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DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING RP-UPLC METHOD FOR THE SIMULTANEOUS QUANTIFICATION OF SOFOSBUVIR AND VELPATASVIR IN FINISHED DOSAGE FORM

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ABSTRACT

A simple, rapid, accurate, precise and economical reverse phase Ultra performance liquid chromatographic method was developed for simultaneous quantification of two anti-viral drugs, viz., Sofosbuvir and Velpatasvir. The separation of both the drugs was achieved on Endeavorsil C 18 column (2.1 × 50 mm, 2.5 µm particle size) as a mobile phase with phosphate buffer (at pH 3): Acetonitrile (50:50 v/v). The flow rate was 0.3 ml/min and detection was done at 240 nm based on isobestic point. The retention time of Sofosbuvir and for Velpatasvir was 2.7 mins and 1.7 mins respectively. The proposed method was validated as per ICH guidelines. The linearity of the method was evaluated at a range of 10 to 50µg/ml and 40 to 200µg/ml for sofosbuvir and Velpatasvir respectively. The Correlation Coefficient of Sofosbuvir and Velpatasvir were 0.999 each. Precision studies were carried out and % RSD of peak areas of Sofosbuvir and Velpatasvir was about 0.6 and 1.6 respectively. The percentage recoveries of both the drugs Sofosbuvir and Velpatasvir from the tablet formulation were 99.79% and 99.95% respectively. Results obtained for LOQ, LOD and Robustness were well within the acceptance criteria. Validation results indicated that the method is linear, accurate, precise, and robust. The simple mobile phase composition makes this method cost effective, rapid, and non-tedious and can also be successfully adopted for simultaneous estimation of both drugs in commercial products.

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INTRODUCTION

The Sofosbuvir and Velpatasvir fixed-dose combination is a direct acting antiviral medication used as in chronic Hepatitis C . Sofosbuvir is a nucleotide hepatitis C virus (HCV) Nonstructural protein (NS) 5B polymerase inhibitor. Velpatasvir is HCV NS5B replication complex inhibitor.

Sofosbuvir is an nucleotide analog with the chemical name propan-2-yl(2S)-2-{[(S)-{[(3R,4R,5R)-5-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl] methoxy}(phenoxy) phosphoryl]amino} propanoate. It is a white to off-white crystalline solid with a solubility of ≥ 2 mg/ ml across the pH range of 2-7.7 at 37 °C and is slightly soluble in water.

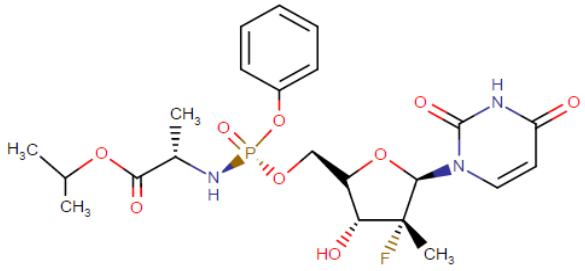

Figure 1: Structure of Sofosbuvir.

Velpatasvir is a selective NS5A inhibitor which bind domain 1 of NS5A consisting of amino acids 33-202. NS5A inhibitors compete with RNA for binding at this site. The chemical name is (2S)-2-{[hydroxy(methoxy)met hylidene]amino}-1-[(2S,5S)-2-(17-{2-[(2S,4S)-1-(2R)-2-{[hydroxy(methoxy)methylidene]amino}-2-phenylacetyl]-4-(methoxymethyl)pyrrolidin-2-yl]-1H-imidazol-5-yl}-21-oxa-5,7- diazapentacyclo[11.8.0.0^{3,11}.0^{4,8}.0^{14,19}]henicosa1(13),2,4(8),6,9,11,14(19)-yl)-5-methylpyrrolidin-1-yl]-3-methylbutan-1-one . It is practically insoluble above pH 5, slightly soluble at pH 2, and soluble at pH 1.2.

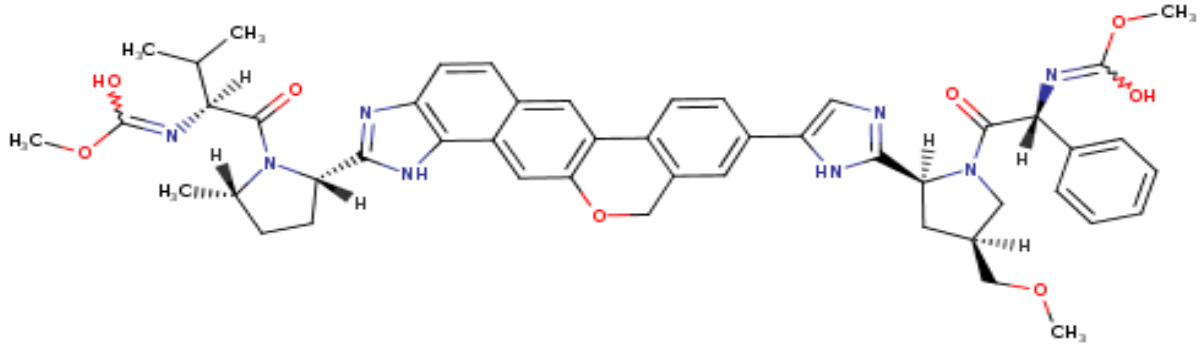

Figure 2: Structure of Velpatasvir.

Some spectroscopic and chromatographic methods have been reported for the estimation of these drugs in individual dosage forms. Similarly a few more methods were reported for their estimation in combinations with other molecules .Literature also reveals reports on bio-analysis of these drugs from various biological matrices .However the methods are cumbersome. But a very few RP-UPLC methods have been reported till date for the quantification of Sofosbuvir and Velpatasvir combination . Based on the above observations and the significance of this drug combination in the management of Hepatitis C treatment we assumed worthy to develop a simple, reliable, economical and validated RP-UPLC method that can be conveniently adopted for the routine analysis.

MATERIALS AND METHODS

Instruments

A Waters, acquity UPLC system with Photodiode Array detector 2996 with data handling system Empower 2 solutions was utilized for the study. Chemicals were weighed using electronic balance Denver. All pH measurements were done on Thermo scientific pH meter.

Chemicals and Solvents:

UPLC grade solvents methanol, orthophosphoric acid and Acetonitrile were obtained from Merck Specialties Pvt Ltd, India. AR grade Potassium dihydrogen Orthophosphate and UPLC grade water, obtained from MERK Pharmaceuticals India Ltd. Sofosbuvir and Velpatasvir were obtained as pure standards from Pharmatrain Labs Pvt Ltd, Hyderabad, India and samples were obtained as [tablets of Sofosbuvir (100mg) and Velpatasvir (100mg)]. (Brand name- Velasof, Epclusa).

Selection of detection wavelength (λmax)

A solution of 10µg/ml of Sofosbuvir and Velpatasvir were prepared in methanol. The solution was scanned in UV-Visible spectrophotometer. The isobestic point was observed at 240 nm. Hence the wavelength of 240 nm was adopted for chromatographic detection. The UV spectrums were summarized in fig no, 3.

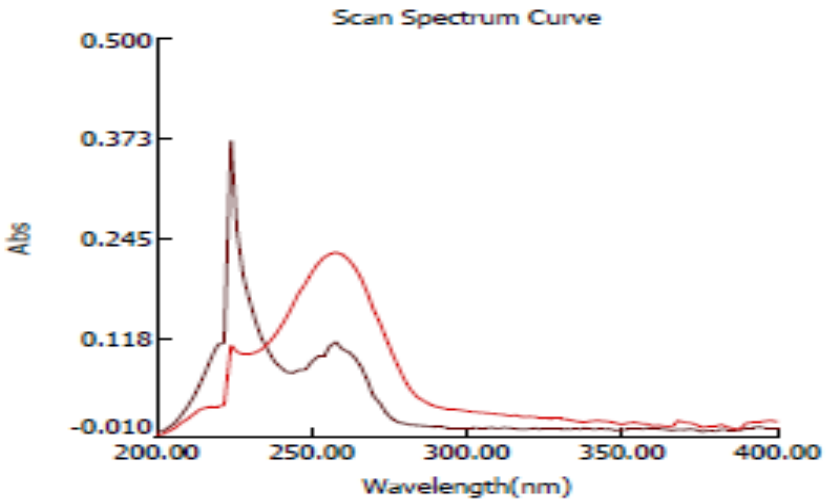

Fig no: 3 Overlay UV Spectrums of Sofosbuvir and Velpatasvir.

Preparation of standard solution:

Accurately weighed and transferred 10 mg of Velpatasvir and 40 mg of Sofosbuvir working standard into a 10ml clean dry volumetric flask Diluents were added and sonicated to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 1 ml of Velpatasvir & Sofosbuvir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Further pipette 3 ml of Velpatasvir & Sofosbuvir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Preparation of sample solution:

Accurately weigh and transfer Equivalent to 10mg of Velpatasvir and 40 mg Sofosbuvir was weighed and transferred equivalent weight of the sample into a 10ml clean dry volumetric flask add about 7ml of Diluents and sonicated to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 1 ml of Velpatasvir & Sofosbuvir of the above stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with Diluents.

Further pipette 3 ml of Velpatasvir & Sofosbuvir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Preparation of placebo:

The amount of powdered inactive ingredient supposed to be present in 10 tablets was accurately weighed and transferred in to 10 ml volumetric flask, 7 ml of diluent was added and sonicated for about 10 minutes. Diluted up to the mark with diluent and allowed to stand until the residue settles before taking an aliquot for dilution. 0.6 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluents up to the mark and the solution was filtered through 0.45 □m filter before injecting into UPLC system.

Diluent: Mobile phase

Chromatographic conditions:

A reverse phase UPLC column Endevorsil, C18, (2.1mm x 50mm, 1.8 µm) particle size was used for elution at ambient temperature. The mobile phase was pumped through the column at a flow rate of 0.3ml/min. The sample injection volume was 4 µl. The detector was set to a wavelength of 240 nm and the chromatographic run time was set to 5 minutes.

Method Development:

With all the initial conditions set and from the preliminary information drawn from previous literature method development and optimization of the same was initiated. During optimization a blend of conditions were tried to achieve better chromatographic elution and resolution as well. Particularly the mobile phase compositions were changed in order to overcome the problems in the previous run. The first trial was initiated with methanol and water at a ratio 60:40 v/v. Unwanted peaks were observed. In order to rectify this phosphate buffer was included in the mobile phase and the composition is PH 3.5 phosphate buffer: methanol at a ratio 40:60v/v. Peaks were eluted with less resolution. In order to improve the resolution organic portion was increased and the mobile phase is PH 3.5 phosphate buffer: acetonitrile at a ratio 35:65%v/v. still the resolution was not good and the peak symmetry was not good as well. Finally the method was optimized by changing the PH of the buffer to 3.0 and by decreasing the portion of acetonitrile. The final mobile phase composition is buffer: phosphate buffer: acetonitrile at a ratio 50:50%v/v. with this mobile phase composition peaks were eluted with good resolution and the retention times for both the analytes were satisfactory i.e. 2.793 & 1.765for Sofosbuvir and Velpatasvir respectively. The chromatogram for this optimized trial is given in fig no: 4.

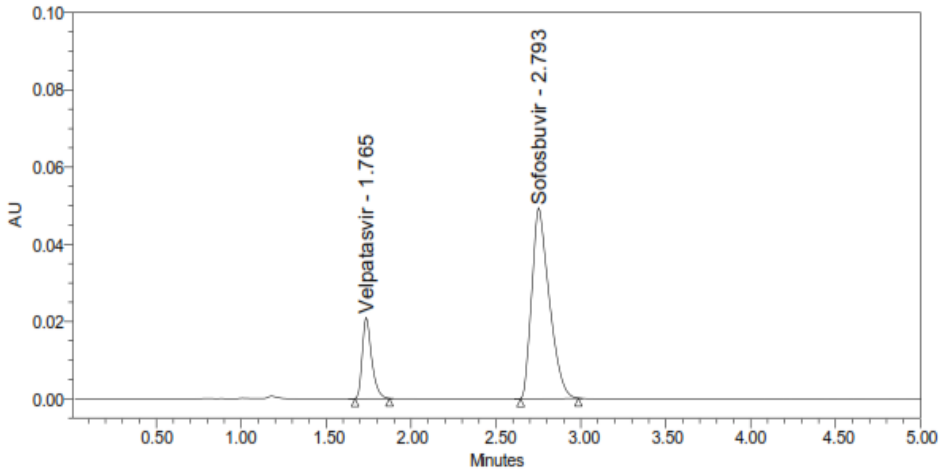

Fig no: 4 Chromatogram of optimized trial.

Method validation:

System Suitability:

Sample solutions injected in triplicates as per the procedure and the chromatogram were recorded. System suitability parameters like tailing factor, theoretical plates and peak areas were checked. The results are given in table-**1&2.**

Table no: 1 system suitability results of Sofosbuvir.

Injection	Rt	Peak area	Plate count	Tailing
1	2.793	7777238	2547.35	1.58
2	2.797	7804773	2546.34	1.57
3	2.624	7799447	2535.26	1.49
4	2.523	7831588	2545.28	1.52
5	2.627	7838873	2547.32	1.48
6	2.568	7836836	2539.32	1.53

Table no: 2 System suitability results of Velpatasvir.

Injection	Rt	Peak area	Plate count	Tailing
1	1.765	134670	3877.36	1.35
2	1.745	131398	3878.65	1.34
3	1.795	131921	3825.52	1.35
4	1.892	136731	3852.63	1.36
5	1.764	132141	3895.86	1.37
6	1.795	132852	3845.21	1.39

Specificity:

It is the ability of the method to measure the analytes of interest specifically in presence of matrix and other components. Samples of blank and placebo were injected as per the test procedure. The chromatograms of blank and placebo were represented as Fig no: **5&6**.

Specificity:

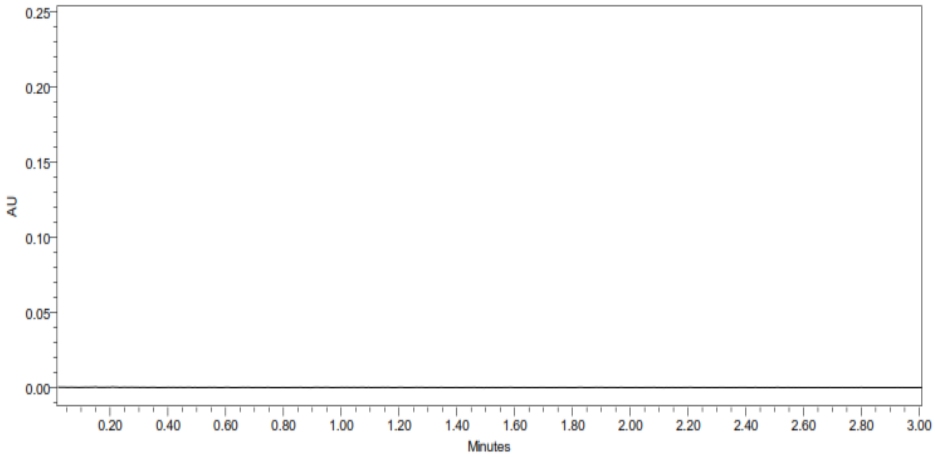

Fig no: 5 Chromatogram of blank.

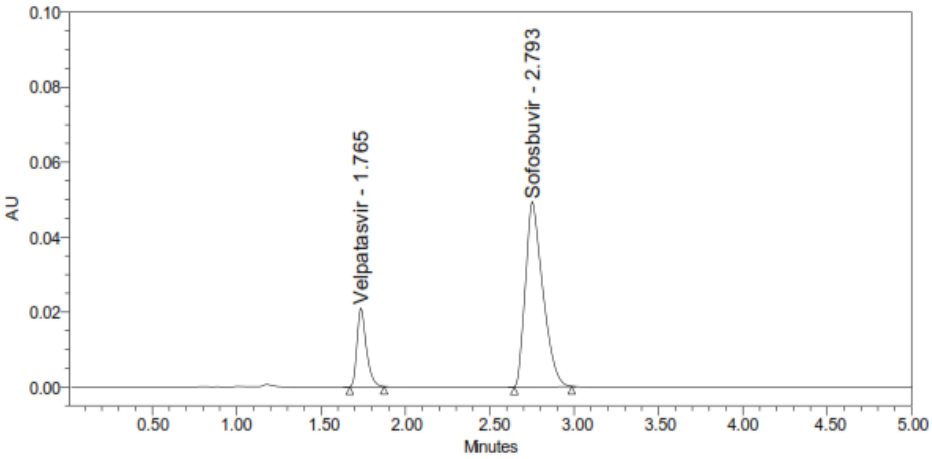

Fig no: 6 Standard Chromatogram for Sofosbuvir and Velpatasvir.

No peaks were observed near the retention time

Linearity:

Aliquots of standard stock solutions of Sofosbuvir and Velpatasvir were transferred into 10ml volumetric flasks and diluted up to the mark by diluents to achieve the concentrations of 40 to 200 µg/ml for Sofosbuvir and 10 to 50 µg/ml for Velpatasvir. Each sample solution was injected into UPLC system and the peak areas were measured. A graph of peak areas vs concentrations was plotted and the r^2 values were calculated. The results were shown in fig 7&8, table – 3, 4.

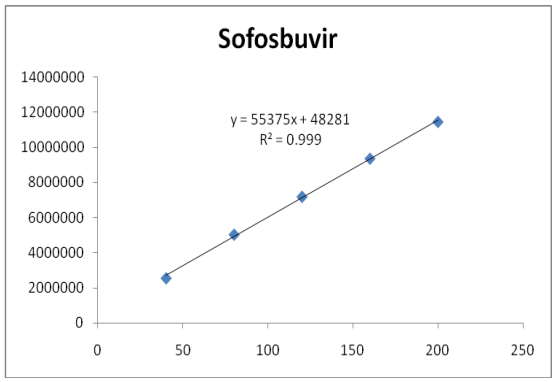

Fig: 7 Calibration curve of Sofosbuvir.

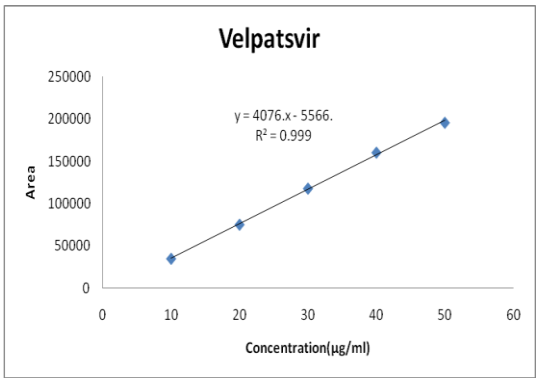

Fig: 8 Calibration curve of velpatasvir.

Table no: 3: Linearity data of Sofosbuvir and Velpatasvir.

S.No	Sofosbuvir(%)	Peak Area	Velpatasvir (%)	Peak Area
1	40	2558079	10	34657
2	80	5042405	20	75042
3	120	7198342	30	117770
4	160	9371867	40	160425
5	200	11468323	50	195811

Table no: 4 Calibration parameter for Sofosbuvir and velpatasvir.

Parameter	Sofosbuvir	velpatasvir
Slope (m)	55375	4076.9
Intercept (c)	482818	5566.3
Correlation co-efficient (R^2)	0.999	0.999

Accuracy

The accuracy of the proposed method was evaluated by recovery studies at various concentrations of Sofosbuvir and Velpatasvir equivalent to 50,100&150%. The percentage recovery at each level was calculated and reported in table – **5**.

Table no: 5 Accuracy results of Sofosbuvir and Velpatasvir.

Sample	Spike level	Amount added	Amount found	%recovery	Mean
Sofosbuvir	50	20	20.02	100.10	99.95
	100	40	39.58	98.96	
	150	60	60.48	100.80	
Velpatasvir	50	5	5.05	100.94	99.79
	100	10	9.90	98.98	
	150	15	14.92	99.46	

The % Recoveries were in the range 97-103 for Sofosbuvir and Velpatasvir respectively.

Precision:

Repeatability (Intraday) was evaluated by injecting the sample solutions in to the UPLC system in replicates and the chromatograms were recorded. The peak areas were observed and reported in terms of % RSD. The results are given in tables **6.**

Table no: 6: Intra-day precision results for Sofosbuvir and Velpatasvir.

Injection. No	Sofosbuvir		Velpatasvir	
	Peak Area	Retention Time	Peak area	Retention Time
1	7661234	2.793	129201	1.765
2	7688065	2.693	127703	1.789
3	7685789	2.893	124262	1.758
4	7706819	2.563	128917	1.765
5	7714821	2.793	126660	1.756
6	7707648	2.569	125980	1.789
Avg	7694063	2.512	127121	1.765
SD	19800.7		1875	
%RSD	0.3		1.5	

Limit of Detection and Limit of Quantification:

The detection limit of an analytical method is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated**.**

LOD = 3.3×Standard deviation

Slope

Limit of quantification is the lowest concentration of analyte in a sample which can be quantitatively determined with acceptable precision and accuracy.

LOQ = 10×Standard deviation

Slope

Where,

σ = standard deviation of the response

S= slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte.

The results were given in table – **7.**

Table no: 7 LOD & LOQ data of Sofosbuvir and Velpatasvir.

S.no	Parameter	Sofosbuvir	Velpatasvir
1	LOD(µg/ml)	1.48	1.69
2	LOQ(µg/ml)	4.49	5.13

Robustness:

The robustness of the proposed method was determined by recording the chromatograms with small deliberate changes in parameter like flow rate, wave length etc.

Effect of variation of flow rate:

The optimized flow rate is 0.3ml/min. Sample solutions were prepared and injected into UPLC system and the chromatograms are recorded.

Effect of variation in flow rate:

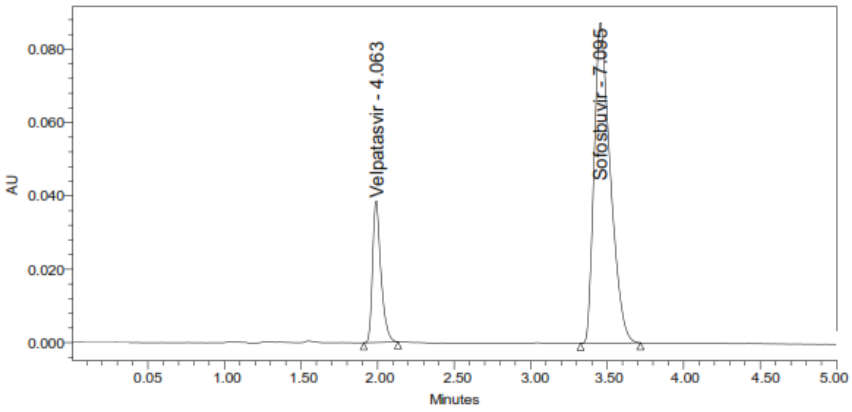

Fig no: 9 Chromatogram showing less flow.

Effect of variation of wave length:

Sample solutions were prepared and injected into UPLC system and the chromatograms were recorded at three different wavelengths. The results are summarized in table-**8**

The chromatograms for robustness studies are given in fig no: 9-12.

Effect of variation in wave length:

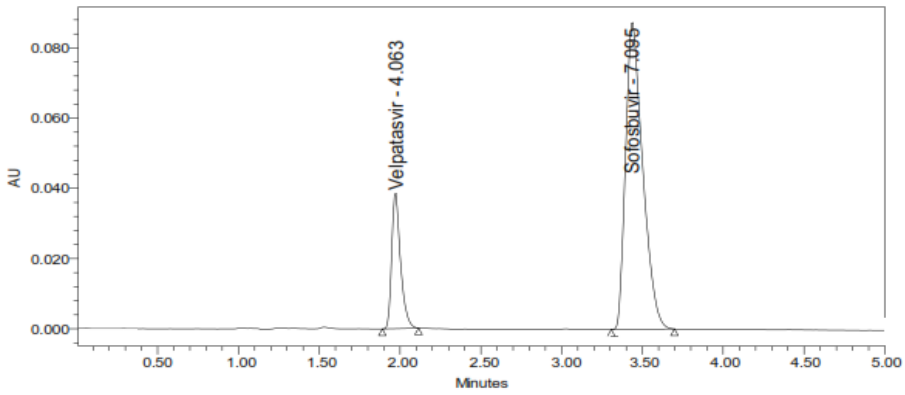

Fig no: 11 Chromatogram showing less organic composition.

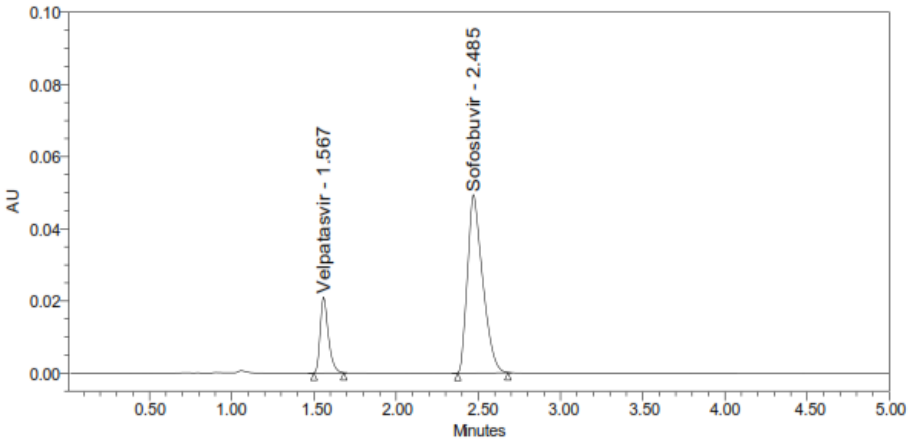

Fig no: 12 Chromatogram showing more organic composition.

Table no: 8 Chromatogram values for Robustness.

Altered parameter	USP Plate count	T Tailing Factor	USP Plate Count
Flowrate-0.27ml	Sof-2496.4	1.49	2496.4
	Vel-3741.8	1.29	3741.8
Flowrate-0.3 ml	Sof-2547.35	1.58	2547.35
	Vel-3877.36	1.35	3877.36
Flowrate-0.33ml	Sof-2684.3	1.35	2684.3
	Vel-3956.1	1.22	3956.1

The retention time, efficiency and asymmetry were within limits for variation in flow rate & wave length (±0.2ml/±2nm). Hence the allowable flow rate should be within 0.27 ml to 0.33 ml and allowable detection wave length is 235-240 nm.

Forced Degradation studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the forced degradation studies on the Sofosbuvir and Velpatasvir using the proposed method. The results are given in table-**9.**

Preparation of stock:

10 tablets were accurately weighed and crushed in mortor and pestle and transferred equivalent to 10 mg of Velpatasvir and 40 mg Sofosbuvir in sample into a 10ml clean dry volumetric flask 7 ml of Diluent was added and sonicated it up to 5 mins to dissolve it completely and made volume up to the mark with the same solvent. Then it is Filtered through 0.44 micron Injection filter. (Stock solution).

Further pipette 1ml of Velpatasvir & Sofosbuvir the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Hydrolytic degradation under acidic condition

Pipette 3 ml of above solution was pipetted into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1 N NaOH and made up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and placed in vials

Hydrolytic degradation under alkaline condition

Pipette 3 ml of above solution was pipetted into a 10ml volumetric and 3ml of 0.1N NaOH was added . Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1N HCl and made up to 10ml with diluent. The solution was filtered with 0.44 microns syringe filters and placed in vials

Thermal induced degradation

Velpatasvir and Sofosbuvir sample was taken in petridish and kept in Hot air oven at 110^0 C for 3 hours. Then the sample was taken and diluted with diluents and injected into UPLC and analyzed**.**

Oxidative degradation

Pipette 3 ml above stock solution was pipette into a 10ml volumetric flask and 1ml of 12.5% v/v of hydrogen peroxide added and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. The solution was filtered with 0.45 microns syringe filters and place in vials

Photo degradation:

The photochemical stability of the drug was also studied by exposing the 3ml stock solution in to 10ml volumetric flask and expose to UV Light by keeping the beaker in UV Chamber for 24 hours. For UPLC study, filter the solution was filtered with 0.45 microns syringe filters and place in vials were injected into the system and the chromatograms were recorded to assess the stability of sample

Table no: 9 Values for forced degradation.

S.No	Sample condition	Analytes	% Assay	radation
1	Untreated sample	Sof	99	-
		Vel	98	-
2	Peroxide treated	Sof	94.7	5.3
		Vel	94.2	5.8
3	Acid treated	Sof	94.4	5.6
		Vel	93.5	6.5
4	lkali treated	Sof	94.8	5.2
		Vel	94.8	5.2
5	rmal /Dry heat exposed	Sof	97	5.3
		Vel	98	4.2
6	lytic degradation	Sof	94.5	5.5
		Vel	93	7.0

CONCLUSION

An attempt was made to develop a simple, accurate, economical and precise method for the routine analysis of Sofosbuvir and Velpatasvir. During optimization a new combination of mobile phase was tried to overcome the drawbacks of the previous run. Finally the method was optimized by using KH_2PO_4 buffer: ACN (50:50) (pH 3) and validated as per ICH guidelines. The method was validated for system suitability, linearity, precision, accuracy, specificity, robustness, LOD and LOQ. From the validation results the methods were found to be linear, precise, accurate, sensitive and robust. Hence it can be concluded that the proposed method was a good approach for obtaining reliable results and found to be suitable for the routine analysis of Sofosbuvir and Velpatasvir in Bulk drug and Pharmaceutical formulation. We further recommend this method for future research to come out with a bio-analytical method development for these analytes .

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