HPLC-MS/MS METHOD DEVELOPMENT AND VALIDATION FOR DETERMINING STABILITY OF ELBASVIR IN HUMAN PLASMA SAMPLES

SREELAKSHMI, M., R.L.C. SASIDHAR

Department of Pharmaceutical Analysis, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur, India

AND

RAVITEJA, B.

Department of Pharmaceutical Analysis, M.A.M. College of Pharmacy, Kesanupalli, Narasaraopet, A.P., India

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Objective: The objective of this research was to develop and validate bioanalytical method for elbasvir by LC-MS/MS.

Method: The validated protein precipitation method was applied for estimation of elbasvir in human plasma with elbasvir D6 as an internal standard (ISTD) by using HPLC-ESI-MS/MS. The chromatographic separation was achieved with 0.1% formic acid in combination with methanol (25:75 v/v) using the C_{18} column Ascentis Express (50 mm × 4.6 mm, 2.7 µm).

Results: The total analysis time was 3 min and flow rate was set to 0.6 ml/min. The mass transitions of elbasvir obtained were m/z 883.4/656.3. The standard curve shows correlation coefficient (r^2) greater than 0.9983 with a range of 5.00-10000.00 pg/ml using the linear regression model.

Conclusion: The developed method was characterized with an rapid, adequate accuracy, precision, selectivity and stability make it an attractive procedure in high-throughput bioanalysis of elbasvir.

Keywords: Elbasvir; Human plasma; HPLC-ESI-MS/MS; Bioanalysis.

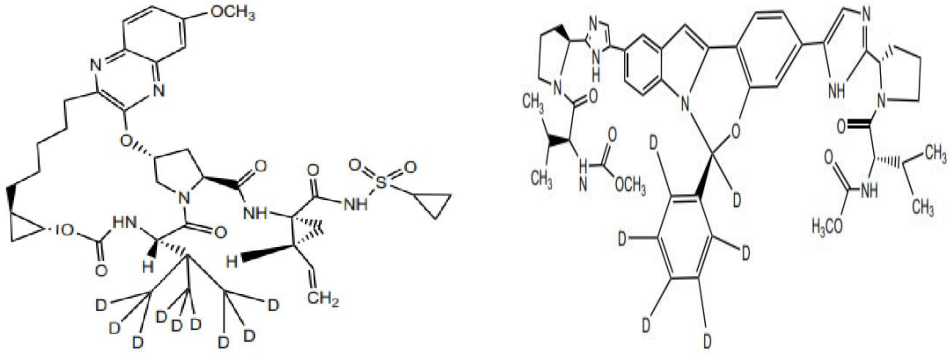
INTRODUCTION

Hepatitis C virus (HCV) infection is a major global health issue. According to some estimates, three to four million persons are newly infected each year, 190 million people are chronically infected and at risk of developing liver disease including cirrhosis and liver cancer, and 350,000 deaths occur each year due to all HCV-related causes [1-8].

HCV, identified in 1989, is an enveloped virus with a 9.6 kb single-stranded RNA genome, a member of the Flaviviridae family, genus Hepacivirus. HCV displays a high degree of genetic heterogeneity and can be classified into six major genotypes (GT1- 6) and a series of subtypes, with genotypes 1a and 1b accounting for ~60% of all infections. The HCV NS5A protein has no known enzymatic function, but has showed multiple functions at various stages of the life cycle, including viral replication and virion assembly. It was demonstrated that a single dose of the NS5A inhibitor could have a rapid virologic response in GT1-infected

patients, which offers potential as part of a therapeutic regimen based on combinations of HCV inhibitors [9-11].

Elbasvir (Fig. 1), is an HCV NS5A inhibitor. 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 [12-13].



(A) **(B)**

Fig. 1. Chemical structures of (A) Elbasvir (EL) and (B) Elbasvir-D6 (ELD6)

Literature survey reveals that limited methods were developed for Quantification of Elbasvir in rat plasma by using LC-MS/MS. It is important to develop the good bio analytical method with proper deuterated or analogue based internal standards in terms of matrix effect and reproducibility. Moreover, should not consider the runtime always to minimize the analysis rather than reproducibility and stability for long analytical batches [14-23].

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 in human plasma by LC-MS/MS with a small amount of sample volume. Moreover, it has to be developed simple extraction method, with a highly sensitive, good linear method with the small amount of plasma usage.

MATERIALS AND METHODS

Chemicals and Reagents

The drugs elbasvir (EL) and elbasvir d6 (ELD6) were procured as a gift samples from Varda Biotech, Mumbai, India, methanol and acetonitrile (J.T Baker, USA), formic acid (Merck, Mumbai, India), Ultra pure water (Milli-Q system, Millipore, Bedford, MA, USA), human plasma (Doctors pathological labs, Hyderabad, India). The chemicals and solvents were used in this study analytical and HPLC grade.

Instrumentation

The 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany). 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 pure drug of EL and ELD6 were prepared in methanol (10.00 ng/mL) and injected with a flow rate of 5 µL/min into positive ion mode mass spectrometer for optimization of mass parameters like source temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (all gas channels were purged with ultra high pure nitrogen gas), EP, DP, CE, FP and CXP were optimized. Analysis was performed using MRM positive ion mode with mass transitions of m mass transitions were selected as *m/z* 883.4/656.3 and *m/z* 888.4/662.3 for quantification of EL and ELD6. The mass fragmentation pattern of parent and product ions mass spectras were depicted in Figure 2 and 3.

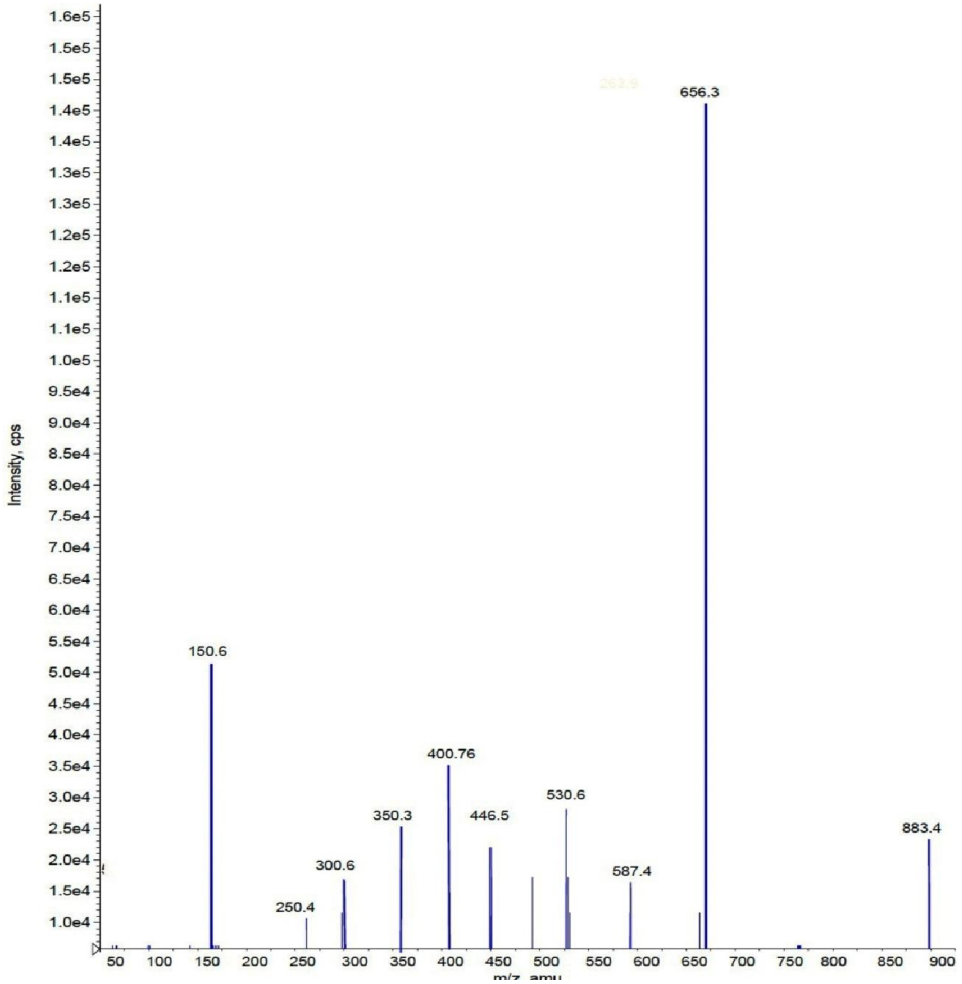


Fig. 2. Parent and product ion mass spectrum of Elbasvir (EL)

Chromatographic conditions

The chromatographic separation was achieved with 0.1% formic acid in combination with methanol (25:75 v/v), gave the best peak shape and low baseline noise was observed using the Ascentis Express C_{18} (50 mm × 4.6 mm, 2.7 µm). The total analysis time was 3 min and flow rate was set to 0.6 ml/min. The temperature was set to 40°C for the column oven. The sample

volume for the injection into mass spectrometry was adjusted to 10 µl for better ionization and chromatography.

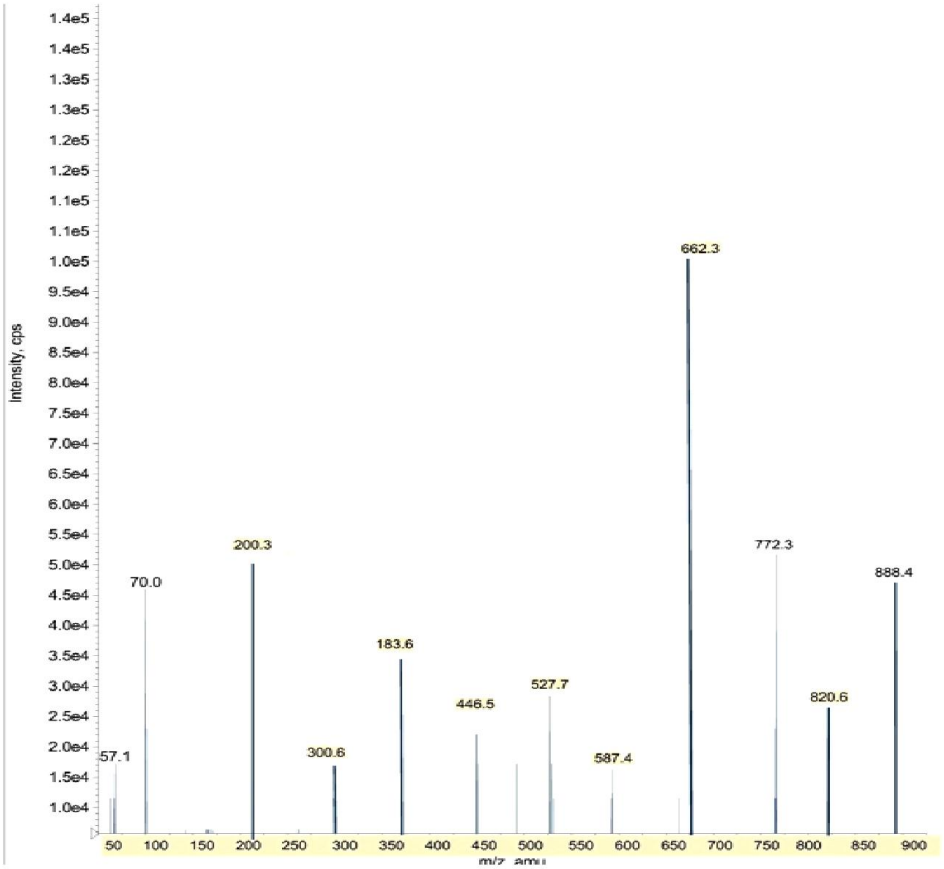


Fig. 3. Parent and Product ion mass spectrum of Elbasvir-D6 (ELD6)

Calibration standards and quality control Samples

Stock solutions of EL (1000.00 µg/ml) and ELD6 (1000.00 µg/ml) were prepared in methanol. The internal standard (ELD6) spiking solution (500.00 ng/ml) was prepared in 75% methanol from ELD6 stock solution. Stock solutions of ES, ESd6 and intermediate spiking solutions were stored in refrigerated conditions (2-8°C) until analysis.

Calibration standards (5.00, 10.00, 50.00, 100.00, 500.00, 1000.00, 2000.00, 4000.00, 6000.00, 8000.00 and 10000.00 pg/ml), quality control samples of lower limit QC, low QC, mid QC, high QC (5.00, 15.00, 3000.00, 7000.00 pg/ml) were used by spiking the appropriate amount of standard solution in the drug free plasma and stored at -30°C till analysis.

Sample Preparation

The protein precipitation method was applied to extract ES and ELD6. To each labelled polypropylene tube 50 µl of ELD6 (500.00 ng/ml) was mixed with the 100 µl plasma sample, then 0.25 ml of acetonitrile were added, vortexed for 5 min and centrifuged at 4000 rpm for 10 min at 20°C. The organic phase was transferred to auto sampler vials containing 100 µl of 0.1% formic acid and injected into the HPLC-ESI-MS/MS for analysis.

Method Validation

The developed method was validated over a linear concentration range of 5.0–10000.0 ng/ml. The validation parameters include selectivity and specificity, LOQ, Linearity, precision and accuracy, matrix effect, recovery, stability (freeze–thaw, auto sampler, bench top, long term) was evaluated under validation section.

Selectivity and Specificity

Ten lots of blank plasma samples were analyzed out of which six lots free from interference were selected for assessing the selectivity and specificity. The endogenous/potential interfering peak areas for blank samples must be less than 20% of the LLOQ peak area of ES retention time and less than 5% for ESD6 retention time.

Precision and Accuracy

One set of calibration standards and one set contains four different concentrations of quality control standards of Lower limit QC (5.00 pg/ml), Low QC (15.00 pg/ml), Mid QC (3000.00 pg/ml) and High QC (7000.00 pg/ml) concentrations were prepared in screened plasma and analyzed each quality control (QC) standards in six replicates on the same day (Intra day) and five different days (Inter day).

Matrix effect

Six extracted blank plasma samples in three replicates were spiked with the un-extracted concentration of mid QC (3000.00 pg/ml) and compared with un-extracted standards of the same concentration.

Recovery

The recovery of samples was performed by protein precipitation method. The extraction recovery was determined in sextuplicate by comparing the extracted QC standards with un-extracted QC standards at three different concentrations of low (15.00 pg/ml), medium (300.00 pg/ml), high (7000.00 pg/ml).

Limit of detection and quantification (LOD and LOQ)

Six LLOQ standards were prepared in screened plasma lot along with IS (500.00 ng/ml) and signal to noise ratio (S/N) was calculated using analyst software.

Stability Studies

Bench top Stability (Room Temperature Stability, 24 h)

Six replicates of spiked low and high concentrations (BT stability samples) were set aside at ambient temperature up to 24 h. Samples were processed and compared with newly prepared low and high concentrations (comparison samples).

Freeze and thaw stability (after 3rd cycle at – 30°C)

Six replicates of low and high concentrations (FT stability samples) were frozen at – 30°C and subjected to three freeze-thaw cycles of 24, 36 and 48 h (– 30°C to room temperature) and compared with newly prepared low and high concentrations (comparison samples).

Autosampler Stability (2-8°C, 65 h)

Six replicates of low and high concentrations (As stability samples) were stored in auto-sampler up to 65 h at 2-8°C. Stability samples were compared with newly prepared low and high concentrations (comparison samples).

Long-term Stability (-30°C, 45 Days)

After completion of the stability period stored at -30 °C (45 days) six replicates of low and high concentrations (LT stability samples) were compared with newly prepared low and high concentrations (comparison samples).

RESULTS AND DISCUSSION

Method development : On the way to develop a simple and easy applicable method for determination of EL in human plasma, HPLC-MS/MS was selected as the method of choice. During method development process chromatographic (mobile phase composition, column, flow rate, injection volume, sample volume), mass spectrometric, sample extraction and internal standard parameters were optimized in logical and sequential manner to achieve the best results.

Separation of the EL was performed with different branded RP-HPLC C_{18} columns. Initial separation was performed with isocratic elution of 10mM ammonium formate and acetonitrile was selected as a mobile phase in varying combinations were tried, but a low response was observed. A mobile phase consisting of 0.1% acetic acid: acetonitrile (20:80 v/v) and 0.1% acetic acid: methanol (20:80 v/v) gave the best response, but poor peak shape was observed.

After a series of trials a mobile phase consisting of 0.1% formic acid in combination with methanol and acetonitrile in varying combinations were tried. Using a mobile phase containing 0.1% formic acid in combination with methanol (25:75 v/v), gave the best signal along with a marked improvement in the peak shape and low baseline noise was observed using the Ascentis Express C_{18} (50 mm × 4.6 mm, 2.7 µm) analytical column with a flow rate of 0.6 ml/min and reduced runtime to 3 min. The column oven temperature was kept at a constant temperature of about 40 °C and temperature of auto sampler was maintained at 4°C.

Injection volume of 10 µl sample was adjusted for better ionization and chromatography. For selection of internal standard, Grazoprevir, Abacavir, Acyclovir, Famciclovir, Penciclovir and Valacyclovir were tried with optimized mobile phase and column conditions. Finally ELD6 was selected as internal standard in terms of better chromatography and extractability.

The retention times of analyte (EL) and internal standard (ELD6) were eluted at 1.42 ± 0.2 min and 1.44 ± 0.2 min respectively with 3 min total runtime. Different procedures like PPT (Protein precipitation), SPE (solid phase extraction) and LLE (liquid-liquid extraction) methods were optimized. Out of all, it was observed that the PPT was suitable due to simple extraction, high recovery and the less ion suppression effect on drug and internal standard.

Electro spray ionization (ESI) provided a maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at a flow rate of 20 µl/min. Elbasvir gave more response in positive ion mode as compare to the negative ion mode.

To get high intense productions source dependent parameters were optimized like nebulizer gas flow 30 psi, CAD gas and curtain gas flow 25 psi, ion spray voltage 5500 V, and

temperature 500°C. The compound dependent parameters such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) were optimized during tuning as 35, 25, 10, 20, 12 eV for Elbasvir and ElbasvirD6, respectively. The collision activated dissociation (CAD) gas was set at 4 psi using nitrogen gas. Quadrupole-1 and quadrupole-3 were both maintained at a unit resolution and dwell time was set at 200 ms for Elbasvir and Elbasvir D6.

The predominant peaks in the primary ESI spectra of EL and ELD6 correspond to the MH^+ ions at *m*/*z* 883.4 and 888.4 . Productions of EL and ELD6 scanned in quadrupole-3 after a collision with nitrogen in quadrupole-2 had a *m/z* 656.3 and 662.3.

Method Validation

Selectivity and Specificity, Limit of Quantification (LOQ)

No significant response was observed at retention times of EL and ELD6 in blank plasma as compared to LLOQ and blank with IS samples. The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 5.0 ng/ml. Represent chromatograms were shown in Figure 4.

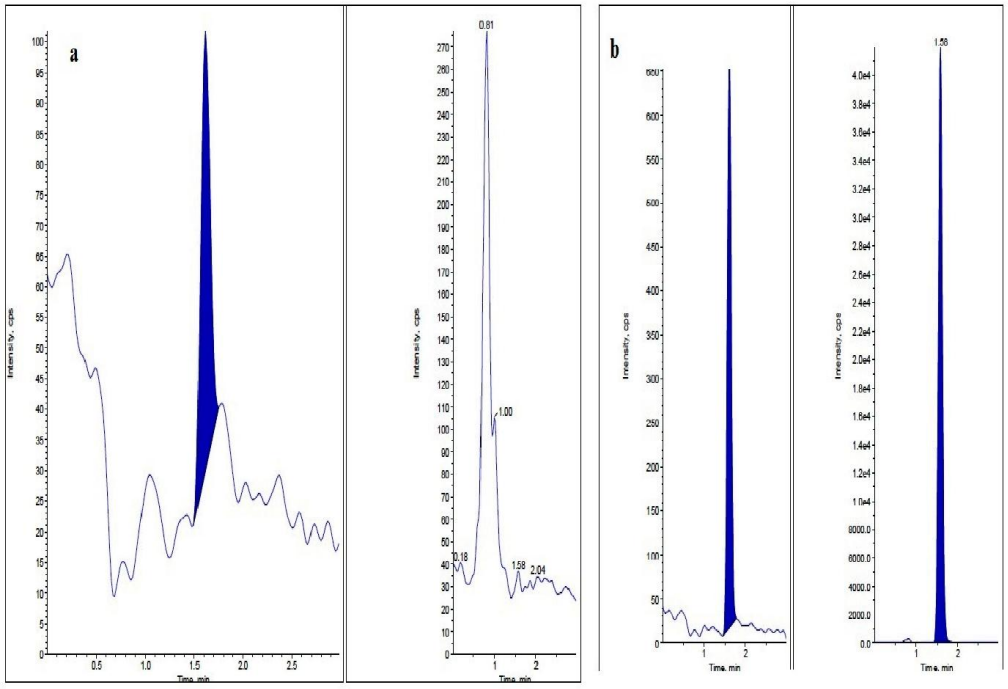


Fig. 4. Representative chromatograms of Elbasvir in plasma (a) Blank plasma chromatogram for interference free EL and ELD6 (b) Chromatogram of LLOQ sample (EL with ELD6).

Linearity

Linearity was plotted as a peak area ratio (EL peak area / ELD6 peak area) on the *y*-axis against EL concentration (pg/ml) on the x-axis. Calibration curves were found to be consistently accurate and precise for EL over a linearity range of 5 to 10000.00 pg/ml. The correlation coefficient was greater than 0.9980 for EL. The %CV was less than 15% and mean % accuracy was ranged between 99.40 - 102.67%. Results were presented in Table 1.

Table 1. Calibration curve details

Spiked plasma Concentration (pg/ml)	Concentration measured (pg/ml) (Mean ± S.D)	%CV (n = 5)	%Accuracy
5.00	4.99 ± 0.01	1.4	99.9
10.00	10.24 ± 0.02	3.6	101.7
50.00	49.89 ± 0.15	2.7	101.3
100.00	100.24 ± 0.22	2.5	100.1
500.00	501.6 ± 0.27	3.8	100.1
1000.00	1004.22 ± 0.21	2.6	101.7
2000.00	1999.18 ± 1.02	3.1	99.4
4000.00	4001.35 ± 1.10	3.4	101.7
6000.00	6003.76 ± 1.11	1.7	102.6
8000.00	8001.12 ± 1.96	3.8	101.5
10000.00	10000.07 ± 1.23	2.5	100.5

Precision & Accuracy

Intra and inter batch % accuracy for EL was ranged between 94.17-96.00 and 91.66 to 99.34. %CV is 2.16 to 5.64 and 1.64% - 8.00%. Results are presented in Table 2.

Table 2. Precision and accuracy (Analysis with spiked samples at three different concentrations)

Spiked Plasma Concentration (pg/ml)	Within-run (Intra-day)			Between-run (Inter-Day)		
	Concentration measured (*n* = 6; pg/ml; mean ± S.D)	%CV	%Accuracy	Concentration measured (n = 6; pg/ml; mean ± S.D)	% CV	% Accuracy
15.00	14.8±0.07	5.6	98.9	14.9±0.08	3.2	99.93
3000.00	3002.34±1.23	2.1	102.4	2999.78±2.56	1.6	99.45
7000.00	6999.47±2.45	3.7	99.8	7004.33±3.61	2.4	103.45

Recovery

The mean % recovery for LQC, MQC, HQC samples of EL were 99.85%, 95.30% and 93.54% respectively. The overall mean % recovery and % CV of EL across QC levels is 96.23% and 3.38%. For the ELD6 (internal standard) the mean % recovery and %CV is 91.68% and 7.09%.

Matrix Effect

No significant matrix effect found in different sources of rat plasma tested for EL and ELD6. The %CV was found to be 3.71.

Stability (freeze–thaw, auto sampler, bench top, long term)

Quantification of the EL in plasma subjected to three freeze–thaw cycles (−30°C to room temperature), autosampler (processed), room temperature (Benchtop), long-term stability details were shown in Table 3.

Table. 3. Stability studies of Elbasvir in plasma

Spiked Plasma concentration (pg/ml)	Room temperature Stability		Processed sample Stability		Long term stability		Freeze and thaw stability	
	24h		65h		45 days		Cycle (48h)	
	Concentration measured (n = 6; pg/ml; mean ± .D)	%CV (n = 6)	Concentration measured (n = 6; pg/ml; mean ± S.D)	%CV (n=6)	Concentration measured (n = 6; pg/ml; mean ± S.D)	%CV (n = 6)	Concentration measured (n = 6; pg/ml; mean ± S.D)	%CV (n = 6)
15.00	14.9 ± 0.12	7.8	15.5 ± 2.16	5.3	15.2 ± 1.54	8.8	14.8 ± 0.12	5.8
7000.00	7005.3 ± .14	8.9	7001.3 ± 1.23	9.5	6999.563 ± 0.12	9.4	7001.4 ± 2.55	2.7

Conclusion

The method described in this manuscript has been developed and validated over the concentration range of 5.0–10000.0 pg/ml in human plasma. The intra and inter-batch precision (%CV) was less than 6.0% and %accuracy ranged from 98.9%–102.4%. The overall %recovery for EL, ELD6 was greater than 90%. The selectivity, sensitivity, precision and accuracy obtained with this method make it suitable for the purpose of the present study. In conclusion, 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 protein precipitation extraction with less run time of 3.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of Elbasvir.

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CONFLICT OF INTEREST: Authors declare that, there is no conflict of interest.

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