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A Bioanalytical Method Development and Validation for Simultaneous Determination of Velpatasvir and Sofosbuvir in Spiked Human Plasma

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A simple, precise, accurate and sensitive, isocratic RP-HPLC method has been developed and validated for the estimation of velpatasvir and sofosbuvir in plasma. Method was developed with, Intersil ODS C18 column (250 mm × 4.6 mm × 5 µ), mobile phase mixture of acetonitrile and water having pH of 6.5 in the ratio of (80:20 v/v) at flow rate 1 mL/min. at UV detector wavelength of 240 nm. Mobile phase pH was adjusted with 1 % *o*-phthalaldehyde (OPA). The retention times are 3.49 min for sofosbuvir and 6.15 min for velpatasvir, Quantitative linearity was obeyed in the concentration range of 0.1 to 0.8 µg/mL for sofosbuvir and 0.025 to 0.2 µg/mL for velpatasvir. The limit of detections are 0.006 µg/mL for sofosbuvir, 0.0017 µg/mL for velpatasvir and limit of quantifications are 0.0125 µg/mL for sofosbuvir, 0.003 µg/mL for velpatasvir for which indicates the sensitivity of the method. The average recovery of sofosbuvir is 100.75 and 99.25 % for velpatasvir. The high percentage recovery indicates that the proposed method is highly accurate. Bench top, Auto sampler, Freeze and Thaw stability test are performed.

Keywords: Velpatasvir, Sofosbuvir, HPLC Method, Bioanalytical method.

INTRODUCTION

Sofosbuvir (Fig. 1) is a prodrug of 2'-deoxy-2'-fluoro-2'-C-methyl-uridine monophosphate that is phosphorylated intra cellularly to the active triphosphate form, used for the treatment of chronic hepatitis C. The nucleoside triphosphate is a non-obligate chain-terminating analogue of UTP that competes for incorporation at the HCV NS5B polymerase active site. Viral RNA synthesis is inhibited secondary to incorporation of the phosphorylated metabolite into nascent viral RNA by the HCV RNA-dependent RNA polymerase [1]. Velpatasvir (Fig. 1) is both an inhibitor and a substrate of the transporter proteins P-glycoprotein (Pgp), ABCG2, OATP1B1 and OATP1B3. It is partly degraded by the liver enzymes CYP2B6, CYP2C8 and CYP3A4 [2].

Literature survey revealed that very few analytical methods were available for analysis of sofosbuvir and velpatasvir. Most of the methods were reported for analysis of sofosbuvir. Raj Kumar and Subrahmanyam [3] developed a HPLC method for the simultaneous quantification of sofosbuvir in pharmaceutical dosage form. In this reported method, mobile phase was a mixture of acetonitrile:water (75:25 % v/v). Method was developed at a wavelength of 253 nm with C18 column. The linearity range was 18.2-91 mg/mL for sofosbuvir. Zaman *et al.* [4] reported a simultaneous method for the determination of sofosbuvir. In this method, the mobile phase consists of CH_3COONH_4 and acetonitrile (35:65 % v/v). Method was developed with C8 column, at 245 nm. Vikas *et al.* [5] reported a method for the determination of sofosbuvir in pure form. In this method mobile was phosphate buffer and methanol in 50:50 %v/v, flow rate is 0.8 mL/min. Method developed at 262 nm. The linearity range was reported between 5-30 µg/mL. Hassouna *et al.* [6] reported a method for simultaneous determination of sofosbuvir and ledipasvir by RP-HPLC method in tablet dosage forms. This method was developed with 0.02 M KH_2PO_4 and 5.7 mM hexane sulfonate, acetonitrile as mobile phase at 1.5 mL/min flow rate. The UV detector wavelength is 254 nm. Vejendla *et al.* [7] submitted a method for the estimation of sofosbuvir in bulk and tablet dosage form. The method was developed with methanol and trifluoroacetic acid as mobile phase and 150 mm ODS column as stationary phase. The linearity was 100-600 µg/mL. Chakravarthy *et al.* [8] reported an ultraviolet-visible spectroscopic method for estimation of daclatasvir and sofosbuvir. In this method, 99.7-100.6 % recovery was reported for sofosbuvir. Bioanalytical methods are simultaneous estimation in plasma methods are not reported. The present study is concerned with the development and validation of sofosbuvir and velpatasvir in spiked human plasma by high performance liquid chromatography.

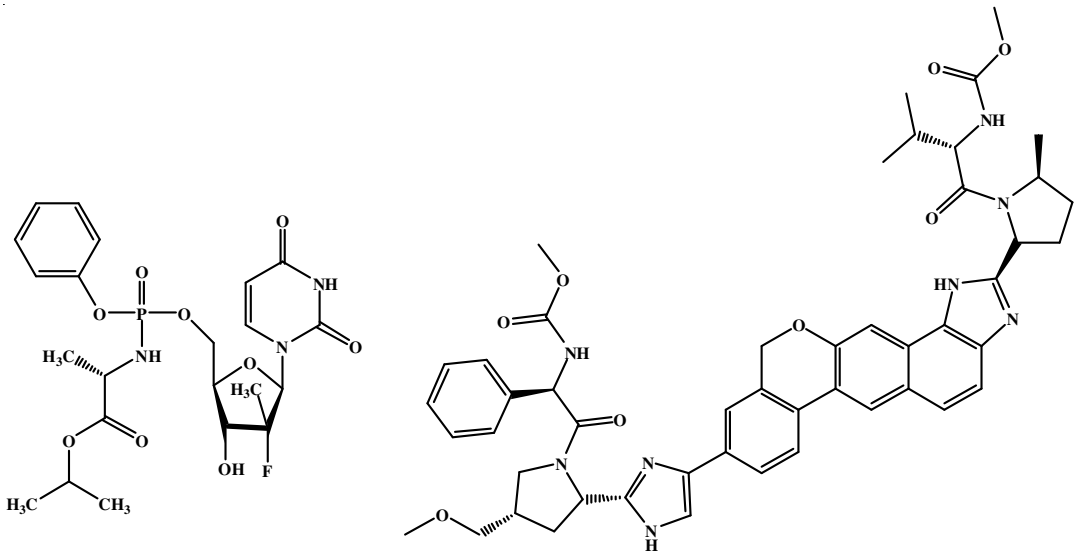

Fig. 1. Structure of sofosbuvir and velpatasvir

EXPERIMENTAL

The method was developed and validated with Peak LC P7000HPLC (Isocratic) system rheodyne injector with 20 µL and UV/visible detector UV7000 and PEAK chromatographic version 1.06. The sofosbuvir and velpatasvir were scanned with UV-visible spectrophotometer (Tech comp-UV 2301, Japan) with Hitachi software. Sofosbuvir and velpatasvir were obtained from Alcon Laboratories, Mumbai. HPLC grade solvents water, acetonitrile, methanol and ortho phosphoric acid (OPA) were procured from Merck, Mumbai. Method was developed with Intersil ODS C18 column (250 mm × 4.6 mm × 5 µ) at 240 nm. The overlay scanning spectra showed in Fig. 2.

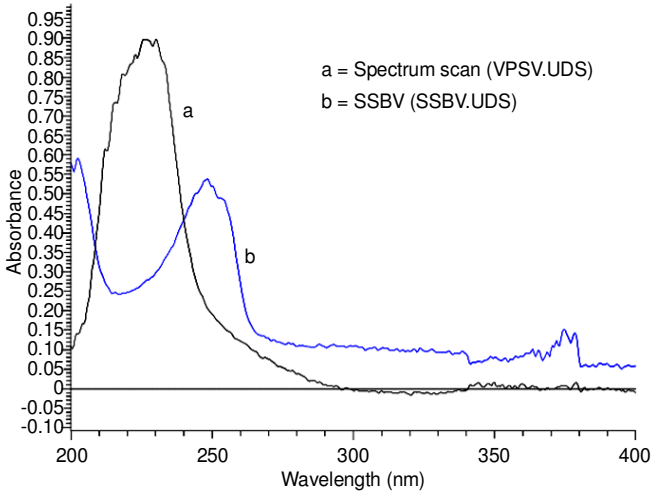

Fig. 2. Overlay of sofosbuvir and velpatasvir

Chromatographic conditions

Optimization of chromatographic conditions: Drugs solubilities were examined and mobile phase was fixed after trail with different ratios of mobile phase combinations. The detection wavelength was optimized in the double beam spectrophotometer, by scanning sample in the range of 200-400 nm. From the overlaid spectrum of sofosbuvir and velpatasvir, 240 nm was selected for the simultaneous quantification of sofosbuvir and velpatasvir in HPLC method. The finalized HPLC conditions are showed in Table-1.

TABLE-1
CHROMATOGRAPHIC CONDITIONS
OF DEVELOPED METHOD

Parameter	Condition
Mobile phase	20 % Water and 80 % acetonitrile v/v
pH	6.5 (Adjusted with OPA)
Column	Intersil ODS C_{18} column (250 mm × 4.6 mm × 5 µ)
Flow rate	1.0 mL/min
UV detector wavelength	240 nm
Run time	10 min
Sample volume	20 µL

Standard stock solution preparation [9]: The extraction of the plasma samples involved liquid-liquid extraction process. The fixed dosages (10 mg) are spiked in to 10 mL plasma and stored for 24 h. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 500 µL was transferred to prelabeled 10.0 mL polypropylene centrifuge tubes. Extraction solvent, 5.0 mL of ethyl acetate, was then added to extract the drug. The samples were then kept on a vibramax unit and vortexed for 15 min. Samples were then centrifuged at 5000 rpm for 5 min in a refrigerated centrifuge (4 °C). Supernatant solution, 1 mL was then transferred into pre-labeled polypropylene tubes and was allowed to evaporate to dryness under nitrogen at 40 °C. The dried residue was then dissolved in 200 µL of mobile phase and transferred into shell vials containing vial inserts for analysis. Samples, 20 µL by volume, were then injected into the column and analyzed by HPLC on the same day to avoid any degradation. The column temperature oven was maintained at ambient temperature. The blank chromatogram and standard chromatograms are showed in Figs. 3 and 4.

Validation

Linearity test: The linearity of calibration curves (peak area *vs.* concentration) in pure solution was checked over the concentration ranges of about 0.1-0.8 µg/mL and 0.025-0.2

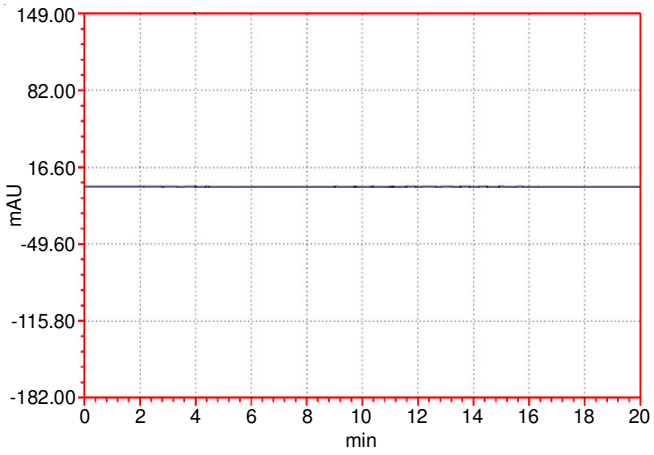

Fig. 3. Blank chromatogram

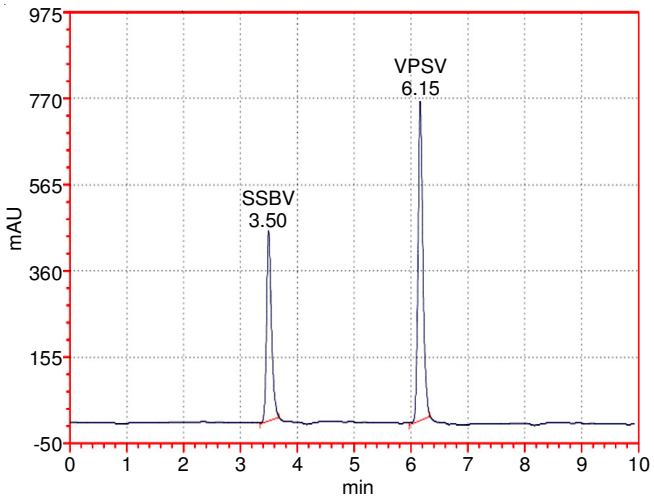

Fig. 4. Standard chromatogram of sofosbuvir and velpatasvir

µg/mL for sofosbuvir and velpatasvir. The total runtime was 10 min. The regression line relating standard concentrations of drug using regression analysis, the calibration curves were linear in the studied range and equations of the regression analysis were obtained (Table-2). The linearity graphs of sofosbuvir and velpatasvir are showed in Figs. 5 and 6, respectively.

TABLE-2
LINEARITY RESULTS OF DEVELOPED METHOD

Conc. (%)	Sofosbuvir conc. (µg/mL)	Peak area	Velpatasvir conc. (µg/mL)	Peak area
25	0.1	174546	0.025	218928
50	0.2	210703	0.05	313402
100	0.4	270338	0.1	450194
150	0.6	324035	0.15	606346
200	0.8	393474	0.2	748597
r^2	0.9990		0.9993	
Slope	305580.1		2995120	
Intercept	146275.5		153005.8	

Precision: Intra-day precision was investigated by replicate applications and measurements of peak area of sofosbuvir and velpatasvir for six times on the same day under similar conditions. Inter-day precision was obtained from % RSD values obtained by repeating the assay six times on two different days. The % RSD was calculated (Tables 3 and 4), which was within the acceptable limit *i.e.* less than 2.0.

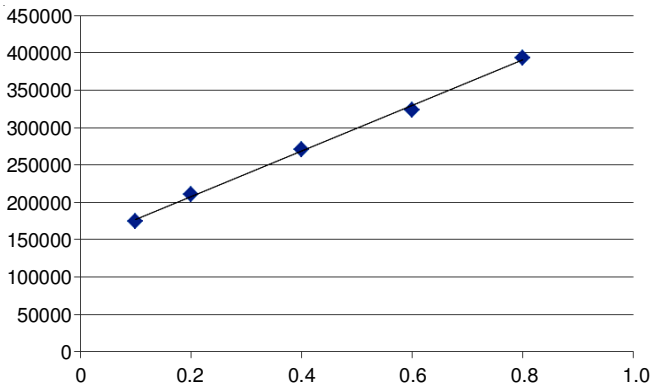

Fig. 5. Linearity graph of sofosbuvir

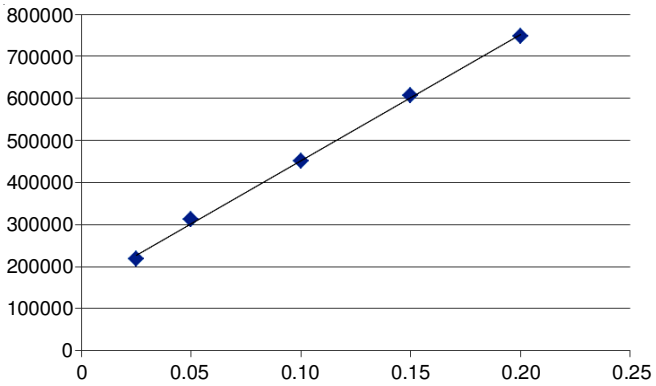

Fig. 6. Linearity graph of velpatasvir

TABLE-3
INTRA-DAY RESULTS OF DEVELOPED METHOD

S. No.	Sofosbuvir conc. (µg/mL)	Peak area	Velpatasvir conc. (µg/mL)	Peak area
Injection-1	0.40	268737	0.10	449503
Injection-2	0.40	279772	0.10	453586
Injection-3	0.40	276166	0.10	451094
Injection-4	0.40	269261	0.10	455082
Injection-5	0.40	277882	0.10	450686
Injection-6	0.40	278001	0.10	449168
RSD (%)	1.73		0.51	

TABLE-4
INTER-DAY RESULTS OF DEVELOPED METHOD

S. No.	Sofosbuvir conc. (µg/mL)	Peak area	Velpatasvir conc. (µg/mL)	Peak area
Injection-1	0.4	269525	0.1	459062
Injection-2	0.4	271142	0.1	456229
Injection-3	0.4	274015	0.1	445129
Injection-4	0.4	274157	0.1	443597
Injection-5	0.4	264785	0.1	458365
Injection-6	0.4	274505	0.1	445404
RSD (%)	1.39		1.61	

Recovery: Accuracy of the method was determined by recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 50 %, 100 %, 150 %. The recovery studies were carried out three times and the percentage recovery and percentage relative standard deviation of the recovery were calculated for both drugs and shown in the Table-5.

TABLE-5
RECOVERY RESULTS OF DEVELOPED METHOD

Concentration level (%)	Sofosbuvir	True area	Recovery (%)	Velpatasvir	True area	Recovery (%)
50	216099	210703	102.5	308121	313402	98.31
100	267879	270338	99.09	451643	450194	100.32
150	326223	324035	100.67	601180	606346	99.14
Average recovery		100.75			99.25	

Sensitivity: The sensitivity was determined by signal to noise (S/N) ratio. The resolution solution was serially diluted and injections were made to obtain chromatogram. Similarly, blank plasma samples were also processed and injected in to chroma-tograph. The results were showed in Table-6.

TABLE-6
LOQ AND LOD RESULTS OF DEVELOPED METHOD

Test	Sofosbuvir (µg/mL)	Velpatasvir (µg/mL)
LOQ	0.0125	0.0030
LOD	0.0060	0.0017

Stability: The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. Short term bench-top stability was evaluated after 24 h at room temperature. Auto sampler stability was evaluated on QCs extracts maintained in the auto sampler at 10 °C for 24 h, by comparing their concentrations with fresh extracts. Freeze and thaw stability have been evaluated after three cycles at -20° to room temperature, by comparison with freshly prepared. Stability of sofosbuvir and velpatasvir solutions was observed at room and temperature and in refrigerated conditions for period of 48 h. Acceptable stability has been considered as percent difference in concentration lower than 5 %. The stability study reports were showed in Table-7. The blank chromatogram and the stability tests chromatograms are showed in Figs. 7-10.

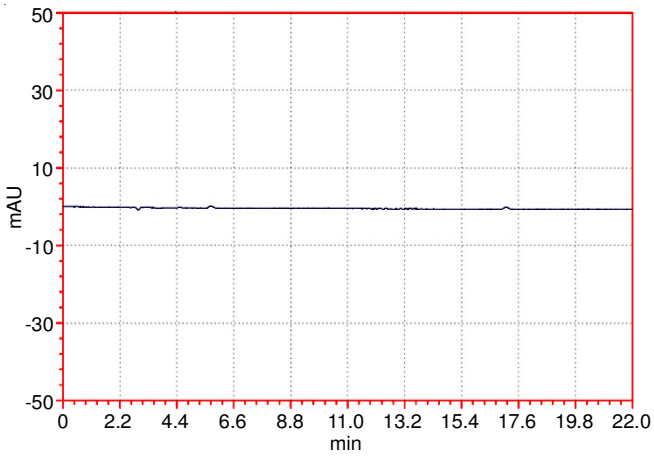

Fig. 7. Blank chromatogram of stability test

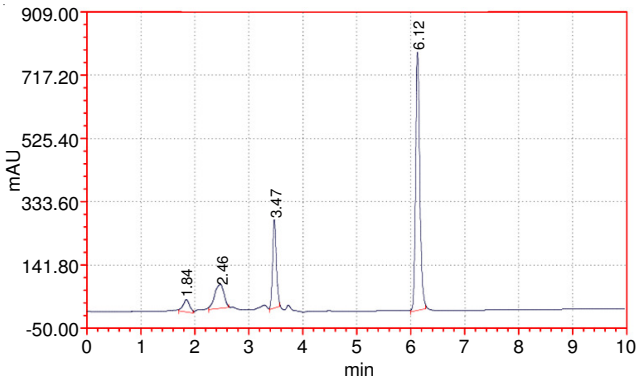

Fig. 8. Bench-top stability test chromatogram of sofosbuvir and velpatasvir

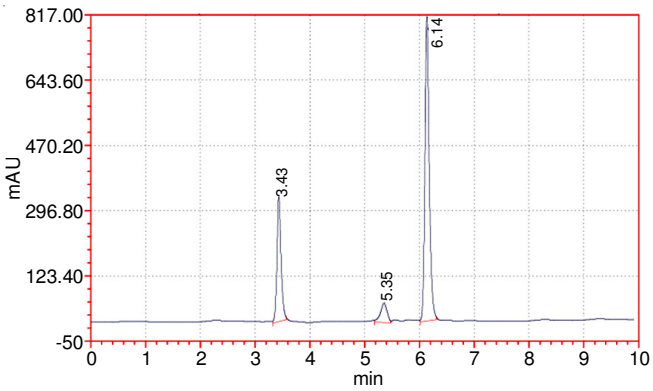

Fig. 9. Auto sampler stability test chromatogram of sofosbuvir and velpatasvir

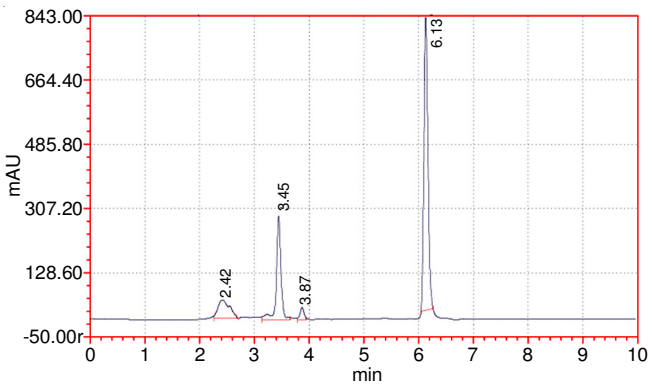

Fig. 10. Freeze and Thaw stability test chromatogram of sofosbuvir and velpatasvir

TABLE-7
STABILITY RESULTS OF DEVELOPED METHOD

S. No.	Stability test	Sofosbuvir	Percentage of change	Velpatasvir	Percentage of change
1	Blank	00	00	00	00
2	Freshly prepared	270338	00	450194	00
3	Bench top	128534	52.45	419692	6.77
4	Auto sampler	171722	36.4	440446	2.16
5	Freeze & Thaw	167866	37.90	415005	7.81

RESULTS AND DISCUSSION

New RP- HPLC method had been developed for simultaneous estimation of sofosbuvir and velpatasvir in plasma. It was shown that the method was precise, accurate, reproducible, linear, selective and specific providing the reliability of the method. The method was validated over the concentration range of 0.1-0.8 µg/mL for sofosbuvir and 0.025-0.2 µg/mL for velpatasvir. The mean percent recovery of sofosbuvir is 100.75 % and for velpatasvir is 99.25 %. The intra- and inter-day precision were conducted at standard concentration, the percen-tage of RSD is below 2 %. Stability of compounds was estab-lished in a series of stability studies. The LOQ concentration of sofosbuvir is 0.0125 µg/mL and velpatasvir is 0.003 µg/mL. The LOD concentration of sofosbuvir is 0.006 µg/mL and velpatasvir is 0.0017 µg/mL. Bench-top stability, auto sampler stability and freeze & thaw stability are conducted with the sample solution. The change in stability test of sofosbuvir is 52.45, 36.4 and 37.90 % in Bench-top stability, Auto sampler stability and freeze and thaw stability respectively. The change in stability test of velpatasvir is 6.77, 2.16 and 7.81 % in bench-top stability, auto sampler stability and freeze and thaw stability, respectively.

Conclusion

The bioanalytical method developed is simple and good accuracy and reproducible. It can be used for the estimation of sofosbuvir and velpatasvir in plasma. The method was validated for linearity, accuracy, precision, LOD, LOQ and recovery. The method was fully validated and showing satisfactory data for all the method validation parameters tested. The recoveries achieved are good by the method. The developed method could be applied in bioequivalence, pharmacokinetic, regular analysis and toxicokinetic studies.

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