Research Article

Stability Indicating Validated RP-UPLC Method for Simultaneous Determination of Elbasvir and Grazoprevir in Bulk and Pharmaceutical Dosage Form

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Received: 07-03-2017; **Revised:** 12-04-2017; **Accepted:** 08-05-2017.

ABSTRACT

A stability indicating Reversed Phase-ultra Performance Liquid Chromatography (RP-UPLC) method was developed for simultaneous determination of elbasvir and grazoprevir in bulk and in combined tablet dosage form. The separation of elbasvir and grazoprevir was achieved within 5 minutes on an oligonucleotide C18 (50 mm x 2.1 mm, 1.7 µm particle size) column using phosphate buffer (pH3): Acetonitrile (30:70 v/v) as the mobile phase. Ultraviolet (UV) detection was carried out using wavelength 264 nm. The method showed adequate sensitivity concerning linearity, accuracy and precision over the range 10-50 µg/ml and 20-100 µg/ml for elbasvir and grazoprevir, respectively. Careful validation demonstrated high sensitivity, accuracy, precision, selectivity, robust and suitability for quality control laboratories. Both the drugs were subjected to acid, alkali, oxidation, thermal and photolytic degradation. The degradation products produced in all the stress conditions were well resolved from the elbasvir and grazoprevir with significant differences in their retention time values.

Keywords: Elbasvir and Grazoprevir, forced degradation, stability indicating ultra-performance liquid chromatography.

INTRODUCTION

Elbasvir is an inhibitor of the Hepatitis C Virus (HCV) and it is a highly potent and selective Non-Structural protein 5A (NS5A inhibitor) of the hepatitis C virus NS5A replication complex.[1,2] It is highly potent against most HCV genotypes tested with EC50 values in the low-picomolar range and modest potency shifts in the presence of 40% NHS. Meanwhile, it also demonstrated a favorable genotypic virologic profile.[3, 4] The chemical name of elbasvir is Dimethyl N,N'-([(6*S*)-6-phenylindolo[1,2-c][1,3]benzoxazine-3,10-diyl]bis{1H-imidazole-5,2-diyl-(2*S*)-pyrrolidine-2,1-diyl[(2*S*)-3-methyl-1-oxobutane-1,2 diyl]}) dicarbamate, it is a tetracyclic indole-based HCV NS5A inhibitor with broad genotype activity. The molecular formula $C_{49}H_{55}N_9O_7$ and has a relative molecular mass 882.02 g/mol. It is a white to off-white powder. It is practically insoluble in water, heptanes, very soluble in ethyl acetate and acetone exists as a weakly dibasic amorphous compound. It is hygroscopic, but remains amorphous after absorption and desorption of moisture. Extensive polymorph screening has not identified any non-solvated crystalline forms of elbasvir and it s exhibits stereoisomerism due to the presence of five chiral centres. Enantiomeric purity is controlled routinely by specific optical rotation.[5]

Grazoprevir is a second generation hepatitis C virus protease inhibitor acting at the NS3/4A protease targets.[7,8] It has good activity against a range of HCV genotype variants, including some that are resistant to most currently used antiviral medications.[9,10] The mechanism of action is blocks NS3, a serine protease enzyme the virus needs for splitting its polyprotein into the functional virus proteins, and NS4A, a cofactor of NS3.Grazoprevir,chemically known as (1aR, 5S, 8S, 10R, 22aR)- N-[(1R, 2S)-1-[(Cyclopropyl sulfonamide) carbonyl]-2-ethenylcyclopropyl]-14-methoxy-5-(2-methylpropan-2-yl)-3, 6-dioxo1,1a, 3, 4, 5, 6, 9, 10, 18, 19, 20, 21, 22, 22a-tetradecahydro-8H-7,10-methanocyclopropa (18, 19) (1, 10, 3, 6) dioxadiazacyclononadecino (11, 12-b) quinoxaline-8-carboxamide with molecular formula $C_{38}H_50N_6O_9S$ and molecular mass 766.90 g/mol. It is a white to off-white powder, practically insoluble in water and its slightly hygroscopic. It exhibits stereoisomerism due to the presence of seven chiral centres.[6]

Various methods of literatures involve determination of elbasvir and grazoprevir by UPLC/MS/MS[11], LC–ESI-MS/MS[12, 13]. However, no method is available for stability indicating UPLC method of elbasvir and grazoprevir in bulk drug and pharmaceutical dosage form. In the present work we have developed a new, simple, precise and stability indicating method for determination of elbasvir and grazoprevir in bulk drug and pharmaceutical dosage form in accordance with the International Conference on Harmonization (ICH) guidelines.[9]

MATERIALS AND METHODS

UPLC instrumentation and chromatographic condition

The UPLC was carried out on the waters with empower 2695 separation module, auto Sampler and photo diode array (PDA) detector were used in the analysis. Ultraviolet-visible spectrophotometer (Lab india), Balance (Afcoset ER-200A) and pH meter (Adwa – AD 1020). All the drugs and chemicals were weighed in the afcoset ER-

200A electronic balance, a pH meter (Adwa – AD 1020) and a sonicator (Frontline FS 4, Mumbai, India). The mobile phase was degassed by ultrasonic vibrations prior to use. All determinations were performed at ambient temperature.

Chromatographic conditions: [11]

Mobile Phase: phosphate buffer (p^H3): acetonitrile (30:70 v/v)

Column: oligonucleotide BEH C18 (50 mm x 2.1 mm, 1.7 µm particle size)

Flow Rate: 0.25 ml/min

Column temperature: Ambient

Injection Volume: 5 µl

Detection wavelength: 264 nm

Runtime: 5 min

Chemicals and reagents

Elbasvir and grazoprevir were obtained as gift sample from pharma treain research solutions, Hyderabad, India. Pharmaceutical tablet formulation was purchased from local pharmacy. Methanol (HPLC Grade; MERCK), Orthophosphoric acid (HPLC grade, Merck), acetonitrile (HPLC Grade; Molychem), potassium hydrogen orthophosphate (Finer chemical ltd) and HPLC grade water were used for the entire study.

Preparation of standard solutions for UPLC

Preparation of Phosphate buffer

Pipetted out 1ml of ortho phosphoric acid into a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water (adjusted to pH 3 with 0.1 M NaOH)

Preparation of Mobile phase

Mobile phase was prepared by mixing ortho phosphoric acid (pH-3) and Acetonitrile (30:70). It was filtered through 0.45µ membrane filter to remove the impurities which may interfere in the final chromatogram and it was sonicated for 15min to remove the undisolved gases.

Preparation of standard solutions elbasvir and grazoprevir Stock solution

Accurately weighed and transferred 10 mg of elbasvir and 20 mg of grazoprevir into 10 ml volumetric flasks separately, added required amount of diluents and sonicated for 5min and made up the final volume with mobile phase (stock solution). Further pipetted out 0.3 ml of the above stock solutions into a 10ml volumetric flask and diluted up to the mark with diluents to get working standard solution containing 30 µg/ml elbasvir and 60 µg/ml grazoprevir.

Preparation of sample solutions of elbasvir and grazoprevir Stock solution

Accurately weighed 10 tablets crush in mortor, transferred equivalent to 10 mg of elbasvir and 20 mg grazoprevir sample into a 10 ml clean, dry volumetric flask added about 7 ml of diluent and sonicated it up to 30 min to dissolve it completely and made volume up to the mark with the same solvent. Then it was filtered through 0.45µ membrane filter (Stock solution). Further pipetted out 0.3 ml of elbasvir and grazoprevir from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent. (30 ppm ELBA and 60 ppm of GRAZO). 0.25 µL of the standard, sample injected into the chromatographic system and measures the areas for elbasvir and grazoprevir peaks which were shown in Figure 1.

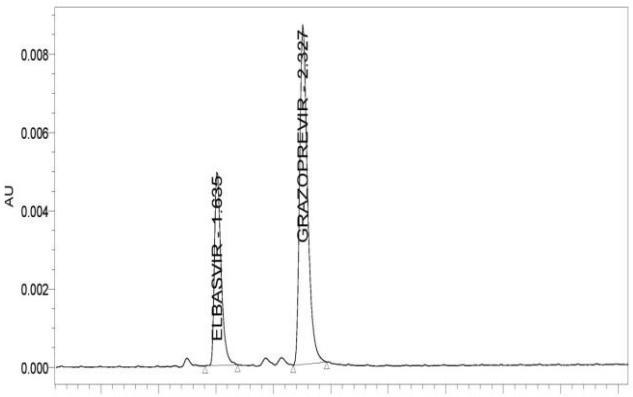

Figure 1: Standard chromatogram of elbasvir and grazoprevir

Forced degradation and stability-indicating tests[16]

Weighed accurately 10 mg of elbasvir and 20 mg of grazoprevir transferred to 10 ml volumetric flasks separately, added required amount of diluents and sonicated for 5min and made up the final volume with mobile phase to get a working standard solution containing 1000 µg/ml elbasvir and 2000 µg/ml grazoprevir. To determine acid degradation, 3 ml of 0.1 N HCl was added to 0.3 ml of stock solution, kept at 60° for about 24 h in a water bath, cooled, mixture was neutralized by the addition of 0.1 N NaOH and volume was made up to 10 ml with the mobile phase and filtered through 0.45 µ membrane filters.To determine alkali-induced degradation, 3 ml of 0.1 N NaOH was added to 0.3 ml of stock solution, heated at 60°C for 24h in a water bath, allowed to cool, the mixture was neutralized by the addition of 0.1 N HCl and volume was made up to 10 ml with the mobile phase and filtered through 0.45 µ membrane filters. To determine oxidative degradation 1 ml of 3% w/v H_2O_2 was added to 0.3 ml of stock solution, the volumetric flask was then kept at room temperature for 15 min, volume made up to 10 ml with the mobile phase and filtered through a 0.45 µ membrane filter. Thermal degradation was determined by keeping 0.3 ml of stock solution at 110°C for 3 hours, allowed to cool,

volume made up to 10 ml with the mobile phase and filtered through a 0.45µ membrane filter. Photo degradation was determined by keeping 0.3 ml of stock solution expose to sunlight for 24hrs, volume made up to 10 ml with the mobile phase and filtered through a 0.45 µ membrane filter.

RESULTS AND DISCUSSION

Method development[11, 12]

The RP-UPLC procedure was optimized to develop a stability indicating assay method that can be used for quality control analysis of elbasvir and grazoprevir simultaneously in laboratories. For method optimization, various conditions such as different columns and mobile phase mixtures were tried. For the separation of elbasvir and grazoprevir and their forced degradation products, oligonucleotide (50 x2.1 mm, 1.7µm) column maintained an ambient temperature was found to be efficient. Different mobile phase mixtures like water: methanol (50:50), phosphate buffer: methanol (30:70),0.1%Octane sulphonic acid: acetonitrile (50:50), 0.1% octane sulphonic acid: acetonitrile (55:45), phosphate buffer(p^H 4.5): acetonitrile (40:60), orthophosphoric acid (pH3): acetonitrile (50:50), orthophosphoric acid (pH4.5): acetonitrile (50:50), phosphate buffer (pH3): acetonitrile (30:70). Finally, a mobile phase composed phosphate buffer (pH3): acetonitrile in the ratio of 30:70 v/v with a flow rate of 0.25 ml/min was chosen for analysis that showed proper separation of drug peaks, good peak shape and resolution. For the detection and quantification of elbasvir and grazoprevir, 264 nm was selected as the optimum detection wavelength.

Method validation

Method validation was done in accordance with ICH recommendation.[14, 15]

System suitability

Chromatographic parameters associated with the developed method must pass the system suitability limits before the analysis of sample. The relative standard deviation of peak area, theoretical plates, resolution and tailing factor for elbasvir and grazoprevir peaks was evaluated using a solution containing 30 µg/ml of elbasvir and 60 µg/ml of grazoprevir. All the results (Table 1) assure the satisfactory of the proposed method for routine analysis of elbasvir and grazoprevir simultaneously.

Table 1: System suitability results

Parameters	Elbasvir	Grazoprevir	Recommended limits
Peak area	48537.7 (%RSD – 0.5)	98690.7 (%RSD – 1.0)	RSD ≤2
USP resolution	--	6.48	> 1.5
USP plate count	2911.87	4790.46	> 2000
USP tailing factor	1.28	1.43	≤ 2

Accuracy

Accuracy of the method was established by performing recovery studies of ICH guidelines. Spiked samples were prepared by preanalyzed sample solutions with the pure drug at three different concentration levels each in triplicate. The percentage recoveries for three replicates were calculated. According to the results revealed in Tables 2, good accuracy was observed for the proposed method and there is no interference from the often encountered tablet excipients. The recovery statistical results are within the acceptable range (% Recovery 98.0 to 102.0%) value for ELBA and GRAZO. Results are shown in table 2.

Table 2: Accuracy determination of elbasvir and grazoprevir.

ELBASVIR				
Spiked Level (%)	**Area**	**Amount added (mg)**	**Amount found (mg)**	**% recovery**
50%	24492.3	5	5.04	100.92
100%	48487.7	10	9.98	99.89
150%	73486.0	15	15.13	100.93
GRAZOPREVIR				
50%	49702.3	10	10.07	100.72
100%	99718.0	20	20.20	101.04
150%	148140	30	30.02	100.07

Precision

The method of precision and intermediate precision of the developed method was determined using the standard solution with a concentration of 30 µg/ml of elbasvir and 60 µg/ml of grazoprevir. Six injections of the solution were made in the UPLC system. Peak areas and their relative standard deviation were calculated. Small values of the relative standard deviations gave a good indication of the high precision of the proposed method. Results are shown in table 3.

Linearity

The measurement of linearity was evaluated by analyzing different concentrations of the standard solution of elbasvir and grazoprevir. For both the drugs, the Beer-lamberts law was obeyed in the concentration range 10-50 µg/ml and 20-50 µg/ml for elbasvir and grazoprevir respectively.

Table 3: Precision and Intermediate Precision results

Sample No	Elbasvir		Grazoprevir	
	Set-1	Set-2	Set-1	Set-2
1	48997	48673	98709	98783
2	48348	48720	98962	98674
3	48957	48793	98700	98647
4	48487	48657	98687	98359
5	48674	48082	98901	98747
6	48691	48956	98960	98911
Mean	48962.3	48646.8	98819.8	98686.8
Std. Dev	254.5	297.4	134.7	185.7
% RSD	0.5	0.6	0.1	0.2

The linearity of the proposed UPLC method was constructed by considering concentration (µg/ml) on X–axis and peak area on Y-axis. The regression coefficient was considered to be 0.98 over a concentration range of 10–50 µg/ml (ELBA) and 0.99 over a concentration range of 20–50 µg/ml (GRAZO). The representative linearity equation was found to be y = 1492x + 2219 (ELBA) and y = 1733x - 2487 (GRAZO) as showed in table 4. For both the drugs the % RSD was found to be within the acceptable theoretical limits of ≤ 2%, which meet the method validation acceptance criteria and hence the method was said to be linear for both the drugs.

Table 4: Linearity, LOD and LOQ data of elbasvir and grazoprevir

Parameters	Elbasvir	Grazoprevir
Linearity (µg/ml)	10-50	20-100
Regression equation (y= mx + c)	y = 1492x + 2219	y = 1733x-2487
Slope (m)	1492	1733
Intercept (c)	2219	2487
Regression coefficient (R^2)	0.989	0.999
LOD(µg/ml)	0.99	3.30
LOQ(µg/ml)	1.26	3.96

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ values determine the sensitivity of the method. Both were calculated as signal-to-noise ratio of 3:1 (LOD) and 10:1 (LOQ). The LOD and LOQ values for elbasvir and grazoprevir were calculated and are presented in Table 4.

Robustness

The robustness of the proposed method were investigated via an analysis of working standard sample under a variety of experimental conditions, such as small changes in mobile phase composition (±10 change) or changing the flow rate of the mobile phase (0.25±0.0.025 ml/min). The effect on retention time, peak area, USP plate count, USP tailing and USP resolution was studied. The results are summarized in Table 5. It was found that the method was robust when the mobile phase composition and the mobile phase flow rate were varied. Results are shown in table 5.

Table 5: Method robustness results

Parameter varied	Retention time	Peak area	USP plate count	USP Tailing	USP resolution
ELBASVIR					
Less flow rate (0.225 ml/min)	1.718	49921	3013.80	1.3	-
More flow rate (0.275 ml/min)	1.366	40784	2845.18	1.43	-
Mobile phase(10% less)	1.723	48576	2963.42	1.3	-
Mobile phase(10% more)	1.469	53765	2841.98	1.44	-
GRAZOPREVIR					
Less flow rate (0.225 ml/min)	2.573	104595	4951.17	1.46	6.64
More flow rate (0.275 ml/min)	2.008	86268	4596.34	1.42	6.34
Mobile phase(10% less)	2.565	104652	3425.02	1.34	6.64
Mobile phase(10% more)	1.946	107107	4160.01	1.42	4.04

Forced degradation studies

In forced degradation studies, the tablet sample is subjected to different stress conditions such as acidic/basic hydrolysis, oxidation, thermal and photolytic degradation. The results of degradation study are summarized in Table 6. Degradation of elbasvir and grazoprevir was seen in all the applied stress conditions. The percentage of degradation of elbasvir was more in thermal, acid and peroxide induced degradation and less in base, photolytic degradation. In case of grazoprevir

percentage of degradation was more in base, photolytic, thermal, acid and less in peroxide induced degradation. The degradation products produced due to stress did not interfere with the detection of elbasvir and grazoprevir. The proposed method can consequently be regarded as stability-indicating.

Table 6: Stress degradation studies

Type of stress	Peak area	% Assay	% Degradation
ELBASVIR			
Undegraded	48274	100.00	0.00
Acid	44953	93.12	6.88
Base	47130	97.63	2.37
Peroxide	45836	94.95	5.05
Heat	44789	92.78	7.22
Sunlight	47130	97.63	2.37
GRAZOPREVIR			
Undegraded	99926	100.00	0.00
Acid	95659	95.73	4.27
Base	92821	92.89	7.11
Peroxide	97498	97.57	2.43
Heat	93151	93.22	6.78
Sunlight	92821	92.89	7.11

CONCLUSION

A simple and sensitive stability-indicating RP-UPLC method was explored for the simultaneous determination of elbasvir and grazoprevir in pure form and in commercially available tablet dosage forms. The method was validated as per ICH guidelines. Forced degradation studies were also conducted using different stress condition as per ICH guidelines. The method proved the selectivity, precision, accuracy and mobile phase used to provide simple and economic application. The method was capable to resolve the peak of selected drugs from stress degradation products. Consequently, the stability indicates the power of the method can be assessed. Therefore, the method was found to be suitable for the routine quality control analysis of elbasvir and grazoprevir simultaneously in laboratories with no interference from the excipients or the stress degradation products.

Acknowledgements: The authors are thankful to Pharma train research solutions, Hyderabad for providing active pharmaceutical and support during work.

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Source of Support: Nil, **Conflict of Interest:** None.