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Analytical Method Development and Validation for Elbasvir and Grazoprevir in Combine Phaarmaceutical Dosage forms by RP-HPLC

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A B S T R A C T

HPLC is at present one of the most sophisticated tool of the analysis. The estimation of elbasvir and grazoprevir was done by RP-HPLC. The Phosphate buffer was p^H 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30 % v/ v. InertsilC18(ODS)column (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 1.0 ml/min. the linearity range of elbasvir and grazoprevir were found to be from 100-500 µg/ml of elbasvirand 1-5µg/ml of grazoprevir. Linear regression coefficient was not more than 0.999.The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of elbasvir and grazoprevir. LOD and LOQ were found to be within limit.

Keywords: Elbasvir and Grazoprevir, RP-HPLC

A R T I C L E I N F O

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1. Introduction

Elbasvir, diethyl *N, N'*-([(6S)-6*H*-indolo[1, 2-c][1, 3] benzoxazine-3, 10-diyl] bis{1*H*-imidazole-5,2-diyl-(2*S*)-pyrrolidine-2,1-diyl[(2*S*)-1-oxo-3-methylbutane-1, 2-diyl]}) biscarbamate, it is a highly potent and selective NS5A inhibitor of the hepatitis C virus NS5A replication complex. [3] 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. Nevertheless, combination products of this type represent the most successful approach yet developed for actually curing hepatitis C, rather than merely slowing the progression of the disease [4].

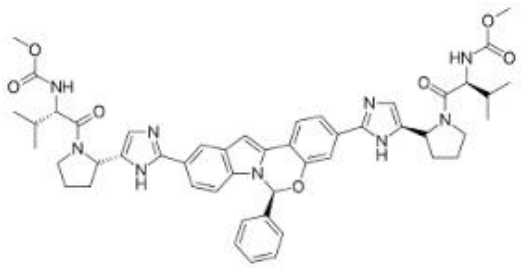

Figure 1: Structure of Elbasvir

Grazoprevir (MK-5172), 1*R*,18*R*,20*R*,24*S*,27*S*)-*N*-{(1*R*,2*S*)-1-[(cyclopropylsulfonyl)carbamoyl]-2-vinylcyclopropyl}-7-methoxy-24-(2-methyl-2-propanyl)-22, 25-dioxo-2, 21-dioxa-4,11,23,26-tetraazapentacyclo[24.2.1.03,12.05,10.0 18,20] nonacosa-3,5,7,9,11-pentaene-27-carboxamide, it is a drug [1] approved for the treatment of hepatitis C. It was developed by Merck and completed Phase III trials, used in combination with the NS5A replication complex inhibitor elbasvir under the trade name Zepatier, either with or without ribavirin [2]. Grazoprevir is a second generation hepatitis C virus protease inhibitor acting at the NS3/4a protease targets.[3] It has good activity against a range of HCV genotype variants, including some that are resistant to most currently used antiviral medications.[4, 5]

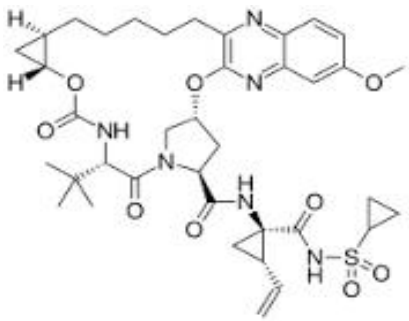

Figure 2: Structure of Grazoprevir

Due to the lack of reported HPLC methods describing determination of the mixtures under investigation, it was deemed useful to develop simple, sensitive and selective HPLC method that could be useful for the simultaneous determination of Elbasvir and Grazoprevir. The proposed method was designed to be suitable for the quality assessment of these mixtures in a tablet dosage form.

2. Materials and Methods

Table 1: Chemicals used

S.No	Chemical	Brand
1	Elbasvir	Mylon
2	Grazoprevir	Cipla
3	KH_2PO_4	Finer chemical LTD
4	Water and Methanol	Lichrosolv (Merck)
5	Acetonitrile for HPLC	Molychem
6	Ortho phosphoric Acid	Merck

Table 2: Solubility profile

Solvents	Metformin	Empagliflozin
Methanol	Soluble	Soluble
ACN	Soluble	Soluble
Water	Partially soluble	Insoluble
Chloroform	Soluble	Partially soluble

Table 3: UV Table

Drug name	Wave length	Absorbance
Elbasvir	250	0.651
Grazopravir	235	0.250

Instrumentation:

The HPLC system was an LC Waters (Waters, Milford, MA, USA) consisting of quaternary gradient system (600 Controller), in line degasser (Waters, model AF), photodiode array detector (Water, 2998 model) and auto sampler (Waters, model 717 plus). Data was processed using Empower Pro software (Waters, Milford, MA, USA). Chromatographic separation assay was performed with a Water's C-18 analytical column (150 mm × 4.6 mm inner diameter, 5 µm particle size, Waters, Dublin, Ireland) maintained at 45 °C. The mobile phase was pumped at a flow rate of 1 mL min^{-1}.

Optimized Chromatogram is Obtained by Following Conditions:

Mobile phase : Water: Methanol (50:50%v/v)
Column : symmetry C8 (4.6*150mm) 5µm
Flow rate : 1.0 ml/min
Wavelength : 270 nm
Column temp : Ambient
Sample Temp : Ambient
Injection Volume: 10 µl
RT : 2.45

HPLC Method Development: [2,3,4]

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Ammonium acetate buffer and Methanol: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to potassium dihydrogen phosphate with buffer (pH 3.0), Methanol in proportion 30: 70 v/v respectively.

Wave length selection:

UV spectrum of 10 µg/ml Elbasvir and Grazoprevir in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 225. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. Inertsil ODS (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 0.8ml/min flow.

Optimized Chromatographic Conditions:

Instrument used : Waters HPLC with auto sampler and PAD or detector.
Temperature : Ambient
Column : Inertsil ODS (4.6 x 150mm, 5µm)
Buffer : 6.8 grams of potassium dihydrogenortho phosphate in 1000 ml water pH adjusted with orthophaosparic acid.
pH : 3.0
Mobile phase : 30% buffer 70% Methanol
Flow rate : 1 ml per min
Wavelength : 260 nm
Injection volume : 10 µl
Run time : 10min.

Preparation of Buffer and Mobile Phase:

Preparation of Phosphate buffer [4, 5]:

Accurately weighed 6.8 grams of KH_2PO_4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.0 with Orthophosphoric acid.

Preparation of mobile phase:

Accurately measured 300 ml (30%) of above buffer and 700 ml of Methanol HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: The Mobile phase was used as the diluent.

Preparation of the Elbasvir & Grazoprevir Standard & Sample Solution:

Standard Solution Preparation:

Accurately weigh and transfer 10 mg of Elbasvir and10mg Grazoprevir working standard into a 10 ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Stock solution: Further pipette 3ml& 0.3ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10 mg of Elbasvir and Grazoprevir (marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Stock solution: Further pipette 3 ml of Elbasvire and Grazoprevir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for Elbasvir and Grazoprevir peaks and calculate the %Assay by using the formulae.

System Suitability: 6,7

Tailing factor for the peaks due to Elbasvir and Grazoprevirin Standard solution should not be more than 2.0. Theoretical plates for the Elbasvir and Grazoprevir peaks in Standard solution should not be less than 2000.

Calculation: (For Elbasvir)

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:
AT = average area counts of sample preparation.
As= average area counts of standard preparation.
WS = Weight of working standard taken in mg.
P = Percentage purity of working standard
LC = label claim of Elbasvir mg/ml.

Calculation: (For Grazoprevir)

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt.}}{\text{Label Claim}} \times 100$$

Where:
AT = average area counts of sample preparation.
As= average area counts of standard preparation.
WS = Weight of working standard taken in mg.
P = Percentage purity of working standard
LC = label claim of Grazoprevir mg/ml.

Method Validation Summary:

Precision:

Preparation of stock solution:

Accurately weigh and transfer 25 mg of Elbasvir and Grazoprevir working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Stock solution: Further pipette 3 ml of Elbasvir & Grazoprevirof the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

The standard solution was injected for five times and measured the area for all five Injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Acceptance Criteria:

The % RSD for the area of five standard injections results should not be more than 2%**.**

Intermediate Precision / Ruggedness:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.

Preparation of stock solution: Accurately weigh and transfer 25 mg of Elbasvir and 10mg of Grazoprevir working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Stock solution: Further pipette 3ml of Elbasvir & Grazoprevir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the

area of five replicate injections was found to be within the specified limits.

Acceptance Criteria:

The % RSD for the area of five standard injections results should not be more than 2%.

Accuracy:

Preparation of Standard stock solution: Accurately weigh and transfer 10 mg of ElbasvirandGrazoprevir10mg of working standard into a 10mL& 100ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Stock solution: Further pipette 3ml& 0.3ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):

Accurately weigh and transfer 5mg of Elbasvir and 5.3mg of Grazoprevir working standard into a 10mL and 100 ml 0f clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Stock Solution:

Further pipette 3 ml of Elbasvire& 0.3 ml of Grazoprevir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% solution (With respect to target Assay concentration):

Accurately weigh and transfer 10 mg of Elbasvir and 10 mg of Grazoprevir working standard into a 10mL and 100 ml 0f clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Stock Solution:

Further pipette 3 ml of Elbasvir& 0.3 ml of Grazoprevir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 150% solution (With respect to target Assay concentration): Accurately weigh and transfer 14.4mg of Elbasvir and 14.5mg of Grazoprevir working standards into a 10mL and 100ml of clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Stock solution:

Further pipette 3 ml of Elbasvir& 0.3 ml of Grazoprevir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Elbasvir & gemigliprin and calculate the individual recovery and mean recovery values.

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%.

Linearity:

Preparation of stock solution: Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10 mg of Elbasvir and Grazoprevir (marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Preparation of Level – I (100ppm ofElbasvir&1ppm of Grazoprevir): 1ml and 0.1 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II (200ppm of Elbasvir&2ppm of Grazoprevir): 2ml and 0.2 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III (300ppm of Elbasvir&3ppm of Grazoprevir): 3ml and 0.3 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – IV (400ppm of Elbasvir&4ppm of Grazoprevir): 4ml and 0.4 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

Preparation of Level – V(500ppm of Elbasvir&5ppm of Grazoprevir): 5ml and 0.5 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Procedure: Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

3. Results and Discussion

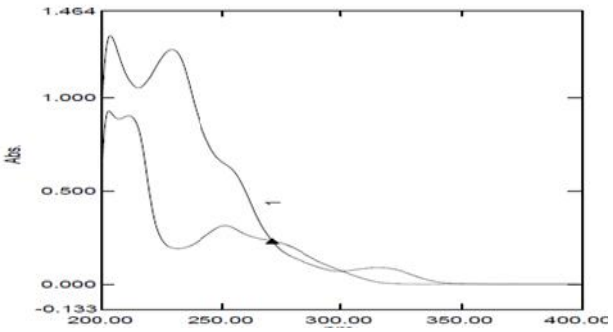

Figure 3: Spectrum showing overlapping spectrum of elbasvir and grazopravir

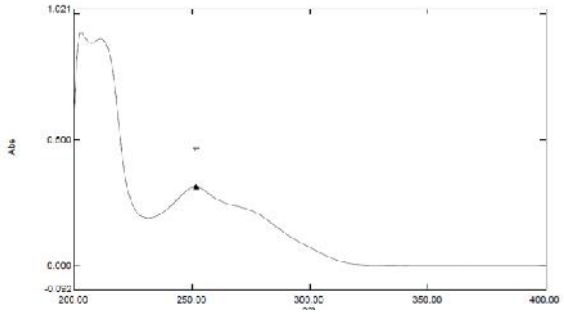

Figure 4: Spectrum showing overlapping spectrum of elbasvir and grazopravir

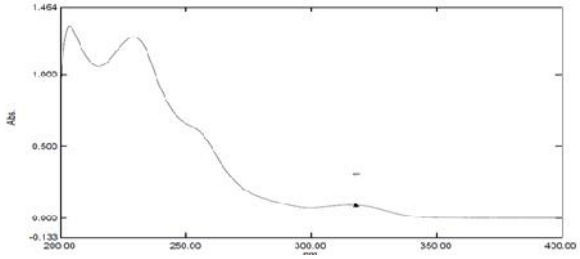

Figure 5: Spectrum showing wavelength of elbasvir

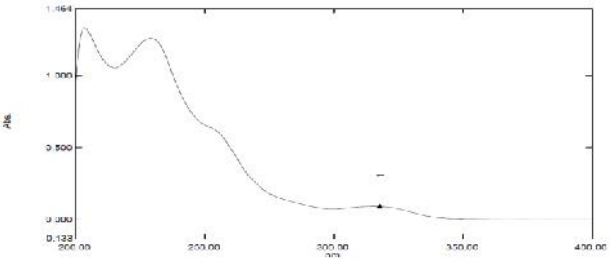

Figure 6: Spectrum showing wavelength of grazopravir

Trial 1: Optimized Chromatogram is Obtained by Following Conditions:

Mobile phase : Water: Methanol (50:50%v/v)
Column : symmetry C8 (4.6*150mm) 5µm
Flow rate : 1.0 ml/min
Wavelength : 270 nm
Column temp : Ambient
Sample Temp : Ambient
Injection Volume: 10 µl
RT : 2.45

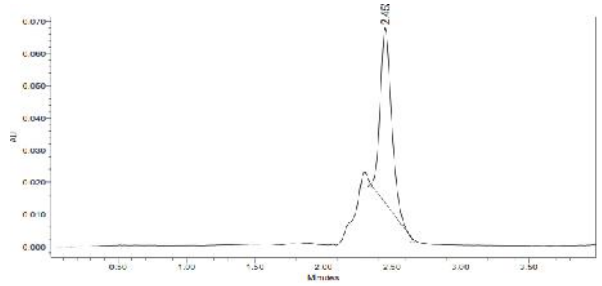

Figure 7: Trial chromatogram for Elbasvir and Grazoprevir

Chromatogram for Elbasvir and Grazoprevir

Column : Inertsil ODS C18 (4.6 x 150mm, 5µm)
Buffer pH: 4.0
Mobile phase: % buffer 70% Methanol
Flow rate : 1.0ml per min
Wavelength : 225 nm
Temperature: ambient.
Run time: 10min.

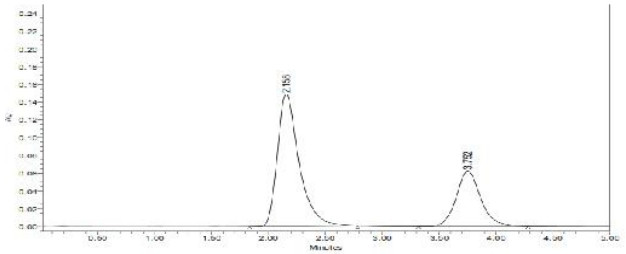

Fig. 8: Chromatogram for Elbasvir and Grazoprevir sample

Preparation

From the above chromatogram it was observed that the Elbasvir and Grazoprevir peaks are well separated
Retention time of Elbasvir – 2.158min
Retention time of Grazoprevir - 3.752 min.

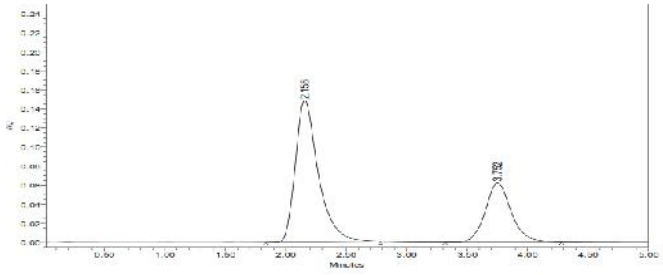

Figure 9: Chromatogram for Elbasvir and Grazoprevir Standard Preparation

Retention time of Elbasvir – 2.158 min
Retention time of Grazoprevir - 3.752 min.

System Suitability:

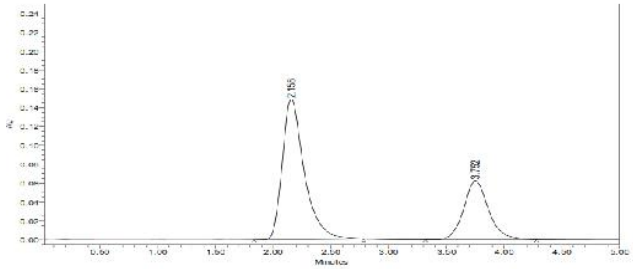

Figure 10: Chromatogram for system suitability injection-1

Validation Parameters:

Precision: Precision of the method was carried out for standard solutions as described under experimental work. The corresponding chromatograms and results are shown below.

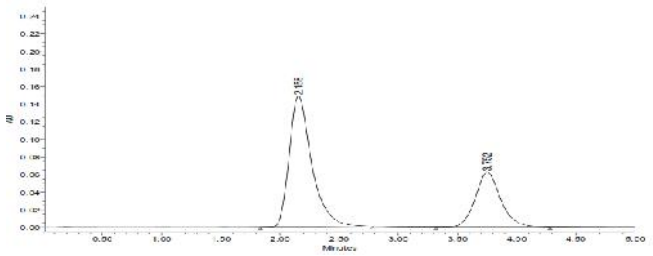

Figure 11: Chromatogram for standard injection-1

Table 4: Results of method precession for Elbasvir

Injection	Area
Injection-1	1302729
Injection-2	1302947
Injection-3	1303236
Injection-4	1303977
Injection-5	1309759
Average	1304529.8
Standard Deviation	2961.1
%RSD	0.2

Table 5: Results of method precession for Grazoprevir

Injection	Area
Injection-1	123149
Injection-2	123766
Injection-3	124271
Injection-4	124691
Injection-5	124956
Average	124162.7
Standard Deviation	725.6
%RSD	0.6

Acceptance criteria:

%RSD for sample should be NMT 2

The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate Precession (Ruggedness):

There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation.

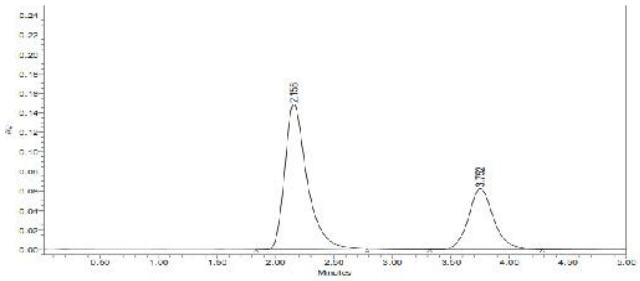

Figure 12: Chromatograms for sample injectiocn-1

Table 6: Results of Intermediate precision for Elbasvir

Injection	Area
Injection-1	1300148
Injection-2	1304520
Injection-3	1305937
Injection-4	1306476
Injection-5	130871
Average	1305070.2
Standard Deviation	3061.8
%RSD	0.2

Table 7: Results of Intermediate precision for Grazoprevir

Injection	Area
Injection-1	122487
Injection-2	122626
Injection-3	122632
Injection-4	122252
Injection-5	122962
Average	122681.8
Standard Deviation	174.8
%RSD	0.1

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

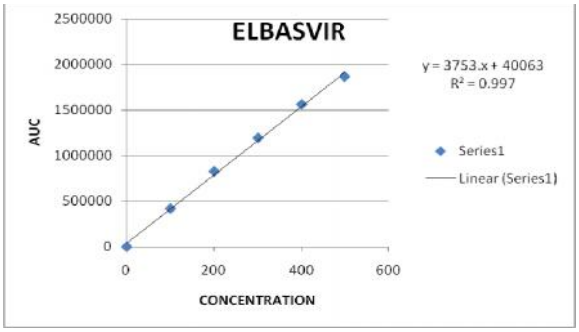

Figure 13: Calibration graph for Elbasvir at 260 nm

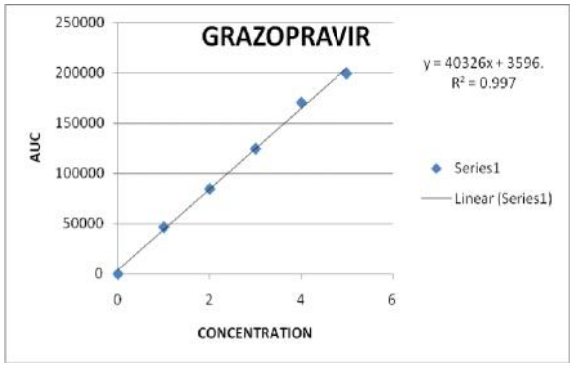

Figure 14: Calibration graph for Grazoprevir at 260 nm.

Table 8: Showing results for system suitability for injection-1

S.No	Name	Retention time(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	**Elbasvir**	2.158	124505	213642		1.2	4673.4
2	**Grazoprevir**	3.752	1308495	154566	6 0	1.3	6090.3

Table 9: Accuracy (recovery) data for Elbasvir

% Concentration (at specification Level)	Average Area	Average Amount Added (mg)	Average Amount Found (mg)	Average % Recovery	Mean Recovery
50%	656659.5	5.0	5.036	100.7%	99.84%
100%	1304258	10.0	10.003	100.0%	
150%	1854608	14.4	14.224	98.780%	

Table 10: Accuracy (recovery) data for Grazoprevir

% Concentration (at specification Level)	Average Area	Average Amount Added (mg)	Average Amount Found (mg)	Average % Recovery	Mean Recovery
50%	65800	5.3	5.34	100.8%	100.51%
100%	124353	10	10.10	100.01%	
150%	177940	14.2	14.45	99.68%	

Table 11: Area of different concentration of Elbasvir

S.No.	Linearity Level	Concentration	Area
1	I	100ppm	418934
2	II	200ppm	826781
3	III	300ppm	1193873
4	IV	400ppm	1563458
5	V	500ppm	1867084
Correlation Coefficient			0.997

Table 12: Area of different concentration of Grazoprevir.

S.No	Linearity Level	Concentration	Area
1	I	1ppm	46510
2	II	2ppm	84701
3	III	3ppm	124802
4	IV	4ppm	170731
5	V	5ppm	199732
Correlation Coefficient			0.997

Table 13: Analytical performance parameters of Elbasvir and Grazoprevir

Parameters	Elbasvir	Grazoprevir
Slope (m)	3753	40326
Intercept (c)	40063	3569
Correlation coefficient (R^2)	0.997	0.997

Table 14: Flow Rate (ml/min) data for Elbasvir

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.6	5339.9	1.4
2	0.8	4673.4	1.3
3	1.0	5216.0	1.4

Table 15: Flow rate (ml/min) data for Grazoprevir

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	7063.3	1.3
2	1.0	6090.3	1.2
3	1.2	6998.0	1.3

Table 16: Change in Organic Composition in the Mobile Phase for Elbasvir

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	4508.4	1.3
2	*Actual	4673.4	1.4
3	10% more	4318.1	1.3

Table 17: Change in Organic Composition in the Mobile Phase for Grazoprevir

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	6387.7	1.2
2	*Actual	6090.3	1.2
3	10% more	6232.5	1.2

4. Conclusion

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of elbasvir and grazoprevir was done by RP-HPLC. The Phosphate buffer was p^H 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30 % v/ v. Inertsil C_{18} (ODS)column (4.6x150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 1.0 ml/min. the linearity range of elbasvir and grazoprevir were found to be from 100-500 ppm of elbasvir and 1-5ppm of grazoprevir. Linear regression coefficient was not more than 0.999.The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of elbasvir and grazoprevir. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear.

5. References

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