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

M. Lakshmi Surekha*, Muggu Muralikrishna and R. Padma

A. M. Reddy Memorial College of Pharmacy, Dept. of Pharmaceutical Analysis, Uppalapadu, Andhra Pradesh 522601. India.

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***Corresponding Author**
M. Lakshmi Surekha
A. M. Reddy Memorial College of Pharmacy, Dept of Pharmaceutical Analysis, Uppalapadu, Andhra Pradesh 522601. India.

ABSTRACT

A simple, rapid, accurate, precise and reproducible RP-HPLC method was developed for the estimation of Elbasvir and Grazoprevir in liquid dosage forms. The method was carried out using Inertsil ODS 3V(150mm x 4.6 mm), 5µm column in an binary mode with mobile phase comprising gradient mixture of pH 3.0 Potassium Di-Hydrogen phosphate and Acetonitrile. The flow rate was 1.2 ml/min and detection was carried out at 260 nm using a UV detector. The retention time for Elbasvir and Grazoprevir was found to be at 3.76 min and 9.74 min. The method for Elbasvir showed linearity in the concentration range of 153.7- 461µg/ml (R^2=1.000) and for Grazoprevir showed linearity in the concentration range of 12.6-37.8µg/ml (R^2=1.000). The recovery studies for Elbasvir and Grazoprevir also carried out and %RSD for reproducibility was found to be below 2%. The method was simple, sensitive and specific. Hence method can be used for the quantification of Elbasvir and Grazoprevir in pharmaceutical dosage form.

KEYWORDS: Elbasvir and Grazoprevir, RP-HPLC, validation.

INTRODUCTION

Elbasvir[1-9]**:** methyIN-(2S)-2{4-[(9S)-5-{2-[(2S)-1-[(2S)-2- [(methoxycarbonyl)amino]-3-methylbutanoyl]pyrrolidin-2-yl]-1H-imidazol-4-yl}-9-phenyl-8-oxa-10-azatetracyclo[8.7.0.$0^{2,7}$.$0^{11,16}$]heptadeca-1(17),2(7),3,5,11(16),12,14-heptaen-14-yl]-1H-imidazol-2-yl}pyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl]carbamate.

Molecular formula	**:**	**$C_{49}H_{55}N_9O_7$**
Molecular weight	**:**	**882.035** [g/mol]
Drug Category	**:**	BCRP/ABCG2 inhibitors
Appearance	**:**	White solid
Solubility	**:**	Soluble in alcohol and water.
pK_a value	**:**	12.42
Melting point	**:**	102-106ºc

Mechanism of action

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. By combining two direct-acting antiviral agents with distinct mechanisms of action and non-overlapping resistance profiles (elbasvir and grazoprevir) into the fixed dose combination product Zapatier. This medication targets HCV at multiple steps in the viral lifecycle with improved resistance rates.

Grazoprevir: 17- (cyclopropylmethyl)- 4, 5α-epoxy-3, 14-dihydroxymorphinan-6-one hydrochloride. Antagonists that can reverse the actions of opioids are also very important and it is used as an Antidote for opioid Poisoning fig .1

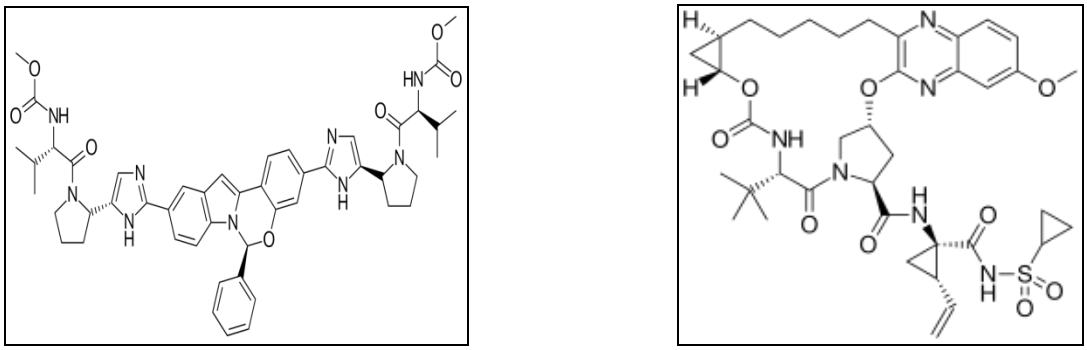

Fig 1: Chemical structure of (a) Elbasvir (b) Grazeprivir Chemical structure of Grazoprevir.

IUPAC name :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[24.2.1.03,12.05,10.018,20]nonacosa-3,5,7,9,11-pentaene-27carboxamide.

Molecular formula	**:**	**$C_{38}H_{50}N_6O_9S$**
Molecular weight	**:**	766.901[g/mol]
Drug Category	**:**	Ns3/4A Protease inhibitors

Appearance : white – off- white solid

Solubility : Soluble in Water, alcohal

pK_a value : 5.31

Mechanism of action

Grazaprevir is a second generation NS3/4a protease inhibitor use to inhibit viral HCV replication. NS3/4a protease is an integral part of viral replication as it is responsible for cleaving the long polypeptide produced following translation of the viral genome. By inhibiting protease activity, grazoprevir prevents the formation of structural and nonstructural proteins required for replication and assembly (E1, E2, NS2, NS3, NS4A, NS4B, NS5A and NS5B).

MATERIALS AND METHODS[10-15]

Instrument used was an UV-Visible double beam spectrophotometer, SHIMADZU (model UV-1800, software – UV probe, version 2.42) with a pair of 1 cm matched quartz cells. All weighing was done on Sartorius electronic analytical balance.

Instrumentation and Chromatographic conditions[16-27]

The analysis was performed by using Chromosil C-18 column, 250 X 4.6mm internal diameter with 5 micron particle size column and UV detector set at 286.9 nm, in conjunction with a mobile phase of Acetonitrile and Water in the ratio of 60:40 v/v (pH 5 adjusted with OPA) at a flow rate of 0.8 ml/min. The retention time of Elbasvir and Grazoprevir Hydrochloride was found to be 2.136 and 5.485 minute. The 10µl of sample solution was injected into the system.

Preparation of standard solution

Accurately weigh and transfer 10 mg of Elbasvir and Grazoprevir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.6ml of the above Elbasvir and 0.3ml of the Grazoprevir stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Mobile Phase Optimization

Initially the mobile phase tried was Methanol: Water, Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Acetonitrile and water in proportion 75:25 v/v respectively.

Optimization of Column

The method was performed with various columns like C18 column, X- bridge column, Xterra. Phenomenex Luna C18 (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Optimized chromatogram)

Column	: Phenomenex Luna C18 (4.6×250mm) 5µ
Column temperature	: 35˚C
Wavelength	: 285nm
Mobile phase ratio	: Acetonitrile:Water(75:25 v/v)
Flow rate	: 1ml/min
Injection volume	: 10µl
Run time	: 7minutes

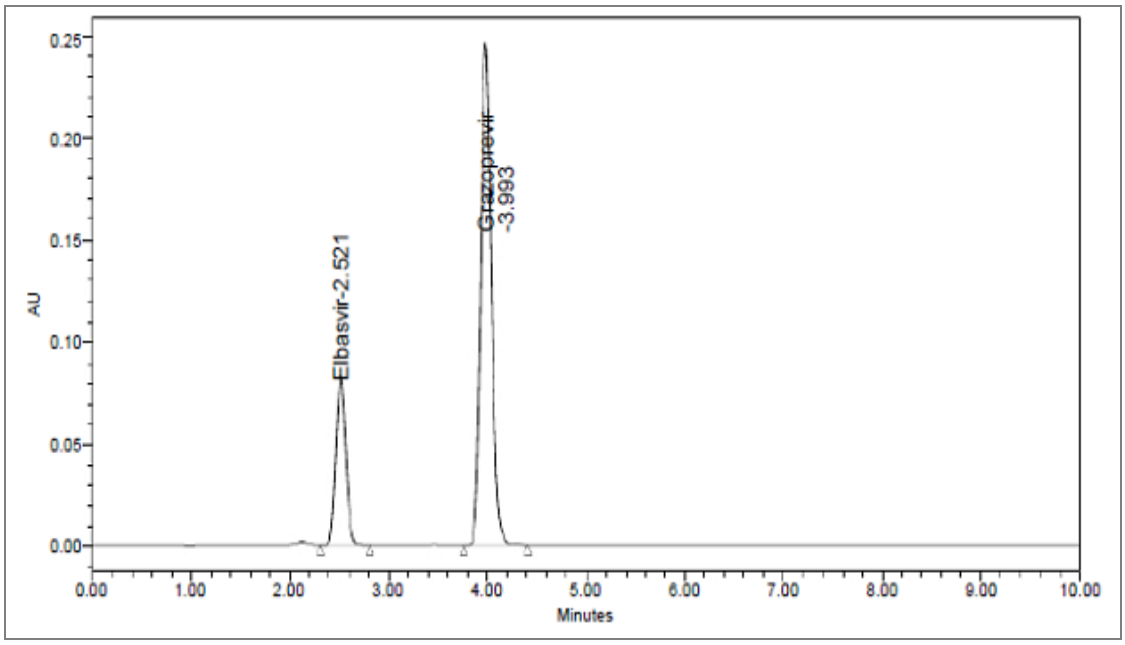

Fig 2: Typical chromatogram of mixture of Standard solution.

VALIDATION

PREPARATION OF MOBILE PHASE

Preparation of mobile phase

Accurately measured 750ml (75%) of HPLC Acetonitrile and 250ml of Water (25%) were mixed and degassed in a digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

Linearity

The linearity of was obtained in the concentration ranges from 20-100 and 10-50 for

Table 1: Linearity data of Elbasvir.

Concentration Level (%)	Concentration µg/ml	Average Peak Area
60	20	909889
80	40	1583641
100	60	2395378
120	80	3185089
140	100	3943725

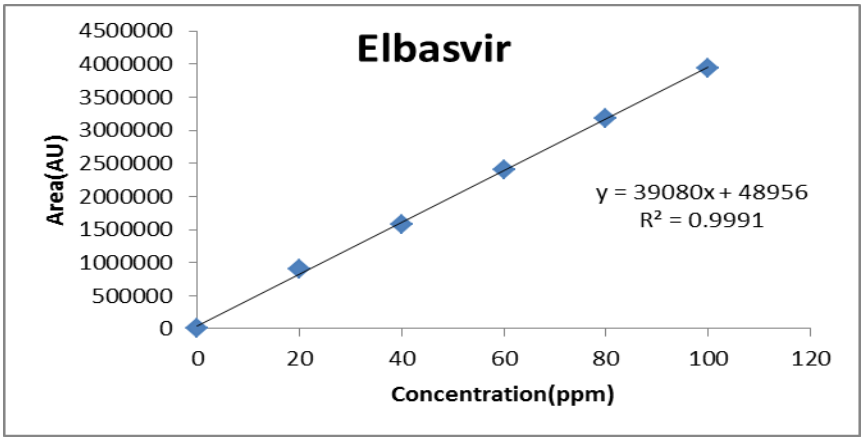

Fig 3: calibration graph of Elbasvir.

LINEARITY PLOT

Linearity of detector response of assay method was found by injecting seven standard solutions with concentration ranging from 20-100 and 10-50µg/mL for Elbasvir and Grazoprevir respectively. The graph was plotted for concentration versus peak area. The results were shown in Table-2 & 3 and fig 2 & 3.

Table 2: Linearity data of Grazoprevir.

Concentration Level (%)	Concentration µg/ml	Average Peak Area
60	10	61953
80	20	130213
100	30	198697
120	40	267002
140	50	321658

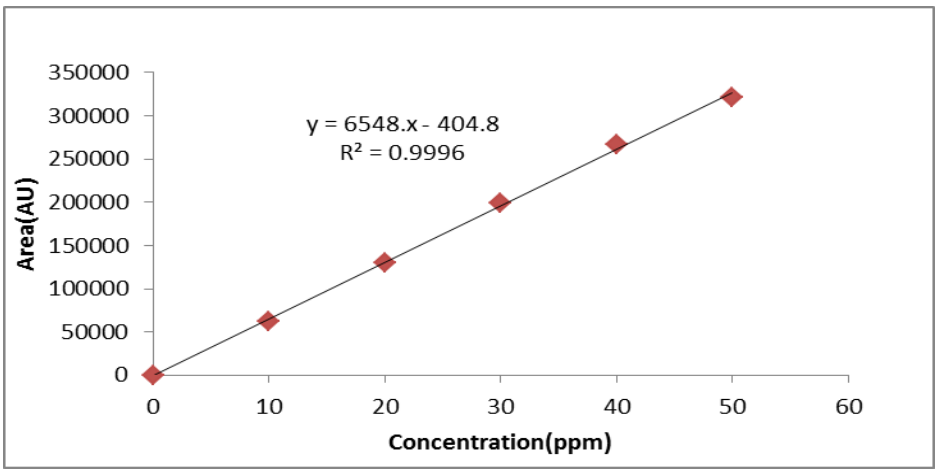

Fig 4: Calibration graph of Grazoprevir.

Precision

Repeatability

The precision of test method was determined by preparing six test preparations using the product blend and by mixing the active ingredient with excipients as per manufacturing formula. And the relative standard deviation of assay results was calculated. The results were shown in Table 3 & 4.

Table 3: Results of repeatability for Elbasvir.

S. No	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Elbasvir	3.213	2397164	381741	8155	1.2
2	Elbasvir	3.253	2391741	371742	9174	1.2
3	Elbasvir	3.297	2371846	391746	7154	1.2
4	Elbasvir	3.215	2361748	391847	9917	1.2
5	Elbasvir	3.254	2371649	384622	9247	1.2
Mean			2378830			
Std.dev			14958			
%RSD			0.628797			

Table 4: Results of repeatability for Grazoprevir.

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Grazoprevir	5.441	198464	7291	6274	1.1
2	Grazoprevir	5.442	193643	7219	6592	1.1
3	Grazoprevir	5.409	196462	7194	6028	1.1
4	Grazoprevir	5.520	194644	8174	6927	1.1
5	Grazoprevir	5.424	198464	8653	5920	1.1
Mean			196335.4			
Std.dev			2190.191			
%RSD			1.115536			

Accuracy

Elbasvir and Grazoprevir tablets content were taken at various concentrations ranging from 50% to 150% (50%, 75%, 100%, 125%, and 150%) to accurately quantify and to validate the accuracy. The assay was performed in triplicate. The results were shown in Table-5 & 6.

Table 5: The accuracy results for Elbasvir.

%Concentration (at specification Level)	Peak area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1217218	30	29.4	99.1	99.5
100%	2397141	60	59.5	99.6	
150%	3514547	90	89.7	99.8	

Table 6: The accuracy results for Grazoprevir.

%Concentration (at specification Level)	Peak area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	98598.67	15	14.6	99.9	99.6
100%	198359.7	30	30.0	100	
150%	291512.3	45	44.7	99	

LIMIT OF DETECTION (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The LOD and LOQ values for Elbasvir and Grazoprevir 3.3µg/ml and 2.5µg/ml respectively.

Quantitation limit (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined. The LOQ values for Elbasvir and Grazoprevir 7.4µg/ml and 10.1µg/ml.

ROBUSTNESS

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Elbasvir and Grazoprevir. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard sample of Elbasvir and Grazoprevir were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor and plate count. Table 7 & 8.

Table 7: Results for Robustness of Elbasvir.

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0mL/min	2391746	3.202	9028	1.2
Less Flow rate of 0.9mL/min	2371831	3.639	7381	1.2
More Flow rate of 1.1mL/min	2218319	2.859	9311	1.1
Less organic phase (about 5 % decrease in organic phase)	2294821	3.460	7462	1.2
More organic phase (about 5 % Increase in organic phase)	2394811	3.022	6817	1.1

Table 8: Results for Robustness of Grazoprevir.

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.1mL/min	194627	5.463	7398	1.1
Less Flow rate of 0.9mL/min	183738	6.250	6883	1.1
More Flow rate of 0.8mL/min	198373	4.863	9917	1.2
Less organic phase (about 5 % decrease in organic phase)	178471	6.196	8372	1.1
More organic phase (about 5 % Increase in organic phase)	189462	5.010	7716	1.2

SUMMARY AND CONCLUSION

The developed HPLC method offers several advantages such as rapidity, usage of simple mobile phase and easy sample preparation steps. Further, improved sensitivity makes it specific and reliable for its intended use. Hence, this method can be applied for the analysis of pure drug and pharmaceutical dosage forms.

From the present study it can be concluded that the proposed method is simple, sensitive, precise, specific, accurate and reproducible. Results of validation parameters demonstrated

that the analytical procedure is suitable for its intended purpose. The results are shown in Table 9.

Table 9: Summary data for Grazoprevir and Elbasvir.

Parameters	Grazoprevir	Elbasvir
Retention Time (min.)	5.463	3.202
Linearity (µg/ml)	10-50µg/ml	20-100µg/ml
Correlation Coefficient (r2)	0.999	0.999
Slope	6548	39080
Y - intercept	454.8	48956
LOD (µg/ml)	2.5	3.3
LOQ (µg/ml)	7.6	10.1
Repeatability (% RSD) n=6	1.1	0.6
Intraday Precision (% RSD) n=6	0.7	1.6
Interday Precision (% RSD) n=6	0.4	0.2
Accuracy (%)	99.6	99.3

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