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LC-MS/MS METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS QUANTIFICATION OF ELBASVIR AND GRAZOPREVIR IN HUMAN PLASMA

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ABSTRACT
A simple, sensitive and fast throughput liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been developed for the simultaneous estimation of Elbasvir and Grazoprevir, in human plasma, using respective deuteriated drug as internal standards. The method involved Solid Phase Extraction (SPE) of the analytes and internal standards from human plasma. The chromatographic separation was achieved on a Gemini, C18, (50×4.6mm and 5µm particle size) analytical column using isocratic mobile phase, consisting of 10mM Ammonium Acetate, Acetonitrile and Methanol (30:56:14 v/v), at a flow-rate of 1.0 mL/min with 75% flow splitting. The parent product ion transitions m/z 882.51 → 656.42 and m/z 888.49 → 662.43 for Elbasvir and Elbasvir-D6 respectively and the grazoprevir was detected at m/z 767.3/553.2 and Grazoprevir-D6 at m/z 773.3/559.2. The analytes and internal standards were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) positive ion modes. The method was validated over the concentration range of 2.00-600 ng/mL and 0.5-150 µg/mL for Elbasvir and Grazoprevir respectively. The mean recovery values for both the drugs from spiked plasma samples were reproducible. The method was rugged and rapid with a total run time of 2.0 minutes.

KEYWORDS: Elbasvir; Grazoprevir; Elbasvir-D6; Grazoprevir-D6; LC–MS/MS; Liquid/liquid extraction.

1. INTRODUCTION
Elbasvir is an HCV NS5A inhibitor, and grazoprevir is an HCV NS3/4A protease inhibitor The IUPAC name for elbasvir is Dimethyl N,N'-([(6S)-6-phenylindolo[1,2-c][1,3] benzo xazine-3,10- diyl]bis{1H-imidazole-5,2-diyl-(2S)- pyrrolidine-2,1-diyl[(2S)-3-methyl-1-xobutane-1,2-diyl]}) dicarbamate. It has a molecular formula of $C_{49}H_{55}N_9O_7$ and a molecular weight of 882.02. Elbasvir is practically insoluble in water (less than 0.1 mg per mL) and very slightly soluble in ethanol (0.2 mg per mL), but is very soluble in ethyl acetate and acetone. The IUPAC name for grazoprevir is (1aR,5S,8S,10R,22aR) -N-[(1R,2S)-1-(Cyclopropylsulfonamido) carbonyl]-2-ethenylcyclopropyl]-14-methoxy-5-(2-methylpropan-2-yl)-3,6-dioxo-1,1a,3,4,5,6,9,10,18,19,20,21,22,22a-tetradeca hydro-8H-7,10-methanocyclopropa[18,19][1,10,3,6] dioxadiaza cyclone onadecino[11,12-b]quinoxaline-8-carboxamide. It has a molecular formula of $C_{38}H_{50}N_6O_9S$ and a molecular weight of 766.90. Grazoprevir is practically insoluble in water (less than 0.1 mg per mL) but is freely soluble in ethanol and some organic solvents (e.g., acetone, tetrahydrofuran and N,N-dimethyl formamide). The combination product, ZEPATIER® contains two direct-acting antiviral agents with distinct mechanisms of action and non-overlapping resistance profiles to target HCV at multiple steps in the viral lifecycle. Elbasvir is an inhibitor of HCV NS5A, which is essential for viral RNA replication and virion assembly. Grazoprevir is an inhibitor of the HCV NS3/4A protease which is necessary for the proteolytic cleavage of the HCV encoded polyprotein (into mature

forms of the NS3, NS4A, NS4B, NS5A, and NS5B proteins) and is essential for viral replication. In a biochemical assay, grazoprevir inhibited the proteolytic activity of the recombinant NS3/4A protease enzymes from HCV genotypes 1a, 1b, 3 and 4a with IC50 values ranging from 4 to 690 pM.. Each tablet contains 50 mg elbasvir and 100 mg grazoprevir. The tablets include the following inactive ingredients: colloidal silicon dioxide, copovidone, croscarmellose sodium, hypromellose, lactose monohydrate, magnesium stearate, mannitol, microcrystalline cellulose, sodium chloride, sodium lauryl sulfate, and vitamin E polyethylene glycol succinate. The tablets are film-coated with a coating material containing the following inactive ingredients: carnauba wax, ferrosoferric oxide, hypromellose, iron oxide red, iron oxide yellow, lactose monohydrate, titanium dioxide, and triacetin. Very few quantitative methods for the estimation of Elbasvir and Grazoprevir have appeared in literatures using HPLC with UV and Mass detection.[4–6] All these methods are for the estimation of either of the drugs instead of simultaneous estimation of both Elbasvir and Grazoprevir. In addition these methods are with large quantity of plasma samples usage, having long run times. However there was no single method reported for the combination of these drugs and these combinations are relatively new in the market as only few companies recently started marketing them. All the individual methods required to work and operate twice for the estimation of the drugs independently, while in reality the samples having the combined drug concentrations in plasma. The combination method will provide simple, rapid and fast throughput simultaneous estimation of Elbasvir and Grazoprevir. Thus, in the present study the objective was to develop a simple, sensitive and fast throughput method for simultaneous determination of Elbasvir and Grazoprevir in human plasma to support the pharmacokinetic testing. The proposed validated LC-MS/MS method provides useful approach into the quantification of the drug concentrations simultaneously in human plasma and exhibited excellent performance in terms of sensitivity, selectivity, ruggedness and efficiency with run time of 2.0 min per sample and broad range of Quantification (from 2.00-600 ng/mL and 0.5-150 µg/mL for Elbasvir and Grazoprevir.

2. EXPERIMENTAL

2.1 Chemicals and materials: Working reference standards of Elbasvir was procured from Dr. Reddy's Laboratories Ltd., India as a gift sample, Grazoprevir was procured from Clearsynth Laboratories, India whereas Elbasvir-D6 and Grazoprevir-D6 were procured from Neucon Pharma, India. HPLC grade methanol, acetonitrile, Analytical Reagent (AR) grade ammonium sulphate and sodium hydrogen carbonate were procured from Merck, India. Water used in the entire analysis was obtained from the in-house Milli Q water purification system. Solid Phase Extraction (SPE) cartridges, DVB LP 30mg/1mL were procured from Orochem Technologies. USA. Blank human plasma was obtained from the blood bank of Supratech Micropath Laboratory, India and this drug free plasma was stored at –20°C until use.

2.2 Liquid chromatographic conditions: A Shimadzu LC system (Japan) consisting of binary gradient pumps, auto-sampler and column oven was used for setting the reverse-phase liquid chromatographic conditions. The analysis of Elbasvir and Grazoprevir was performed on analytical column, Gemini, C18 (50x4.6mm with 5µm particle size) and maintained at 40°C in column oven. The mobile phase consists of 56% acetonitrile, 14% of methanol and 30% of 10mM ammonium acetate buffer. The flow rate of the mobile phase was kept at 1.0 mL/min with 75% flow splitting. The total chromatographic run time was 2.0min. The samples were maintained at a temperature of 5°C in the auto-sampler.

2.3 Mass Spectrometric conditions: Analyst software with version 1.4.1 was used to control all parameters of HPLC and MS. Ionization and detection of analytes and internal standards were carried out on a triple quadrupole mass spectrometer, MD Sciex API 4000 Mass Spectrometer equipped with electro spray ionization and operating in negative ion mode. Quantification was performed using selected ion monitoring (SIM) mode to monitor the parent→product ion transitions (m/z) transitions m/z 882.51 → 656.42 and m/z 888.49 → 662.43 for Elbasvir and Elbasvir-D6 respectively and the grazoprevir was detected at m/z 767.3/553.2 and Grazoprevir-D6 at m/z 773.3/559.2 respectively. The source dependent parameters were maintained for Curtain gas (CUR) at 40.00, Ion spray voltage (IS) at -4500.00, Temperature (TEM) at 450.00, Nebulizer gas(GS1) at 40.00, Heater gas(GS2) at 60.00, Interface Heater(ihe) at ON and Collision gas (CAD) at 5.00. The optimum analyzer parameters are given in **Table 1**.

2.5 Analytical data processing

Peak area ratios of Elbasvir/Elbasvir-D6 (ISTD) and Grazoprevir/ Grazoprevir-D6 (ISTD) were obtained from multiple reaction monitoring and utilized for the construction of calibration curves, using weighted ($1/x^2$) linear least squares regression of the plasma concentrations. Data collection, peak integration, and calculations were performed using Analyst software version 1.4.1. The regression equation for the calibration curve was also used to back calculate the measured concentration at each standard and control sample.

2.6 Standard stock, calibration standards and control sample preparation: The standard stock solutions of Elbasvir (1 mg/mL), Grazoprevir (20 mg/mL), Elbasvir-D6 (1 mg/mL) and Grazoprevir-D6 (1 mg/mL) were prepared by dissolving requisite amount of drug in methanol. Diluted combined stock solution was prepared by diluting the individual stocks with methanol to obtain 50 and 15000 µg/mL of Elbasvir and Grazoprevir respectively. Calibration standards and control samples were prepared by spiking in drug free blank plasma with

combined stock solution. Eight calibration curve standards were made for both Elbasvir (at 2.00, 4.00, 20.0, 40.0, 80.0, 160, 300 and 600 ng/mL) and Grazoprevir (0.500, 1.00, 5.00, 10.0, 20.0, 40.0, 75.0 and 150 µg/mL) while control samples were prepared at four levels for Elbasvir (2.00, 6.00, 240 and 480 ng/mL) and Grazoprevir (0.500, 1.50, 60.0 and 120 µg/mL). Combined internal standard stock solution of Elbasvir-D6 (1.0 µg/mL) and Grazoprevir-D6 (5.0 µg/mL) was prepared by diluting Elbasvir-D6 and Grazoprevir-D6 stock solutions in methanol. All the aqueous solutions (standard stock, spiking solutions of calibration standards and control samples) were stored at 2–8°C and used as per the requirement of the experiments. All the plasma spiked samples were stored in deep freezer at below -20^{0}C and at below -70^{0}C and used as per the requirement of the experiments.

2.7 Sample processing: All frozen samples, calibration standards and control samples were thawed by allowing them to equilibrate to room temperature. To an aliquot of 200 µL of spiked plasma sample, 50µL of mixed ISTD dilution (1µg/mL Elbasvir-D6 & 5µg/mL Grazoprevir-D6) was added to all the samples except STD Blank and vortexed for about 30 seconds. 200µL of extraction buffer (0.1N sodium hydrogen carbonate in water) was added to all samples and vortexed for about 30 seconds. All the samples were centrifuged at 4000rpm for 2 minutes by using refrigerated centrifuge maintained at 10±2°C. Required number of pre-labeled Orochem DVB LP 30mg/1mL extraction cartridges were arranged on EZYPRESS® 48 – 48 Position Positive Pressure Processor. All the cartridges were conditioned with 1.0mL of methanol followed by 1.0mL water. 400µL of the prepared samples were loaded carefully on the conditioned cartridges. The cartridges were washed with 1mL of water followed by 1mL of 10% methanol in water, and were dried for 2 minutes by applying positive pressure at maximum flow rate. The contents from the cartridges were eluted with 1mL of methanol into pre-labeled tubes and vortexed for mixing. All the contents were transferred into pre-labeled auto-sampler vials, and injected in to HPLC System.

2.8 Bioanalytical method validation

Bioanalytical method validation was carried out as per the USFDA Method Validation guidelines.[11] Following parameters were evaluated during the course of Method Validation.

2.8.1 System Suitability and Auto- sampler Carryover: System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of Elbasvir, Grazoprevir, Elbasvir-D6 and Grazoprevir-D6 at start of each batch during the method validation. The carryover test was performed by injecting a sequence of samples consisting of aqueous standards (Drugs and ISTDs), reconstitution solution, extracted standard (Drugs and ISTDs) equivalent to highest standard and standard blank.

2.8.2 Linearity: The linearity of the method was determined by analysis of standard plots associated with an eight point standard calibration curve. Three linearity curves containing eight non-zero concentrations were analyzed. The ratio of area response for Elbasvir to Elbasvir-D6 and Grazoprevir to Grazoprevir-D6 was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression which was selected and finalized during method development. Back calculations were done from these curves to determine the concentration of Elbasvir and Grazoprevir in each calibration standard.

Acceptance criterion set for linearity standard were as follows - Correlation coefficient (r) for all the analytical batches should be greater than 0.99. In the lower limit of quantification (LLOQ), the analyte response should be at least five times more than the response obtained from drug free (blank) extracted plasma sample. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with a precision (%CV) not greater than 20.0 and accuracy within 80.0–120.0%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0%.

2.8.3 Selectivity: The selectivity of the method towards endogenous plasma matrix components was assessed in ten plasma lots (7 lots of normal of K3 EDTA plasma, 1 haemolysed, 1 lipidemic and 1 heparinised) of blank human plasma which were processed as per the proposed sample preparation protocol and then chromatographed to determine the extent to which endogenous plasma components may contribute towards interference at the retention time of analytes and internal standards. The cross talk of MRM for analytes and internal standards was checked using highest standard on calibration curve and working solution of internal standard.

2.8.4 Recovery: The absolute recovery of Elbasvir, Grazoprevir, Elbasvir-D6 and Grazoprevir-D6 was performed at low, middle and high quality control levels. It was evaluated by comparing the mean area response of five replicates of extracted samples (Blank plasma spiked with analyte followed by Solid Phase Extraction) to that of unextracted samples (Solid Phase extraction of blank plasma followed by spiking the drug to the extract) at each quality control levels. The recovery of internal standards was estimated similarly. As per the acceptance criteria, the recovery of the analytes need not be 100.0%, but should be consistent, precise and reproducible.

2.8.5 Precision and Accuracy: For determining the intra-day accuracy and precision, replicate analysis of plasma samples of Elbasvir and Grazoprevir was performed on the same day. The run consisted of a calibration curve and five replicates each of LLOQ, low, middle, high quality control samples. The inter-day accuracy and precision were assessed by analysis of three precision and accuracy batches on three consecutive validation days. The precision of the method was determined by

calculating the percent coefficient of variation (%CV) for each level. The deviation at each concentration level from the nominal concentration was expected to be within ±15.0% except for LLOQ, for which the acceptance criteria is not be more than 20.0%. Similarly, the mean accuracy should not deviate by ±15.0% except for the LLOQ where it can be ±20.0% of the nominal concentration.

2.8.6 Ion Suppression: To study the ion suppression/ enhancement, the post column infusion was used during the method development. To study the effect of matrix on analytes quantification with respect to consistency in signal enhancement/ suppression, it was checked in six different lots. Six samples of LLOQ levels were prepared from six different lots of plasma and checked for the % accuracy and precision. This was assessed by comparing the back calculated value from the control samples to nominal concentration. The deviation of the standards should not be more than ±15.0% and at least 80% of the lots should be within the aforementioned criteria.

2.8.7 Stability: Stability experiments were carried out to examine the stability of analytes in stock solutions and in plasma samples under different conditions. Short term and long term stock solution stability at room temperature was assessed by comparing the area response of stability sample of analytes and internal standards with the area response of sample prepared from fresh stock solutions. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Auto-sampler stability, bench top stability, dry extract stability and freeze-thaw stability were performed at low and high quality control samples using three replicates at each level. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed control samples was within ±15.0. Auto-sampler re-injection reproducibility was assessed by re-injecting one accepted precision and accuracy batch which was stored in the auto-sampler.

2.8.8 Ruggedness: To authenticate the ruggedness of the proposed method, it was done on three precision and accuracy batches. The first batch was analyzed by different analyst, second batch with different column and the third batch was analyzed on different LC-MS/MS system.

Dilution Integrity: Dilution integrity experiment was evaluated by diluting the stock solution prepared as spiked standard at concentrations of 1080 ng/mL for Elbasvir and 270 µg/mL for Grazoprevir. The precision and accuracy were found within ±15% from its nominal values for dilution integrity standards 540 ng/mL after 1:2 dilution and 108 ng/mL after 1:10 dilution for Elbasvir and 135 µg/mL after 1:2 dilution and 27µg/mL after 1:10 dilution for Grazoprevir. Back calculated concentrations were determined by analyzing the samples against calibration curve standards.

3. RESULTS AND DISCUSSIONS

Table. 1: Analysis Condition in ESI.

Compound	Transition(m/z)	DP	EP	CE	CXP
Elbasvir	344.10/194.00	-55	-10	-25	-6
Elbasvir-D6	347.30/197.00	-55	-10	-24	-18
Grazoprevir	229.20/169.00	-60	-10	-50	-15
Grazoprevir-D6	232.20/169.00	-40	-10	-35	-35

Table. 2: Summary of Linearity Standards for Elbasvir and Grazoprevir.

	Elbasvir							
STD ID	**STD 8 (2.00 ng/mL**	**STD 7 (4.00 ng/mL)**	**STD 6 (20 .00 ng/mL)**	**STD 5 (40 .00 ng/mL)**	**STD 4 (80.00 ng/mL)**	**STD 3 (160.00 ng/mL)**	**STD 2 (300 .00 ng/mL)**	**STD 1 (600 .00 ng/mL)**
n	28							
Mean	1.96	4.09	21.8	42.8	79.7	156	288	552
SD	0.0851	0.149	0.548	1.86	3.32	7.11	9.86	27.2
%CV	4.34	3.64	2.51	4.35	4.17	4.56	3.42	4.93
% Bias	-2.00	2.25	9.00	7.00	-0.38	-2.50	-4.00	-8.00
	Grazoprevir							
STD ID	STD 8 (500 ng/mL	STD 7 (1000 ng/mL)	STD 6 (5000 ng/mL)	STD 5 (10000 ng/mL)	STD 4 (20000 ng/mL)	STD 3 (40000 ng/mL)	STD 2 (75000 ng/mL)	STD 1 (150000 ng/mL)
n	28							
Mean	498	998	5200	10200	19700	39500	74400	147000
SD	11.3	27.9	167	379	568	2330	3190	7760
%CV	2.27	2.80	3.21	3.72	2.88	5.90	4.29	5.28
% Bias	-0.40	-0.20	4.00	2.00	-1.50	-1.25	-0.80	-2.00

Table. 3: Intra-day and Inter-day quality control samples for Elbasvir and Grazoprevir.

QC	Elbasvir				Grazoprevir			
Intra-batch	LLOQ QC (2 ng/mL	LQC (6 ng/mL)	MQc (240 ng/mL)	HQC (480 ng/mL)	LLOQ QC (5000 ng/mL)	LQC (1500 ng/mL)	MQc (60000 ng/mL)	HQC (120000 ng/mL)
Mean	1.91	6.25	220.00	500.00	465.00	1520.00	56100.00	131000.00
SD	0.0841	0.239	2.88	7.09	16.70	37.00	1210.00	548.00
%CV	4.40	3.82	1.31	1.42	3.59	2.43	2.16	0.42
% Bias	-4.50	4.17	-8.33	4.17	-7.00	1.33	-6.50	9.17
Mean	1.64	6.64	227.00	496.00	441	1570.00	59100.00	134000.00
SD	0.0851	0.222	4.30	6.80	25.3	50.30	1290.00	3030.00
%CV	5.19	3.34	1.89	1.37	5.74	3.20	2.18	2.26
% Bias	-18.00	10.67	-5.42	3.33	-11.80	4.67	-1.50	11.67
Mean	1.82	5.83	215	451	508	1440	56700.00	124000.00
SD	0.182	0.301	4.49	11.5	32.8	45.1	444.00	1100.00
%CV	10.00	5.16	2.09	2.55	6.46	3.13	0.78	0.89
% Bias	-9.00	-2.83	-10.42	-6.04	1.60	-4.00	-5.50	3.33
Inter-batch								
Mean	1.79	6.24	221.00	482.00	471	1510	57300.00	130000.00
SD	0.163	0.418	6.23	24.30	37.0	71.7	1640.00	4490.00
%CV	9.11	6.70	2.82	5.04	7.86	4.75	2.86	3.45
% Bias	-10.50	4.00	-7.92	0.42	-5.80	0.67	-4.50	8.33

Table. 4: Stability of Elbasvir and Grazoprevir in Human plasma at two QC levels (n=5).

Stability Condition	Compound	Nominal Concentration (ng/mL)	Calculated concentration	
			Mean ± SD	% Bias
Bench Top stability	Elbasvir	6	5.94±0.240	-1.00
		480	477±9.560	-0.63
	Grazoprevir	1500	1360±27.9	-9.33
		120000	117000±836.66	-2.50
Wet extract Stability	Elbasvir	6	6.07±0.113	1.17
		480	481±7.530	0.21
	Grazoprevir	1500	1410±42.1	-6.00
		120000	122000±1140	1.67
Freeze thaw stability after 5 cycles at -20°C	Elbasvir	6	6.69±0.243	11.50
		480	463±8.44	-3.54
	Grazoprevir	1500	1630±74.8	8.67
		120000	119000±2610	-0.83
Freeze thaw stability after 5 cycles at - 78°C	Elbasvir	6	6.56±0.254	9.33
		480	469±8.26	-2.29
	Grazoprevir	1500	1650±53.9	10.00
		120000	122000±1870	1.67
Autosampler Stability	Elbasvir	6	6.18±0.166	3.00
		480	498±15.3	3.75
	Grazoprevir	1500	1580±56.3	5.33
		120000	128000±4760	6.67

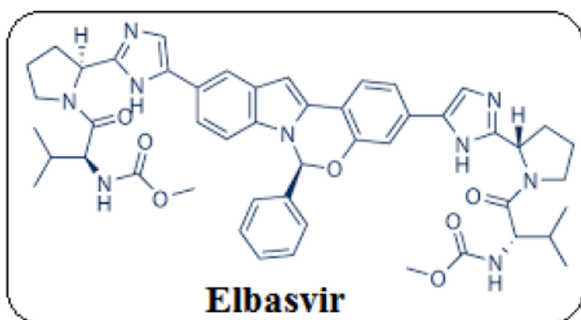

Figure. 1: Structure of Elbasvir.

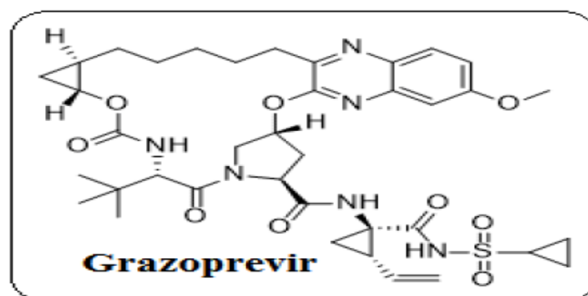

Figure. 2: Structure of Grazoprevir.

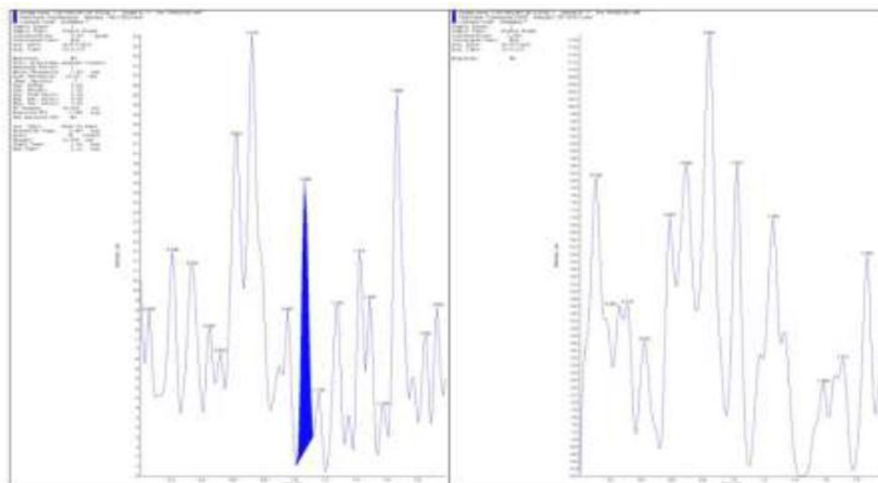

Figure. 3: Representative Chromatogram of Blank Sample of Elbasvir.

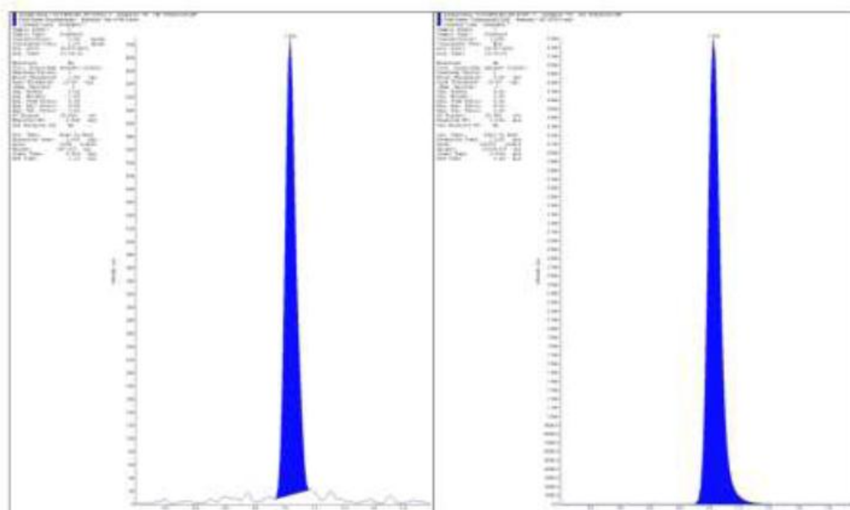

Figure. 4: Representative Chromatogram of LLOQ Sample of Elbasvir.

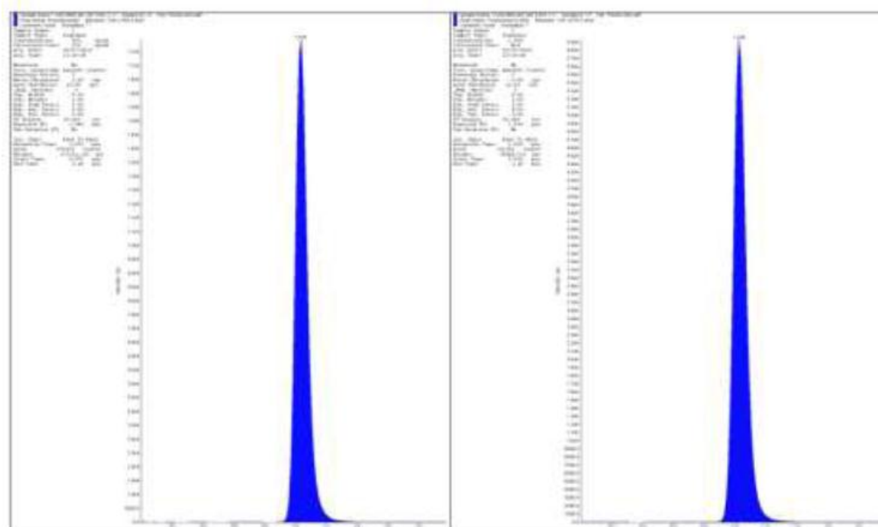

Figure. 5: Representative Chromatogram of UOQ Sample of Elbasvir.

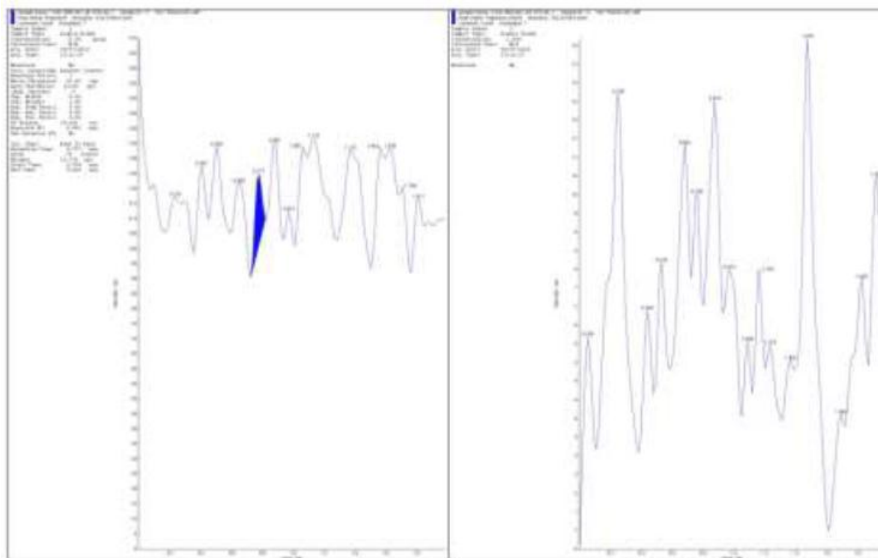

Figure. 6: Representative Chromatogram of Blank Sample of Grazoprevir.

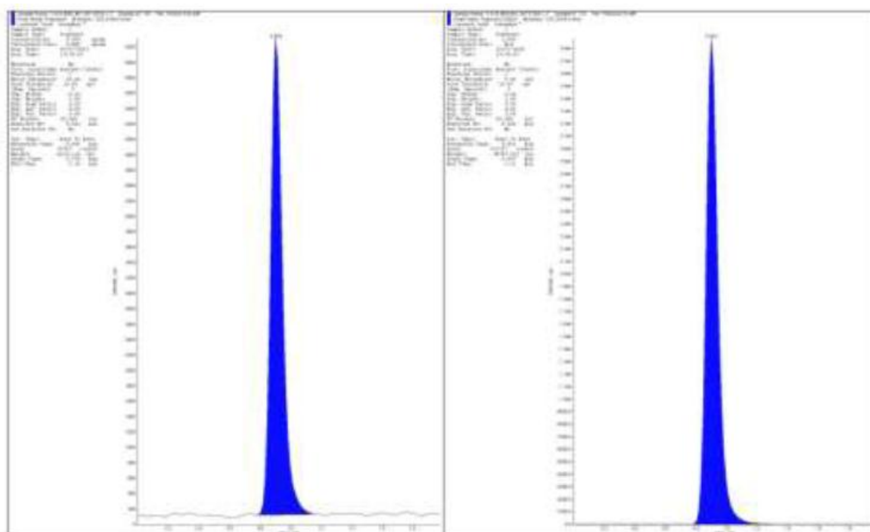

Figure. 7: Representative Chromatogram of LLOQ Sample of Grazoprevir.

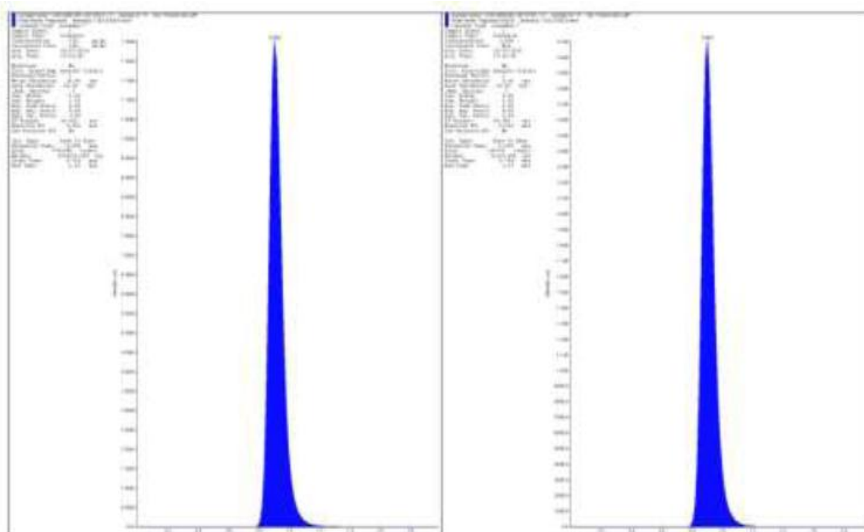

Figure. 8: Representative Chromatogram of ULOQ Sample of Grazoprevir.

3.1 Bioanalytical method development

To develop a rapid, rugged and precise method it was important to optimize the chromatographic and mass spectrometric conditions, as well as to have an efficient and simple extraction procedure for Elbasvir and Grazoprevir. Chromatographic analysis of Elbasvir, Grazoprevir and Internal standards was initiated under isocratic conditions to obtain adequate response, sharp peak shape and a short run time. It was also observed that the pH of extraction buffer is an important criterion. The analytes showed poor reproducibility for proposed linear range except of Gemini column that offered superior peak shape, efficient separation, desired linearity and reproducibility for Elbasvir and Grazoprevir and internal standards from endogenous plasma matrix. The mobile phase consisting of 10mM ammonium acetate in water: acetonitrile: methanol (30:56:14 v/v) ratio was found most suitable for eluting the Elbasvir, Grazoprevir, Elbasvir-D6 and Grazoprevir-D6 at 1.0, 0.9, 1.0 and 0.9 min respectively. A flow-rate of 1.0 mL/min with 75% flow splitting produced good peak shapes and permitted a run time of 2.0 min per analysis.

The present study was conducted using ESI as the ionization source as it gave high intensity for the Elbasvir, Grazoprevir and its internal standards, and a good linearity in regression curves. Initially, the parent and product ions were optimized by infusing 100 ng/mL solutions in the mass spectrometer, both in the positive and negative polarity modes between m/z 50 and 400 range. The intensity found was much higher in the negative ion mode for Elbasvir and Grazoprevir and the internal standards as they have similar sites for deprotonation. Also, the use of 10mM ammonium acetate in the mobile phase further enhanced the response of deprotonation precursor $[M-H]^-$ ions at m/z 344.10, 229.20, 347.30 and 232.20 in the Q1 MS full scan spectra for Elbasvir, Grazoprevir, Elbasvir-D6 and Grazoprevir-D6 respectively. The most abundant product ions at m/z m/z 882.51 → 656.42 and m/z 888.49 → 662.43 for Elbasvir and Elbasvir-D6 respectively and the grazoprevir was detected at m/z 767.3/553.2 and Grazoprevir-D6 at m/z 773.3/559.2. The MRM state file parameters like nebulizer gas, CAD gas, ion spray voltage and temperature were suitably optimized to obtain a consistent and adequate response for the analytes and internal standards. A dwell time of 200 ms for Elbasvir, Grazoprevir and their respective internal standards was adequate and no cross talk was observed between their MRMs.

Initially, the extraction of Elbasvir and Grazoprevir was carried out by protein precipitation with common solvents like acetonitrile, methanol and the obtained results are not satisfactory with respect to the sample cleanup as the solvents usable for the protein precipitation are not strong enough to clean up the sample fully and this may lead to column clogging, source contamination and finally contamination to the mass detector. Liquid-liquid extraction technique was also tested to isolate the drugs from plasma using diethyl ether, dichloromethane, methyl tertiary butyl ether, ethyl acetate and dichloromethane (alone and in combination) as extracting solvents. However, the recovery was inconsistent with the possible ion suppression. The extraction was tried in different pH but recovery was found to be less in the acidic condition and is mainly due to the degradation of the Elbasvir in acidic conditions. Finally development of the solid phase extraction method was opted and the results obtained with the optimized solid phase extraction conditions exhibited the required precision and accuracy. According to the internal standard selection guidelines, the internal standard should ideally mirror the analytes in as many ways as possible. It should preferably belong to the same class, with same physicochemical and spectral properties to significantly improve the method ruggedness. Different drugs like Lansoprazole, Ibuprofen and Mefenamic acid were tested as potential candidates for internal standard and finally, deuterated internal standard superseded all other candidates in terms of consistency and reproducibility. There was no significant effect of internal standards on analytes recovery, sensitivity or ion suppression. Moreover, there was no matrix effect of internal standards on Elbasvir and Grazoprevir. Also, the validation results obtained from this LC-MS/MS method encouraged its selection as internal standards for the present study.

System Suitability and System Performance

Throughout the method validation, the % CV of system suitability was ≤ 2.75 for retention time of Elbasvir, Elbasvir-D6 and their area ratio and ≤ 1.90 for the retention time of Grazoprevir, Grazoprevir-D6 and their area ratio, which are much less than the acceptance criteria of not more than 4%. System performance was monitored by signal to noise ratio, analyte and internal standard carryover during the method validation. The observed values were ≥ 59.6, ≤ 4.96% and ≤ 0.10% for Elbasvir and ≥ 68.8, ≤ 6.44 and ≤ 0.00 for Grazoprevir, which is with in acceptance limits of Signal to Noise ratio ≥5.0 for LLOQ sample, carryover ≤20.0% for the analyte and ≤5.0% for internal standard.

Linearity and Lower Limit of Quantification (LLOQ)

All the three calibration curves analyzed during the course of validation were linear for the standards ranging from 2.00 to 600 ng/mL and 0.500 to 150 µg/mL for Elbasvir and Grazoprevir respectively. A straight-line fit was made through the data points by least square regression analysis and a constant proportionality was observed. In order to establish the best weighting factor back-calculated calibration concentration was determined. The model with the lowest total bias and most consistent bias across the range was considered as the best fit. Weighting factor of 1/x2 was giving best possible results. Using weighted least squares with weights that are inversely proportional to the variance at each level of the explanatory variables yields the most precise parameter estimates possible. The mean values

for slope, intercept and correlation coefficient (r) observed during the course of validation were 0.0057, 0.0027 and 0.9940 for Elbasvir and 0.2938, 0.0129 and 0.9974 for Grazoprevir respectively. The %bias and precision (%CV) observed for the calibration curve standards was -8.0 to 9.0 and ≤ 4.93 for Elbasvir, and -2.00 to 4.00 and ≤ 5.00 for Grazoprevir respectively. The lower limit of quantification (LLOQ) achieved was 2.00 ng/mL for Elbasvir and 0.5μg/mL for Grazoprevir. The mean bias (%) for back calculated concentration was -2.00 with precision (%CV) of 4.34 for Elbasvir, and the mean bias (%) for back calculated concentration was -0.40 with precision (%CV) of 2.27 for Grazoprevir. **Table 2** summarizes the mean back calculated concentration with % bias and precision data for all the fourteen linearity curves.

Selectivity, Recovery, Precision and Accuracy (Bias)

The selectivity of the method towards endogenous plasma matrix was ascertained in six batches of human plasma by analyzing blanks and spiked plasma samples at LLOQ concentration. No endogenous peaks were observed at the retention time of the analytes for any of the batches. **Fig. 3&6.** Five replicates each at low, middle and high levels were prepared for recovery determination. The % mean recovery was 112.8% and 110.9% for Elbasvir and Grazoprevir respectively. The recovery of internal standards, Elbasvir-D6 and Grazoprevir-D6 was 111.9% and 113.5% respectively. The intra-batch and inter-batch accuracy and precision was determined in three batches at LLOQ, low, middle and high levels with six replicates for each batch.

For Elbasvir, the precision (%CV) for intra batch and inter batch is < 10.00 and < 9.11 respectively for all control samples. For Grazoprevir, the precision (%CV) for intra batch and inter batch is < 6.46 and < 7.86 respectively for all control samples.

For Elbasvir, the % bias for intra batch ranged from -18.00 to 10.67 and for inter-batch bias was from -10.50 to 4.00. For Grazoprevir, the % bias for intra batch ranged from -11.80 to 11.67 and for inter-batch bias was from -5.80 to 8.33. The detailed results are presented in **Table 3**.

Matrix effect and Stability

Matrix effect is due to co-elution of some components present in biological samples. These components may not give a signal in MRM of target analytes but can certainly decrease or increase the analytes response dramatically to affect the sensitivity, accuracy and precision of the method. Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC-MS/MS method for supporting pharmacokinetics studies. No significant signal suppression/enhancement was observed due to endogenous plasma matrix at the retention times of Elbasvir, Grazoprevir, Elbasvir-D6 and Grazoprevir-D6using post column infusion. The % mean accuracy of back calculated concentration for LLOQ samples from six different matrix lots was 93% with precision of 5.59% for Elbasvir and 96% with precision of 5.98% for Grazoprevir.

Stock solutions of Elbasvir, Grazoprevir, Elbasvir-D6 and Grazoprevir-D6 were stable at room temperature for minimum period of 7.0 hours and when stored between 2-8°C they were stable for 7 days. Elbasvir, Grazoprevir, Elbasvir-D6 and Grazoprevir-D6 in control human plasma (bench top) at room temperature was stable for at least 7.0 hours at 25°C and for minimum of five freeze and thaw cycles at temperatures -20^{0}C and -78^{0}C. Auto sampler stability of the spiked control samples maintained at 5^{0}C was determined up to 46.0 hours. Long term stability of the spiked control samples stored at -78^{0}C was found stable for 65 days. Different stability experiments in plasma and the values for the precision and percent change are shown in **Table 4**.

Ruggedness and Dilution Integrity

Ruggedness was performed by using three precision and accuracy batches. The first batch was analyzed by different analyst, the second batch was analyzed on different column and the third batch was analyzed on different equipment.

For all the experiments for Elbasvir, the precision was ≤ 8.04 and the bias is varied within ±12.92% and for Grazoprevir, the precision was < 3.22% and bias is ±8.00% respectively which is within the acceptance limit of 15% in precision and ±8.00% in mean bias.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analytes concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis.

For Elbasvir, the precision for dilution integrity of 1/2 and 1/10th dilution were 1.12 and 7.27%, while the bias was -9.72 and -6.48% respectively and for Grazoprevir, the precision for dilution integrity of 1/2 and 1/10th dilution were 1.51 and 6.76%, while the bias was -13.33% and -10.74% respectively, which is within the acceptance limit of 15% for precision (CV) and ± 15% of bias.

CONCLUSIONS

The bioanalytical methodology for determination of Elbasvir and Grazoprevir described in this manuscript is highly specific, rugged and rapid for therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for clinical trial samples with desired sensitivity, precision, accuracy and high throughput. The method involved a simple and specific sample preparation by solid phase extraction followed by isocratic chromatographic separation in 2.0 min. The overall analysis time is promising compared to other reported procedures for

Elbasvir and Grazoprevir. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with any marketing formulation of Elbasvir and Grazoprevir in human volunteers.

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