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

Pharmaceutical Science

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ABSTRACT

A simple, Accurate and precise method was developed for the simultaneous estimation of Grazoprevir and Elbasvir Tablets. Chromatogram was run through Std Discovery C8 250 x 4.6 mm, 5m. Mobile phase containing Buffer 0.1% OPA: Acetonitrile taken in the ratio 45:55 was pumped through column at a flow rate of 1 ml/min. Buffer used in this method was 0.1% OPA buffer. Temperature was maintained at 30°C. Optimized wavelength selected was 260 nm. Retention time of Elbasvir and Grazoprevir and were found to be 2.503 min and 3.004. %RSD of the Elbasvir and Grazoprevir were and found to be 0.3 and 0.4 respectively. %Recovery was obtained as 98.17% and 99.83% for Grazoprevir and Elbasvir respectively. Obtained LOD, LOQ values from regression equations of Grazoprevir and Elbasvir were 0.24, 0.73 and 0.06, 0.19 respectively. Regression equation of Grazoprevir is y = 24933.x + 37206 and that of Elbasvir is y = 21941x +756.6. Results show that the retention and run time were decreased, so its evident that the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

KEYWORDS

AIM

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 tablets in bulk.

Objective and Plan:

objectives of the present work includes

- To develop a new stability indicating HPLC method for simultaneous estimation of `Grazoprevir and Elbasvir and to develop the validated method according to ICH guidelines.
- To apply the validated method for the simultaneous estimation of Grazoprevir and Elbasvir in pharmaceutical formulation

3. MATERIALS AND METHODS

Materials:

Grazoprevir and Elbasvir, Combination of Grazoprevir and Elbasvir tablet dosage forms, 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.

Instrument:

HPLC instrument used was of WATERS HPLC 2965 SYSTEM with Auto Injector and PDA Detector. Software used is Empower 2. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz was be used for measuring absorbance for Grazoprevir and Elbasvir solutions.

METHODS:

Preparation of buffer:

Buffer: (0.1% OPA)

Accurately 1ml of OPA 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.

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, 50mL 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.

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.

Preparation of buffer:

%OPA Buffer:

1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water.

Validation:

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

Specificity:

Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision:

Preparation of Standard stock solutions:

Accurately weighed 10mg of Grazoprevir, 5mg of Elbasvir and transferred to 10ml volumetric flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (1000µg/ml of Grazoprevir and 500µg/ml Elbasvir)

Preparation of Standard working solutions (100% solution):

1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (100µg/ml of Grazoprevir and 50µg/ml of Elbasvir)

Linearity:

25% Standard solution:

0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (25µg/ml of Grazoprevir and 12.5µg/ml of Elbasvir)

50% Standard solution:

0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (50µg/ml of Grazoprevir and 25µg/ml of Elbasvir)

75% Standard solution:
0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (75µg/ml of Grazoprevir and 37.5µg/ml of Elbasvir)

100% Standard solution:
1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (100µg/ml of Grazoprevir and 50µg/ml of Elbasvir)

125% Standard solution:
1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (125µg/ml of Grazoprevir and 62.5µg/ml of Elbasvir)

150% Standard solution:
1.5ml each from two standard stock solutions was pipettede out and made up to 10ml (150µg/ml of Grazoprevir and 75µg/ml of Elbasvir)

Accuracy:
Preparation of Standard stock solutions:
Accurately weighed 10mg of Grazoprevir, 5mg of Elbasvir and transferred to 10ml volumetric flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (1000µg/ml of Grazoprevir and 500µg/ml Elbasvir)

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.

Acceptance Criteria:
The % Recovery for each level should be between 98.0 to 102

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 effected 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, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

Degradation studies:
Oxidation:
To 1 ml of stock solutionof Grazoprevir and Elbasvir, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60^{0}c. For HPLC study, the resultant solution was diluted to obtain 100µg/ml&50µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:
To 1 ml of stock s solution Grazoprevir and Elbasvir, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^{0}c .The resultant solution was diluted to obtain 100µg/ml&50µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:
To 1 ml of stock solution Grazoprevir and Elbasvir, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^{0}c. The resultant solution was diluted to obtain 100µg/ml&50µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:
The standard drug solution was placed in oven at 105°C for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 100µg/ml&50µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Photo Stability studies:
The photochemical stability of the drug was also studied by exposing the 1000µg/ml& &500µg/ml solution to UV Light by keeping the beaker in UV Chamber for 1days or 200 Watt hours/m^2 in photo stability chamber For HPLC study, the resultant solution was diluted to obtain 100µg/ml 50µg/ml solutions 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 1h r s at a temperature of 60º. For HPLC study, the resultant solution was diluted to 100µg/ml&50µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

METHOD DEVELOPMENT PROCEDURE
The wide variety of equipment's, columns, eluent and operation preparations involved high performance liquid chromatography (HPLC) method development seems complex. The processes influenced by the nature of analytes and generally follow the following steps

Steps:
- Step 1 - Selection of initial system and the HPLC method
- Step 2 - Selection of initial conditions
- Step 3 - Selectivity optimization
- Step 4 - Optimizing system parameter
- Step 5 - Validation of method

Step 1 - selection of initial system and the HPLC method
When developing an HPLC method, the first step is always to consult the literature to ascertain whether the separation has been previously performed and if so, under what conditions - this will save time doing unnecessary experimental work. While selecting an HPLC system, it must have a high probability of being able to analyse the sample; for example, if the sample includes polar analytes then reverse phase HPLC would offer both adequate retention and resolution, whereas normal phase HPLC wouldn't be much feasible

Step 2: Selection of initial conditions
This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detectability). Selection of the following is then required.

Step 3 - selectivity optimization:
The aim of this step is to achieve adequate peak spacing. Both the mobile phase and stationary phase compositions need to be taken into account. Only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined to minimize the number of trial chromatograms involved. To select these the nature of the analytes must be considered. The relevant optimization parameters

may be selected once the analyte types are identified. Note that the optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization.

Step 4 – optimization of system parameter

This is used after satisfactory selectivity has been achieved to find out the desired balance between resolution and analysis time. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

Step 5 - Validation of method

Proper validation of analytical methods is important for pharmaceutical analysis when ensure of the continuing efficacy and safety of each batch manufactured relies solely on the determination of quality. The ability to control this quality is dependent upon the ability of the analytical methods, as applied under well-defined conditions and at an established level of sensitivity, to give a reliable demonstration of all deviation from target criteria.

Analytical methods should be used in accordance with good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols which are set out in the international conference on harmonization (ICH) guidelines (Q2A and Q2B). The most widely applied validation characteristics are accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantitation limit, linearity, range, robustness and stability of analytical solutions. Method validation must have a written and approved protocol prior to use.

RESULTS AND DISCUSSION

Optimized wavelength selected was 260nm.

Method development:

Method development was done by changing various, mobile phase ratios, buffers etc.

Trial 1:

Chromatographic conditions:

Mobile phase : Water and Methanol taken in the ratio 50:50
Flow rate : 1 ml/min
Column : BDS C18 (4.6 x 250mm, 5µm)
Detector wave length : 260nm
Column temperature: 30°C
Injection volume : 10mL
Run time : 10 min
Diluent : Water and Acetonitrile in the ratio 50:50
Results: Grazoprevir peak was not eluted so, further trial is carried out.

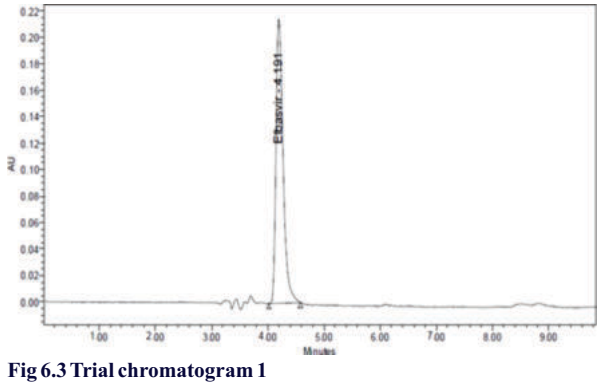


Fig 6.3 Trial chromatogram 1

Trial 2:

Chromatographic conditions:

Mobile phase: 0.1% OPA**:** Acetonitrile (40:60)
Flow rate: 1 ml/min
Column: BDS C18 (4.6 x 250mm, 5µm)
Detector wave length: 260nm
Column temperature: 30°C
Injection volume: 10mL
Run time: 10 min
Diluent: Water and Acetonitrile in the ratio (50:50)
Results: Grazoprevir peak shape and retention time were not good so further trial is carried out

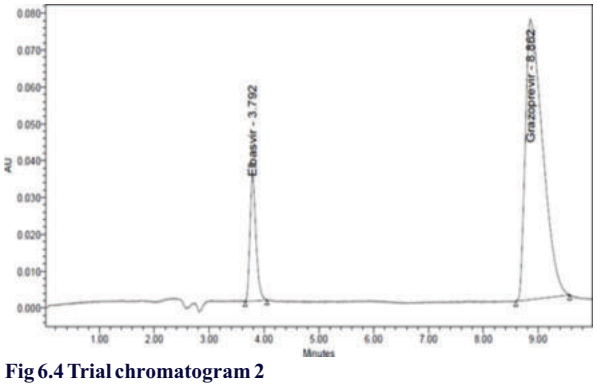


Fig 6.4 Trial chromatogram 2

Trial 3:

Chromatographic conditions:

Mobile phase: 0.1% OPA 40%: 60% Acetonitrile
Flow rate: 1 ml/min
Column: BDS C18 (4.6 x 150mm, 5µm)
Detector wave length: 260nm
Column temperature: 30°C
Injection volume: 10mL
Run time: 10 min
Diluent: Water and Acetonitrile in the ratio 50:50
Results: Grazoprevir **&** Elbasvir both peak are eluted but Grazoprevir peak not good so, further trail was carried out.

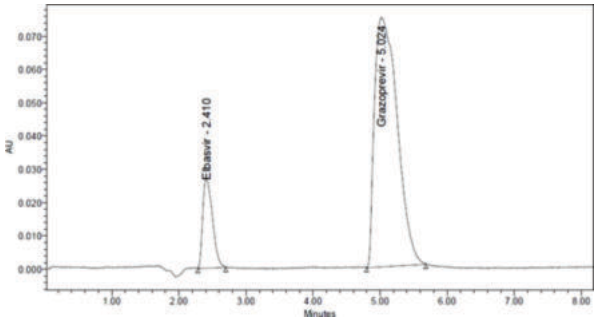


Fig 6.5 Trial chromatogram 3

Trial 4:

Chromatographic conditions:

Mobile phase: 0.1%OPA 40%: 60% Acetonitrile
Flow rate: 1 ml/min
Column: Discovery C18 (4.6 x 150mm, 5µm)
Detector wave length: 260nm
Column temperature: 30°C
Injection volume: 10mL
Run time: 10 min
Diluent: Water and Acetonitrile in the ratio 50:50
Results: Both peak shape &USP plate count were not good Elbasvir eluted at void time so, further trial is carried out.

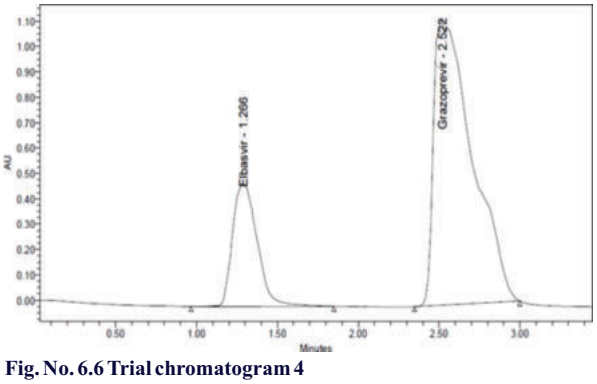


Fig. No. 6.6 Trial chromatogram 4

Optimized method:

Chromatographic conditions:

Mobile phase: 0.1% 45: 55% Acetonitrile**Flow rate:** 1

ml/min
Column: Discovery C8 (4.6 x 250mm, 5µm)
Detector wave length: 260nm
Column temperature: 30°C
Injection volume: 10mL
Run time: 7 min
Diluent: Water and Acetonitrile in the ratio 50:50
Results: Both peaks have good resolution, tailing Factor, theoretical plate count and resolution.

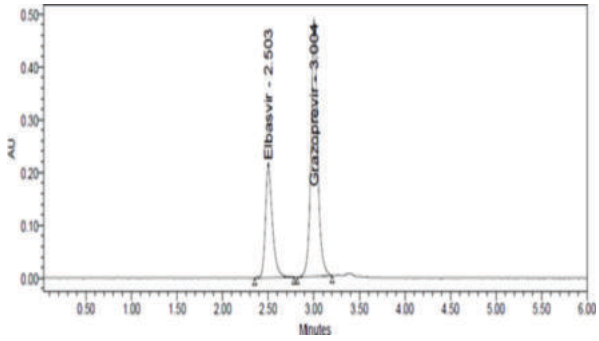
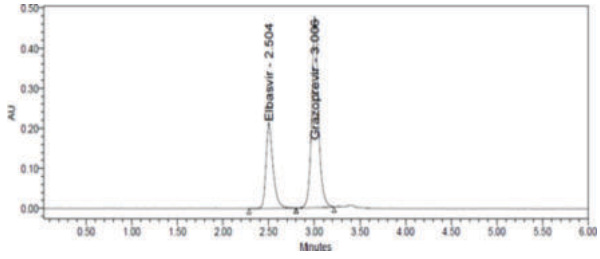


Fig 6.8 Optimized Chromatogram
Observation:
Grazoprevir and Elbasvir were eluted at 3.004 min and 2.503 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

System suitability:
All the system suitability parameters were within the range and satisfactory as per ICH guidelines

Table: 6.1 System suitability parameters for Grazoprevir and Elbasvir

S no	Grazoprevir				Elbasvir		
Inj	RT(min)	USP Plate Count	Tailing	USP Resolution	RT(min)	USP Plate Count	Tailing
1	3.004	8691	1.12	3.7	2.501	5321	1.33
2	3.005	8373	1.13	3.7	2.503	5385	1.30
3	3.006	8409	1.13	3.7	2.503	5157	1.34
4	3.006	8467	1.13	3.7	2.503	5123	1.34
5	3.006	8384	1.12	3.6	2.504	5157	1.35
6	3.007	8733	1.10	3.5	2.505	5156	1.35



DISCUSSION:
According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Linearity:
Table 6.2 Linearity table for Grazoprevir and Elbasvir.

Grazoprevir		Elbasvir	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
0	0	0	0
25	658032	12.5	283657
50	1347776	25	547451
75	1934411	37.5	799555
100	2491185	50	1111600
125	3130786	62.5	1385268
150	3788046	75	1637364

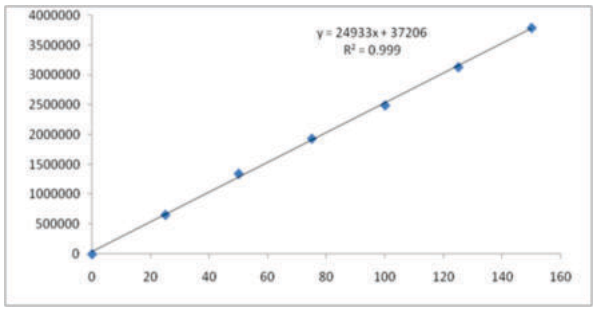


Fig No. 6.13 Calibration curve of Grazoprevir

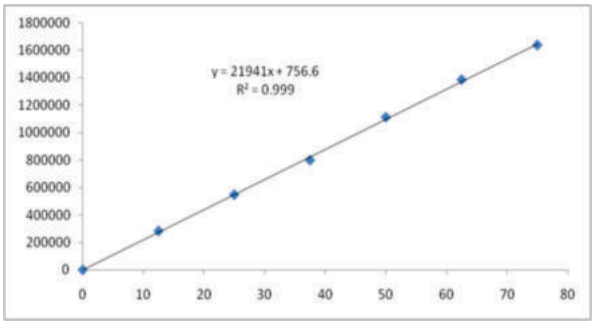


Fig No. 6.14 Calibration curve of Elbasvir
DISCUSSION:
Six linear concentrations of Grazoprevir (25-150µg/ml) and Elbasvir (12.5- 75µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Grazoprevir was y = 24933.x + 37206and of Elbasvir was y = 21941.x + 756.6 Correlation coefficient obtained was 0.999 for the two drugs.

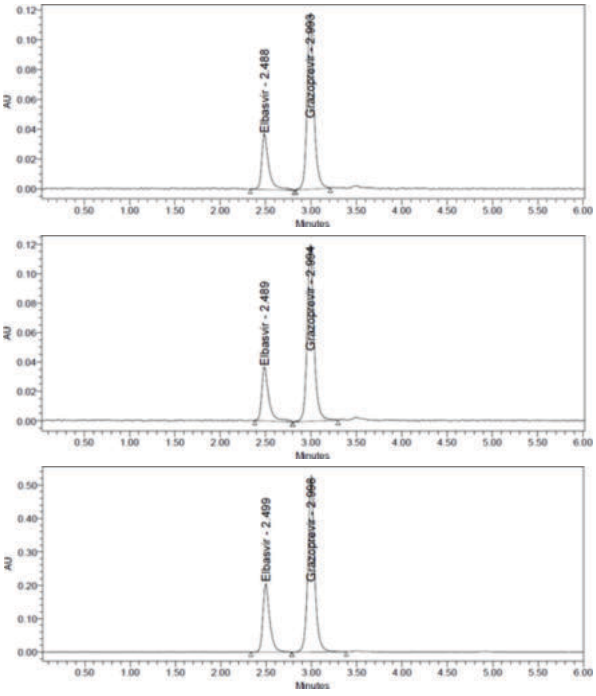


Fig No. 6.18 Linearity 100% Chromatogram of Grazoprevir and Elbasvir

Precision:
System Precision:

Table 6.3 System precision table of Grazoprevir and Elbasvir

S. No	Area of Grazoprevir	Area of Elbasvir
1.	2472663	1139108
2.	2457936	1141894
3.	2471406	1138977
4.	2484912	1137119
5.	2462272	1145541

6.	2445053	1152528
Mean	2465707	1142528
S.D	13773.0	5710.4
%RSD	0.6	0.5

DISCUSSION:

From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.6% and 0.5% respectively for Grazoprevir and Elbasvir. As the limit of Precision was less than "2" the system precision was passed in this method.

Accuracy:

Table 6.6 Accuracy table of Grazoprevir

% Level	Amount Spiked (µg/mL)	Amount recovered(µg/mL)	% Recovery	Mean %Recovery
50%	50	49.27554	98.55	99.17%
	50	49.36205	98.72	
	50	50.16528	100.33	
100%	100	99.54017	99.54	
	100	100.117	100.12	
	100	100.5057	100.51	
150%	150	147.3853	98.26	
	150	147.3525	98.24	
	150	147.3391	98.23	

Table 6.7 Accuracy table of Elbasvir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	25	24.7406	98.96	99.83%
	25	25.17932	100.72	
	25	25.16172	100.65	
100%	50	50.38295	100.77	
	50	50.3068	100.61	
	50	50.3577	100.72	
150%	75	74.36386	99.15	
	75	73.69611	98.26	
	75	73.9683	98.62	

DISCUSSION:

Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.17% and 99.83% for Grazoprevir and Elbasvir respectively.

Sensitivity:

Table 6.8 Sensitivity table of Grazoprevir and Elbasvir

Molecule	LOD	LOQ
Grazoprevir	0.24	0.73
Elbasvir	0.06	0.19

3.SUMMARY AND CONCLUSION

Summary Table

Parameters		Grazoprevir	Elbasvir	LIMIT
Linearity Range(µg/ml)		25-150 µg/ml	12.5-75µg/ml	R< 1
Regressioncoefficient		0.999	0.999	
Slope(m)		24933	21941	
Intercept(c)		37206	756.6	
Regression equation (Y=mx+c)		y = 24933.x + 37206	y = 21941x +756.6	
Assay(%mean assay)		99.28%	99.29 %	90-110%
Specificity		Specific	Specific	No interference of any peak
System precision %RSD		0.6	0.5	NMT 2.0%
Method precision %RSD		0.4	0.3	NMT 2.0%
Accuracy %recovery		98.17%	99.83%	98-102%
LOD		0.24	0.06	NMT 3
LOQ		0.73	0.19	NMT 10
Robustness	FM	0.5	0.4	%RSD NMT 2.0
	FP	1.1	0.6	
	MM	1.8	0.4	
	MP	0.3	0.4	
	TM	0.2	0.5	
	TP	0.4	0.4	

CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Grazoprevir and Elbasvir in Tablet dosage form. Retention time of Elbasvir and Grazoprevir and were found to be 2.503 min and 3.004. %RSD of the Elbasvir and Grazoprevir were and found to be 0.3 and 0.4 respectively. %Recovery was obtained as 98.17% and 99.83% for Grazoprevir and Elbasvir respectively. LOD, LOQ values obtained from regression equations of Grazoprevir and Elbasvir were 0.24, 0.73 and 0.06, 0.19 respectively. Regression equation of Grazoprevir is y = 24933.x + 37206, y = 21941x +756.6 of Elbasvir. Retention times were 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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