International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319-6505, Impact Factor: 6.614
Available Online at www.journalijcar.org
Volume 7; Issue 6(J); June 2018; Page No. 13779-13783
DOI: http://dx.doi.org/10.24327/ijcar.2018.13783.2474

Research Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF GRAZOPREVIR AND ELBASVIR IN BULK AND PHARMACEUTICAL DOSAGE FORM

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A R T I C L E I N F O

Article History:

Received 12th March, 2018
Received in revised form 24th April, 2018 Accepted 5th May, 2018
Published online 28th June, 2018

Key words:

Grazoprevir, Elbasvir, RP-HPLC, Kromosil.

A B S T R A C T

A simple, Accurate, precise method was developed for the simultaneous estimation of the Grazoprevir and Elbasvir in Tablet dosage form. Chromatogram was run through Kromosil (250mm 4.6mm, 5µ). Mobile phase containing 0.01n Kh2po4 Buffer and Acetonitrile and methanol in the ratio of 45:55 was pumped through column at a flow rate of 1.0 ml/min. Temperature was maintained at 30°C. Optimized wavelength for Grazoprevir and Elbasvir was 260nm. Retention time of Grazoprevir and Elbasvir were found to be 2.938min and 2.100min. %RSD of the Grazoprevir and Elbasvir were and found to be 0.9and 0.5 respectively. %Recover was Obtained as 99.68% and 99.34% for Grazoprevir and Elbasvir. LOD, LOQ values were obtained from regression equations of Grazoprevir and Elbasvir were 0.26ppm, 0.78ppm and 0.18ppm, 0.55ppm respectively. Regression equation of Grazoprevir is y = 43118x+27765, and of Elbasvir is y =31499 + 35670.Retention times are decreased and that run time was decreased so the method developed was simple and economical that can be adopted in regular Quality control test in Industries

INTRODUCTION

Elbasvir is an inhibitor of the Hepatitis C Virus (HCV) Non-Structural protein 5A (NS5A), which is essential for viral RNA replication and virion assembly. Elbasvir is a compound with molecular formula C49H55N9O7 and molecular weight of 882.035 gm/mol1. It is sparingly soluble in methanol and insoluble in water.

Grazoprevir is a direct acting second generation antiviral medication used as part of combination therapy to treat chronic Hepatitis C, which is a liver disease caused by infection with Hepatitis C Virus (HCV). Grazoprevir is a compound with molecular formula C38H50N6O9S molecular weight of 766.903 gm/mol2. Grazoprevir is very slightly soluble in water, soluble in methanol and acetonitrile. Elbasvir, when used in combination with grazoprevir as the combination product Zapatier3,4, is indicated for use with or without ribavirin for the treatment of chronic hepatitis C virus (HCV) genotypes 1 or 4 infections in adults.

Literature survey revealed that there were no any official or reported methods available for the estimation of both the drugs in combination.

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The main aim of the present study is to develop an accurate, precise, sensitive, selective, reproducible and rapid analytical technique for simultaneous estimation of Grazoprevir, Elbasvir in bulk ant tablet dosage form.

MATERIALS AND METHODS

Chemicals and Reagents

Grazoprevir and Elbasvir pure drugs (API), Combination Grazoprevir and Elbasvir tablets (ZEPATIER), distilled water, acetonitrile, phosphate buffer, ammonium acetate buffer, glacial acitic acid, methanol, potassium dihydrogen phosphate buffer, tetra hydrofuran, tri ethyl amine, ortho-phosphoric acid etc.

Instruments and Chromatographic Conditions

Electronics Balance-Denver, P^H meter -BVK enterprises, India, Ultrasonicator-BVK enterprises, WATERS HPLC Aquity system equipped with quaternary pumps, UV detector and Auto sampler integrated with Empower 2 Software was used for LC peak integration and Data processing. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz cells integrated with UV-win 6 Software was used for measuring absorbance of Grazoprevir and Elbasvir solutions. The mobile phase used was 0.01N Kh2po4: Acetonitrile (45:55A) at a flow rate of 1ml/min. samples were analyzed at 260 nm detector wave

length and at an injection volume of 10 µL using Kromosil 250x4.6mm, 5µ with run time of 6 min.

Preparation of Solvents and Solution

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50.

Buffer: 0.01N Potassium dihyrogen ortho phosphate

Accurately weighed 1.36gm of Potassium dihyrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then added 1ml of Triethylamine then PH adjusted to 3.5 with dil. Orthophosphoric acid solution

Standard Preparation

Accurately Weighed and transferred 10mg of Grazoprevir and 5mg of Elbasvir working Standards into a 10ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents. 1ml from the above stock solution was taken into a 10ml volumetric flask and made up to 10ml.

Sample Preparation

1tablet was weighed ,powdered and then was transferred into a 100mL volumetric flask, 10mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 1 ml was pipeted out into a 10 ml volumetric flask and made upto 10ml with diluent.

Method Validation

As per ICH guidelines the method was validated and the parameters like Linearity, Specificity, Accuracy, Precision, Limit of Detection (LOD) and Limit of Quantitation (LOQ) were assessed.

Specificity: It is the ability of analytical method to measure the response of the analyte and have no interference from other extraneous components and well resolved peaks are obtained.

Linearity

Linearity: Linearity solutions are prepared such that 0.25, 0.5, 0.75, 1, 1.25, 1.5ml from the Stock solutions of Grazoprevir and Elbasvir are taken in to 6 different volumetric flasks and diluted to 10ml with diluents to get 25ppm, 50ppm, 75ppm, 100ppm, 125ppm, 150ppm of Grazoprevir, and 12.5ppm, 25ppm, 37.5ppm, 50ppm, 62.5ppm, 75ppm of Elbasvir.

Accuracy:

Preparation of Standard stock solutions: Accurately Weighed and transferred 10mg of Grazoprevir and 5mg of Elbasvir working Standards into a 10ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents. 1ml from the above stock solution was taken into a 10ml volumetric flask and made up to 10ml

Preparation of 50% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Robustness: Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

LOD sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of Grazoprevir, Elbasvir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

LOQ sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each of Grazoprevir, Elbasvir, and solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

System suitability parameters

The system suitability parameters were determined by preparing standard solutions of Grazoprevir (100ppm) and Elbasvir (50ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined to check whether the results complies with Recommended limits.

Assay of Grazoprevir and Elbasvir

An Accurately measured weight equivalent to (ZEPATIER) 10 mg and 5mg of Grazoprevir and Elbasvir respectively was used to perform assay by utilizing the method developed and under the optimized chromatographic conditions. Sample solutions were injected in to the HPLC system and scanned at 260 nm from which the % of drug was estimated.

RESULTS & DISCUSSIONS

Optimization of Chromatographic Conditions

To develop and establish a suitable RP-HPLC method for simultaneous estimation of Grazoprevir and Elbasvir in bulk

Table 1 Optimized Chromatographic Conditions

Parameter	Condition
RP-HPLC	WATERS HPLC SYSTEM equipped with quaternary pumps with PDA detector
Mobile phase	50% OPA (0.1%) : 50% Acetonitrile
Flow rate	1ml/min
Column	Kromosil 250x4.6mm, 5µ
Detector wave length	260nm
Column temperature	30°C
Injection volume	10µL
Run time	6 min

Diluent	Water and Acetonitrile in the ratio 50:50
Retention Time	Grazoprevir 2.938 min and Elbasvir 2.100min
Theoretical Plates	Grazoprevir 3820 and Elbasvir 2941

and Tablet dosage forms, different preliminary tests were performed and different chromatographic conditions were tested and optimized chromatographic conditions were developed which were given in Table-1. The final analysis was performed by using 50% Ortho phosphoric acid:50% Acetonitrile at a flow rate of 1ml/min. samples were analyzed at 220 nm detector wave length and at an injection volume of 10 µL using BDS C18 4.6 x 150mm, 5µm.with run time of 6 min. The proposed method was optimized to give sharp peak with good resolution and minimum tailing effect for Grazoprevir and Elbasvir, the optimized chromatogram was obtained as shown in (Figure-3).

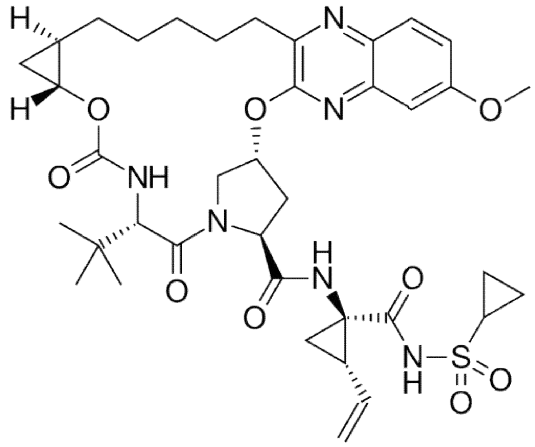

Figure 1 Chemical Structure of Grazoprevir

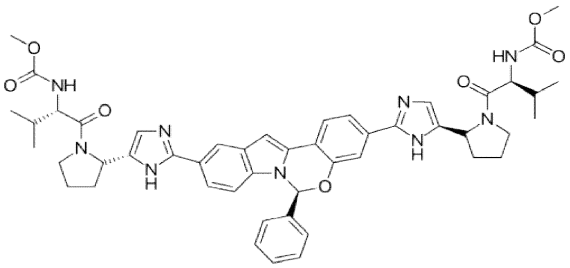

Figure 2 Chemical Structure of Elbasvir

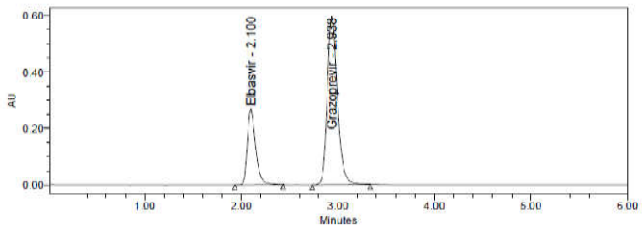

Figure 3 Optimized Chromatogram of Grazoprevir and Elbasvir

Chromatographic conditions

Mobile phase : 0.01N Kh2po4: Acetonitrile (45:55A)
Flow rate : 1 ml/min
Column : Kromosil 250x4.6mm, 5µ
Detector wave length : 260nm
Column temperature : 30°C
Injection volume : 10µL
Run time : 6min
Diluent : Water and Acetonitrile in the ratio 50:50

Validation

Linearity was established for Grazoprevir (25-150µg/ml) and Elbasvir (12.5-75 µg/ml) at six different concentrations each were injected in a duplicates and average areas were determined and linearity equations were obtained as y = 43118.x + 27765 for Grazoprevir and y = 31499.x + 35670 for Elbasvir, Correlation coefficient (R^2) was determined as 0.999 for the two drugs. The Linearity calibration curves were plotted as shown in (Figure-4&5) for Grazoprevir and Elbasvir, respectively. Retention times of Grazoprevir and Elbasvir were 2.938min and 2.100 min respectively. Where no interfering peaks in blank and placebo at retention times of these drugs were not found in this method.

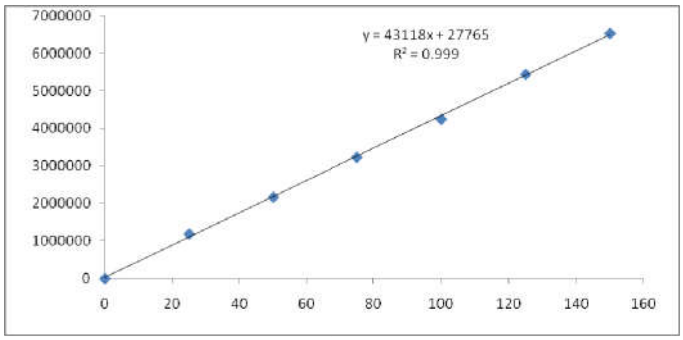

Figure 4 Linearity Curve of Grazoprevir

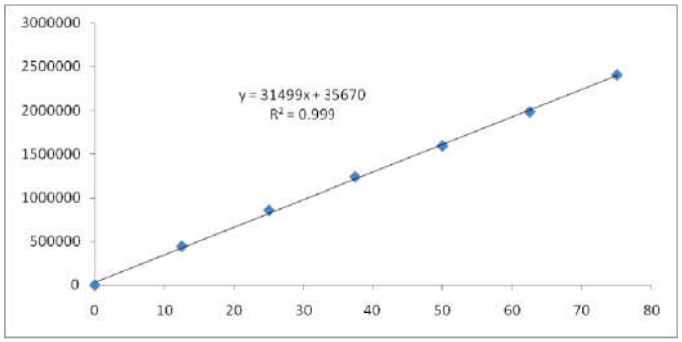

Figure 5 Calibration Curve of Elbasvir

So this method holds its specificity. Three levels of Accuracy samples 50%, 100%, 150% were prepared and Triplicates of injections were given for each level of accuracy and mean % Recovery was obtained as 99.68% and 99.34% for Grazoprevir and Elbasvir respectively were shown in (Table-2).% RSD was calculated from the corresponding peaks obtained by injecting six times a known concentration of Grazoprevir and Elbasvir the repeatability was obtained as 0.5% and 0.6% respectively for Grazoprevir and Elbasvir and the % RSD for intermediate Precision was obtained as 1.0%, 1.3% for Grazoprevir and Elbasvir, Low % RSD values indicates that the method developed was precise as shown in (Table-3).

Table 2 Accuracy results of Grazoprevir and Elbasvir

Sample	Amount added (µg/ml)	Recovery (%)	% RSD
Grazoprevir	50	100.67	0.41
	100	99.74	1.18
	150	98.62	0.54
Elbasvir	25	100.54	0.20
	50	98.61	0.80
	75	98.86	0.75

Table 3 Precision Results of Grazoprevir and Elbasvir

S.No	Repeatability		Intermediate precision	
	Area of Grazoprevir	Area of Elbasvir	Area of Grazoprevir	Area of Elbasvir
1.	4271994	1576476	4418306	1541949
2.	4276767	1566637	4462894	1552437
3.	4232383	1566824	4542584	1589898
4.	4297430	1563224	4446796	1544228
5.	4267353	1578781	4423559	1533359
6.	4271590	1587778	4456254	1548121
Mean	4269586	1573287	4458399	1551665
S.D	21098.4	9355.2	44872.8	19797.7
%RSD	0.5	0.6	1.0	1.3

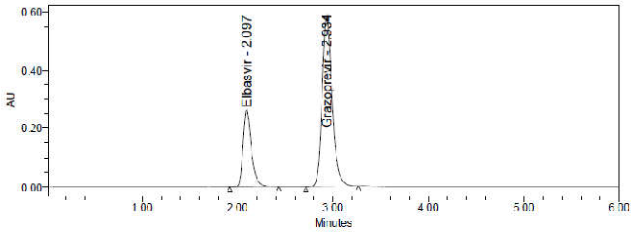

Figure 6 Standard Chromatogram of Grazoprevir and Elbasvir

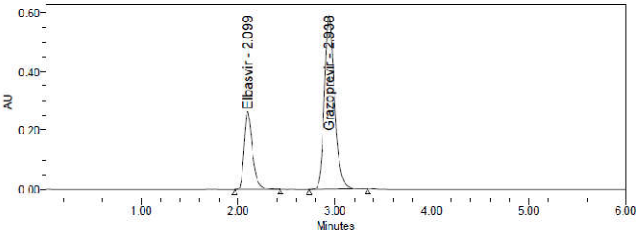

Figure 7 A Sample Chromatogram of Grazoprevir and Elbasvir in Pharmaceutical Dosage Form

The LOD and LOQ values were evaluated based on Relative standard deviation of response and slope of the calibration curve Grazoprevir and Elbasvir. The detection limit values were obtained as 0.26 and 0.18 and Quantitation limit were fund to be 0.78 and 0.55 for Grazoprevir and Elbasvir Respectively as given in (Table-4).

Table 4 LOD and LOQ values of Grazoprevir and Elbasvir

Molecule	LOD	LOQ
Grazoprevir	0.26	0.78
Elbasvir	0.18	0.55

Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus (65:35) mobile phase plus (55:45) temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner Table-5).

Table 5 Robustness Data of Grazoprevir and Elbasvir

S.no.	Condition	%RSD of Grazoprevir	%RSD of Elbasvir
1	Flow rate (-) 0.9ml/min	0.2	0.6
2	Flow rate (+) 1.1 ml/min	0.9	0.9
3	Mobile phase (-) 60B:40A	0.3	0.5
4	Mobile phase (+) 50B:50A	0.1	0.2
5	Temperature (-) 25°C	1.6	1.6
6	Temperature (+) 35°C	0.9	1.0

System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit (Table -6). Grazoprevir and Elbasvir pure drugs (API) were obtained from spectrum Pharma research solutions and Asian Med Enterprises (Zepatir), bearing the label claim Grazoprevir 10mg, Elbasvir 5mg. Assay was performed with the above formulation.

Table 6 System Suitability Parameters Results of Grazoprevir and Elbasvir

S no	Grazoprevir			Elbasvir			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	Resolution
1	2.802	5623	1.33	2.257	2612	0.98	3.2
2	2.803	5703	1.33	2.261	2701	0.96	3.1
3	2.803	5261	1.37	2.266	2708	0.96	3.0
4	2.804	4997	1.39	2.270	2570	0.95	3.0
5	2.804	5232	1.32	2.272	2506	1.03	3.0
6	2.805	5717	1.37	2.275	2477	1.00	3.1

Average % Assay for Grazoprevir and Elbasvir obtained was 99.23% and 98.87% respectively the results were shown in (Table-7) and the chromatograms for Grazoprevir and Elbasvir standard drugs and pharmaceutical dosage forms were shown in (Figure-6, 7) Respectively.

Table 7 Assay Results of Grazoprevir and Elbasvir

S. No.	Grazoprevir %Assay	Elbasvir %Assay
1	99.28	99.07
2	99.39	98.45
3	98.36	98.46
4	99.88	98.23
5	99.18	99.21
6	99.27	99.78
AVG	99.23	98.87
STDEV	0.49	0.59
%RSD	0.49	0.59

Degradation Studies: Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation (Table 8&9).

Table 8 Degradation Data of Grazoprevir & Elbasvir

S.NO	Degradation Condition	%Drug Degraded	
		Grazoprevir	Elbasvir
1	Acid	4.85	4.46
2	Alkali	2.59	2.57
3	Oxidation	1.57	1.92
4	Thermal	0.85	0.70
5	UV	0.74	0.59
6	Water	0.85	0.82

CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Grazoprevir and Elbasvir in Tablet dosage form. Retention time of Grazoprevir and Elbasvir were found to be 2.938min and 2.100min. %RSD of the Grazoprevir and Elbasvir were and found to be 0.5and 0.6 respectively. % Recover was Obtained as 99.68% and 99.34% for Grazoprevir and Elbasvir. LOD, LOQ values were obtained from regression equations of Grazoprevir and Elbasvir were 0.26ppm, 0.78ppm and 0.18ppm, 0.55ppm respectively. Regression equation of Grazoprevir is y = 43118x+27765, and of Elbasvir is y =31499 + 35670. Retention times are decreased and that run time was decreased so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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How to cite this article:

Padmavathi.Sakinala *et al* (2018) 'Rp-Hplc Method Development and Validation for the Simultaneous Estimation of Grazoprevir and Elbasvir in Bulk And Pharmaceutical Dosage Form', *International Journal of Current Advanced Research,* 07(6), pp. 13779-13783. DOI: http://dx.doi.org/10.24327/ijcar.2018.13783.2474

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