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

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ABSTRACT

A rapid, simple and precise RP-HPLC method has been described for simultaneous estimation of antiviral drugs namely Elbasvir and Grazoprevir in bulk and tablet dosage forms by make use of waters symmetry Shield C18 column , 4.6×250 mm, 5.0 µm with mobile phase consisting of 0.1 % Ortho Phosphoric acid (pH 2.2) : Acetonitrile, (45:55, v/v) premixed. The flow rate was maintained at 0.9ml/min. The column effluents were monitored by using PDA detector at 260 nm. The retention times of Grazoprevir and Elbasvir was found to be 2.311 min and 3.848 min. The correlation coefficients of Elbasvir and Grazoprevir was 0.999 with equation y=21841x + 1868 for grazoprevir and y=22881x + 3308 for Elbasvir. The performance of the developed method has been validated in terms of accuracy, specificity, linearity, precision and robustness according to ICH guidelines. Recovery of the method was found to be 98-102%. The detector response was found to be linear over a concentration range of 12.5–150 µg/mL for both the drugs. The described method can be successfully employed for the quality control analysis of Elbasvir and Grazoprevir in formulations.

1. INTRODUCTION:

Grazoprevir is a second generation NS3/4a protease inhibitor used to inhibit viral HCV replication. It is mainly used in the treatment of chronic hepatitis C of the genotypes 1 and 4 for adults. Its molecular formula is $C_{38}H_{50}N_6O_9S$ and molecular mass is 766.90 g/mol.It is insoluble in water and is slightly hygroscopic. Elbasvir is a NS5a protein inhibitor used to inhibit viral HCV replication complex. Its molecular formula is $C_{49}H_{55}N_9O_7$ and molecular mass of 882.02 g/mol.It is practically insoluble in water and is slightly hygroscopic.[1-3].

Literature review revealed that this drug combination has been approved by FDA in the year 2016 and very few RP-HPLC methods have been available for the simultaneous estimation of these drugs in bulk and pharmaceutical dosage forms. Hence a new HPLC method for simultaneous determination of elbasvir and grazoprevir in bulk and pharmaceutical

dosage forms has been developed and validated according to ICH guidelines.

2. MATERIALS AND METHODS

2.1. Materials and Methods

Elbasvir and Grazoprevir were obtained from sinfa chemicals as gift sample. All the chemicals and reagents employed such as ortho phosphoric acid, methanol, acetonitrile were of analytical grade and were procured from sd fine, qualigens and merck chemicals, India.

2.1.1. HPLC instrumentation and Chromatographic conditions

The experiments were carried out on the chromatographic system AGILENT consisting of HPLC Pump, auto sampler and PDA detector. The partial loop injection volume was 10µL. Chromatographic separations were performed on waters symmetry shield, C_{18}, 4.6×250 mm, 5.0 µm column with UV detection at 260 nm. The flow rate was 0.9 mL/min and the column temperature was set at 30 ^{0}C and the injection volume was 10µL. Empower software was used for data collection and analysis [4, 5].

2.1.2. Selection and preparation of mobile phase

Various mobile phases containing orthophosphoric acid and acetonitrile in different ratios were tried by different columns, flowrates. Good symmetrical peaks, resolution and retention time was observed with mobile phase 0.1 % orthophosphoric acid: acetonitrile, (55:45, v/v) premixed. The mobile phase was sonicated for 10min and filtered through 0.45µm membrane filter.

2.1.3. Preparation of standard stock solution

Accurately weighed 5 mg& 10 mg of elbasvir and grazoprevir working standards were transferred into a 10mL clean dry volumetric flask respectively and 5mL of diluent was added and sonicated for 30 minutes and made up the final volume with diluents to get a concentration of 100 µg/mL of grazoprevir and 50µg/mL of elbasvir. From the above stock solutions, 1mL was pipetted out in to a 10mL volumetric flask and then made up to the final volume with diluent.

2.1.4. Selection of wavelength

Ideal wavelength is the one that gives good response for the drugs that are to be detected. UV spectra of both the drugs showed that Grazoprevir and Elbasvir absorbed appreciably at 260 nm.So detection was carried out at 260 nm.

2.1.5. Solutions for the estimation of linearity and accuracy

For the estimation of accuracy and linearity eight solutions containing Elbasvir and grazoprevir were prepared in the concentration range of 12.5–150 µg/mL in the mixture of acetonitrile and water (50:50 v/v). These solutions were further sonicated for 30 min and were stirred using the magnetic stirrer for 2 h. The accuracy of Elbasvir and grazoprevir is estimated by performing the recovery experiment at 50%, 100% and 150% of the label claim of the drug and the linearity of the method was estimated by plotting a calibration graph and slope of the method has been found out.

2.1.6. Solutions for the precision estimation

A quantity of tablet content corresponding to 5 mg of grazoprevir and 10 mg of elbasvir were placed into a 100 mL volumetric flask and extracted with the mixture of acetonitrile and water (50:50 v/v) followed by sonication and filtration. The final volume was made to the mark with the same solvent and the solution was

Filtered. From that stock solution, six solutions containing 150µg/mL of elbasvir and grazoprevir were prepared.

2.2. Method Validation: In accordance with the ICH Q2A guidelines validation studies

were conducted using the optimized assay conditions. Important validation parameters including specificity, accuracy, precision, linearity, detection limit and quantification limit were evaluated [6-8].

2.2.1. System suitability Test

In a view to ensure its effectiveness and to verify reproducibility certain system suitability test parameters were monitored by repetitively injecting the drug solution into the HPLC at the start of study of each validation parameter since these tests are an essential part of chromatographic analysis method and are ferformed to evaluate the behavior of chromatographic system such as capacity factor (k^1), plate number (N) and Tailing factor (T).

Acceptance criteria

•Asymmetry <1.0

•Theoretical plates >2000

• RSD <2.0%

2.2.2. Specificity:

Specificity was performed by comparing the chromatograms of both the drugs with recorded chromatogram of placebo and blank. In all tested conditions interference of peaks was determined.

2.2.3. Limit of detection and limit of Quantification

LOD is the lowest amount of analyte that can be detected, but not necessarily quantitated and LOD is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

The limit of detection of instrumental procedures is carried out by determining the signal-to-noise ratio. A signal-to-noise ratio of 2:1 or 3:1 is generally accepted

LOD = 3.3 σ/S

LOQ was calculated using the following formula

LOQ = 10 σ/S

2.2.4. Precision and intermediate precision[9]

The degree of repeatability of an analytical method under normal conditions is precision. The precision of the method was determined by repeatability (intraday) and intermediate precision (interday) and reported as % RSD for a statistically significant number of replicate measurements. **Repeatability (intra-day):**

The intra-day precision for the determination of Elbasvir and grazoprevir was carried out by analyzing dosage form sample solutions prepared at 100% of test concentration stated in the method (0.1 mg/mL) using the proposed method, within the same laboratory, using the same analyst, with the same equipment, on the same day .

Intermediate precision (inter-day):

The inter-day precision for the determination of Elbasvir and grazoprevir was carried out as under repeatability but on three consecutive days, at each day the determination was done twice (n =6).

2.2.5. Accuracy: Accuracy of the method was determined by applying the proposed method to synthetic mixture containing known amount of each drug to 50%, 100% and 150% of the label claim. The test results were compared with the standard values and % recovery was calculated. Accuracy of the method was confirmed by recovery studies.

2.2.5. Linearity:

Linearity studies were performed in the concentration range of 12.5-75 µg/mL for Grazoprevir (corresponded to 50% to 150 %, respectively, of the test solution concentration) and 25-150 µg/mL for Elbasvir (corresponded to 50% to 150 %, respectively, of the test solution concentration).

2.2.6. Robustness: To determine the robustness of a HPLC method, few parameters were deliberately varied. The parameters include variation of flow rate. At standard concentrations for both the drugs

Elbasvir and Grazoprevir various parameters were modified. Variation in flow rate by ±0.2 ml/min. Variation in temperature by ±0.5ºC. Variation in Organic phase concentration by ± 10 %

RESULTS AND DISCUSSION

3.1. Method development and validation :

3.1.1. Method development [10-12]

The main objective of the present study is to develop simple, accurate, precise method for quantitative determination of Elbasvir and Grazoprevir in bulk and pharmaceutical dosage forms. For the developed method in order to obtain good symmetrical peaks, better resolution and less retention time various mobile phases containing Orthophosphoric acid and acetonitrile in different ratios were tried by different columns and flowrates. In HPLC methd mobile phase comprising of premixed 0.1 % Ortho Phosphoric acid : Acetonitrile, (55:45,v/v) resulted in high sensitivity ,short analysis time and good peak symmetry.

3.2. Method Validation:

3.2.1. Specificity: From Specificity test it was evident that there is no interference of placebo at the retention time of Elbasvir and Grazoprevir. Interference from blank and placebo was not observed at any peak of interest.

Acceptance Criteria: The % relative standard deviation of Elbasvir and Grazoprevir individual, from the six units should be not more than 2.0%. The individual assays of Elbasvir and Grazoprevir were found to be not less than 98% and not more than 102.0%.

3.2.2. Limit of detection and quantitation: The lowest amount of analyte that can be detected and quantified was calculated by employing suitable formula and the chromatograms were recorded.

3.2.3. Linearity: Different concentrations of elbasvir and grazoprevir were prepared and linearity graph was plotted with concentaration on X-axis and peak area on Y-axis.

3.2.4. Precision: Repeatability of the method was assessed by injecting multiple injections of the sample in same concentration and interday and intraday precision was performed.

3.2.5. Accuracy: Accuracy of the method was confirmed by recovery studies by standard addition method and the results obtained were tabulated.

Acceptance Criteria:

The mean % recovery of the Elbasvir and Grazoprevir at each spike level was found to be not less than 98.0% and not more than 102.0% for both the drugs separately.

3.2.6. Robustness: To determine the robustness of a HPLC method, few parameters were deliberately varied and the results obtained were tabulated. The results obtained for accuracy and precision studies shows that recovery results were well within the range of 98-102% and low values of % RSD indicate the accuracy and precision of the method. Chromatograms of blank and placebo solutions showed no interfering peak at retention times of drugs indicating specificity. Graphs plotted as detector response as a function of labeled claim shows a linear relationship over 25%-150 %. This has indicated the capability of method to estimate accurately the drug over a wide range. The variation of Flow rate, mobile phase composition and temperature has not shown any significant change in 12 hrs indicating robustness of the method.

CONCLUSION: The proposed simple, accurate, precise RP-HPLC method can quantitatively determine both the antiviral drugs Elbasvir and Grazoprevir in bulk and pharmaceutical dosage forms with good resolution, peak symmetry and shoter analysis times. The method was validated for accuracy, linearity, precision, accuracy, specificity and robustness and the results obtained clearly demonstrates that this method provides appropriate information for monitoring the quality of bulk samples and identifies the samples even at low concentrations.

Table 1: Optimized chromatographic conditions

Parameters	Optimized conditions
Mobile Phase	0.1 % Ortho Phosphoric acid (pH 2.2) : Acetonitrile, (45:55, v/v) premixed
Column	Waters Symmetry Shield, C18, 4.6×250 mm, 5.0 µm
Flow Rate	0.9 ml/Min
Temperature	30˚C
Injection Volume	10µl
Run time	6 min
Detector	PDA

Table 2: Linearity Results of Grazoprevir and Elbasvir

Grazoprevir		Elbasvir	
Concentration of drug(µg/mL)	**Peak Area**	**Concentration of drug(µg/mL)**	**Peak Area**
12.5	286145	25	555601
25	563560	50	1115990
37.5	866694	75	1662623
50	1180049	100	2262279
62.5	1434926	125	2748639
75	1698143	150	3252043

Table 3: Data of Repeatability using Multiple Determinations at the Test Concentration for Grazoprevir and Elbasvir

Grazoprevir			Elbasvir		
Determination	**Area of Analyte**	**% Assay**	**Determination**	**Area of Analyte**	**% Assay**
100	2244219	99.12	50	1184892	100.00
100	2240298	98.85	50	1172082	98.92
100	2240010	99.49	50	1182586	99.81
100	2246007	100.19	50	1174020	99.08
100	2239950	99.24	50	1179760	99.57
100	2238469	99.52	50	1180156	99.60
Average	2241492	99.40	Average	1178916	99.50
SD	2931.2	0.46	SD	4941.8	0.42

Table 4: Recovery Result for Elbasvir

Analyte Level	Analyte Peak Area	Nominal Concentration	Actual Concentration	Individual % Recovery	Mean % Recovery	% RSD
Level 1	1714244	25	24.7754	99.10	99.60	0.43
	1718715	25	24.97081	99.88		
	1718324	25	24.95372	99.81		
Level 2	2286191	50	49.77199	99.54	99.96	0.57
	2298321	50	50.30213	100.60		
	2288387	50	49.86797	99.74		
Level 3	2864963	75	75.06687	100.09	100.05	0.22
	2860304	75	74.86325	99.82		
	2867680	75	75.18561	100.25		

Table 5: Recovery Result of Grazoprevir

Analyte Level	Analyte Peak Area	Nominal Concentration	Actual Concentration	Individual % Recovery	Mean % Recovery	% RSD
Level 1	3283739	50	49.49192	98.98	99.28	0.29
	3289931	50	49.77542	99.55		
	3287308	50	49.65533	99.31		
Level 2	4406492	150	100.8977	100.90	100.00	0.82
	4371719	150	99.30557	99.31		
	4382254	150	99.78792	99.79		
Level 3	5461965	100	149.223	99.48	99.50	0.21
	5469805	100	149.5819	99.72		
	5455996	100	148.9497	99.30		

Table 6: Robustness data

FACTORS	ELBASVIR (Rt min)	GRAZOPREVIR (Rt min)
A. Flow rate (mL/min)		
O.7 mL	3.77	2.30
1.1 mL	3.78	2.31
B. Temperature (ºC)		
25 ºC	3.79	2.31
35 ºC	3.80	2.31
C. Organic phase composition		
ACN (35 %)	3.76	2.30
ACN (55 %)	3.78	2.31

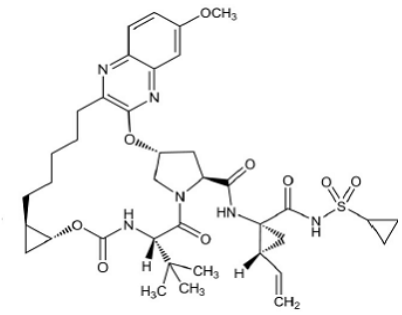


Figure1: Structure of Grazoprevir

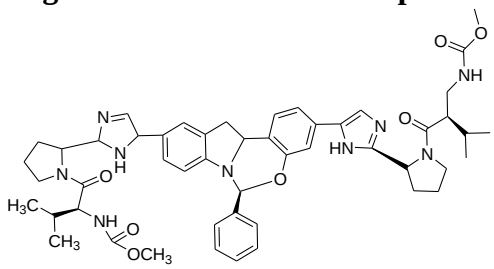


Figure 2: Structure of Elbasvir

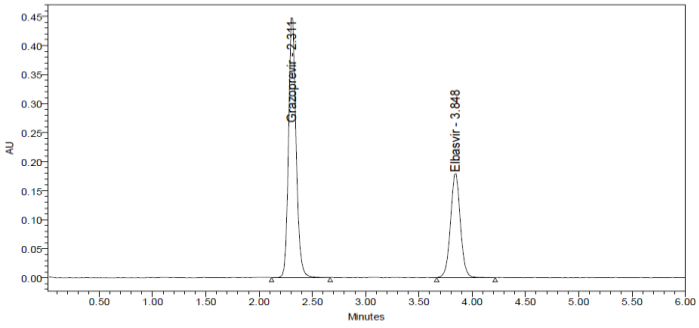


Figure 3: Chromatogram of Elbasvir and Grazoprevir obtained under optimised conditions

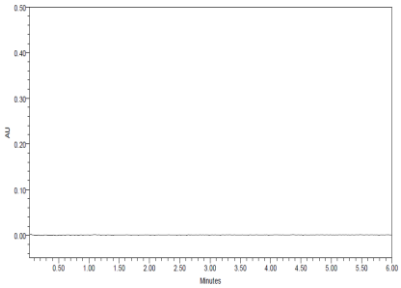


Figure 4: Chromatogram of Placebo

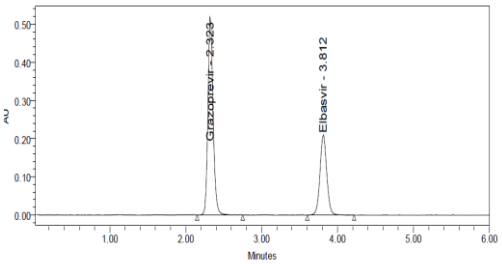


Figure 5: Chromatogram of sample

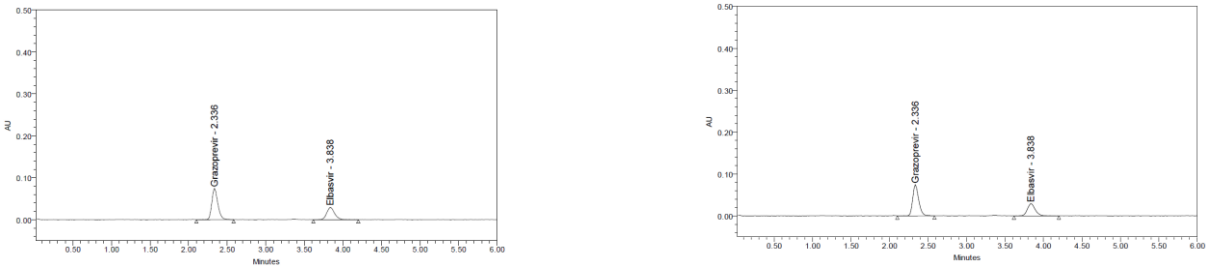


Figure 6: Chromatograms for Limit of Detection and Limit of Quantification

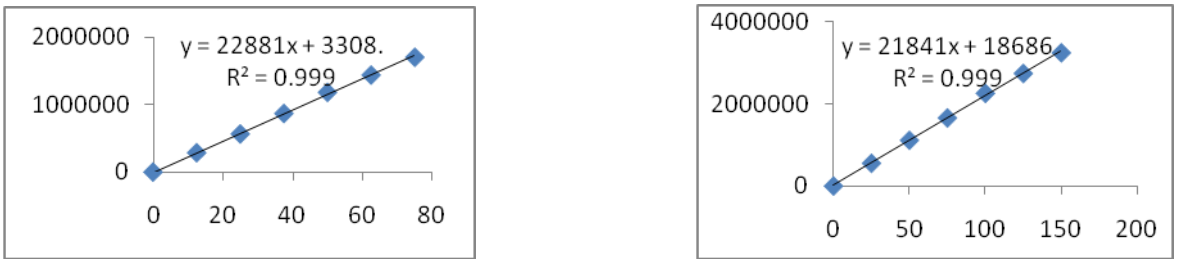


Figure 7: Calibration curve for Elbasvir and Grazoprevir

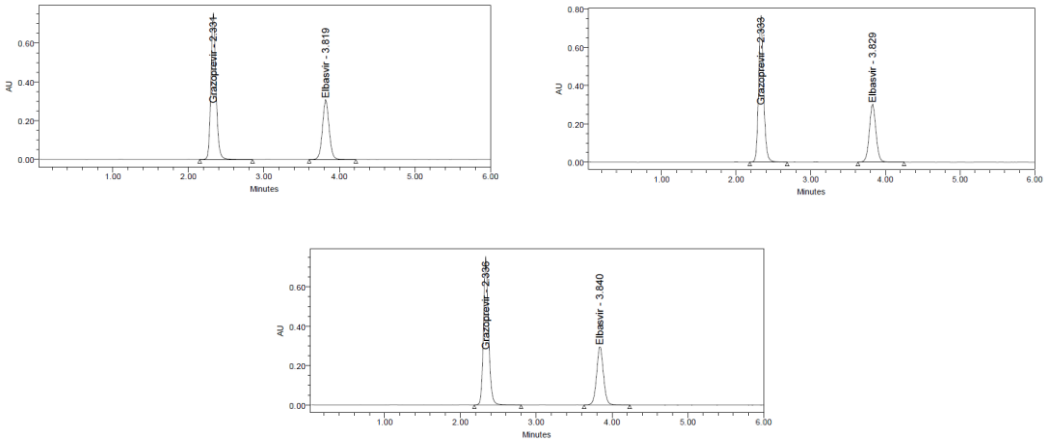
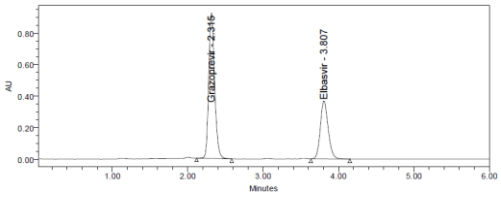


Figure 8: Chromatograms for Accuracy (50%) standard



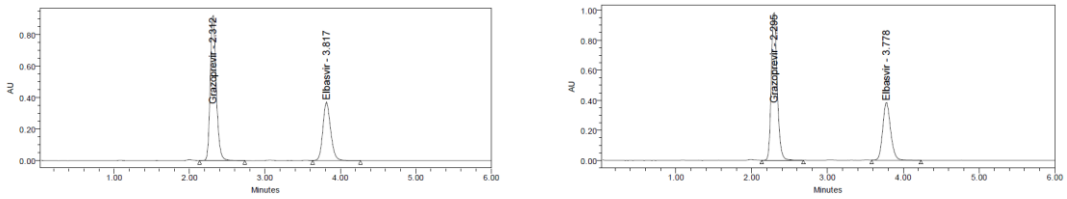


Figure 9: Chromatograms for Accuracy (100%) standard

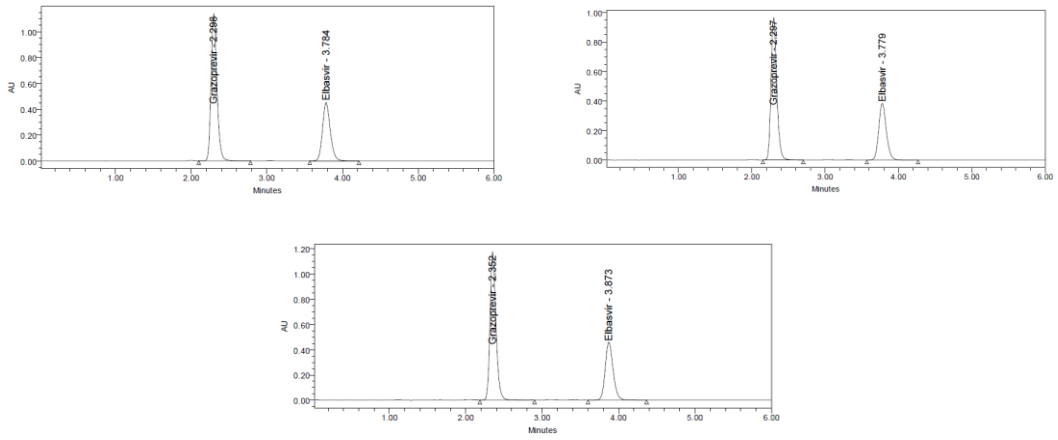


Figure 10: Chromatograms for Accuracy (150%) standard

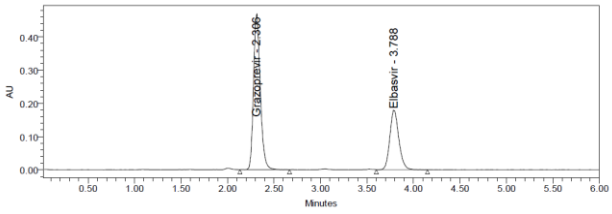


Figure 11: Effect of Variation of Flow Rate (0.7 ml/min)

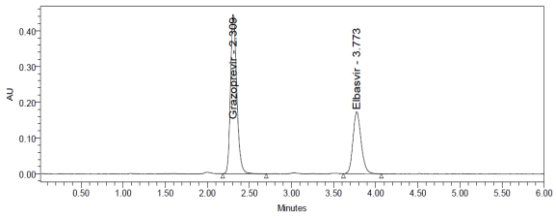


Figure 12: Effect of Variation of Flow Rate (1.1 ml/min)

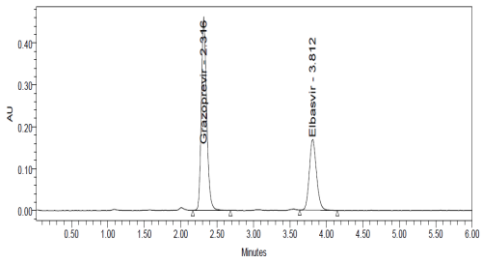


Figure 13: Effect of Variation of Column temp (35 ºC)

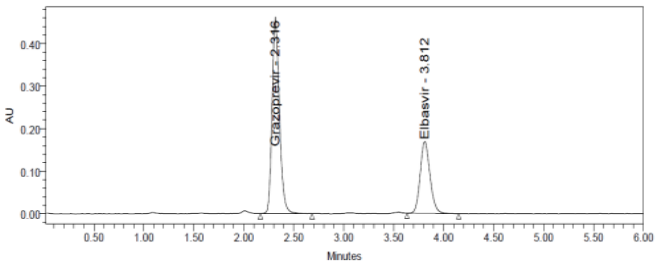


Figure 14: Effect of Variation of Column temp (25 ºC)

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