate sample solutions from the reconstructed formulations at the same concentration level of intra-day repeatability; the mean peak areas and RSD% values were calculated from peak areas. The results were shown in table 4.

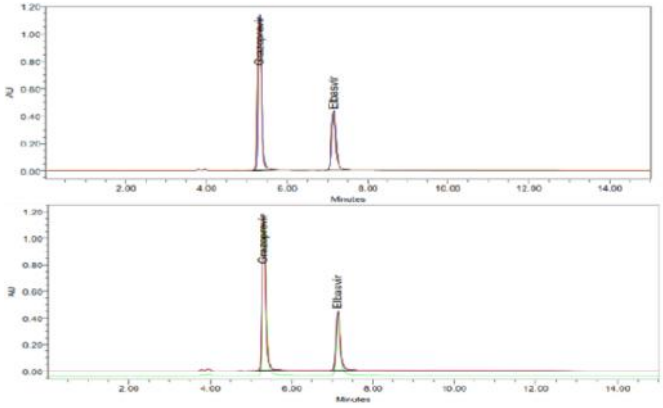

Fig 4: Precision Chromatograms (Intraday & Interday) of Grazoprevir and Elbasvir

Accuracy:

The accuracy of the method was determined by measuring the recovery of the drugs by the method of standard additions. Known amounts of each drug (10% standard drug solution composed of Grazoprevir 150 µg/mL and Elbasvir 75 µg/mL) corresponding to 80% dilution (composed of Grazoprevir 1200 µg/mL and Elbasvir 600 µg/mL), 100% dilution (composed of Grazoprevir 1500 µg/mL and Elbasvir 750 µg/mL), and 120% dilution composed of Grazoprevir 1800 µg/mL and Elbasvir 900 µg/mL) of the target test concentrations were added to a placebo mixture to determine whether the excipients present in the formulation led to positive or negative interferences. The results of accuracy studies from standard solution and process related impurity were shown in Table 5.

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

The LOQ can be determined by a signal-to-noise ratio of 10:1, or approximated by multiplying the LOD by 3.3. This method is commonly applied to analytical methods that exhibit baseline noise. Limit of detection (LOD) for Grazoprevir and Elbasvir were 0.15 µg/mL and 0.3 µg/mL respectively. Limit of quantification (LOQ) for Grazoprevir and Elbasvir were 0.45 µg/mL and 0.9 µg/mL respectively.

Robustness:

The robustness of the method was evaluated through the studies of influence of small and premeditated alteration of analytical parameters. The parameters selected were different column, and variation in mobile phase composition with organic phase solvent system. The above deliberate changes mobile phase flow rate at ± 0.2 mL/ minute. The % assay for the active pharmaceutical ingredients in robustness study were evaluated and found to be well within the acceptable criteria of98.0-110. The % RSD was found to less than 2% in case of active pharmaceutical ingredients. The results of the robustness studies are shown in Table 7 & 8.

4. Conclusion

In this study, a validated simple and reliable RP-HPLC-PDA procedure was described for the assay of a complex multidrug combination consisting of Zepatier® Tablet composed of 50 mg of Elbasvir and 100 mg of Grazoprevir which is indicated is indicated for the treatment of chronic hepatitis C virus (HCV) genotype 1 or 4 infection in adults.. All the three active ingredients were successfully resolved and quantified using Eclipse XDB -C18 150x4.6mm,5 micron in a relatively short run time of 15 minutes in isocratic mode of chromatographic method. The proposed method provides a good resolution between active ingredients. The developed method reported herein was validated by parameters as described in ICH-Q2B guideline. System suitability, specificity, linearity, LOD, LOQ values, within- and between-day precision and accuracy of the proposed technique were obtained during the validation studies. The proposed method has the advantages of simplicity, repeatability, sensitivity and requires less expensive reagents.

Table 1: System suitability data of Grazoprevir and Elbasvir

Parameter	Grazoprevir	Elbasvir
Retention time (min)	5.313	7.158
Theoretical plates	17640.80	22515.89
Tailing Factor	1.27	1.29
HETP	$1.4172x10^{-5}$	$1.110x10^{-5}$
USP plates/meter	70563.2	90063.56
Resolution	---	10.25
Peak area	7264033	3298549
% Peak Area	68.77	31.23

***** Average of six determination

Table 2: Specificity Study of Grazoprevir and Elbasvir

Sample solution	Retention time (min)	Peak area	% Peak area	RT-Ratio	USP Resolution	USP Plate count	USP Tailing
Blank	0	--------	----	---	------	---	--
Grazoprevir	5.307	29614719	99.17	0.75	8.17	12031	1.21
Elbasvir	7.148	18976974	99.20		6.74	21924	1.29

Table 3: Linearity data of Grazoprevir and Elbasvir

Target level	Grazoprevir		Elbasvir	
	Conc (µg/ml)	Peak area	Conc (µg/ml)	Peak area
40	600	2850511	300	1257978
60	900	4344054	450	1925309
80	1200	5697137	600	2523638
100	1500	7082863	750	3158726
120	1800	8539547	900	3783997
Regression equation	Y=4705x+56070		Y=4190.303x+15747.6	
Correlation coefficient	0.999		0.999	

Table 4: Precision study of Grazoprevir and Elbasvir

Injection No	Intraday precision		Interday precision	
	Grazoprevir	Elbasvir	Grazoprevir	Elbasvir
1	7251105	3195882	7298435	3212949
2	7292812	3191109	7249553	3221922
3	7256680	3186000	7284833	3223196
4	7244666	3199952	7282774	3235038
5	7280844	3184730	7258032	3211061
6	7273777	3194682	7273378	3225821
Mean	7266647.3	3192059.2	7274500.8	3221664.5

Std. dev	18767.3	5918.2	18126.9	8797.0
% RSD	0.3	0.2	0.2	0.3

Table 5: Accuracy studies of Grazoprevir and Elbasvir in Zepatier®

Accuracy level	Injection no	Grazoprevir	Elbasvir
Accuracy study at 80% target level	1	6506123	2935357
	2	6507335	2931867
	Mean	6516372	2929704
	Std.dev	6509943.3	2932309.3
	% RSD	5600.3	2852.3
	% Recovery	0.1	0.1
Accuracy study at 100% target level	1	7906619	3569363
	2	7933779	3580909
	Mean	7936288	3576000
	Std.dev	7925562.0	3575424
	% RSD	16453.0	5794.5
	% Recovery	0.2	0.2
Accuracy study at 100% target level	1	9380813	4210940
	2	9379823	4231121
	Mean	9403083	4234337
	Std.dev	**9387906**	**4225466**
	% RSD	13152.7	12682.2
	% Recovery	0.1	0.1

Table 6: LOD and LOQ studies of Grazoprevir and Elbasvir

Parameter	Grazoprevir	Elbasvir
Concentration Range (µg/mL)	600-1800	300-900
Limit of Detection(LOD)	0.3 µg/mL	0.15µg/mL
Limit of Quantification(LOQ)	0.9 µg/mL	0.45µg/mL

Table 7: Robustness study with different column of Grazoprevir and Elbasvir

Injection No	Grazoprevir	Elbasvir
1	7383989	3230989
2	7258648	3210706
3	7278860	3226397
Mean	**7307165.7**	**3222697**
Std. Dev	67294.1	10635.6
%RSD	**0.9**	**0.3**
% Assay	99.04	99.87

Table 8: Robustness study of variation in mobile phase flow rate of Grazoprevir and Elbasvir

Condition at increased flow rate	Grazoprevir	Elbasvir
1	7095844	3195875
2	6921405	3193255
3	7022003	3124501
Mean	7013084	3171210.3
Std. Deviation	87560.9	40472.7
% RSD	1.2	1.3
% Assay	99.20	99.69
Condition at decreased flow rate	Grazoprevir	Elbasvir
1	8184538	3045521
2	8128955	3058293
3	8111952	3085646
Mean	8141815	3063153
Std. Deviation	37963.4	20499.3
% RSD	0.5	0.7

% Assay	99.04	99.69

Table 9: Assay results of Grazoprevir and Elbasvir

Formulation	Label Claim (mg/tablet)		Amount found in (mg)	
Zepatier® is a fixed-dose tablet	Grazoprevir	Elbasvir	Grazoprevir	Elbasvir
	100 mg	50 mg	99 mg	49 mg

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