Original Research Article

A Novel Stress Indicating RP-HPLC Method Development and Validation for the Simultaneous Estimation of Velpatasvir and Sofosbuvir in bulk and its Tablet Dosage Form

Abstract

Aim: The objective of the study was simplest, accurate, precise and robust reversed phase high performance chromatographic (RP-HPLC) method was developed for the estimation of Velpatasvir (VEL) and Sofosbuvir (SOF) in the bulk and its tablet dosage form.
Study Design: The Quantitative and Qualitative estimation and designed forced degradation study of Velpatasvir & Sofosbuvir by RP-HPLC.
 Place and Duration of Study: The study was carried at Santhiram College of Pharmacy and time taken 4 months.
Method : The method was attained by used Waters(5Âμ, C18 250 x 2.6 mm) column with mobile phase consists of 0.5 mM disodium hydrogen phosphate buffer pH adjusted to 6.5, with Ortho phosphoric acid and Methanol in the ratio of 78:22 v/v, a flow rate of 1.0 mL/min and ultraviolet detection at 258 nm.
Results: The method was validated as per ICH guidelines with different parameters, the mean retention times of VEL and SOF were found to be 2.8 & 4.7 min respectively. The resolution between VEL and SOF was found to be 10.66. The Correlation coefficients for calibration curves within the detection range of 32.5 - 97.5 and 125 - 375 Âμg/mL were 0.999 for VEL and SOF respectively. The LOD and LOQ for VEL and SOF were found to be 0.0068-0.104 Âμg/mL and 0.029-0.342 Âμg/mL respectively.
Conclusion: The results were indicated that the developed method was used for the routine analysis of VEL & SOF combined form in bulk and its commercial formulation. To the best of our knowledge there was no method on RP-HPLC for the determination of VEL alone or in combination with SOF molecule.

Keywords:- Velpatasvir, Sofosbuvir, HPLC, commercial formulations.

1. Introduction

Hepatitis C virus (HCV) infection is a major public health challenge now a days. It has been estimated that the global prevalence of HCV infection is around 2%, with 170 million persons chronically infected with the virus and 3 to 4 million persons newly infected each year. [1, 2]
It is a pathogen that is already responsible for a significant proportion of liver disease in various regions of India.[3]
Velpatasvir (VEL) is a novel HCV nonstructural Protein 5A (NS5A) inhibitor that was developed in combination with other drugs, which are directly acting antiviral for the treatment of HCV infections.[4] The IUPAC name for velpatasvir is Methyl {(1R)-2-((2S,4S)-2-(5-{2-((2S,5S) -1-{(2S)-2 -((methoxycarbonyl) amino)-3- methyl butanoyl} -5-methyl pyrrolidin-2-yl)-1,11 dihydro(2) benzopyrano (4',3':6,7) naphtha (1,2-d) imidazol-9-yl}-1H –imidazol -2-yl) -4-

44 (methoxy methyl) pyrrolidin-1-yl)-2-oxo-1-phenylethyl} carbamate. It is a white to off-white
45 powder, slightly soluble in water. It has a molecular formula of $C_{49}H_{54}N_8O_8$.[5]
46 Sofosbuvir SOF is a nucleotide pro-drug that effectively inhibits 1-6 HCV RNA replicons in
47 vitro and has proved to have a high sustained virologic response (SVR) rates.[5,6] Sofosbuvir is a
48 prodrug of 2'-deoxy-2'-fluoro-2'-C-methyluridine mono phosphate that is phosphorylated intra
49 cellularly to the active triphosphate form.[7] Chemically it is (S)-Isopropyl 2-((S)-
50 (((2R,3R,4R,5R)-5- (2,4- dioxo- 3,4-di hydro pyrimidin-1 (2H)-yl) -4- fluoro-3- hydroxy-4-
51 methyl tetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate. It is a white to
52 off-white crystalline powder, found to be slightly soluble in water and freely soluble in alcohol
53 and acetone. It has a molecular formula of $C_{22}H_{29}FN_3O_9P$.[8] Chemical structures of VEL and SOF
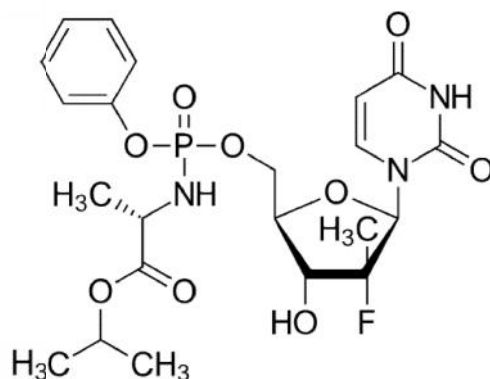54 were shown in figure 1 and 2 respectively.
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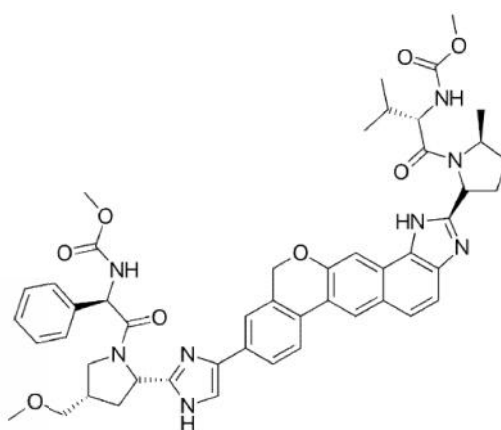
Figure 1: Chemical structure of velpatasvir

Figure 2: Chemical structure of Sofosbuvir

65 The combined dosage form (Tablet – Velpanat, Natco Pharma) consists of 100 mg of VEL and
66 400 mg of SOF was indicated for the treatment of chronic hepatitis C virus (HCV) infection in
67 adults.[4, 6] LC-MS/MS method has been reported for the estimation of SOF with Ledipasvir in

68 human plasma.[9] Two UPLC-MS/MS methods have been reported for determination of SOF[10]
69 and in combination of Ledipasvir (11,12) in human plasma for determination of bioequivalence
70 studies. Few RP-HPLC methods has been reported for the estimation of SOF alone [12, 13] or with
71 combination of other drugs like Ledipasvir [14, 15] and Simeprevir used in the combination for the
72 treatment of HCV infection.[16]
73
74 **2. EXPERIMENTAL**

75 **2.1 Reagents**

76 All the chemicals and reagents were of analytical grade. Water was redistilled and filtered with a
77 membrane filter. Methanol – HPLC grade (Merck, India), Ortho phosphoric acid and disodium
78 hydrogen phosphate (SD finechem, India) were used to prepare mobile phase. Pharmaceutical
79 grade standard drugs viz., Velpatasvir and Sofosbuvir were kindly gifted by Natco Pharma Ltd,
80 Hyderabad, India. The combined tablet formulation contains 100 mg of Velpatasvir and 400mg
81 of Sofosbuvir (Velpanat, Natco) purchased from local market of Nellore.

82 **2.2 Chromatographic Conditions**

83 The method was developed by using HPLC system consisted of a LC Waters (Waters, Milford,
84 MA, USA) using a Water's C_{18} 250 x 4.6 mm, 5μ column, a quaternary gradient system (600
85 Controller), in line degasser (Waters, model AF). The system was equipped with a photodiode
86 array detector (Water, 2998 model) and auto sampler (Waters, model 717 plus). Data was
87 processed using Empower Pro software (Waters, Milford, MA, USA). The Isocratic mobile
88 phase consist of a mixture of 0.5 mM disodium hydrogen phosphate buffer pH adjusted to 6.5,
89 with Ortho phosphoric acid and Methanol in the ratio of 78:22% v/v was used throughout the
90 analysis. The mobile phase was pumped at a flow rate of 1.0 mL/min. UV detection wavelength
91 for analytes was 285 nm. Column temperature was kept ambient and injection volume was 10μL.
92
93 **2.3 Solution Preparation**
94 **2.3.1 Standard stock solution preparation**
95 10 mg of VEL and SOF each was weighed accurately and transferred to individual 10 ml
96 volumetric flasks. Dissolved and diluted with methanol to get a concentration of 1000 μg/ml.
97 **2.3.2 Working standard Solution**
98 1.625 mL of VEL and 6.25 mL of SOF standard stock solutions were accurately measured and
99 transferred to a 25 mL volumetric flask, mixed well and diluted to final volume with diluent, so
100 as get the final concentrations of 65 μg/mL of VEL and 250 μg/mL of SOF.
101 **2.3.3 Sample solution preparation (Assay)**
102 Twenty tablets were weighed and finely powdered. The average weight of tablets was
103 determined. A portion of powder equivalent to 98.96 mg of VEL was weighed and transferred to
104 a 10 mL volumetric flask. 10 mL of methanol was added to disintegrate tablets completely by
105 using ultra sonicated for 10 min.
106 **2.3.4 Working sample solution**

107 1.625 mL was further diluted to get final concentrations of 65 µg/mL of VEL and 250 µg/mL of
108 SOF. This solution was filtered through 0.45 µm membrane filter. The 10µL of this solution was
109 injected in to HPLC system.
110
111 **2.4 Method Validation**
112
113 The method was validated according to ICH guidelines for the estimation of velpatasvir and
114 sofosbuvir. The following validation parameters are enveloped precision, accuracy, linearity,
115 limit of detection & limit of quantification, robustness and force degradation studies. The
116 standard solution was prepared at six concentrations ranging from 32.5- 97.5µg/mL for VEL and
117 125-375 µg/mL for SOF solutions were prepared for linearity. The reggration of the curve was
118 obtained by peak area vs concentration. The method sensitivity was measured by limit of
119 detection and limit of quantification. The limit of detection and limit of quantification were
120 determined by signal to noise ratio 3:1& 10:1.The precision of the method was assessed by
121 measured six times standard solution of VEL & SOF and measured the area of all six injections
122 in the HPLC chromatographic system. The accuracy of the method was determined by standard
123 addition and recovery method. The accuracy of the method was evaluated in triplicate at three
124 concentration levels, i.e. 50%, 100% and 150% of target test concentration and the percentages
125 of recoveries were calculated. The robustness of the method was manifested by deliberate
126 changes in experimental conditions. The changes made in the chromatographic conditions like
127 flow rate by ±0.1 mL/min, mobile phase composition change ± 3 and the column temperature ±5
128 °C. The drugs were subjected to different stress conditions like acid (refluxed 0.1N HCL for 1 hr
129 at 80°C),base (refluxed 0.1N NaOH for 4 hrs at 80°C), H_2O_2(stored 3% H_2O_2 room temp for 2
130 hrs) light and water near UV ≥200 FOR 10 days) forced degradation studies were conducted on
131 the VEL & SOF.
132 **3. RESULTS AND DISCUSSION**
133 **3.1 Method development and optimization of chromatographic conditions**
134 During the optimization of the method, different columns (Inertsil C8, 250 mm×4.6 mm, 5 µm;
135 Zorbax C18 250 mm×4.6 mm, 5 µm; Symmetry C18 250 mm×4.6 mm, 5 µm) and two organic
136 solvents (acetonitrile and methanol) were tested. The chromatographic conditions were also
137 optimized by using different buffers like phosphate, acetate and citrate for mobile phase
138 preparation. After a series of screening experiments, it was concluded that phosphate buffers
139 gave better peak shapes than their acetate and citrate counterparts. With acetonitrile as solvent
140 both the peaks shows less theoretical plates and more retention time compared to methanol. The
141 chromatographic separation was achieved on a Waters C18, 250 mm×4.6 mm, 5µm column, by
142 using a mixture of 0.5 mM disodium hydrogen phosphate buffer pH adjusted to 6.5, with Ortho
143 phosphoric acid and Methanol in the ratio of 78:22 v/v, as mobile phase. Temperature was
144 maintained ambient to facilitate mass exchange with the corresponding decrease of peak
145 broadening and increase in sensibility. The flow rate kept was 1.0 mL/min to achieve adequate
146 retention time of two peaks 2.80 min and 4.78 for VLE and SOF respectively. Figure 3, 4 and 5
147 shows blank, standard and sample chromatograms. The Table1 shows the optimized
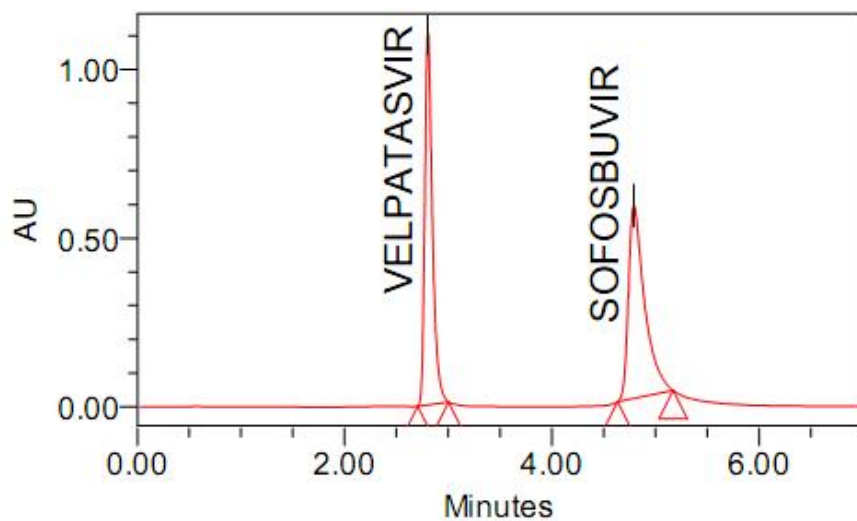148 chromatographic conditions.
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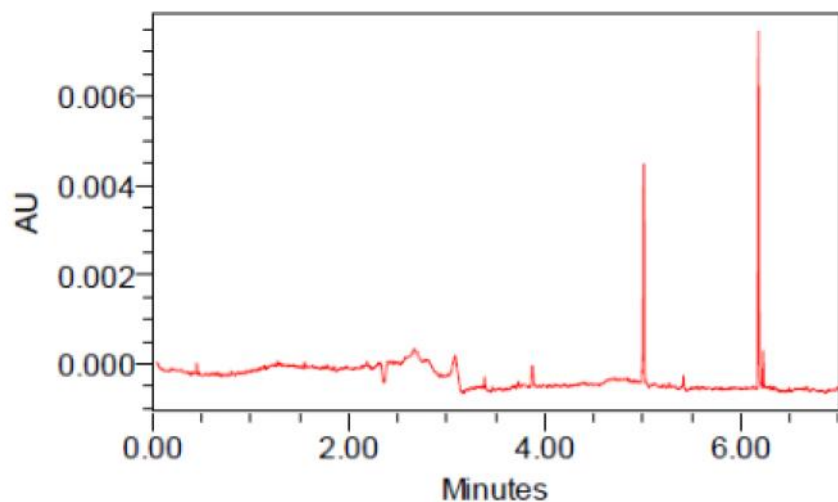
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Figure 3: Blank Chromatogram
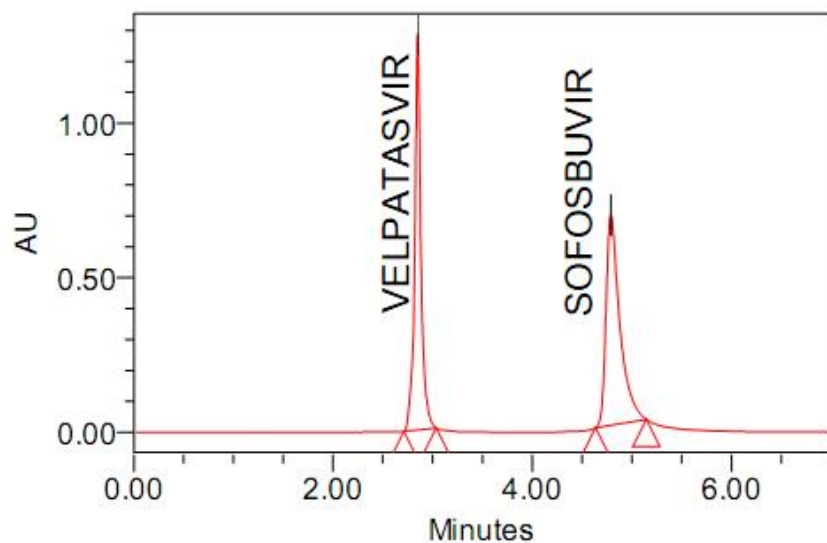
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Figure 4: Standard Chromatogram of VEL & SOF
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Figure 5: Sample Chromatogram of VEL & SOF

Table 1: Optimized HPLC conditions for simultaneous estimation of Velpatasvir and Sofosbuvir

S. No	Parameter	Description/Value
1.	Stationary Phase	Water's C18 (250X4.6X5)
2	Mobile Phase	0.5 mM Disodium Phosphate buffer (pH 6.5, adjusted with OPA) and MeOH in the ratio of 78:22 v/v
3	Flow rate	1 mL/min
4	Detection Wavelength (Isosbestic Point)	285nm
5	Detector	Photo diode array
6	Injection	Autosampler -Waters, model 717 plus
7	Injection volume	10 µl
8	Column Temperature	Ambient
9	Run time	6 mins
10	Diluent	Methanol
11	Rt's	Velpatasvir: 2.806 min Sofosbuvir: 4.780 min

3.2 Method validation

When a method has been optimized it must be validated before practical use. By following ICH guidelines for analytical method validation, Q2 (R1), the validation characteristics were addressed.[17]

3.3 System Suitability

The system suitability test ensures the validity of the analytical procedure as well as confirms the resolution between different peaks of interest. All critical parameters tested met the acceptance criteria on all days. As shown in the chromatograms (figure 4 & 5), two analytes were eluted by forming symmetrical single peaks well separated from each other and from excipients. Table 2 shows the System Suitability results.

Table 2: System suitability results

S.No	Parameters	Results		Limits
		Velpatasvir	Sofosbuvir	
1	RSD of peak area	0.20	0.86	<2 n ≥ 6
2	Retention times	2.849	4.786	-
3	RSD of retention time	0.56	0.89	<2 n ≥ 5
4	USP plate count	13196	6255	>2000
5	USP tailing factor	1.06	1.75	T<2
6	USP resolution	-	10.66	R >2

3.4 Linearity

For the construction of calibration curves, five calibration standard solutions were prepared over the concentration range of 32.5 – 97.5 µg/ml for VEL and 125.0 – 375 µg/mL for SOF. The

176 results, summarized in Table 3, showed a good correlation between analytes peak area and
177 concentration with r > 0.999 (n = 5). Linearity curve was shown in figure 6 and 7.
178

Table 3: Linearity results of VEL & SOF

S. No	Linearity Level	Velpatasvir		Sofosbuvir	
		Concentration (µg/mL)	Peak Area	Concentration (µg/mL)	Peak Area
1	50	32.5	2813066	125	3077228
2	75	48.75	4253268	187.5	4629475
3	100	65	5613521	250	6143585
4	125	81.25	7052657	312.5	7688257
5	150	97.5	8406053	375	9285177
Slope		86064		24759	
Intercept		33568		25128	
R^2		0.9999		0.9999	

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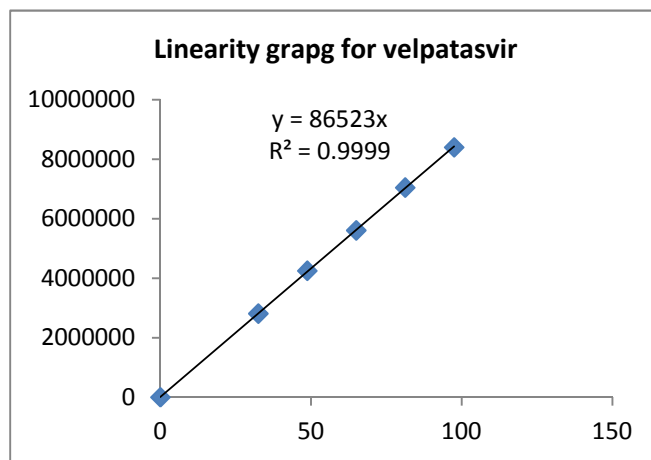181
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Figure 6: Linearity curve of VEL

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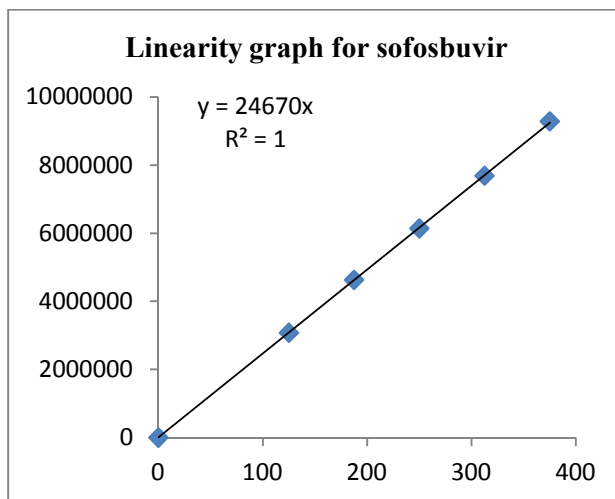193 Figure 7: Linearity curve of SOF
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3.5 Precision

The assay was investigated with respect to repeatability and inter-day precision. The repeatability of the system (while keeping the operating conditions identical) was examined by injecting analyte solution with 6 replicate injections. The RSD values varied from 0.47 to 0.86% Showed, that the inter-day precision of the method was satisfactory. Table 4 shows the precision results.

Table 4: Results of Method Precision

S. No	Velpatasvir		Sofosbuvir	
	Peak Area	% Assay	Peak Area	% Assay
1	5516391	100.55	6556728	100.84
2	5518106	100.58	6531198	100.44
3	5518136	100.58	6475888	99.59
4	5555471	101.26	6429678	98.88
5	5565122	101.43	6412642	98.62
6	5570645	101.53	6481227	99.67
Average	5540645.17	100.99	6481226.83	99.67
SD	25775.38	0.47	55834.05	0.86
%RSD	0.47	0.47	0.86	0.86

3.6 Accuracy

To govern the accuracy of the proposed method, recovery studies has been performed, known amount of pure drug sample solution at three different concentration levels, ie, 50%,100%,150% was calculated. Accuracy was calculated as percentage of recovery. The accuracy results tabulated as 5.

Table 5: Accuracy results of VEL & SOF

Parameters	Peak Area	Amount added(µg)	Amount recovered (µg)	% of recovery	% mean recovery
Velpatasvir					
50%	2807301	32.33	32.58	100.79	100.79
100%	5644767	64.66	65.52	101.33	101.33
150%	8332433	96.99	97.26	99.72	99.72
Sofosbuvir					
50%	276869	32.33	32.14	99.41	99.41
100%	5548876	64.66	64.41	99.61	99.61
150%	8506216	96.99	98.74	101.80	101.80

3.7 Limit of detection (LOD) and Limit of quantification (LOQ)

The Limit of detection and limit of quantification were considered as the signal- to- noise ratio 3:1 and 10:1 respectively. The limit of detection and limit of quantitation to be determined 0.0068µg/ml & 0.029µg/ml for VEL and 0.104 µg/ml & 0.347 µg/ml for SOF respectively.

3.8 Robustness

The robustness of the method was unaffected when small, deliberate changes like, flow rate change, mobile phase composition, column temperature were performed at 100% test

218 concentration. The method was found to be robust for the said conditions. Results were tabulated
219 in table 6.
220
221 <div align="center">Table 6: Results of Robustness</div>

S. No	Parameter	Condition	Velpatasvir			Sofosbuvir		
			RT	Peak Area	% Assay	RT	Peak Area	% Assay
1	Flow	0.8 ml/min	3.52	5476665	99.82	5.90	6564947	100.96
2		1 ml/min	2.85	5570645	101.53	4.79	6481226	99.67
3		1.2 ml/min	2.39	5526688	100.73	4.04	6410579	98.59
4	Temp	25 °C	2.85	5498542	100.22	4.80	6647497	102.23
5		30 °C	2.85	5570645	101.53	4.79	6481226	99.67
6		35 °C	2.84	5449293	99.32	4.76	6597781	101.47
7	Mobile Phase	B:M 75:19 v/v	2.81	5498542	100.22	4.22	6452436	99.23
8		B:M 78:22 v/v	2.85	5570645	101.53	4.79	6481226	99.67
9		B:M 81:25 v/v	2.68	5556765	101.28	5.26	6643379	102.17

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223 **3.9 Analysis of tablet formulation**
224 The proposed method was applied for the analysis of velpatasvir and sofosbuvir in tablet dosage
225 forms, the results were found to be between 99.67-100.99%, the results summarized in table 7.
226
227 <div align="center">Table 7: Assay results of VEL and SOF</div>

Drug	Labled amount mg/tab	Amount found mg/tab	% Assay
Velpatasvir	100	99.01	100.99
Sofosbuvir	400	401.32	99.67

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230 **3.10 Forced degradation and stability indicating studies**

231 Non interference of blank and degradants, the developed HPLC method proves the capability
232 stability indicating method for the analysis of VEL and SOF. Purity angle was less than the
233 purity threshold and hence the proposed method was the specific and revealed its stability-
234 indicating power. The results were summarized in Table 8. Figure 8 (a-e) shows chromatograms
235 of different stress degradation conditions.

236 <div align="center">Table 8: Degradation studies of VEL & SOF</div>

Stress conditions	% Assay of active moiety			
	Velpatasvir	% degradation	Sofosbuvir	% degradation
Acid (0.1 N HCl, refluxed for 1 H at 80ºC)	92.68	-7.32	92.06	-7.94
Base (0.1 N NaOH refluxed for 4H at 80ºC)	92.68	-7.32	92.98	-7.02
H_2O_2 (3% H_2O_2 Stored at room	93.05	-6.95	92.71	-7.29

temperature for 2 H)				
Water	89.69	-10.31	93.27	-6.73
UV light (near UV ≥200 for 10 days)	92.20	-7.80	92.81	-7.19

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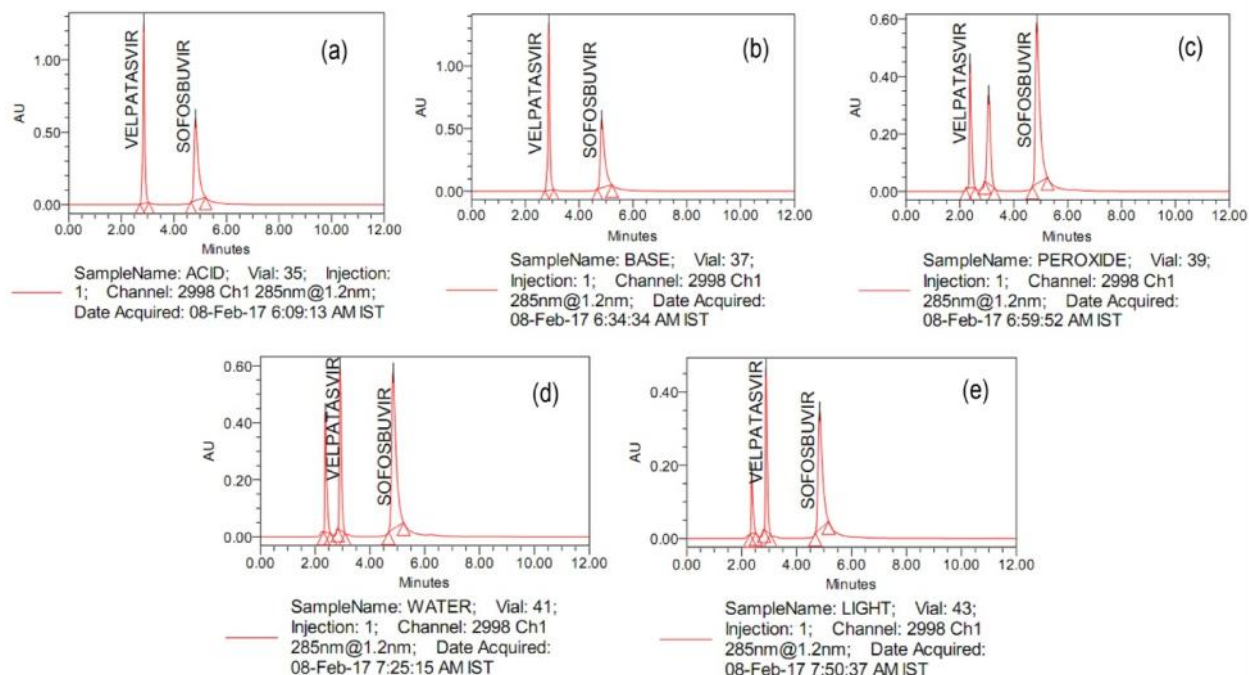
240 **Figure 8: Degradation Chromatogram working standard solutions of valpatasvir &**
241 **sofosbuvir after (a) Acid hydrolysis (0.1 N HCl, refluxed for 1 H at 80ºC) (b) Alkali (0.1 N**
242 **NaOH refluxed for 4H at 80ºC) and (c) Oxidative degradation (3% H₂O₂ Stored at room**
243 **temperature) (d) Water degradation (e) UV light degradation (near UV ≥200 for 10 days).**

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246 SOF. Two drugs were subjected to various stress conditions like acid (0.1 N HCl, refluxed for 1
247 H at 80ºC), base (0.1 N NaOH refluxed for 4H at 80ºC), peroxide (3% H₂O₂ Stored at room
248 temperature for 2H), water and light (near UV ≥200 for 10 days) stability studies were conducted
249 on these samples. Hence the proposed method was the specific and revealed its stability-
250 indicating power.

251 **4. Conclusion**
252 A simple, specific, precised and accurate isocratic HPLC-UV method was developed for the
253 estimation of velpatasvir and Sofosbuvir in their pharmaceutical formulation. The two
254 compounds were subjected to forced degradation applying several stress conditions. The
255 proposed method was successfully separated two compounds with degradants, estimate the
256 pharmaceutical active contents. The Proposed method was specific and stability-indicating
257 power. Hence the developed method can be adapted to regular quality control analysis.

258
259

CONSENT

It is not applicable

ETHICAL APPROVAL

It is not applicable

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