Development and validation of a stability-Indicating RP-HPLC method for simultaneous estimation of sofosbuvir and velpatasvir in fixed dose combination tablets and plasma

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Abstract: A simple, specific, sensitive, robust, accurate and precise reverse-phase high performance liquid chromatographic (RP-HPLC) method was developed and validated for simultaneous determination of sofosbuvir (SOF) and velpatasvir (VLP) in fixed dose combination tablets and plasma. Validation parameters, such as system suitability, accuracy, inter-day and intra-day variances, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness and stability were assessed following the standards set by the International Conference on Harmonization (ICH). The isocratic elution of SOF and VLP was carried out under ambient conditions using ammonium acetate buffer (pH = 7.0), acetonitrile and methanol (20:40:40, v/v/v) as mobile phase flowing through a Promosil C18 column at a flow rate of 1.0 mL/min. The average retention time of SOF and VLP was 3.72 min and 7.09 min, respectively. The LOD and LOQ of SOF were 0.23µg/mL and 2.48µg/mL, respectively; while those of VLP were 0.70µg/mL and 7.52µg/mL, respectively. The regression coefficient (r^2) was 0.998. The relative standard deviation (RSD) was less than 2% for precision. The recovery of both the analytes remained within 100±1%. All other validation parameters complied with ICH guidelines. The analytes remained stable throughout the analytical procedure. Moreover, this method was successfully applied to assess the *in vitro* dissolution of SOF and VLP loaded fixed dose combination tablets. Same method with same mobile phase was applied on rat plasma and there was no interference.

Keywords: ICH guidelines, RP-HPLC, simultaneous determination, sofosbuvir, velpatasvir.

INTRODUCTION

According to an approximation, 170-180 million people are infected with Hepatitis C virus (HCV) across the globe. Thus, it has become a major public health concern (Lavanchy, 2011, Choudhary *et al*., 2014, Beumont *et al*., 2018). The patients suffering from HCV may also suffer from complications such as decompensated cirrhosis, hepatocellular carcinoma and in severe cases needs liver transplantation (Poynard and Afdhal, 2010). Before the advent of advance treatment strategies for six genotypes and subtypes of HCV, various amalgams of ribavirin, interferon, PEGylated alpha interferon, boceprevir and telaprevir were present as a standard of care (SOC) with minimal sustained virologic response (SVR). Nevertheless, patients were suffering from poor tolerability and severe side effects (Naggie *et al*., 2010, Magiorkinis *et al*., 2013, Pearlman and Traub, 2011, Ghany *et al*., 2011, Harrison, 2012).

Sofosbuvir, known as GS-7977, is a nucleotide analogue polymerase inhibitor of non-structural protein (NS-5B). It's most effective and promising pro-drug possessing high SVR rate. In the treatment of HCV infection, it is administered in combination with other drugs (Cholongitas and Papatheodoridis, 2014, Keating and Vaidya, 2014, Rose *et al*., 2014). The intention of HCV therapy is to enact SVR, described as HCV-RNA <15 IU/ml, after 3 to 6 months of antiviral treatment. Previously, SVR was predicted after 24 weeks of treatment but new guidelines encourages it at 12 weeks due to similarity between SVR12 and SVR24 (Dugum and O'Shea, 2014, Yoshida *et al*., 2015, Chen *et al*., 2013).

In recent past, numerous randomized control trails (RCTs) have assessed effectiveness of fusion of SOF and VLP for treatment of different HCV genotypes (Everson *et al*., 2015, Pianko *et al*., 2015, Curry *et al*., 2015, Feld *et al*., 2015, Foster *et al*., 2015). Epclusa® is a combination product containing sofosbuvir and velpatasvir. It is prescribed in adults with chronic hepatitis C infection. Sofosbuvir is inhibitor of nucleotide non-structural protein (NS-5B) polymerase of HCV while velpatasvir is HCV NS5A replication complex inhibitor (Carter *et al*., 2017). To manage the genotypes 1 through 6 HCV infected adults, the Food and Drug Administration (FDA) have recommended the combination of SOF and VLP with once daily dose orally (Tanzi, 2016). Sofosbuvir is a non-hygroscopic white to off-white coloured crystalline

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powder with the chemical formula $C_{22}H_{29}FN_3O_9P$ (fig. 1). According to Biopharmaceutics Classification System (BCS), sofosbuvir is placed in class III as it possesses high aqueous solubility and poor permeation across cell membranes. It has a pH-independent solubility over a pH of 1.2 to 7.7 (Amidon *et al*., 1995, Zaman *et al*., 2016). On the other hand, velpatasvir (fig. 2) is placed in class II and show low aqueous solubility and good permeation across cell membranes. However, velpatasvir exhibits pH-dependent solubility. It is completely miscible at pH 1.2, while it is soluble to some extent at pH 2, and highly immiscible at pH > 5.

So far, only few RP-HPLC methods have been developed and validated for quantification of pure sofosbuvir (Vikas *et al*., 2016), in some pharmaceutical dosage forms (Jeyabaskaran *et al*., 2014) and for *in vitro* simultaneous determination of sofosbuvir with other drugs, such as ledipasvir (Hassouna *et al*., 2017, Zaman *et al*., 2016), simeprevir (Kumar and Subrahmanyam, 2016). Similarly, some RP-HPLC methods also have been developed for simultaneous determination of velpatasvir with other drugs such as voxilaprevir (Rani and Devanna).

Here, we propose reliable, sensitive RP-HPLC method in accordance with the guidelines of ICH, for simultaneous quantification of sofosbuvir and velpatasvir in pharmaceutical formulations and plasma samples.

MATERIALS AND METHODS

Reagents and chemicals

Sofosbuvir (Pharmagen Pvt Ltd., Lahore, Punjab, Pakistan) and Velpatasvir (Astron Research Pvt Ltd., Ahmedabad, Gujarat, India) were the presented samples. Tablets containing 400mg of SOF and 100mg of VLP were obtained from (Sofosvel tablet, Beacon Pharmaceuticals Ltd., Dhaka, Bangladesh). Analytical grade ammonium acetate, methanol, acetonitrile and potassium dihydrogen phosphate were purchased from Daejung Chemical Co. (Siheung, South Korea). The remaining reagents employed in this research were of analytical grade. Double distilled water was used throughout the research.

Instrumentation

Shimadzu RP-HPLC equipment (Shimadzu Corporation, Kyoto, Japan), having quaternary LC-20AD pumps equipped with a DGU-$20A_3$ degasser and a variable wavelength programmable UV visible detector SPD-20A, was used to perform chromatographic analyses under ambient conditions. Promosil C18 series column (250 mm × 4.6mm ID, 5µm) was procured from Bonna-Agela technologies (Tianjin, China) and used for analysis. Mobile phase was comprised of ammonium acetate buffer (pH = 7.0), acetonitrile and methanol (20:40:40, v/v/v). Mobile phase flow rate was 1.0 ml/min for isocratic elution. A Shimadzu AW220 electronic balance (Shimadzu Corporation, Kyoto, Japan), James ultrasonic bath (James Products Europe Ltd., Dorset, UK), Shimadzu UV-1601 PC-UV/Visible double beam spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and Millipore vacuum filtration assembly was used throughout the operation. The injection volume was 20 µl. The eluent was monitored at 270 nm for quantification of analytes. The USP type II dissolution apparatus (GHT-1 Galvano-Scientific Co., Lahore, Punjab, Pakistan) was used for *in vitro* dissolution study. Calibrated analytical balance and glassware's were used in all experiments.

Methods

Mobile phase and dissolution medium

Accurately weighed 750mg of ammonium acetate was dissolved in 400ml of distilled water using 500ml calibrated volumetric flask to prepare the ammonium acetate buffer. Its pH was adjusted to 7.0±0.5 by using the 1.0% (w/v) ammonia solution. For preparation of mobile phase ammonium acetate buffer, acetonitrile and methanol was mixed (20:40:40) and filtered through the 0.2µm nylon filter by using Millipore vacuum filtration assembly. 50 mM Sodium acetate buffer was used for dissolution study: Dissolve 4.4 g of sodium acetate trihydrate in about 0.8L of de ionized (DI) water, adjust the pH to 5.0 with glacial acetic acid (about 1.2 mL), then transfer it to a 1 L volumetric flask and bring to volume with DI water. Further dissolve 0.5% w/v cetyltrimethyl ammonium bromide (CTAB) in above solution.

Preparation of standard stock solution

Stock solutions containing 0.4mg/mL of SOF and 0.1mg/mL of VLP were made by using the mobile phase as the dissolution medium. Serial dilution of stock solution leads to standard solutions of following strengths: 10-60µg/mL for SOF and 1-6µg/mL for VLP.

Preparation of sample solution

Ten tablets ofSofosvel were accurately weight and powdered. Tablet powder equivalent to 100 mg of velpatasvir and 400 mg of sofosbuvir was mixed with dissolution media in 100 ml of volumetric flask. Sonicate the solution for thirty minutes. For analyzing the analytes, the above prepared solution was further diluted to 400 µg/mL and 100 µg/mL of sofosbuvir and velpatasvir, respectively with the mobile phase. Before analysis filter the through 0.45 µm membrane

In vitro dissolution studies

In vitro dissolution studies were executed by using USP dissolution apparatus II (GHT-1 Galvano scientific, Lahore, Punjab, Pakistan). The dissolution medium was comprised of 900 mL of 50 mM sodium acetate buffer containing 0.5% w/v cetyltrimethyl ammonium bromide (CTAB) at pH 5.0 (FDA). Paddle rotation was fixed at 75 rpm and temperature of medium was maintained at 37 ± 5°C. Later on samples were collected at the following predetermined time points i.e. 5, 10, 20, 30 and 45

minutes respectively. At each time interval, 10mL of sample was withdrawn, filtered (0.45µm) and diluted suitably. Afterwards, these diluents were analysed chromatographically to determine SOF and VLP titre.

Moreover, 10mL of fresh dissolution medium, maintained at the same temperature, was added to the dissolution vessel after each sampling as compensation.

Method development and optimization

As the structures of sofosbuvir and velpatasvir hold the benzene rings and conjugated groups, they are the UV-active compounds. The absorbance maxima was obtained at wavelength of 261±1nm for SOF and 334±1nm for VLP, however specific absorbance (A=1%) at the utmost wavelength was 178.5±4 (SOF) and 564.3±5 (VLP) respectively. In order to obtain optimal wavelength on UV spectrophotometer at a single wavelength for simultaneous identification, absorbance of mixture composite and separate reference solution composed of sofosbuvir 100µg/mL and velpatasvir 10 µg/mL was calculated from 200 to 400 nm. So, 270nm was selected for both entities where they gave suitable absorbance. By considering the nature and solubility of each analyte, a set of conditions was selected to make the resolution better by systematic elution. Most of all reported HPLC methods till date use C-8 or C-18 columns. They used complex mobile phase compositions. Hence, attempts were directed towards development of a simple and better method on commonly used Agela C18 column with good resolution. Different modifications were tried to get good separation among the two drugs. These changes included change in mobile phase composition and different flow rate (data not shown).

Optimum conditions employed consist of mobile phase where its ratio was kept constant at 40:40:20 of methanol: acetronitrile: ammonium acetate and flow rate of 1.0 mL/min. Because of better resolution, each analyte exhibited different retention time, and they can easily be quantified and identified in the comparison with individual reference solutions. Given set of chromatographic conditions was further ratified according to USP and ICH guidelines.

Method validation

Analytical procedure was affirmed for precision and accuracy, intermediate precision, specificity and linearity, limit of quantification (LOQ), and limit of detection (LOD) in accordance to (CATEGORY 1) USP and ICH guidelines.

Accuracy and recovery

By inspecting six samples with known concentrations of SOF (60, 50, 40, 30, 20 10µg/mL) and VLP (6, 5, 4, 3, 2 and 1µg/mL) the validity of analytical procedure was settled. Each sample was injected in triplicate and RSD was less than 2%.

Precision and accuracy

To determine the fidelity and veracity, QC samples (in addition to calibration standards), were processed with various concentrations covering the whole linearity range. The extent of closeness of different measurements under given set of operational condition for any analytical procedure is referred as precision. Assay precision was done by intermediate precision (inter-day) and repeatability (intra-day) and statistically significant number of replicate measurements were reported as relative standard deviation (Swartz and Krull, 2012).

Assays performed on three different days to study the intermediate precision and were compared; the outcomes were reported as SD and RSD. The analyte recovered from already known added concentration is accuracy. Aanalytical method was validated by testing three concentration levels that overlay the particular range, over three different time periods (Swartz and Krull, 2012).

Linearity and range

Six concentrations (n=3) of SOF (60, 50, 40, 30, 20, 10 µg/mL) and VLP (6, 5, 4, 3, 2, 1µg/mL) were analysed to study the linearity. Graph was plotted having concentration on X-axis and peak response (A) on Y-axis. In account least square linear regression equation i.e. A = slope C + Y intercept, (where A stands for peak area, C corresponds to concentration), response concentration relation was regarded. Limit of quantification (LOQ) and limit of detection (LOD) was calculated by using the expression 10δ/slope and 3.3δ/slope respectively.

Specificity and placebo interference

Specificity of method is essential to analyse the interference of dissolution medium and excipients on the elution of drug substance. Placebo solution was prepared by using the medium which includes all the inactive substance of tablets, and the chromatograms were observed for interference. Analysis of given solution was performed and baseline assessed for peak response using the same chromatographic conditions. The results ensured that there was no interference of placebo with sofosbuvir and velpatasvir.

Stability of solution

Amber coloured flasks were used to store sample solutions. Sample solution stability was evaluated by the analysing solutions having SOF 60µg/mL and VLP 6µg/mL. Different conditions were provided and replicated (n=3) at various temperatures i.e. (2-8°C) and (15-25°C) for 14 and 7 days respectively. By comparing it with the assay of freshly prepared reference standard solution the results were examined. Forced degradation study was also performed.

Plasma spiking

A cost effective and accurate method should also be applied for analysing plasma samples. Our developed

method was used for the analysis of SOF and VLP in rat plasma, meeting all the requirements for the validation of an analytical methodology.

RESULTS

System suitability

The assessment of system suitability was done by selecting a set of conditions as per USP guidelines i.e. mobile phase comprising acetonitrile, methanol and ammonium acetate buffer (40:40:20 % v/v) and flow rate 1.0 mL/min with column Agela C18, 250 mm × 4.6cm, 5µm. Ezichrom chromatography manager software was used to calculate the statistical data of different parameters i.e. retention time (tR), peak area (A), relative standard deviation, symmetry factors (As), theoretical plates (N), and resolution of SOF and VLP for peak response. Results of table 1 elaborate the execution framework of analytical procedure for system suitability and complied with the USP specifications. Capacity factor (K) was 2-10, tailing factor was less than 2.0 and the amount of theoretical plates exceeds 2000.

Method validation

Accuracy and recovery

The accuracy of the developed method was computed by calculating the percentage recoveries of three different concentrations of each component in the ternary mixture. The percentage recovery values are presented in table 2.

Precision

Inter-day and intra-day precisions used to evaluate the accuracy of developed method. From the results that are reported in table 3, it can be seen that the method is precise. The mean recovery value was 98.393 for SOF, and 98.696 for VLP thus depicting the accuracy of the developed method.

Robustness

Results shown in table 4 specify that there are minute changes of ±2% in the values of chromatographic conditions that do no influence the results for sofosbuvir and velpatasvir. RSD values at several concentration (i.e., 20 and 120%) fall under acceptance criteria of ±2.0% for replicates (n = 3).

Linearity

Favourable concentration-peak response relation indicates the confirmation of statistical data imitated from linearity studies as shown from results in table 4. y=59308x-1812.9 is the linear regression equation for linearity of velpatasvir and for sofosbuvir it is y=20517x- 10264, while 0.9987 (SOF) and 0.9985 (VLP) are correlation coefficient. LODs were 2.480 and 0.230µg mL^{-1} and the LOQs were 7.517 and 0.697µg/mL for sofosbuvir and velpatasvir respectively.

Specificity and Placebo Interference

No notable peak of placebo (fig. 3) is observed at the given retention time and the influence of tablet inactive form on the peaks of sofosbuvir and velpatasvir under designated chromatographic conditions is imperceptible.

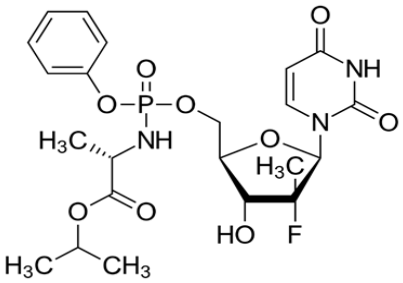

Fig. 1: Chemical structure of sofosbuvir (Drug Bank).

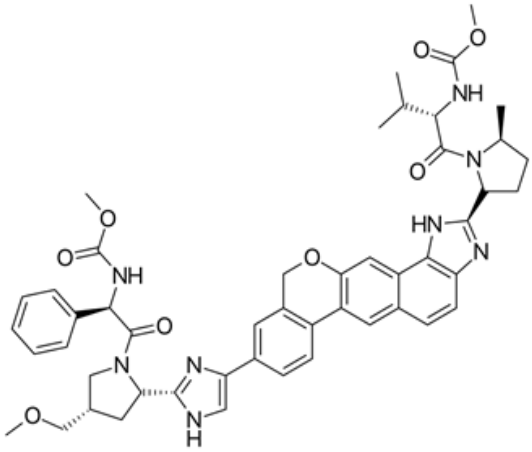

Fig. 2: Chemical structure of velpatasvir (Drug Bank).

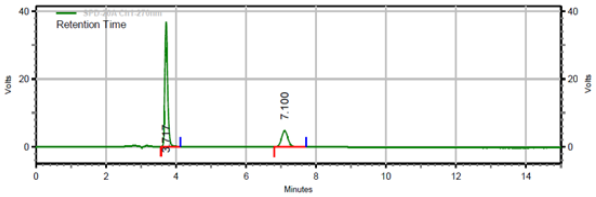

Fig. 3: Chromatogram of 10µg/ml SOF and 1µg/ml VLP standards.

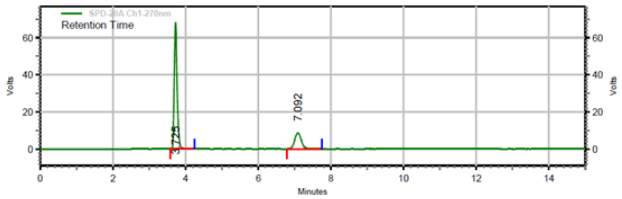

Fig. 4: Chromatogram of 20µg/ml SOF and 2µg/ml VLP standards.

Stability of Solution

Table 5 elaborates and summarizes the stability of sofosbuvir and velpatasvir. Solutions remain stable up to seven days at room temperature (15-25°C) and 14 days at cool temperature and therefore amber colour flask protected it from degradation. These two compounds were

subjected to forced degradation study by applying stress conditions and results are shown in table 7. Force degradation study was conducted and no more than 20 % degradation was observed.

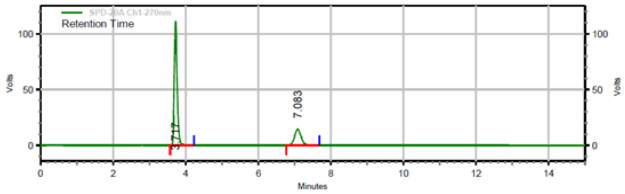

Fig. 5: Chromatogram of 30µg/ml SOF and 3µg/ml VLP standards.

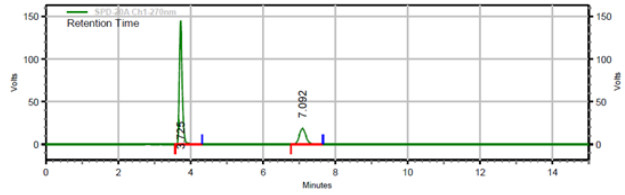

Fig. 6: Chromatogram of 40µg/ml SOF and 4µg/ml VLP standards.

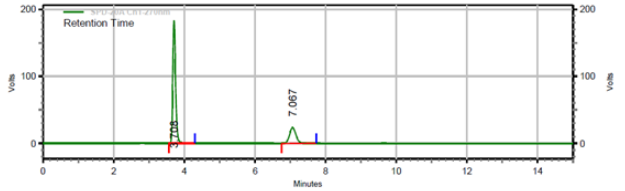

Fig. 7: Chromatogram of 50µg/ml SOF and 5µg/ml VLP standards.

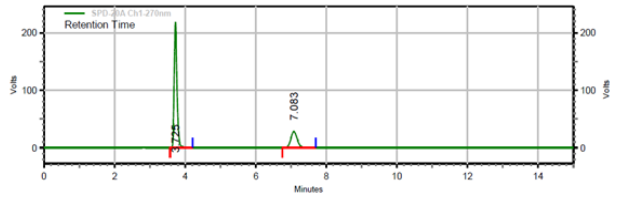

Fig. 8: Chromatogram of 60µg/ml SOF and 6µg/ml VLP standards.

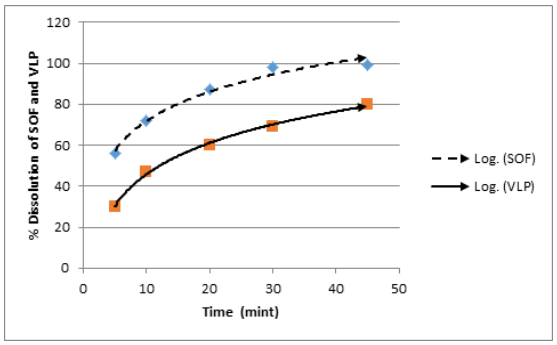

Fig. 9: Dissolution profile of VLP and SOF

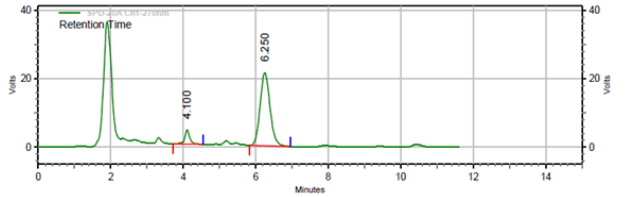

Fig. 10: Chromatogram of SOF and VLP plasma spiking.

Application of method and in vitro dissolution of tablets

For the quantitative assurance of sofosbuvir and velpatasvir in tablet dosage form, this technique was propitiously practiced. Table 2 mentions the recovery studies which assured the recovery of sofosbuvir and velpatasvir. Table 6 shows parallel dissolution studies implemented on brand Sofosvel tablets for the recovery of sofosbuvir and velpatasvir in the dissolution samples, while applying dissolution process and conditions as illustrated by the FDA. Aggregated % of sofosbuvir and velpatasvir released plotted against time, to attain the product in vitro dissolution profile (fig. 9). Graphical form and statistical analysis shows the analogous behaviour shown by dissolution of both generic brands to the prime developer, and all brands meet the criteria of USP (Q = 80 %) within 45 minutes of dissolution.

Plasma spiking

The suggested method was successfully applied to determine the studied mixture in spiked plasma sample. By using same conditions we performed this assay in rat plasma and results have shown that it's a reliable method. There is no interference found during performance (fig. 10).

DISCUSSION

A method of analysis is pivotal in the product development of a drug. RP-HPLC is the most convenient and frequently used method of analysis for drugs in pharmaceutical industry. This work mentions the simultaneous determination of sofosbuvir and velpatasvir by RP-HPLC. We were able to validate the method as per ICH guidelines. The developed assay was precise, specific, linear and robust. It can easily quantify both the drugs in bulk and in the dissolution medium. No interference was observed in any sample, which indicates the suitability of the assay. The forced degradation analysis also reveals the suitability of the method for quantitative and qualitative evaluation of both drugs. Plasma spiking results demonstrated that the process of liquid-liquid extraction was adequate and the rat plasma components did not interfere with the separation of both active moieties. This makes the current method even more attractive for its ability to quantify drugs not only in the pharmaceutical samples but also from the biological fluids.

CONCLUSIONS

For pharmaceutical dosage forms, *in vitro* dissolution studies are fundamental norm for product quality control. Different analytical methods are used to detect and determine the concentration of drug substances in dissolution media. We developed RP-HPLC method for simultaneous detection and estimation of SOF and VLP in dissolution media and plasma. Our method complied with

Table 1: Results of system suitability parameters for SOF 10 µg/ml and VLP 1µg/ml.

Sr. No	Parameters	SOF	VLP
1	Peak Area	203224± 0.621	60331± 0.563
2	Retention time	3.725± 0.107	7.083± 0.291
3	Theoretical plates	More than 2000	More than 2000
4	Asymmetry	1.80% ± 0.348	1.50% ± 0.556

Table 2: Results of recovery study by HPLC methods.

Drug	Amt. present(µg/ml)	Amt. added (µg/ml)	Amt. found* (µg/ml)	Amt. recovered* (µg/ml)	% Recovery*	% RSD
SOF	10	2	11.93	1.93	96.5	1.591
	10	4	13.95	3.95	98.75	1.543
	10	6	15.97	5.97	99.5	1.523
VLP	1	1	1.98	0.98	98.0	1.651
	1	2	2.89	1.89	94.5	1.453
	1	3	3.97	2.97	99.0	1.871

Table 3: Data for intra-day and inter-day precision.

Drug	Amt (µg/ml)	Intra-day variation (n=3)		Inter-day variation (n=3)	
		% Precision	% RSD	% Precision	% RSD
SOF	10	98.431	0.622	99.582	0.451
	10	97.232	0.531	98.182	0.552
	10	99.432	0.432	97.511	0.452
Average precision		98.393			
VLP	6	97.131	0.624	98.383	0.532
	6	98.233	0.533	99.581	0.651
	6	99.484	0.431	99.382	0.484
Average precision		98.696			

Table 4: Regression analysis and sensitivity determination.

Parameters	SOF	VLP
Working λmax	270	270
Regression equation	y=20517x- 10284	y=5938x- 1812.9
Slope	20517	5938
Intercept	- 10284	-1812.9
LODµg/ml	2.480	0.230
LOQµg/ml	7.517	0.697
Regression coefficient (r_2)	0.9987	0.9989
Retention time (min)	3.717	7.100

Table 5: Results of stability studies

Drug	Concentration analyzed (µg/mL)	Concentration recovered % ± S.D	
		15–25 °C, 7 days	2–8 °C, 14 days
SOF	60	99.451± 0.15	98.873 ± 0.22
VLP	6	98.972± 0.16	98.732 ± 0.17

guidelines of USP and ICH. Results of the tests complied with both limits and criteria. This method could be applied for determination of SOF and VLP in fixed dosage form and concurrent determination of dissolution profiles using USP apparatus II. In conclusion, this method was found to be suitable for assessment of SOF and VLP in combination dosage forms, test solutions and plasma samples.

Table 6: Dissolution of tablet %

Time (minutes)	Sofosvel tablet Brand			
	SOF	Average % release	VLP	Average % release
5	45	45.058± 0.097	56	56.109± 0.096
10	59	59.322± 0.285	72	72.050± 0.087
20	84	84.680± 0.843	87	87.115± 0.112
30	98	98.105± 0.600	98	98.212± 0.226
45	99	99.249± 0.217	99	99.379± 0.364

Table 7: Forced degradation study

Stress conditions	Mean area of SOF	% Degradation average	Mean area of VLP	% Degradation
Heat stress	193224± 0.321	4.920	58331± 0.563	3.310
1N HCl	189204± 0.243	6.898	57321± 0.467	4.989
1N NaoH	173222± 0.432	14.763	59311± 0.503	1.690
5% H_2O_2	183314± 0.621	9.797	58332± 0.463	3.313
Humidity and Light	193234± 0.243	4.915	59031± 0.134	2.154

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