A ROBUST UPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF SOFOSBUVIR AND VELPATASVIR IN COMBINED TABLET DOSAGE FORMS

[1]B.V.V.S. Jagadeesh and [2]J.V.L.N. Seshagiri Rao

[1]Research Scholar and [2]Professor

[1, 2] Pharmaceutical Analysis and Quality Assurance Division, A.U. College of Pharmaceutical Sciences, Andhra University, Visakhapatnam-530003, Andhra Pradesh, India

ABSTRACT: A precise and accurate UPLC method has been developed and validated for the simultaneous quantitative analysis of sofosbuvir and velpatasvir. The separation of the drugs was achieved on an Acquity UPLC CSH C18 column (100 x 2.1 mm; 1.7 µm) using a mobile phase consisting of a mixture of ammonium acetate buffer and acetonitrile in the ratio of 50:50 v/v. The flow rate was 0.3 mL/min and the column temperature was maintained at 30°C. The drugs were detected at 260 nm. The retention times obtained for sofosbuvir and velpatasvir were 1.197 and 1.723 min respectively. The method was validated for specificity, linearity, precision, accuracy and robustness. The specificity of the method was determined by checking the interference from the placebo and by stress studies on the drug substances. The quantitation was linear over the concentration ranges of 25–150 µg/mL for sofosbuvir and 6.25 – 37.5 µg/mL for velpatasvir. The accuracy of the method was between 98–102%. The method was found to be robust and suitable for the quantitative analysis of sofosbuvir and velpatasvir in tablet formulation. Forced degradation studies were conducted on the drugs and the resulting degradation products did not interfere with the quantitation of sofosbuvir and velpatasvir, demonstrating that the proposed method is specific for their estimation.

Keywords: Sofosbuvir, Velpatasvir, Simultaneous estimation, UPLC

1. INTRODUCTION: Sofosbuvir, a nucleotide prodrug[1-2], is a direct acting antiviral drug used to treat chronic infectious liver disease caused by Hepatitis C Virus (HCV). HCV is a single-stranded RNA virus which is categorized into nine distinct genotypes. Sofosbuvir after getting metabolized into 2'-deoxy-2'-α-fluoro-β-C-methyluridine-5'-triphosphate becomes a defective substrate for non-structural protein 5B (NS5B), which is an RNA polymerase responsible for the transcription of Hepatitis C viral RNA and its replication. Sofosbuvir and other direct acting antiviral drugs are preferred for the treatment of Hepatitis C as they do not exhibit resistance unlike other antiviral agents that target viral enzyme protease.

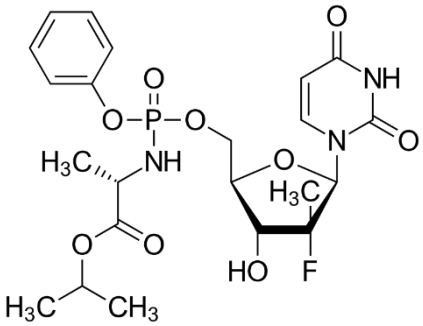

Fig. 1: Chemical structure of sofosbuvir

Velpatasvir is a complex organic hetero pentacyclic compound, an NS5A inhibitor[4-5], which is used in combination therapy for treatment of chronic hepatitis C infection of all six major genotypes. Non-Structural Protein 5A(NS5A), a non-enzymatic viral protein, plays a key role in hepatitis C virus replication. Velpatasvir acts as a defective substrate for NS5A protein. Velpatasvir is indicated for the treatment of adult patients with chronic hepatitis C virus genotype 1, 2, 3, 4, 5, or 6 infections without cirrhosis or with compensated cirrhosis.

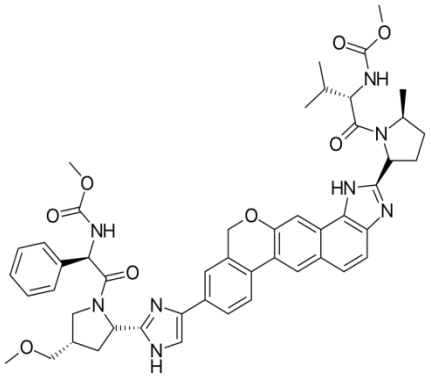

Fig. 2: Chemical structure of velpatasvir

2. CHEMICALS AND EQUIPMENT USED IN THE STUDY

2.1 Chemicals and solvents

Table 1: Chemicals and solvents

Chemical Name	Make	Grade
Water	Merck	HPLC
Acetonitrile	Merck	HPLC
Ammonium Acetate	Sigma-Aldrich	ACS Reagent
Conc.Hydrochloric acid	S.D. Fine Chemicals	AR (35.4 %)
Sodium hydroxide	S.D. Fine Chemicals	AR
Hydrogen peroxide	S.D. Fine Chemicals	AR (30 % w/v)

2.2 Equipment

Table 2: Equipment

Equipment	Make
UPLC: Waters Acquity 2996 with Empower 2 software	Waters Corporation, Milford, USA
Electronic balance	Mettler Toledo
pH meter	Mettler Toledo
Ultra sonicator	Lab India Instruments
Thermal oven	Thermostat
Micro pipettes	Brand and Eppendorf

2.3 Reference standards and commercial tablets

Reference standard samples of sofosbuvir and velpatasvir were obtained from Mylan Laboratories Ltd, Hyderabad. The commercial formulation "MyHep All" Tablets (400 mg Sofosbuvir + 100 mg Velpatasvir; Mylan Laboratories Ltd.) was purchased from the local pharmacy.

3. METHOD DEVELOPMENT

3.1 Preparation of standard stock solutions (1.0 mg/mL): Independent stock solutions of the drugs were prepared by transferring 10 mg of sofosbuvir and 10 mg of velpatasvir into separate 10 mL volumetric flasks and adding 7 mL of the diluent. The flasks were sonicated for 5 min to dissolve the drugs completely. The final volumes were made up with the diluent to get 1.0 mg/mL concentration of the drugs.

3.2 Preparation of mixed working standard solution: From the above stock solutions, 0.25 mL of velpatasvir solution and 1.0 mL of sofosbuvir solution were pipetted into a 10 mL volumetric flask. The final volume was made up with the diluent to get concentrations of 25 µg/mL of velpatasvir and 100 µg/mL sofosbuvir.

3.3 Diluent: A mixture of water and acetonitrile in the ratio of 50:50 v/v was used as the diluent for the preparation and dilution of the drug solutions.

3.4 Optimized chromatographic conditions: Simultaneous chromatographic separation of sofosbuvir and velpatasvir was achieved on an Acquity UPLC CSH C18 column (100 x 2.1 mm; 1.7 µm) by using a 50:50 v/v mixture of 10 mM ammonium acetate buffer and acetonitrile. The mobile phase was optimized in order to get better peak shapes and resolution between the peaks with accepted peak tailing and reproducibility of the response. The flow rate was set at 0.3 mL/min. The column temperature was maintained at 30°C. The injection volume was 3µL. Run time was chosen as 3 min. The drugs in the eluates were monitored at 260nm. Under the above optimized chromatographic conditions, the retention times obtained for Sofosbuvir and Velpatasvir were 1.197 and 1.723 min respectively. The corresponding system suitability parameters are given in Table 3.

Table 3: System suitability parameters

Parameter	Sofosbuvir	Velpatasvir
Peak Area	2031521	807378
Retention Time (min)	1.203	1.724
USP Plate count	2734	4187
USP Tailing	1.26	1.28
% CV	0.5	0.5
Resolution	5.2	

4. VALIDATION OF THE PROPOSED METHOD

The optimized HPLC method was validated as per the ICH guidelines by determining the following parameters:

1. Specificity
2. Precision
3. Accuracy
4. Linearity
5. Robustness
6. Forced degradation studies

Specificity: The specificity of the method was established by analyzing the blank, placebo and standard solutions separately. No interfering peaks were observed at the retention times of sofosbuvir and velpatasvir (Fig. 5-7). Hence, the developed method was found to be specific for the simultaneous estimation of sofosbuvir and velpatasvir.

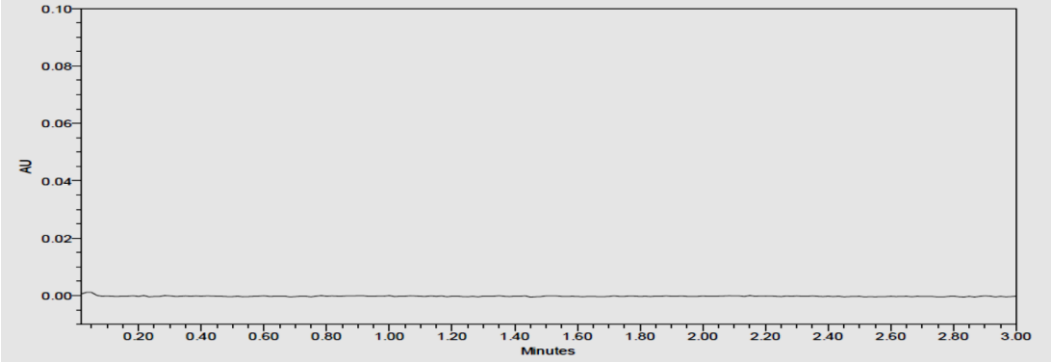

Fig. 3: Chromatogram of the blank

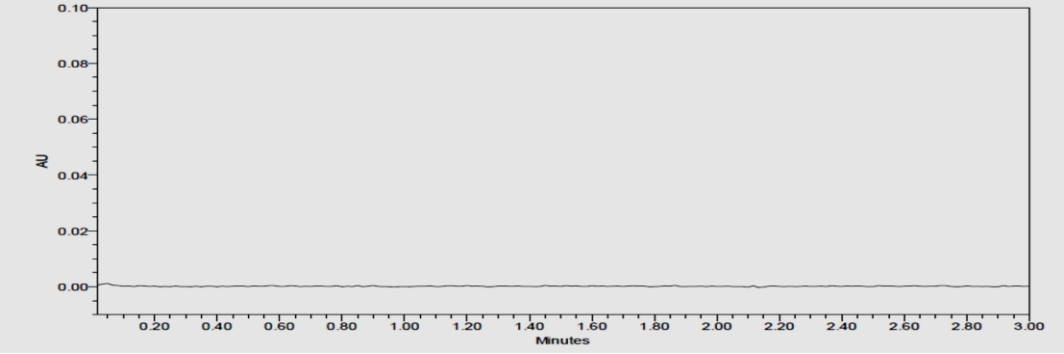

Fig. 4: Chromatogram of the placebo

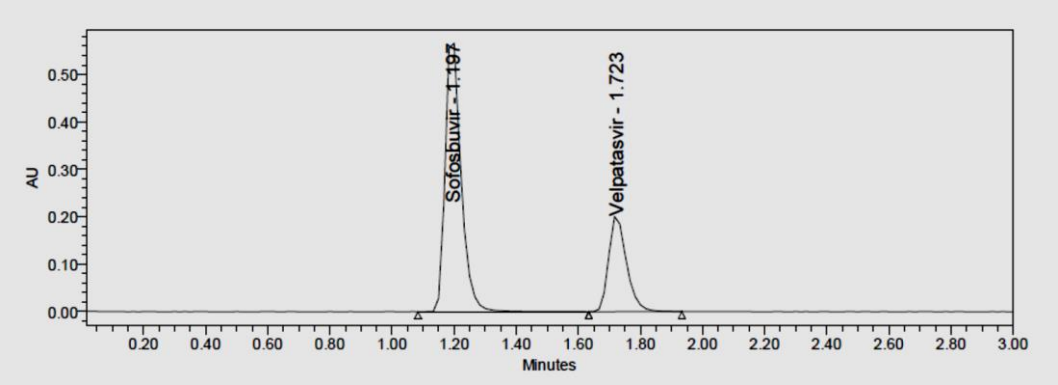

Fig. 5: Chromatogram of the mixed standard solution

Precision: The precision of the proposed UPLC method was evaluated by conducting intra-day and inter-day precision studies.

Intra-day precision (Repeatability): The precision of the method was assessed by performing six independent assays of the test sample, calculating the % RSD of the peak area values. The relevant results are tabulated in Table 4.

Table4: Intra-day precision of sofosbuvir and velpatasvir

S. No.	Sofosbuvir			Velpatasvir		
	Peak area	USP Plate count	USP Tailing	Peak area	USP Plate count	USP Tailing
1	2030376	2747	1.26	812045	3833	1.29
2	2057728	2727	1.26	821638	3975	1.22
3	2039601	2740	1.26	817612	4078	1.21
4	2069867	2754	1.25	805804	4155	1.21
5	2036334	2786	1.26	807374	4237	1.21
6	2049314	2687	1.26	810620	4294	1.21
Average (n=6)	**2047203**	-		**812516**	-	
SD	**14751**			**6074**		
% RSD	**0.7**			**0.7**		

Intermediate Precision (Inter-day precision): The inter-day precision of the method was checked by performing the above procedure on two consecutive days under the same experimental conditions, taking the overall average and calculating the % RSD. The corresponding resultsare shown in Table 5.

Table5: Inter-day precision of sofosbuvir and velpatasvir

Day	Sofosbuvir			Velpatasvir		
	Average Peak Area (n=6)	USP Plate count	USP Tailing	Average Peak Area (n=6)	USP Plate count	USP Tailing
Day 1	2030583	2735	1.25	804011	4123	1.22
Day 2	2029513	2740	1.25	803331	4189	1.21
Overall Average	**2030048**	-		**803671**	-	
SD	**757**			**481**		
% RSD	**0.0**			**0.1**		

Accuracy: The accuracy of the proposed UPLC method was evaluated by determining the recoveries of sofosbuvir and velpatasvir after adding pre-determined amounts to the mixed standard solution. Solutions ofsofosbuvir and velpatasvir at 50, 100 and 150% concentration levels were prepared, analyzed and the corresponding chromatograms obtained. The mean percent recoveries were calculated from the peak areasof the drugs. The corresponding resultsare shown in Table 6.

Table 6: Accuracy data of sofosbuvir and velpatasvir

Conc. Level	Sofosbuvir				Velpatasvir			
	Amount added (µg/mL)	Difference amount recovered (µg/mL)	Mean % Recovery	% RSD	Amount added (µg/mL)	Difference amount recovered (µg/mL)	Mean % Recovery	% RSD
50%	50.000	50.540	100.0	0.97	12.500	12.510	99.9	0.22
		49.753				12.490		
		49.660				12.456		
100%	100.00	101.609	100.6	0.85	25.000	24.871	99.5	0.27
		100.297				24.952		
		99.995				24.819		
150%	150.000	152.761	101.5	0.40	37.5000	37.7684	100.3	0.36
		151.593				37.5357		
		152.495				37.5289		

Linearity: Linearity of the proposed analytical method was assessed by injecting three replicates of the mixed working standard solutions at six different concentration levels in the ranges of 25 to 150 µg/mL for sofosbuvir and 6.25 to 37.5 µg/mL for velpatasvir. The corresponding responses were found to be linear. The results are summarized in Tables 7.

The linear regression equation for sofosbuvir was found to be y = 20174x + 15873 (r^2=.0.9997) (Fig. 6). The linear regression equation for velpatasvir was found to be y = 31833x + 14553 (r^2=0.9998) (Fig. 7).

Table7: Linearity data of sofosbuvir and velpatasvir

Sofosbuvir				Velpatasvir			
Conc. (µg/mL)	**Peak Area**	**Average Peak Area (n=3)**	**% RSD**	**Conc. (µg/mL)**	**Peak Area**	**Average Peak Area (n=3)**	**% RSD**
25.00	510242	511537	0.2	6.25	214179	214158	0.6
	511790				212791		
	512579				215503		
50.00	1024086	1024477	0.3	12.50	407885	406313	1.0
	1027340				409419		
	1022005				401635		
75.00	1527490	1526576	0.1	18.75	612861	613544	0.3
	1527184				612170		
	1525055				615601		
100.00	2045969	2050606	0.3	25.00	816028	814921	0.3
	2057188				812457		
	2048662				816278		
125.00	2551519	2554118	0.1	31.25	1015543	1015253	0.0
	2555769				1015277		
	2555067				1014938		
150.00	3018695	3019457	0.0	37.50	1202970	1201214	0.1
	3019964				1200716		
	3019711				1199956		

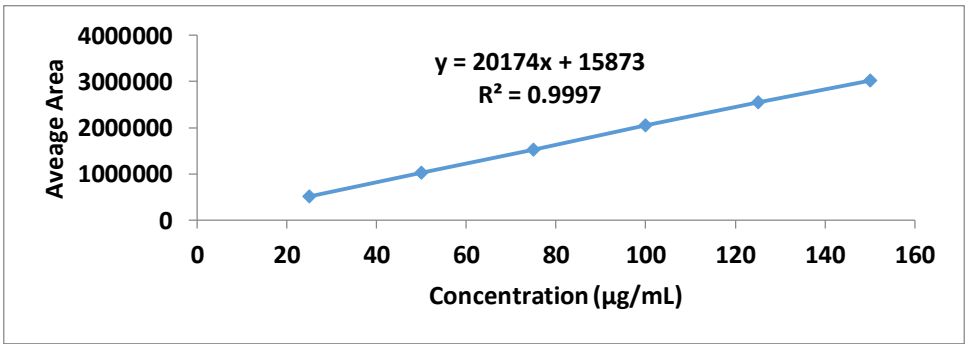

Fig. 6: Linearity curve for sofosbuvir

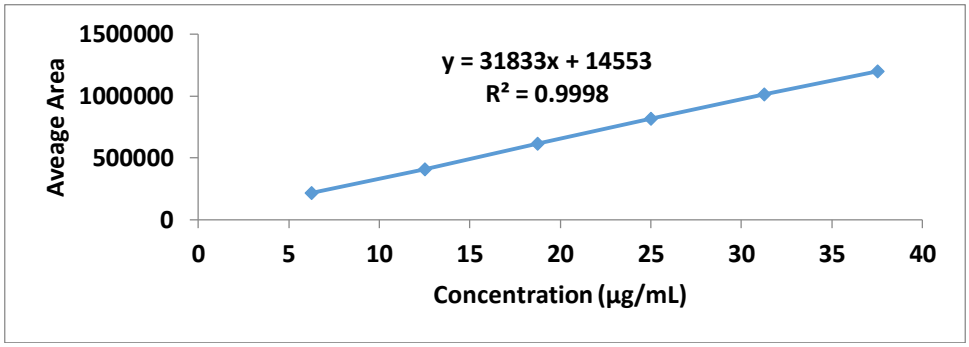

Fig. 7: Linearity curve for velpatasvir

Robustness: Small deliberate changeswere made in the methodconditions like flow rate, mobile phase composition and column temperature and the corresponding chromatograms were obtained. No appreciabledifferenceswere observed in the system suitability results, which were within the acceptable limitsas per the ICH guidelines. The results are shown in Tables 8, 9 and 10.

Table8: Robustness data of change in flow rate

Flow rate (mL/min)		Drug	Retention time (min)	Asymmetry	Plate count	Resolution
Actual	Change					
0.3	0.2	Sofosbuvir	1.331	1.3	2542	5.1
		Velpatasvir	1.918	1.3	4258	
	0.4	Sofosbuvir	1.093	1.3	2730	5.1
		Velpatasvir	1.574	1.3	4156	

Table9: Robustness data of change in mobile phase composition

Mobile Phase (Buffer-ACN v/v)		Drug	Retention time (min)	Asymmetry	Plate count	Resolution
Actual	Change					
60:40	55:45 v/v	Sofosbuvir	1.201	1.2	2845	5.2
		Velpatasvir	1.705	1.2	4563	
	65:35 v/v	Sofosbuvir	1.203	1.3	2845	5.5
		Velpatasvir	1.755	1.3	4474	

Table10: Robustness data of change in column temperature

Column temperature (°C)		Drug	Retention time (min)	Asymmetry	Plate count	Resolution
Actual	Change					
30	28	Sofosbuvir	1.206	1.3	2952	5.4
		Velpatasvir	1.731	1.3	4601	
	32	Sofosbuvir	1.201	1.2	2926	5.4
		Velpatasvir	1.725	1.3	4449	

Limit of Detection and Limit of Quantitation: The LOD values were found to be 0.133 µg/mL for sofosbuvir and 0.010 µg/mL for velpatasvir. The LOQ values were found to be 0.404 µg/mL for sofosbuvir and 0.030 µg/mL for velpatasvir. The relevant LOD and LOQ data are given in Table 11 and the corresponding chromatograms in Fig. 8.

Table 11: LOD and LOQ results

Drug	LOD (µg/mL)	LOQ (µg/mL)
Sofosbuvir	0.133	0.404
Velpatasvir	0.010	0.030

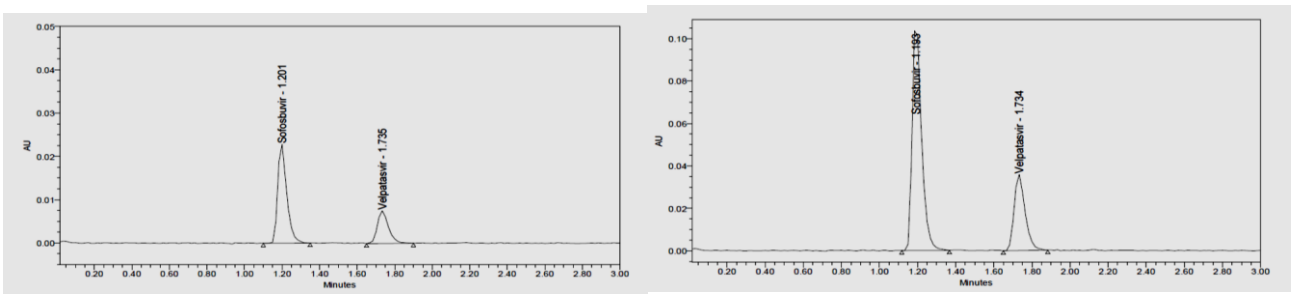

Fig. 8: LOD & LOQ Chromatograms

5. SIMULTANEOUS ESTIMATION OF SOFOSBUVIR AND VELPATASVIR IN TABLET DOSAGE FORM: Twenty tablets were weighed and finely powdered. An amount of powder equivalent to the weight of one tablet was accurately weighed and transferred into a 50 mL volumetric flask. 30 mL of the diluent was added to the flask and sonicated for 30 min.The contents were diluted to volume with the diluent and filtered through a 0.45 µ membrane filter. 125 µL of the filtrate was pipetted into a 10 mL volumetric flask and the volume was made up with the diluent. The resultant solution was chromatographed and analyzed. The average assay value obtained for sofosbuvir was 99.85 % and that for velpatasvir was 99.48%.The results are given in Table 12 and the corresponding chromatogram in Fig. 9.

Table12: Assay of sofosbuvir and velpatasvir

S. No.	Sofosbuvir			Velpatasvir		
	Peak Area from working standard	Peak Area from formulation	% Assay	Peak Area from working standard	Peak Area from formulation	% Assay
1	2017921	2034752	100.06	804159	802550	99.30
2	2047634	2025010	99.58	805682	804792	99.58
3	2027058	2027280	99.69	805387	805936	99.72
4	2032436	2026442	99.65	814160	805526	99.67
5	2038418	2040317	100.33	810972	804033	99.49
6	2025662	2029696	99.81	803912	801227	99.14
Average (n=6)	2031522	2030583	99.85	807379	804011	99.48
SD	10461.748	5865.733	0.288	4198.285	1816.396	0.225
% RSD	0.5	0.3	0.3	0.5	0.2	0.2

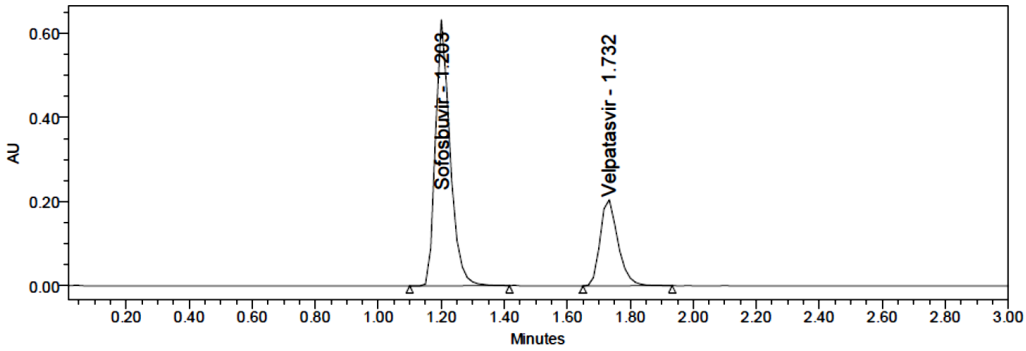

Fig. 9: Chromatogram of marketed formulation

6. FORCED DEGRADATION STUDIES ON THE DRUG SUBSTANCES: The forced degradation studies were conducted on the mixed working standard solution of sofosbuvir and velpatasvir. The drugs were subjected to degradation with acidic, alkaline, peroxide, dry heat, photolytic and neutral stress conditions. The results of the study are given in Table 13.

Table 13: Forced degradation data

Nature of degradation	Stress conditions	Sofosbuvir		Velpatasvir	
		% Assay	% Degradation	% Assay	% Degradation
Acid	2N HCl at 60 °C for 30 min	96.72	3.3	96.57	3.4
Base	2N NaoH at 60 °C for 30 min	96.24	3.8	94.59	5.4
Peroxide	Heated with 20 % H_2O_2 at 60 °C for 30 min	96.43	3.6	97.54	2.5
Dry heat	Heated in an oven at 105 °C for 6 h	95.85	4.1	96.03	4.0
Photolytic	Exposed to UV light at 200 Watts hours/m2 for 7 days	98.84	1.2	98.14	1.9
Neutral	Heated with water at 60 °C for 6 h	99.62	0.4	99.77	0.2

7. **CONCLUSION:** The UPLC method developed for the simultaneous estimation of sofosbuvir and velpatasvir was accurate and reproducible and can be employed for estimation of the drugs in their combined formulations. The chromatographic conditions were optimized prior to validation studies in terms of stationary phase, mobile phase composition, flow rate and column oven temperature, which gave good system suitability.The developed method was validated as per ICH Q2A (R1) guidelines. The developed method was found to be specific because there was no interference from the placebo, matrix, and degradation products at the retention times of the analytes.Hence, the developed method can be used for routine quality control analysis of sofosbuvir and velpatasvir in their combined tablet dosage forms.

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