Picogram Level Quantification of Grazoprevir and Elbasvir with Deuterated Internal Standards in Human Plasma Samples by LC–ESI-MS/MS

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ABSTRACT

A fixed oral dose combination of grazoprevir and elbasvir is used for the treatment of patients with genotypes 1&4 HCV infections. In this manuscript, authors developed a simple, sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method used for quantification of grazoprevir and elbasvir in human plasma. Agilent TC-C18, 4.6 x 75 mm, 3.5 μm, 80 Å column, and 5 mM ammonium acetate: acetonitrile (20:80 v/v) mobilephase was used for Chromatographic separation. MRM positive mode was used to detect the grazoprevir and elbasvir at m/z 767.3/553.2, 883.4/656.3 respectively. Liquid-liquid extraction was employed in the extraction of analytes and internal standard from human plasma. This method is validated over a linear concentration range of 50.0 – 10000.0 pg/ml for grazoprevir and elbasvir. Calibration functions, lower limit of quantitation (LLOQ), stability, intra and inter-day reproducibility, accuracy, and recovery are estimated. Both drugs were stable in plasma samples. This method was free from matrix effects and any abnormal ionization.

Key words: LC-ESI-MS/MS, Grazoprevir, Elbasvir, Human plasma, Bioanalytical.

INTRODUCTION

Hepatitis C infection is a major issue concerned to global health. According to the centers for disease control and prevention estimates, of the people infected with liver diseases, about 3 million deaths occur worldwide each year due to hepatitis C virus (HCV) related causes.[1,2]

Treatment options for patients with hepatitis C infection and stage 4 to 5 chronic kidney disease remain suboptimum. Approved all oral therapies are not ideal regimens because they contain drugs whose metabolites are cleared by the kidney or because they need co-ordination with ribavirin, which is associated with anaemia.[3]

Zepatier (marketed by Merck and co. inc, New jersey) is a fixed dose combination tablet (single dose once daily) containing grazoprevir- 100 mg (GP) and elbasvir- 50 mg (ES) was approved by FDA (Jan 28, 2016) to market globally. It was used for treatment of patients with genotypes 1 and 4 HCV infections with or without use of ribavarin. The safety and efficacy of zepatier was evaluated in clinical trials of 1,373 participants with chronic HCV genotype 1 or 4 infections with or without cirrhosis.[3,4]

Grazoprevir (Figure 1) is a hepatitis C virus NS3/4A protease inhibitor. 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-dio xo1,1a,3,4,5,6,9,10,18,19,20,21,22,22a-

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tetradecahydro-8H-7,10-methanocyclopropa(18,19) (1,10,3,6) dioxadiazacyclononadecino (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.[5]

Elbasvir (Figure 1) is an HCV NS5A inhibitor.[6] The IUPAC name for elbasvir is Dimethyl N,N'-([(6S)-6-phenylindolo(1,2-c)(1,3)benzoxazine-3,10-diyl] bis{1H-imidazole-5,2-diyl-(2S)-pyrrolidine-2,1-diyl[(2S)-3-methyl-1-oxobutane-1,2-diyl]})dicarbamate. It has a molecular formula of $C_{49}H_{55}N_9O_7$ and a molecular weight of 882.02.[5,7]

Literature survey reveals that only one method was developed for quantification of Elbasvir in rat plasma by using LC-MS/MS.[8] There is no method reported for quantification of Elbasvir and Grazoprevir by using LC-MS/MS in human plasma. It is important to develop a good bio analytical method with proper deuterated or analogue based internal standards interms of matrix effect and reproducibility.

The main goal of the present study is to develop and validate the novel simple, sensitive, selective, rapid, rugged and reproducible analytical method for quantitative determination of Elbasvir and Grazoprevir in human plasma by LC-MS/MS with a small amount of sample volume. Moreover, it should be a simple extraction method, which is highly sensitive, and has good linearity with a small amount of plasma usage.[9]

MATERIALS AND METHODS

Chemicals and reagents

The drugs grazoprevir (GP) and grazoprevir d9 (GPd9), elbasvir (ES) and elbasvir d6 (ESd6) were procured as a gift samples from Symed labs, Hyderabad, India and Toronto research chemicals, Canada. Ethyl acetate, HPLC grade methanol and acetonitrile were purchased from J.T. Baker USA. Sodium dihydrogen phosphate (NaH_2PO_4, reagent grade), ammonium acetate (reagent grade) was purchased from Merck Limited, Worli, Mumbai. Human plasma was obtained from Doctors labs, Hyderabad, India. Ultra pure water from MilliQ-system (Millipore) was used throughout the study.

Instrumentation

The 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany) was used. Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. A turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

Detection

The mass transitions were selected as m/z 767.3/553.2, 883.4/656.3 and m/z 776.4/563.3, 888.4/662.3 for quantification of GP, ES and GPd9, ESd6 respectively.

Chromatographic and mass spectrometric conditions

Agilent TC-C18, 4.6 x 75 mm, 3.5 µm, 80 Å column, and 5 mM ammonium acetate: acetonitrile (20:80 v/v) mobile phase with a flow-rate of 0.5 ml/min was used. The column was placed at a temperature of 40°C. 20 L of sample was injected into LC-MS/MS System. The analytes and internal standards were eluted at 1.62 mins (GP), 0.56 mins (GPd9) and 1.94 mins (ES), 1.93 minutes (ESd6) with total runtime of 4 mins for each injection.

Calibration standards and quality control Samples

Standard Stock solutions of GP (100.0 µg/ml), GPd9 (100.0 µg/ml), ES (100.0 µg/ml), ESd6 (100.0 µg/ml) were prepared in methanol. From each stock solution 100.0 ng/ml intermediate dilution was prepared in plasma. Aliquots of 100.0 ng/ml were used to spike blank human plasma in order to obtain calibration curve standards of 50.0, 100.0, 500.0, 1000.0, 2000.0, 4000.0, 6000.0, 8000.0, 10000.0 pg/ml. Four levels of QC concentrations at 50.0, 150.0, 3000.0 and 7000.0 pg/ml (LLOQ, LQC, MQC and HQC) were prepared by using the different plasma. Spiked calibration curve standards and Quality control standards were stored at -30°C. Standard stock solutions of GPd9, ESd6 (100.0 µg/ml) were prepared in methanol. GPd9 and ESd6 was further diluted to 10.0 ng/ml (Spiked concentration of internal standard) using 50% methanol and was stored in the refrigerator at a temperature of 2-8°C until analysis.

Sample preparation

Liquid-liquid extraction was carried out to extract the drug and internal standard. For this purpose, 100 µl of respective concentration of plasma sample was taken into polypropylene tubes and mixed with 50 µl of internal standard (10.0 ng/ml). This was followed by addition of 100 l of 5 mM NaH_2PO4 solution and 3.0 ml of ethyl acetate and vortexed for approximately 10 mins. Then the samples were centrifuged at 4000 rpm for 10 minutes at 20°C. Further, the supernatant was transferred into labeled polypropylene tubes and evaporated with nitrogen gas at 40°C. Then the samples were reconstituted with the mobile phase and vortexed for 2 mins. Finally, Sample was transferred into auto sampler vials to inject into the LC-MS/MS.

Selectivity and specificity

Selectivity was performed by analyzing the human blank plasma samples from six different sources (donors) to test for interference at the retention times of analytes. The peak area of GP and ES in blank samples should not be more than 20% of mean peak area of LOQ of GP and ES. Similarly, peak area of GPd9 and ESd6 in a blank sample should not be more than 5% of mean peak area of LOQ of GPd9 and ESd6.

Precision and accuracy

Precision and accuracy was determined by replicate analysis of quality control samples (n=6) at LQC (low quality control), MQC (medium quality control) and HQC (high quality control) levels. The % CV should be less than 15%, and accuracy should be within 15% except LLOQ where it should be within 20%.

Matrix effect

The matrix effect due to plasma was used to evaluate the ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pretreatment (Liquid-liquid extraction with ethyl acetate) with that of the reconstituted samples. Experiments were performed at MQC levels in triplicate with six different plasma lots. The acceptable precision (%CV) of ≤ 15% was maintained.

Recovery

The extraction efficiencies of GP, ES and GPd9, ESd6 were determined by analysis of six replicates at each quality control concentration level for GP and ES and at one concentration for the internal standard GPd9 and ESd6. The per cent recovery was evaluated by comparing the peak areas of extracted standards to the peak areas of non extracted standards (spiked into mobile phase).

Limit of detection and quantification (LOD and LOQ)

The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal-to-noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples.

The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of GP and ES.

## Stability (Freeze- thaw, Auto sampler, Room temperature, Long term)

Stock solution stability was performed by comparing the area response of analyte and internal standard in the stability sample, with the area response of sample prepared from fresh stock solution.

Stability studies in plasma were performed at the LQC and HQC concentration level using six replicates at each level. Analyte was considered stable if the % Change is less than 15% as per US FDA guidelines. The stability of spiked human plasma samples stored at room temperature (bench top stability) was evaluated for 61 h. The stability of spiked human plasma samples stored at -30°C in autosampler (autosampler stability) was evaluated for 70 h. The autosampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were reinjected after storing in the auto-sampler at 20°C for 70 h. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the autosampler at 20°C for 70 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at -30°C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. For long term stability evaluation the concentrations obtained after 91 days were compared with initial concentrations.

RESULTS AND DISCUSSION

Method development and validation

The goal of this work is to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of GP, ES from plasma samples. LC-MS/MS has been used as one of the most powerful analytical tools in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility. The MS optimization was performed by direct infusion of solutions of GP, ES, GPd9 and ESd6 into the ESI source of the mass spectrometer. The vital parameters like ionization type, temperature, voltage, gas parameters such as nebulizer and heater gases, compound parameters like DP, EP, FP, CE and CXP were optimized to obtain a better spray shape and ionization to form the respective productions from the protonated GP, ES, GPd9 and ESd6 molecules (Figure 2 and Figure 3). Chromatographic conditions, especially, composition of the mobile phase, selection of suitable column were optimized through several trials to achieve the best resolution and increase the
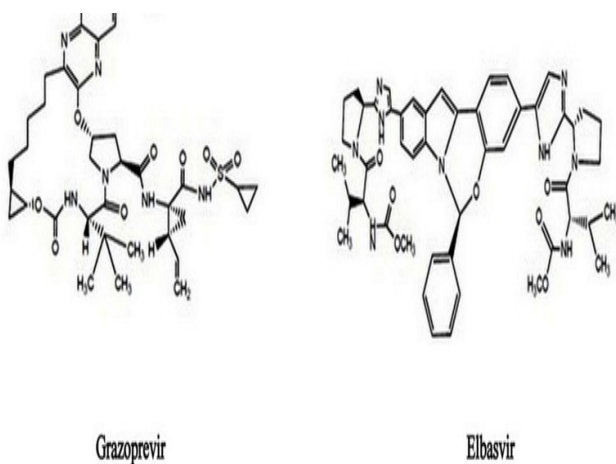
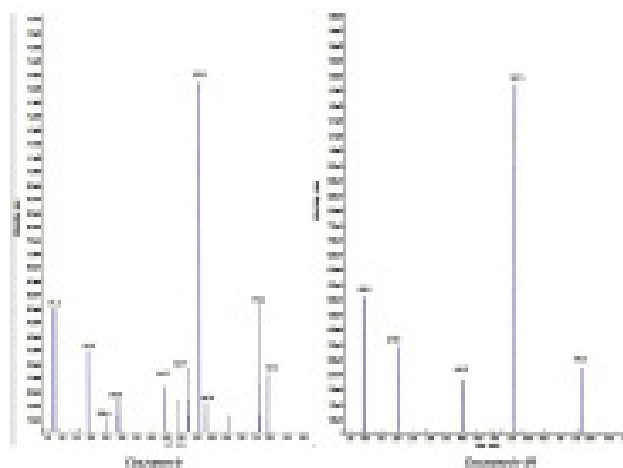**Figure 1: Chemical structures of Grazoprevir and Elbasvir.**

Figure 2: Product ion mass spectrum of Grazoprevir and Grazoprevir-d9.
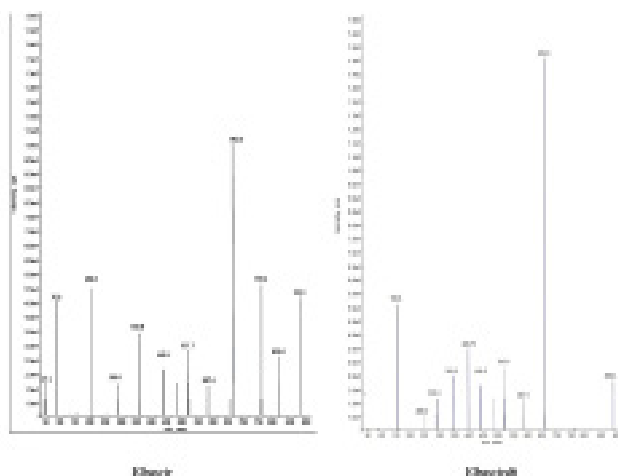

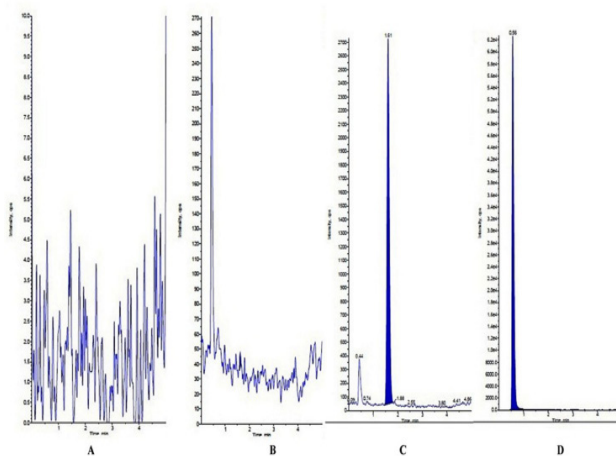**Figure 3: Product ion mass spectrum of Elbasvir and Elbasvir-d6.**

Figure 4: Typical MRM chromatograms. A and B:Blank chromatograms of grazoprevir, grazoprevir-d9; C and D: Grazoprevir in plasma spiked with grazoprevir-d9.
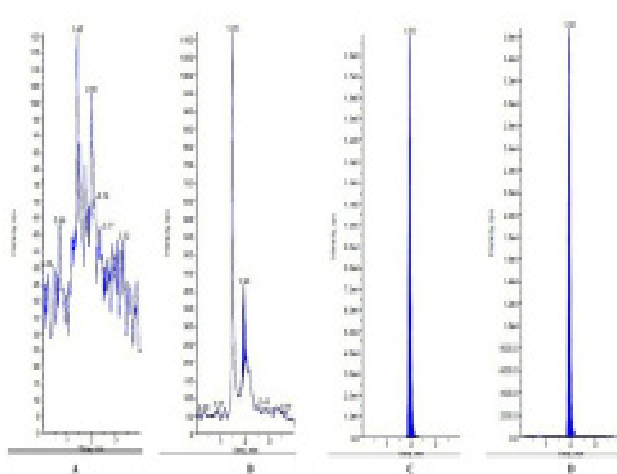

Figure 5: Typical MRM chromatograms. A and B:Blank chromatograms of Elbasvir and Elbasvir-d6; C and D: Elbasvir in plasma spiked with Elbasvir-d6.

Table 1: Calibration curve details of grazoprevir and elbasvir

Spiked plasma concentration (pg/ml)	Concentration measured(mean) (pg/ml), (n = 5)	Precision (CV %) (n = 5)	Concentration measured(mean) (pg/ml), (n = 5)	Precision (CV %) (n = 5)
	Grazoprevir		Elbasvir	
50.0	51.0 ± 1.3	2.5	51.2 ± 1.0	2.0
100.0	96.6 ± 4.7	4.9	95.8 ± 3.4	3.5
500.0	498.4 ± 24.7	5.0	495.1 ± 26.3	5.3
1000.0	1000.0 ± 17.1	1.7	1010.5 ± 28.3	2.8
2000.0	2013.0 ± 74.6	3.7	2019.1 ± 70.0	3.5
4000.0	4008.4 ± 206.6	5.2	4067.1 ± 224.9	5.5
6000.0	5956.5 ± 190.7	3.2	5628.5 ± 735.7	13.1
8000.0	7952.2 ± 165.6	2.1	8162.8 ± 191.2	2.3
10000.0	10317.1 ± 487.6	4.7	10440.0±521.5	5.0

Table 2: Precision and accuracy

Spiked plasma concentration (pg/ml)	Within-run (n=6)			Between-run (n=30)		
	Concentration measured (pg/ml) (mean ± S.D.)	Precision (CV %)	Accuracy %	Concentration measured (pg/ml) (mean ± S.D.)	Precision (CV %)	Accuracy %
			Grazoprevir			
50.0	51.4 ± 2.3	4.5	102.7	55.5 ± 4.1	7.4	110.6
150.0	152.9 ± 1.4	2.2	105.5	151.9 ± 1.7	1.6	102.2
3000.0	3103.8 ± 102.0	3.3	103.2	3133.0 ± 108.2	3.5	104.3
7000.0	7197.1 ± 89.9	1.2	91.7	7178.7 ± 275.5	1.8	103.9
			Elbasvir			
50.0	42.4 ± 1.0	2.4	84.8	49.9 ± 6.9	3.8	99.4
150.0	152.6 ± 2.3	1.4	106.4	151.5 ± 1.6	1.2	101.6
3000.0	3072.4 ± 132.6	4.3	102.4	3216.1 ± 162.6	5.1	107.1
7000.0	7160.1 ± 105.8	1.4	98.0	7174.3 ± 123.9	1.1	102.1

Table 3: Stability of grazoprevir and elbasvir in spiked human plasma samples

Stability experiments	Storage condition	Spiked plasma concentration (pg/ml)	Concentration measured (n=6) Mean ± SD	CV(%) (n=6)	Accuracy (%)
			Grazoprevir		
Bench top (Room temperature)	RT 61 hr	150.0	148.3 ± 8.1	5.5	98.9
		7000.0	6728.3 ± 206.3	3.1	81.5
Processed (extracted sample)	Auto sampler 70 hr	150.0	162.3 ± 2.4	1.5	108.2
		7000.0	7536.7 ± 294.5	3.9	90.4
Freeze & Thaw stability	-30⁰C Cycle-3	150.0	156.5 ± 4.0	2.5	104.3
		7000.0	7381.7 ± 173.4	2.3	90.4
Long term stability	- 30⁰C, 91 days	50.0	160.3 ± 13.2	8.2	106.9
		7000.0	7450.0 ± 229.1	3.1	90.5
			Elbasvir		
Bench top (Room temperature)	RT 61 hr	150.0	156.3 ± 8.7	5.6	104.2
		7000.0	7411.7 ± 213.7	2.9	92.6
Processed (extracted sample)	Auto sampler 70 hr	150.0	161.7 ± 4.9	3.0	107.8
		7000.0	7675.0 ± 473.5	6.2	95.9
Freeze & Thaw stability	-30⁰C Cycle-3	150.0	159.7 ± 7.6	4.7	106.4
		7000.0	7540.0 ± 323.0	4.3	94.3
Long term stability	- 30⁰C,91 days	50.0	159.0 ± 6.3	4.0	106.0
		7000.0	7608.3 ± 297.2	3.9	95.1

signal of analyte and internal standard. Different extraction methods like solid phase extraction, Liquid-liquid extraction, precipitation methods were optimized for extraction of GP, ES, GPd9 and ESd6 from the plasma sample. A good separation and elution were achieved using 5 mM ammonium acetate: acetonitrile (20:80 v/v) as the mobile phase, at a flow-rate of 0.5 ml/min and injection volume of 20 μL. Liquid-liquid extraction was chosen to optimize the drug and internal standard. The retention time was optimized 1.62 mins (GP), 0.56 mins (GPd9) and 1.94 mins (ES), 1.93 mins (ESd6) (Figure 4 and Figure 5).

Linearity

Calibration curve was plotted as the peak area ratio (GP/GPd9 and ES/ESd6) versus (GP and ES) concentration. Calibration was found to be linear over the concentration range of 50.0 –10000.0 pg/ml. The correlation coefficient (r^2) was greater than 0.9997 for all curves (Table 1).

Selectivity

The selectivity of the method was assessed by comparing chromatograms of blank plasma. There were no significant endogenous peaks at respective retention time of GP, ES, GPd9, ESd6. The results indicate that the method exhibited both good specificity and selectivity (Figure 4 and Figure 5).

Precision and Accuracy

Precision and accuracy for this method were controlled by calculating the Within-run and Between-run variations at three concentrations (150.0, 3000.0 and 7000.0 pg/ml) of QC samples in six replicates. As shown in Table 2, the Within-run precision and accuracy were between 1.2 to 4.5 and 91.7 to 105.5% for GP, 1.4 to 4.3 and 84.8 to 106.4% for ES. Similarly, the Between-run precision and accuracy were between 1.6 to 7.4 and 102.2 to 110.6% for GP, 1.1 to 5.1 and 99.4 to 107.1% for ES. These results indicate the adequate reliability and reproducibility of this method within the analytical range (Table 2).

Matrix effect

The ion suppression/enhancement in the signal at MQC level was found %CV 1.27 for GP and %CV 1.20 for ES respectively. These results indicate that there is no effect on ion suppression and ion enhancement.

Recovery

The extraction recoveries of GP were determined at three different concentrations 150.0, 3000.0 and 7000.0 pg/ml, were found to be 99.6 ± 3.53, 88.2 ± 2.7 and 97.60 ± 4.7%,. Similarly, extraction recoveries of ES

were determined at three different concentrations 150.0, 3000.0 and 7000.0 pg/ml, were found to be 95.5 ± 9.7, 92.6 ± 10.21 and 92.3 ± 4.7%. The overall average recoveries of GP, ES, GPd9 and ESd6 was found to be 95.1 ± 6.1, 93.5 ± 1.8, 96.0 ± 2.8 and 98.1 ± 4.47 %. Recoveries of the analyte and internal standard were consistent, precise and reproducible.

Limits of Detection and Quantification (LOD&LOQ)

The LOQ signal-to-noise (S/N) values found for six injections of GP, ES at LOQ concentration was 31.95 pg/ml, 40.23 pg/ml and LOD 2.7 pg/ml and 5.4 pg/ml.

Stability (Freeze - thaw, Auto sampler, Room temperature, Long term)

Stock solution stability was performed to check stability of GP, ES, GPd9 and ESd6 in stock solutions prepared in methanol and stored at 2-8°C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 25 days. The % change for GP, ES, GPd9 and ESd6 were -0.02%, 0.01%, 0.02% and 0.03% respectively indicate that stock solutions were stable at least for 25 days. Room temperature and autosampler stability for GP and ES was investigated at LQC and HQC levels. The results revealed that GP and ES were stable in plasma for at least 60 h at room temperature, and 70 h in an auto sampler. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with GP and ES at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that GP and ES were stable in a matrix up to 91days at a storage temperature of -30°C. The results obtained from all these stability studies are tabulated in Table 3. Precision (%CV) is less than 5 for room temperature, long-term, freeze thaw, auto sampler stability.

CONCLUSION

The proposed research work is highly specific due to the inherent selectivity of tandem mass spectrometry and has significant advantages over other previously described methods. Quantification of grazoprevir and elbasvir were compared with respective isotope labeled internal standards. Extraction of analyte and internal standard were achieved by using LLE. Linearity range, column, mobile phase, flow rate, injection volume, plasma usage volume for analysis was improved. Hence this method has significant advantages over previously reported methods in-terms of selectivity, sensitivity, linearity, reproducibility.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors have none to declare.

ABBREVIATION USED

HCV: Hepatitis C virus; **LC–MS/MS:** Liquid chromatography and mass spectroscopy and mass spectroscopy; **ESI:** Electro spray ionisation; **mm:** Milli meter; **μm:** Micro meter; **mM:** milli molar; **V/V:** volume by volume; **μg/ml:** Micro gram per milli liter; **ng/ml:** Nano gram per milli liter; **pg/mL:** Pico gram per milli liter; **Å:** Angstrom units; **°C:** Degree centigrade; **GP:** Grazoprevir; **GPd9:** Grazoprevir d9; **ES:** Elbasvir; **ESd6:** Elbasvir d6; **LLOQ:** Lower limit of quantification; **LQC:** Limit of quantification; **MQC:** Medium quality, control; **HQC:** High quality control; **QC:** Quality control; **LLE:** Liquid liquid extraction; **SD:** Standard deviation; **% CV:** Percentage coefficient of variation; **DP:** declustering potential; **EP:** Entrance potential; **FP:** Focussing potential; **CE:** Collisional energy; **CXP:** Collisional energy existing potential; **MRM:** Multiple reaction monitoring; **IUPAC:** The International Union of Pure and Applied Chemistry.

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PICTORIAL ABSTRACT

Grazoprevir   Elbasvir
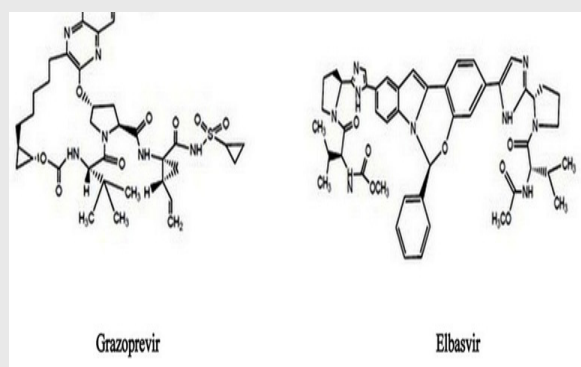
SUMMARY

- The method described in this manuscript has been developed and validated over the concentration range of 50.0 –10000.0 pg/ml in human plasma.

- The intra-batch precision was less than 15.0 % and Accuracy ranged from 91.7 to 105.5% for Grazoprevir and 84.8 to 106.4% for Elbasvir.

- The inter-batch precision was less than 15.0 % and Accuracy ranged from 102.2 to 110.6% for Grazoprevir, 1.1 to 5.1 and 99.4 to 107.1% for Elbasvir.

- The method used in the present study is easy and fast to perform; it is also characterized with an adequate accuracy, precision, selectivity and stability.

- The simplicity of the method, and using rapid liquid–liquid extraction and sample turnover rate of 5.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of Grazoprevir and Elbasvir.

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