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Original Article

Quantification of Sofosbuvir and Velpatasvir in Human Plasma using LCMS/MS Technique -Application to Pharmacokinetic Study

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ABSTRACT

Objective: An effective high-performance Liquid Chromatography-tandem Mass Spectrophotometric (LCMS/MS) method was developed for the simultaneous quantitation of sofosbuvir (SOFOS) and Velpatasvir (VELPA) in spiked human plasma. **Methods:** The extraction of both analytes from plasma was performed by Liquid-Liquid Extraction (LLE) technique. Separation was achieved on a Zorbax C_{18} Stable Bond (SB), C_{18} (4.6mm id x 50 mm) analytical column using acetonitrile: 1% formic acid (50:50) v/v with a flow rate of 600µl/min. The MS/MS analysis was performed in Multiple Reaction Monitoring (MRM) to obtain the product ion *m/z* 530→242.3 for SOFOS, *m/z* 883.8 → 643.0 for VELPA and *m/z* 889.5 →732.6 for internal standard (ledipasvir). **Results:** The calibration curve was found linear over the range of 0.5→5000 ng/ml for SOFOS and 1.5→2000 ng/ml for VELPA. Intra and inerday accuracy (% nominal 98 → 102%), precision (% CV ≤3.8%) was excellent. Matrix effect (matrix factor 1.340 for SOFOS and 1.004 for VELPA), selectivity (% interference = 0) with a extraction recovery of 96.70% →98.30%. The stability (% nominal 95.85→98.90 %) of all types were within acceptable limit. **Conclusion:** The proposed method was applied successfully for the pharmacokinetic study of marketed dosage form in rabbit blood samples with single oral human equivalents dose. The developed method has further applied during clinical and preclinical trials in human and other experimental animals.

Key words: Sofosbuvir, Velpatasvir, Bioanalytical, LC-MS/MS, Pharmacokinetic.

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INTRODUCTION

Hepatitis C is a liver disorder caused by the hepatitis C virus. It is a blood born virus and common modes of transfer is through a small quantity of blood, can be happened through unsafe injection practices, unsafe healthcare, unsafe blood transfusion.[1] Approximately 3 lacks 99 thousand people die each year. Hepatitis C occurs mostly from hepatocellular carcinoma and cirrhosis.[2] Various antiviral agents are available from Hepatitis C but the direct acting antiviral combination medications shows promising effects on chronic hepatitis C[3] sofosbuvir is a direct acting antiviral used in combinations with Velpatasvir. Sofosbuvir with molecular formula $C_{22}H_{29}FN_3O_9P$, is prodrug analogue,[4] which on metabolites into its active form to deoxy-2 ∞ fluoro, ß C- methyl uridine-5 triphosphate.[5] The chemical structure was shown in Figure 1. Velpatasvir is also a direct acting medication used as a part of combination thereby[6] it also place a key role for the inhibition of hepatitis C viral replications.[7] Treatment option for chronic hepatitis C has advanced with direct acting antiviral combination since 2011.[8] Though various analytical methods like UV, HPLC, has been utilised[9] but only two methods has been reported for the estimation of this combination in spiked plasma. In reported method,[10] utilised three mobile phase combination and also utilised protein precipitation technique for the extraction of drug from the matrix. The disadvantage is that most of the time the matrix component is efficiently removed and may co elute with analyte and lowered the signal for the analyte. The linearity range of the reported method is 5-5000 and 10-1500ng/ml. The lower limit can be further reduce to improve the sensitivity. The average extraction recovery is 80-81%, which is not satisfactory. The retention of internal standard and sofosbuvir is too close to each other. In another method[11] used also protein precipitation technique which has several disadvantages that was discussed earlier, therefore the interference was found in the MRM signals. These possible disadvantages in the reported methods limits their acceptability for further clinical and other study. Therefore a sensitive, fast and easy method is spiked human plasma was planned and developed by limiting all disadvantages with the use of liquid-liquid extraction technique for the drugs from spiked plasma with better extraction recovert, analysis time and good recovery. An authentication of the developed method was proven using rabbit plasma sample. So, the present method can be better applicable for further bioequivalence, toxicity and other forensic study.

MATERIALS AND METHODS

Sofosbuvir and Velpatasvir with stated purity of 99.74 % were obtained from Natco Pharmaceutical Pvt. Ltd. Hyderabad, Telangana, India. ledipasvir, purity 99.86% used as Internal Standard (IS) was kindly provided by Natco pharmaceutical Ltd., Hyderabad, India as a gift sample. Acetonitrile and formic acid was purchased from Sigma- Aldrich with ≥ 99.8 % purity. K2 EDTA human plasma was obtained from Valley Biomedical, 121 Industrial Dr, Winchester, VA 22602, USA.

HPLC operating conditions

An isocratic elution technique considered with acetonitrile: 1 % formic acid (50:50) v/v, as a mobile phase and a Zorbax C_{18} Stable Bond (SB),

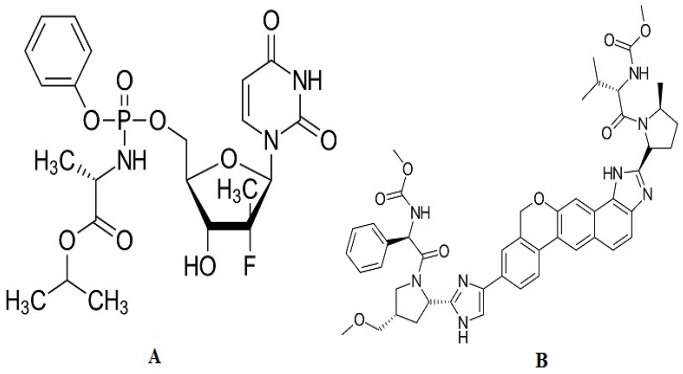

Figure 1: Chemical structure of Sofosbuvir (A) and Velpatasvir (B).

Table 1: Optimized mass spectrophotometric parameters for analytes and internal standard.

Parameters	Sofosbuvir	Velpatasvir	Ledipasvir (Internal standard)
MRM	Parent *m/z*	Parent *m/z*	Parent *m/z*
	Daughter *m/z*	Daughter *m/z*	Daughter *m/z*
Ion spray voltage	5400	5400	5400
Source temperature	140	140	140
Curtain gas1	41	41	41
Dewll time (msec)	202	202	202
Declustering potential (DP)	42	42	42
Capillary (kv)	3.38	3.38	3.38
Cone (v)	40	40	40
Collision energy (CE)	31.5 ev	31.5 ev	31.5 ev
Extractor(v)	6	6	6
RF lens(v)	0	0	0
Desolvation temperature (°C)	800	800	800
Desolvation gas flow (L/h)	800	800	800

C_{18} (4.6mm id x 50 mm) analytical column (Agilent Technologies India Pvt. Ltd. Madhapur, Hyderabad, India). The flow rate 600 µl/min was set in to ESI-MS chamber. Under these condition retention time of SOFOS was 1.13± 0.3 min, 1.32 ± 0.3 min for VELPA and 12.5± 0.3 for internal standard. Column effluent was introduced into mass and run time was maintained up to 2 min.

Mass spectrometry operating conditions

API-3000 triple quadrupole mass spectrometer (AB SCIEX, Foster City, CA/concord and Ontario, Canada) equipped with an electrospray ionisation source (ESI) mass spectrophotometer was used and operated in a positive ion mode at 800°C desolvation temperature. The ion source parameters for examples capillary voltages 3.38 kv, extraction cone voltage 40 KV, source temperature 140°C, desolvation gas flow 800L/h and several other parameters were also fixed for the analysis of both SOFOS, VELPA and IS were summarised in Table 1. Ions were detected in multiple reaction monitoring by monitoring the transition pairs

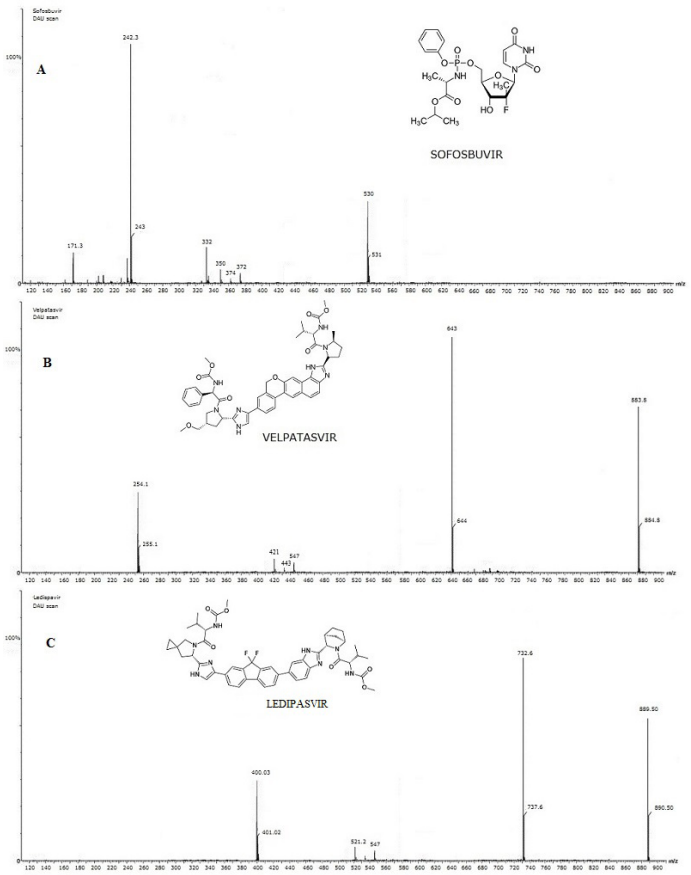

Figure 2: Product ion mass spectra $[M+H]^+$ of Sofosbuvir (A), Velpatasvir (B) and Ledipasvir (C).

of *m/z* 530 → 242.3 for SOFOS, *m/z* 883.8→643.0 for VELPA and *m/z* 889.5→732.6 for internal standard was shown in Figure 2.

Preparation of calibration curve (CC) and quality control (QC) samples

The calibration curve and quality control samples of SOFOS, VELPA and internal standard were prepared from the diluted aqueous stock solutions, which were prepared separately using 4 ml of HPLC grade acetonitrile as a dissolving solvent for 10 mg of SOFOS, VELPA and IS, the volume was made up to 10 mL to achieve the stock concentration 1mg/mL. The plasma spiked CC samples were prepared in the range of 0.5-5000 ng/mL. The solutions were prepared by withdrawing 0.25 mL from the different aqueous calibration curve concentrations and transferred to pre labelled volumetric flasks, finally made the volume up to 5 ml with K2-EDTA human plasma to achieve the concentrations 0.5, 1, 5, 20, 40, 100, 200, 500, 1000, 2000, 3000, 4000 and 5000 ng/ml. For plasma spiked QC samples different concentration of SOFOS, VELPA and internal standard were pipette out from the working stock solutions (40000-100ng/ml) and transferred into pre labelled volumetric flask. The volumes were made up to 5 ml with K2- EDTA human plasma to achieved various levels of QC samples, 1 ng/ml (LLOQ, lower limit of quantitation), 50 ng/ml (LQC, lower quality control), 500 ng/ml (MQC-1, medium quality control-1), 1000 ng/ml (MQC-2, medium quality control-2), 2000 ng/ml (HQC, high quality control), 4000 ng/ml (DIQC, dilution integrity quality control). Individual analyte stock solution was used to spike into CC and QC samples and prepared samples (5ml each) were stored inside the deep freezer at -40°C.

Sample preparation

A liquid-liquid extraction technique was used for the sample preparation. The plasma samples of SOFOS, VELPA and internal standard for the quality control samples and calibration curve samples were thawed at room temperature and vortexed the plasma samples to ensure the complete mixing. 500µl of plasma samples were withdrawn and placed in a different stopper flask. 10 µl of internal standard (100ng/ml) was added to each stoppard flask and vortexes except the blank plasma samples (10µl of diluent) was added. 2 ml ethyl acetate was added as an extracting solvent to each flask and shaken for 20 min with reciprocating shaker at 400 rpm. Samples were then centrifuged at 3000 rpm for 10 min at 25°C. The supernatant layer was transferred into pre labelled tubes and evaporated to dryness under nitrogen gas at 40°C. Samples were reconstituted with 500 µl of mobile phase and transferred into autosampler. 10 µl of each sample were injected into LC-MS/MS system.

Method Validation

The developed method was validated as per the guidelines of bioanalytical method development and validation of by USFDA.[12] The developed method was validated for matrix effect, carry over effect, recovery, sensitivity, accuracy, precision and different stability parameters.

Carry over effect

It was performed to investigate the effect of carrying analytes and internal standard in different sections of the system. Six replicates of the extracted blank matrix and six replicates of the extracted high concentration of analytes at the calibration curve range (ULOQ), six extracted LLOQ concentrations of the analytes and internal standard were prepared and injected as per sequence i.e. first six injections were extracted LLOQ samples, extracted ULOQ samples followed by blank plasma. ULOQ and blank plasma samples were injected alternatively.

Matrix effect

It was performed at two different concentration level (LQC and HQC) in eight replicates for both analytes and internal standard. One hemolytic and one lipemic plasma lots have been selected for the study. One set of each sample were spiked with blank matrices (hemolytic), another set of each the sample (LQC and HQC) with internal standard was spiked with lipemic plasma. Six replicates of aqueous samples equivalent to final LQC and HQC concentrations were prepared by spiking the analytes (SOFOS and VELPA) with internal standard (LEDISPA) to reconstituted solution and injected individually. IS normalized matrix factor was calculated and the variability in IS-normalized matrix factor was measured by the coefficient of variation and it should be should be less than 15%.

Matrix selectivity and specificity

It was evaluated by analysing the plasma of six different lots, including one haemolytic and lipemic plasma to investigate the interference the retention time of the analytes and internal standard. The interference at the retention times of the drugs by comparing the response in the blank plasma, against the response of LLOQ was evaluated. The interference at the retention time of internal standard also evaluated against the response of the extracted internal standard in LLOQ sample.

Analytes selectivity

Analytes selectivity study was performed to investigate the internal standards interference at analyte's retention time. Six replicates of matrix blank with internal standard was injected, if any area for analytes was found was compared with mean area of the analytes obtained with LLOQ concentration injected. Similarly internal standards selectivity was also investigated for this six replicates of matrix blank with drugs was in injected at ULOQ level and area of internal standard if obtained, compared with mean area of internal standard at LLOQ level.

Precision and accuracy

The intra and interday precision and accuracy study of the developed method was conducted using several QC samples at the level of LLOQ, LQC, MQC and HQC in six replicates and the concentrations in these level was calculated followed by standard deviation,% CV for precision and % nominal for accuracy for each replicates.

Linearity

The developed method was assessed for linearity in the concentration range of 0.5-5000 ng/ml, for SOFOS and 1.5-2000ng/ml for VELPA. Several CC (calibration curve) samples has been prepared by spiking with human plasma and processed. The calibration curve was constructed by using a regression equation with a weighing factor 1/ (concentration ratio)^2 of the drug to internal standard concentration to produce the best fit for the concentration/response relationship. The acceptance criteria for the linearity is that the r^2 (coefficient of correlation) should be ≥0.98 and for LLOQ concentration ±20% deviation can be acceptable from nominal value but ±15% deviation should maintain other than LLOQ concentration.

Dilution integrity

For the dilution integrity 12 sets of QC stock solution were prepared by spiking 1.5 times of the highest standard concentration. Six sets of dilution integrity samples were prepared by diluting 2 times and another six samples by 4 times dilution were made. These samples were analysed and concentration were calculated multiplying suitable dilution factors, 2 (for two times dilution) and 4(for four times dilution).

Recovery study

This study was performed to evaluate the extraction efficiency of a analytical process, by comparing the peak response from extracted and non-extracted samples. Six LQC, MQC and HQC samples has been prepared freshly and these samples were processed by adding internal standard and injected. Eighteen blank matrix samples were spiked with six sets of each LQC, MQC and HQC with internal standard for non-extracted samples and injected. Six non extracted samples of each 3 levels were prepared by spiking 10µl of analytes and 10 µl of internal standard in extracted blank plasma. Overall mean % recovery was calculated.

Ruggedness

For the study of the developed method, one precision and accuracy batch of samples were processed and analysed with different columns of same make and with different reagent lots.

Stability Studies

In the Bench top, wet extract, freeze thaw, autosampler, short term and long-term stability study of the developed method, freshly prepared calibration curve samples and quality control samples were prepared and analysed at low middle, high level. Concentration response linearity data was collected and used to calculate the concentration of stability samples.

Room temperature stability study

It was conducted using the prepared stock solution for a period of 6 hr. Fresh stock solution of both analytes and internal standard were prepared. The final dilution of both stock solution (stability samples) and fresh stock solution (comparison sample) was done, which is equivalent to final middle quality control analytes and internal standard. Six replicates of fresh and comparison samples were injected immediately and % of stability was calculated.

Bench top stability

For the bench top stability study six sets of LQC and HQC samples were collected from the deep freezer and placed unprocessed for a period of 12 hr. After that period six sets of fresh quality control samples (low, middle, high) and calibration samples were prepared. Bench top stability samples were processed, analysed along with fresh samples. Concentration was calculated from the linearity data.

Refrigerator stock solution stability

For this type of stability study six replicates of stock solution was prepared and stored at refrigerator at 2-8°C for 4 days. On the day of study fresh standard stock solution was prepared comparison sample similar to final MQC concentration of the analytes with final concentration of internal standard in reconstituted solution. All stability and comparison samples were injected immediately. Percentage of stability was also calculated for both the analytes and internal standard

Autosampler stability

For this stability study six sets of QC samples were prepared in LQC and HQC level and kept in autosampler for 3 days. All the stability samples were quantified against the freshly prepared spiked calibration curve and quality control samples at low middle and high level.

Freeze thaw stability

For this study four freeze thaw cycles was conducted. Six replicates of LQC and HQC samples were prepared and stored in deep freezer at -70°C. After 24 hr of freezing, first six samples were withdrawn and thawed at room temperature and refreeze again. Similar way remaining samples were also withdrawn after next 12 hr followed by another 12 hr and refreeze again. Entire range of samples were processed after suitable 4 cycles. Stability samples were analyzed along with freshly spiked calibration samples and quality control samples at low mid and high level.

Wet extract stability

For this study six LQC and HQC samples of six replicates were prepared, processed and kept 24 hr at room temperature (20±5°C). After the stability period the samples were injected with freshly prepared spiked calibration curve samples and quality control samples at low middle and high level. The amount of analytes in stability samples was calculated in compared with freshly prepared samples.

Short term stability at -20°C

Quality control samples at low and high level were prepared for six sets and stored at deep freezer at -20°C after spiking for 3 days. On the day of study samples were processed along with freshly prepared quality control samples in all levels and calibration curve samples. The concentration of the stability samples were calculated in comparison to freshly prepared samples.

Long term stability at -70°C

This was investigated with LQC and HQC samples kept for 30 days at -70°C. On the day evaluation six sets of long-term quality control samples (LQC and HQC) were withdrawn and processed them with freshly prepared calibration curve and quality control samples. Calibration curve data was utilised to quantify all the samples.

Pharmacokinetic study

This study was performed using NZ white rabbit (n=3, 2.5 kg each, 2 male, male: female 2:1) model using the permission of IAEC permission, no. 1604/PO/Re/S/13/CPCSEA. To assess the applicability of the developed method, it was carried at oral dose of 1.35 mg of SOFOS and for VELPA is 5.3mg for Etizolam. The orally administered dose was human equivalent dose for this particular combination of marketed formulation "Velamen" (sofosbuvir 400 mg and velpatasvir 100mg). The dose was calculated as per the US-FDA guidelines for the calculation of equivalent dose.[13,14] Silicon gastric intubation tube was utilised for the administration of dosage form to their respective group. Blood samples (1ml) was collected from the marginal ear vein into polypropylene tube (K2 EDTA, J.K diagnostic- Rajkot) at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 5, 8, 10 h post dose. Plasma was separated immediately by centrifugation (2000rpm, 7 min) and stored at -80°C until analysis. The plasma samples were spiked with internal standard and processed as per sample preparation technique described earlier. The plasma concentration and time data were analysed with a compartment model, using PK solver. 2 a Manu driven adding programme for MS excel (18).

One batch intraday accuracy and precision study of rabbit blood samples

This was performed to strengthen the applicability of the developed method. The blood samples that were collected for the pharmacokinetic study from the rabbits were also subjected to one batch intraday accuracy and precision study. The collected blood samples were further processed as per the sample preparation method with internal standard, at LLOQ, LQC, MQC-1, MQC-II and HQC level.

RESULTS

Mass spectrometry

Both protonated analyses and internal standard $[M+H]^+$ ion was the parent ion in the quadropole-1 segment and used as precursor ion to obtained product ion spectra in quadropole-3. All the optimized mass parameters has been cited in Table 1 and product ion mass spectra was shown in Figure 2.

Method development

Several combination of buffers and acetonitrile has been utilized in initial trials, different types of columns like C-8, C_{18} of Waters symmetry shield, Zorbax, hypersil, kromasil has been used and finally, Zorbax C_{18} Stable Bond (SB) analytical column with the mobile phase composed of acetonitrile and 1% formic acid in the volume ratio (50:50) v/v has selected. Ledipasvir was selected as an internal standard. Under this optimized condition the retention time of SOFOS was 1.13± 0.3 min, 1.32 ± 0.3 min for VELPA and 12.5± 0.3 for internal standard, shown as MRM chromatograms at LQC and HQC level in Figure 3 and 4.

Prevalidation and validation

In carry over test the response for blank samples was found 0, therefore the calculated % carry over is 0. "Internal standard normalized factor" was calculated to determine the matrix effect on the analytes. The % CV of Is normalized factor was found 5.32 and 7.81 for SOFOS LQC and HQC samples, whereas 6.53 and 5.03 for VELPA LQC and HQC samples. % CV of between batch precision for LLOQ, LQC and MQC-I, MQC-II and HQC samples of SOFOS was found 2.13, 1.03, 1.18, 1.23 and 1.68. Similarly, for VELPA it was 0.63, 2.19, 0.32, 0.06 and 1.23. Between the batch accuracy for LLOQ, LQC and MQC-I, MQC-II and HQC were found 95.83, 96.23, 98.03, 97.53 and 98.71% for SOFOS and for VELPA accuracy values were 95.3, 97.18, 98.63, 98.33 and 98.90%, details were shown in Table 2. In matrix selectivity study the response of interfering peaks at the retention time of both analyses and is was found 0% of the mean drug response. A regression equation of 1/ (concentration ratio)^2 of drugs to internal standard concentration was found 0.99 in the concentration range of 0.5-4000ng/mL for SOFOS and 1.5-200ng/mL for VELPA. In dilution integrity study (2 and 4 times) at ULOQ level, the precision and accuracy of SOFOS for dilution factor 2 were found 0.085 and 98.96%. For VELPA the values were 0.147 and 98.96%. Similarly,

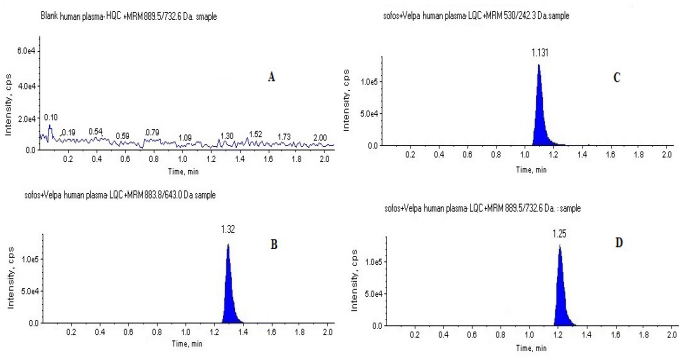

Figure 3: MRM chromatogram of Blank (A), Sofosbuvir (B), Velpatasvir (C) and Ledipasvir (D) At LQC level.

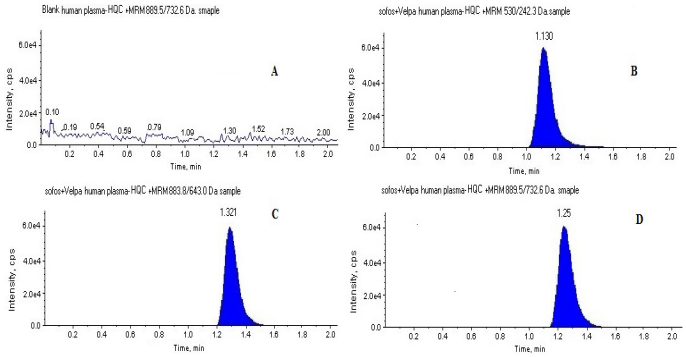

Figure 4: MRM chromatogram of Blank (A), Sofosbuvir (B), Velpatasvir (C) and Ledipasvir (D) At HQC level.

Table 2: Intraday and Interday accuracy and precision of determination of Sofosbuvir and Velpatasvir in human plasma.

QC	Sofosbuvir measured concentration (ng/ml)					Velpatasvir measured concentration (ng/ml)			
	Run*	Mean	SD	% CV	% Nominal	Mean	SD	%CV	%Nominal
LLOQ (1ng/mL)	1	0.96	0.19	3.18	96.02	0.95	0.87	2.05	95.20
	2	0.98	0.36	0.08	98.5	0.93	0.76	1.56	93.65
	3	0.89	0.88	0.19	89.60	0.97	0.03	2.98	97.08
	4	0.91	0.75	0.33	91.05	0.92	0.06	1.75	93.52
LQC (50ng/mL)	1	48.76	0.35	0.69	97.03	49.05	0.11	0.34	98.12
	2	48.97	0.89	1.75	97.08	49.19	0.52	3.50	98.32
	3	49.08	0.12	0.79	98.40	48.76	0.06	0.41	97.51
	4	49.71	0.04	0.66	99.42	48.43	0.06	0.63	98.86
MQC-1 (500ng/mL)	1	480.35	0.31	1.32	97.87	487.51	0.48	0.32	97.50
	2	491.30	0.35	1.03	98.26	492.51	0.07	0.35	98.50
	3	492.47	0.53	1.01	98.49	493.53	0.39	0.26	98.70
	4	489.75	0.89	0.92	97.95	491.86	0.06	0.04	98.37
MQC-II (1000ng/mL)	1	969.13	0.85	1.13	96.91	988.18	1.19	0.07	98.81
	2	983.86	0.73	1.20	98.38	970.20	0.50	0.03	97.07
	3	989.5	1.19	1.23	98.95	978.10	1.16	0.07	97.81
	4	989.20	1.21	1.03	98.12	980.30	1.32	0.08	98.03
HQC (2000ng/mL)	1	1982.21	1.23	1.32	99.11	1969.30	0.08	1.01	98.46
	2	1962.51	1.03	1.87	98.12	1972.59	0.39	1.23	98.62
	3	1953.81	1.08	1.65	97.69	1952.63	0.19	1.29	97.63
	4	1972.72	1.21	1.03	98.63	1988.49	0.11	1.31	99.42
						Interday variations (each concentration include 20 replicates)			
LLOQ		0.97	0.18	0.58	97.0	0.97	0.13	0.59	97.0
LQC		48.93	0.32	0.83	96.36	47.93	0.58	0.72	95.86
MQC-1		489.31	0.51	1.32	97.86	488.13	0.44	1.38	97.22
MQC-II		967.76	0.56	1.53	98.46	945.57	0.78	0.95	97.45
HQC		1972.48	0.03	1.82	97.24	1975.31	0.39	0.87	97.53

*Each run includes six replicate.

for a dilution factor 4 the precision and accuracy of SOFOS values were 0.465 and 156.17 and for VELPA values are 0.183 and 101.06. The mean overall recovery of SOFOS was found 94.04% with a precision range of 2.11% to 4.90% and a percentage difference 5.31. Similarly, overall recovery of SOFOS was found 98.80% with a precision range of 0.929% to 6.69 %, with a percentage difference of 14.48. Internal standard mean overall recovery was found 98.16% which satisfied acceptance criteria. In ruggedness study within batch precision of LLOQ, LQC, MQC-I,

Table 3: Stability data of sofosbuvir and velpatasvir.

QC concent-ration	Stability Type	Sofosbuvir				Velpatasvir			
		Mean*	SD	% CV (Precision)	% Nominal (Accuracy)	Mean	SD	% CV (Precision)	% Nominal (Accuracy)
LQC	Bench top	48.23	0.02	1.62	96.46	47.39	0.05	1.38	94.78
	Freeze thaw	49.17	0.19	1.41	98.34	48.15	0.29	1.32	96.30
	Autosampler	48.73	0.36	1.35	97.46	48.78	0.58	1.45	97.56
	Wet extract	48.55	0.08	1.67	97.1	47.13	0.98	1.76	94.26
	Short term	49.01	0.59	1.21	98.02	48.35	0.76	1.67	96.70
	Long term	47.92	1.01	1.37	95.84	47.37	1.06	1.80	94.74
HQC	Bench top	1972.2	1.08	1.55	98.61	1968.13	1.09	1.54	99.40
	Freeze thaw	1968.25	0.96	1.76	98.43	1959.73	0.99	1.85	97.98
	Autosampler	1978.01	0.92	1.88	98.90	1981.32	0.97	1.62	99.06
	Wet extract	1972.69	0.67	1.26	98.63	1962.59	0.58	1.88	98.12
	Short term	1961.54	0.29	1.10	98.15	1968.11	0.78	1.92	98.40
	Long term	1968.11	0.89	1.57	98.40	1072.18	0.67	1.58	98.60

* Mean of six replicates

Table 4: Pharmacokinetic parameters of SOFOS and VELPA in rabbit plasma.

Pharmacokinetic parameters	Sofosbuvir	Velpatasvir
C_{max}	839.59	1013.03
t_{max}	2.38	3.12
$AUC_{0-\infty}$ (h* ng/ml)	3432.18	5018.2
AUC_{0-t} (h* ng/ml)	3328.72	4388.8
$t_{1/2}$ (hr)	1.27	4.32

MQC-II and HQC ruggedness SOFOS samples were found 2.34%, 1.807%, 0.533% and 0.545%. Similarly, for VELPA ruggedness samples values were 5.208%, 0.563%, 0.410% and 1.37%. The accuracy values for SOFOS were 97.53%, 98.60%, 98.30%, 99.12% and for VELPA values were 98.00%, 98.86%, 98.34% and 99.16%.

Stability studies

The calculated % stability for SOFOS, VELPA and internal standard were 97.4, 98.4 and 97.8. for room temperature stability study. In refrigerator stock solution stability study at 2-8°C for 4 days, the calculated % of stability for SOFOS was found 101.03, for VELPA it was 98.37 and for internal standard 98.45. In bench top stability, the mean % of nominal of LQC and HQC samples were found 96.46% and 98.61% for SOFOS. Whereas% nominal of VELPA was found 94.78 and 99.40 in LQC and HQC. After 72 h of suitable stability period in autosampler, the % nominal (accuracy) was found 97.46% and 98.00% for SOFOS at LQC and HQC level, whereas % nominal (accuracy) values for VELPA were 97.56% and 99.90% at LQC and HQC level. The four cycles of freeze thaw stability sample shows % nominal 98.34% and 98.43% in LQC and HQC samples of SOFOS, VELPA stability samples shown 96.30% and 97.98% in LQC and HQC level respectively. In wet extract stability study, the % nominal value for SOFOS was found 97.1% and 98.63% in LQC and HQC and for SOFOS it was 94.26 % and 98.12 % in both levels, satisfied acceptance criteria for stability study. In short term stability study, the calculated % nominal for SOFOS were 98.02% and 98.15% at LQC and HQC level and for VELPA was 96.70% at LQC and 98.46% at HQC level. The mean % nominal for long term stability were 95.85% for LQC and 98.40% for HQC stability samples of SOFOS. Similarly the % of nominal for VELPA were 94.74% and 98.60 % at LQC and HQC stability samples. The summary of all stability study results were demonstrated in Table 3. In bench top, wet extract, freeze thaw, autosampler, short and long term (-70°C, 30days) stability studies.

In vivo pharmacokinetic study

The results of pharmacokinetic parameters obtained from the study using non compartmental model, were area under the curve (AUC_0-∞) for SOFOS =3432.18±1092.67 hr.ng/ml and for VELPA = 5018.23 ±109.19 hr.ng/ml. Elimination half-life ($t^{1/2}$) for SOFOS = 1.26±1.03 h and VELPA= 4.32±1.08 h. All the other parameters were demonstrated in Table 4.

In one batch accuracy and precision study of rabbit blood samples, the %CV (precision) were found less than 2 and % N(Accuracy) were found within 95-101% in all the study levels of both the SOFOS and VELPA. The details of the result shown in Table 5.

DISCUSSION

The mass parameters has been optimized only after the proper tuning of mass spectrophotometer in both positive and negative ion mode for both the analytes and internal standard, finally positive ion mode with multiple reaction monitoring mode has been selected for better specificity. Several trials were conducted to optimize the chromatographic condition required for the separation of components using different mobile phases, columns.

The obtained chromatographic condition satisfactory peak shape was obtained with reasonable retention times for both analytes and internal standard. Initially several compounds were investigated to select a suitable internal standard, finally ledipasvir was selected because the retention time and other values were very reproducible and selective and lack of interference with other analytes.

The carryover test result shows that there was no interference was found at the retention time of the analytes and internal standard at ULOQ and LLOQ level. No significant effect of matrix was found in eight batch includes haemolytic and lipemic plasma. Within batch accuracy and precision results for both SOFOS and VELPA for all quality control levels were found within acceptance criteria. Therefore, the method was found

Table 5: Interday accuracy and precision data of original rabbit plasma sample.

Analyte	Sofosbuvir					Velpatasvir				
Level	LLOQ 1 ng/mL	LQC 50 ng/mL	MQC-1 500 ng/mL	MQC-2 1000 ng/mL	HQC 2000 ng/mL	LLOQ 1 ng/mL	LQC 50 ng/mL	MQC-1 500 ng/mL	MQC-2 1000 ng/mL	HQC 2000 ng/mL
Mean*	0.96	48.35	482.56	969.52	1979.88	0.97	47.65	473.54	959.52	1949.88
SD	0.34	0.45	0.76	0.88	1.45	1.01	1.45	0.57	0.89	1.03
%CV	1.11	0.94	0.84	1.54	1.87	1.22	1.67	0.83	1.46	1.21
%Nominal	96.01	96.70	96.51	97.53	98.99	97	95.34	94.58	95.95	97.49

*average of six individual run.

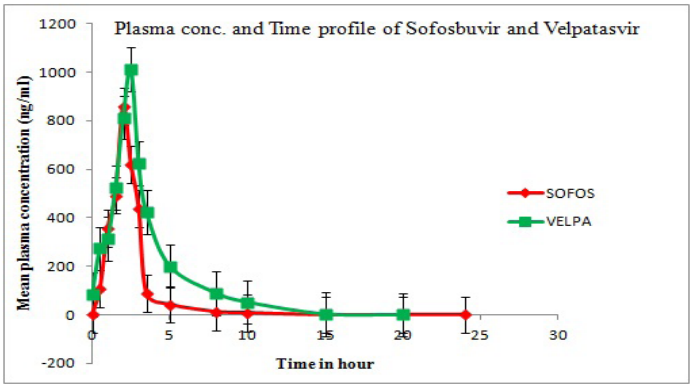

Figure 5: Mean plasma concentration (ng/ml) – time (h) profile of Sofosbuvir and Velpatasvir after oral administration of marketed dosage form.

accurate and precise. Analyte selectivity study results shows that there was no interference at the retention time of both analytes in presence of internal standard and found 0% interference at the retention time of internal standard in the presence of both analytes. In the linearity study the regression equation observed best fit relationship with a correlation coefficient (r^2). The dilution integrity study results confirm the integrity of the developed method up to 4 times of its diluted samples. The developed method was also found rugged as all the empirical evidences were within acceptance limit.

The room temperature (20±5°C) stability study results was found within acceptance criteria. And the results of refrigerator stock solution stability study at 2-8°C for 4 days, indicates the stability of the developed method. The % nominal of both HQC and LQC levels indicted the bench top stability of the developed method. Results of other stability samples were compared with freshly prepared samples and concentration result was back calculated from the calibration curve sample. In all types of stability study, the mean% of nominal values were found within 85%-115% and % CV values were less than 15%, which strongly demonstrated about the stability of all quality control samples as well as stability of the developed method. In pharmacokinetic study on rabbit models the plasma concentration and time curves for both the drugs were found smooth to derive all parameters (Figure 5). The elimination half-life and clearance were well fitted with one compartmental model. The sensitivity, selectivity and specificity of the developed method was sufficient enough for the simultaneous characterization and the study of Pharmacokinetic profile of SOFOS and VELPA in rabbit model. The results of one accuracy and precision study on rabbit blood samples were within the acceptance criteria. Therefore, this study further strengthened the applicability of the developed method

CONCLUSION

The developed method is the first reporting bioanalytical method for the simultaneous estimation of sofosbuvir and velpatasvir in spiked human plasma using LC-MS/MS. The developed method was found simple, fast and convenient. The obtained pharmacokinetic parameters confirm the specific applicability of the developed method. Therefore, the present method can undoubtedly applicable for the simultaneous quantitative analysis of sofosbuvir and velpatasvir during clinical trials and toxicological study in human and other experimental animals.

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CONFLICT OF INTREST

The authors declare no conflict of interest.

ABBREVIATIONS

SOFOS: Sofosbuvir; **VEPLA:** Velpatasvir; **LEDISPA:** Ledipasvir; **LCMS:** Liquid chromatography and mass spectroscopy; **LQC:** Lower quality control; **MQC:** medium quality control; **HQC:** Highest quality control; **ULOQ:** Upper limit of quantitation; **DIQC:** dilution integrity quality control; **% CV:** Percentage coefficient of variation; **AUC:** Area under the curve; **SD:** Standard deviation; **CC:** Calibration curve.

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