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DEVELOPMENT AND VALIDATION OF LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS DETERMINATION OF SOFOSBUVIR, VELPATASVIR AND VOXILAPREVIR IN FIXED TABLET DOSAGE FORM

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ABSTRACT
Developing a single analytical method for the estimation of individual drugs is very challenging due to the formation of drug-drug and drug-excipient interactions. The present study demonstrates the applicability of the chromatographic method to develop a new, sensitive, single HPLC method for the simultaneous quantitative determination of three antiviral agents in the fixed pharmaceutical dosage form. Chromatographic separation of the three antiviral drugs was achieved by using a isocratic elution at a flow rate of 1.0 mL/min on Spursil ODS C18 column (150m×4.6mm, 5µm particle size) at ambient temperature. Potassium dihydrogen orthophosphate KH2PO4 (0.03M) in 1000 ml of water and by adjusting the pH to 3.0 with diluted sodium hydroxide. Mobile phase composition is 85% of buffer and 15% of Acetonitrile and UV detection at 245 nm was employed to monitor the analytes. A linear response was observed for Sofosbuvir over the concentration range 100-600 µg/ mL, 25-150 µg/ mL of Velpatasvir and 25-150µg/ mL of Voxilaprevir.

KEYWORDS: Sofosbuvir, Velpatasvir and Voxilaprevir, Isocratic -HPLC, Vosevi, Method Development, and validation.

INTRODUCTION
Sofosbuvir, Velpatasvir, Voxilaprevir (trade name Vosevi) is a combination drug for the treatment of hepatitis C. It combines three drugs that each act by a different mechanism of action against the hepatitis C virus: sofosbuvir, velpatasvir, and voxilaprevir.

Sofosbuvir
Sofosbuvir (tradename Sovaldi) is a direct acting antiviral medication used as part of combination therapy to treat chronic Hepatitis C, an infectious liver disease caused by infection with Hepatitis C Virus (HCV). HCV is a single-stranded RNA virus that is categorized into nine distinct genotypes, with genotype 1 being the most common in the United States, and affecting 72% of all chronic HCV patients.

The chemical name of sofosbuvir is (S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)- (phenoxy)phosphorylamino)propanoate.

The molecular formula is $C_{22}H_{29}FN_3O_9P$ and the molecular weight is 529.458. The drug substance is Sofosbuvir 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.

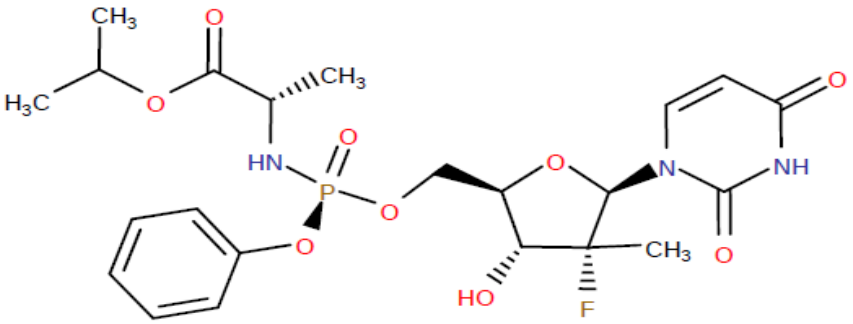

Fig. 1: Structure of Sofosbuvir.

Velpatasvir

Velpatasvir is a new NS5A inhibitor with antiviral activity against all HCV genotypes.

The chemical name of velpatasvir is methyl {(2S)-1-[(2S,5S)-2-(9-{2-[(2S,4S)-1-{(2R)-2-[(methoxycarbonyl)amino]-2-phenylacetyl}-4-(methoxymethyl)pyrrolidin-2-yl]-1H-imidazol-4-yl}-1,11-dihydro[2]benzopyrano[4',3':6,7]naphtho[1,2-d]imidazol-2-yl)-5-methylpyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl}carbamate The molecular formula is $C_{49}H_{54}N_8O_8$ and the molecular weight is 883.019. The drug substance is White to light yellow solid powder Soluble in DMSO, not in water.

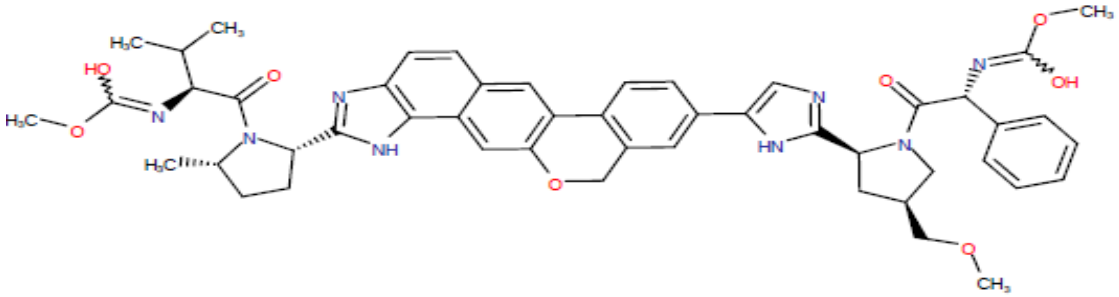

Fig. 2: Structure of Velpatasvir.

Voxilaprevir

Voxilaprevir is a Direct-Acting Antiviral (DAA) medication used as part of combination therapy to treat chronic Hepatitis C, an infectious liver disease caused by infection with Hepatitis C Virus (HCV). HCV is a single-stranded RNA virus that is categorized into nine distinct genotypes, with genotype 1 being the most common in the United States, and affecting 72% of all chronic HCV patients.

The chemical name of Voxilaprevir is (1R,18R,20R,24S,27S,28S)-24-tert-butyl-N-[(1R,2R)-2-(difluoromethyl)-1-{[(1-methylcyclopropyl)sulfonyl] carbamoyl}cyclopropyl]-28-ethyl-13,13-difluoro-7-methoxy-22,25-dioxo-2,21-dioxa-4,11,23,26-tetraazapentacyclo[24.2.1.0^{3,12}.0^{5,10}.0^{18,20}] nonacosa-3(12),4,6,8,10-pentaene-27-carboxamide. The molecular formula is $C_{40}H_{52}F_4N_6O_9S$ and the molecular weight is 868.94. The drug substance is Solid powder and Soluble in DMSO.

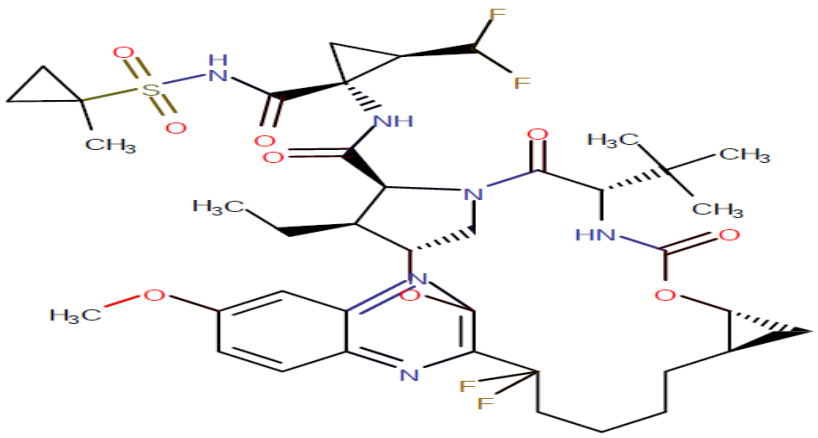

Fig. 3: Structure of Voxilaprevir.

A Survey of Literature has revealed few analytical methods for determination of Sofosbuvir, Velpatasvir and Voxilaprevir in biological fluids and in pharmaceutical products. These include HPLC. On the contrary, to the best of our knowledge, There is no method reporting to simultaeneous determination of Sofosbuvir, Velpatasvir and Voxilaprevir in a fixed dosage form. We report the simple precise and accurate RP-HPLC method for the assay of sofosbuvir, Velpatasvir and Voxilaprevir with solution stability and forced degradation studies in a fixed dosage form. The new method is capable of separating all active ingredients present in the tablet. Validation of current method will be performed according to the requirements of USP for assay determination which include precision, selectivity, linearity and range.

EXPERIMENTAL

Chemicals and reagents: sofosbuvir, Velpatasvir and Voxilaprevir are obtained as kind gift samples from Local laboratories Ltd, Hyderabad. Potassium dihydrogen orthophosphate, from Finer Chemical Ltd. Water and Methanol were obtained from Merck, Mumbai, India. All the solutions were prepared in Milli Q water (Millipore, USA). Test samples composed of Vosevi film-coated fixed-dose tablet contains 400 mg of sofosbuvir, 100mg of Velpatasvir and 100 mg of Voxilaprevir are obtained from pharmatrian.

Equipment and Chromatographic conditions: Waters Alliance 2695 separation module (Waters Corporation, Milford, USA) equipped with 2998 PDA detector with Empower 2 software was used for the analysis. The HPLC system was equipped with a column compartment with temperature control and an on-line degasser. Spursil ODS C18 column (150×4.6mm, 5µm, Waters Corporation, Milford, USA) and a isocratic mixture of solvents were used as stationary and mobile phases, respectively. The buffer contains 3.4 gms of Potassium Dihydrogen Orthophosphate (0.03M) in 1000 ml of water and by adjusting its pH was adjusted to 3.0 with Sodium Hydroxide. The 85% of buffer and 15% of Acetonitrile are used as Mobile phase and was adjusted at 1.0 ml/min flow rate and 20 µL injection volume were maintained. The eluted compounds were monitored at 245 nm. The column oven temperature was maintained at 25°C. Data acquisition, analysis, and reporting were performed by Empower2 (Waters) chromatography software.

Preparation of Solutions

Preparation of 0.025M Phosphate buffer

3.4g of potassium dihydrogen ortho phosphate was weighed and taken in a 1000ml volumetric flask and adjust the P^H with Diluted NaOH upto 3, finally the solution was filtered by using 0.45 Micron membrane filter, sonicate it for 10 mins.

Preparation of mobile phase

Accurately measured 850 ml (85%) of above buffer and 150 ml of Acetonitrile HPLC (15%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

Standard and stock solutions*:* Accurately weigh and Transfer 400 mg of sofosbuvir, 100 mg of Velpatasvir and 100 mg of Voxilaprevir working standard into a 100 ml clean dry volumetric flask. Add about 70 ml of Diluent and sonicate to dissolve it completely and make volume upto the mark with the same solvent (Stock Solution).

Further pipette 1.0 ml of the above stock solutions into a 10 ml volumetric flask and dilute upto the mark with diluent.

Preparation of the Sample solution: accurately weigh 20 tablets crush in mortor and pestle and transfer equivalent to 400 mg of sofosbuvir, 100 mg of Velpatasvir and 100 mg of Voxilaprevir in sample into 100 ml clean dry flask add about 70 ml of diluent and sonicate it upto 30 minutes to dissolve it completely and make volume upto the mark with the same solvent. Then it is filtered through 0.45 micron injection filter.(Stock Solution).

Further pipette 1.0 ml of Sofosbuvir, Velpatasvir and Voxilaprevir from the above stock solution into a 10ml volumetric flask and dilute upto the mark with diluent.

Label claim:- VOSEVI is a fixed-dose combination tablet containing sofosbuvir, velpatasvir, and voxilaprevir for oral administration. Each tablet contains 400 mg sofosbuvir, 100 mg velpatasvir, and 100 mg of voxilaprevir.

RESULTS AND DISCUSSIONS

Mobile Phase Optimization

Initially the mobile phase tried was methanol: OPA buffer and Methanol: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to Phosphate buffer with (pH 3.0) Acetonitrile in proportion 85: 15 v/v respectively.

Wave length selection

UV spectrum of 10 µg/ml Sofosbuvir, Velpatasvir and Voxilaprevir in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 245nm. At this wavelength all these three drugs show good absorbance.

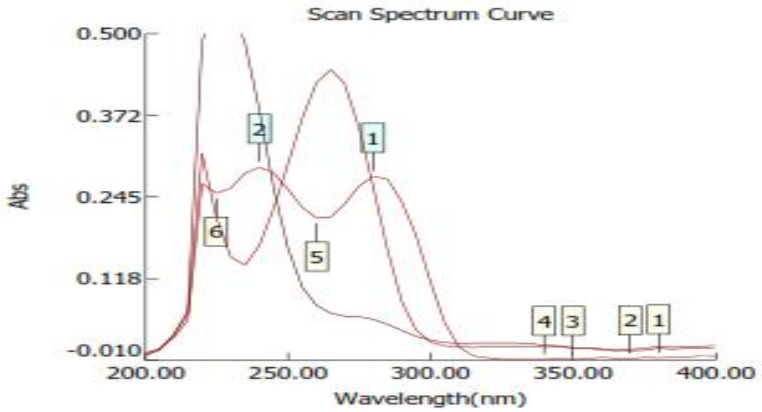

Fig. 4: Wavelength selection.

Optimization of Column

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. Spursil ODS C $_{18}$ (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow.

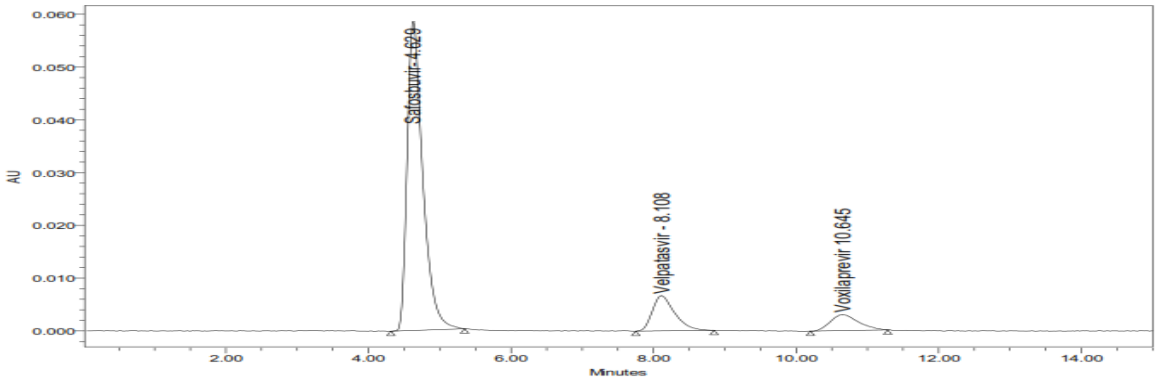

Fig. 5: Chromatogram of Sofosbuvir, Velpatasvir and Voxilaprevir.

METHOD VALIDATION

Linearity

Prepared linearity solutions of Sofosbuvir, Velpatasvir and Voxilaprevir standards at levels of 100,200,300,400,500,600 ppm of Sofosbuvir , and 25,50,75,100,125,150ppm of Velpatasvir and 25,50,75,100,125,150ppm of Voxilaprevir, analyzed as per test method and plotted linearity graphs, calculated Square of correlation coefficient.

Calibration curve with concentration verses peak areas was plotted by injecting the above prepared solutions and the obtained data were subjected to regression analysis using the least squares method.

Table 1: Linearity data of Sofosbuvir.

Level in ppm	Peak area
100	522088
200	734633
300	950658
400	1192066
500	1430452
600	1648027
Correlation Coefficient	0.9997

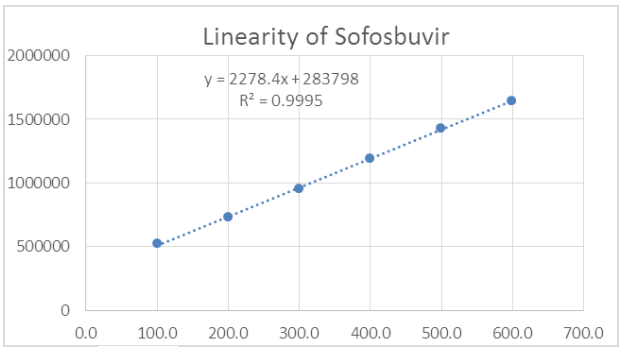

Fig. 6: Linearity graph of Sofosbuvir.

Table 2: Linearity data of Velpatasvir.

Level in ppm	Peak area
25	65477
50	110790
75	153097
100	193120
125	239955
150	271466
Correlation Coefficient	0.9990

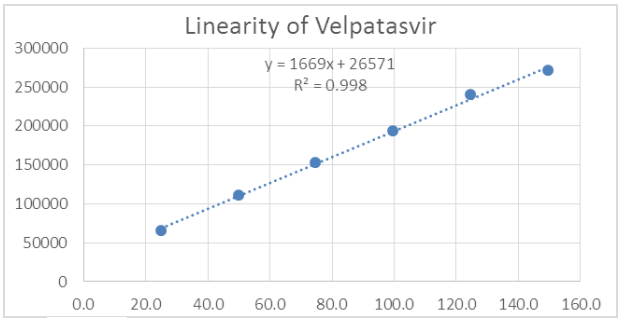

Fig. 7: Linearity curve of Velpatasvir.

Table 3: Linearity data of Voxilaprevir.

Level in ppm	Peak area
25	47257
50	67723
75	89884
100	109712
125	134068
150	154564
Correlation Coefficient	0.9997

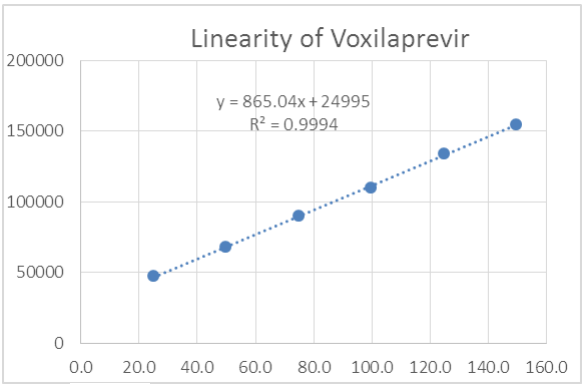

Fig. 8: Linearity curve of Voxilaprevir.

RESULT

A linear relationship between peak areas versus concentrations was observed for sofosbuvir, Velpatasvir and Voxilprevir. Correlation coefficient was 0.9997, 0.9990 and 0.9997 for sofosbuvir, velpatasvir and Voxilaprevir which proves that the method is linear.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on signal to noise ratio. The S/N ratio of sofosbuvir, Velpatasvir and Voxilprevir are given below.

Table 4: LOD and LOQ values Calculated from S/N Ratio.

	Sofosbuvir ppm	**S/N Ratio**	**Velpatasvir** ppm	**S/N Ratio**	**Voxilaprevir ppm**	**S/N Ratio**
LOD	1.33	3:1	2.72	3:1	1.16	3:1
LOQ	4.44	10:1	9.07	10:1	3.92	10:1

RESULT

The detection Limit and Quantification Limit of of Sofosbuvir, Velpatasvir and Voxilprevir are found within the acceptable criteria.

Method precision (repeatability)

The precision of the method was checked by repeated preparation(n=6) of 400ppm of Sofosbuvir, 100 ppm of Velpatasvir and 100 ppm of Voxilaprevir without changing the parameter of the proposed chromatographic method.

Table 5: Summary of method precision.

S. No.	% Assay		
	Sofosbuvir	Velpatasvir	Voxilaprevir
1	99.6	99.4	100.0
2	99.6	99.1	100.0
3	99.5	98.9	99.9
4	99.3	98.8	100.2
5	99.8	99.4	100.1
6	99.7	99.6	100.3
Average	99.6	99.2	100.1
% RSD	0.2	0.3	0.2

RESULT

Results of variability were summarized in the above table. Percentage relative standard deviation (%RSD) was found to be less than 2.0% for all the three drugs which proves that method is precise.

Accuracy (recovery study)

The accuracy of the method was determined by calculating the recoveries of sofosbuvir, Velpatasvir and Voxilaprevir by analyzing solutions containing approximately 50%, 100% and 150% of the working strength of Sofosbuvir, Velpatasvir and Voxilaprevir. The percentage recovery results obtained are listed in Table 6,7 and 8.

Table 6: Recovery data of Sofosbuvir.

Level	Amount Added (mg)	Amount Recovered (mg)	% Recovery	Average % Recovery	% RSD
50	199.80	198.87	99.5	99.5	0.7
	199.80	200.14	100.2		
	199.80	197.63	98.9		
100	399.60	399.30	99.9	100	0.2
	399.60	400.56	100.2		
	399.60	398.98	99.8		
150	599.40	604.89	100.9	100.9	0.3
	599.40	606.57	101.2		
	599.40	602.45	100.5		

Table 7: Recovery data of Velpatasvir.

Level	Amount Added (mg)	Amount Recovered (mg)	% Recovery	Average % Recovery	% RSD
50	49.95	50.31	100.7	100.4	0.4
	49.95	50.25	100.6		
	49.95	49.96	100.0		
100	99.90	99.67	99.8	99.8	0.0
	99.90	99.66	99.8		
	99.90	99.67	99.8		
150	149.85	149.35	99.7	99.7	0.5
	149.85	150.09	100.2		
	149.85	148.85	99.3		

Table 8: Recovery data of Voxilaprevir.

Level	Amount Added (mg)	Amount Recovered (mg)	% Recovery	Average % Recovery	% RSD
50	49.95	50.09	100.3	100.3	0.0
	49.95	50.11	100.3		
	49.95	50.12	100.3		
100	99.90	99.50	99.6	99.4	0.3
	99.90	98.89	99.0		
	99.90	99.39	99.5		
150	149.85	149.07	99.5	99.4	0.1
	149.85	148.79	99.3		
	149.85	148.75	99.3		

RESULT

Results of accuracy study are presented in the above table. All the results indicate that the method is highly accurate**.**

Robustness: Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate, detection wavelength and mobile phase variation on assay of the analyte of interest. Here the detection wavelength varied ±3nm, flow rate was varied ±0.1ml/min and change in organic composition in the mobile phase about 10%. The results were shown in (Table no.9, 10 & 11).

Table 9: Robustness results for Sofosbuvir.

Parameter	Sofosbuvir	
	Theoretical plates	Asymmetry
Flow rate 0.9 mL/min	2264	1.4
Flow rate 1.1 mL/min	2151	1.4
Mobile phase*	2446	1.4
Mobile Phase**	2105	1.4
Wave length at 242 nm	2264	1.4
Wave length at 248 nm	2165	1.4

Table 10: Robustness results for Velpatasvir.

Parameter	Velpatasvir		
	Theoretical plates	Asymmetry	USP Resolution
Flow rate 0.9 mL/min	3331	1.3	7.4
Flow rate 1.1 mL/min	2972	1.4	7.1
Mobile phase*	3595	1.3	9.2
Mobile Phase**	2935	1.5	6.0
Wave length at 242 nm	3331	1.3	7.3
Wave length at 248 nm	3287	1.3	7.3

Table 11: Robustness results for Voxilaprevir.

Parameter	Voxilaprevir		
	Theoretical plates	Asymmetry	USP Resolution
Flow rate 0.9 mL/min	3035	1.4	3.9
Flow rate 1.1 mL/min	3466	1.4	3.9
Mobile phase*	5095	1.3	4.7
Mobile Phase**	3253	1.4	3.6
Wave length at 242 nm	3036	1.4	3.3
Wave length at 248 nm	3523	1.4	3.4

***Buffer solution and Acetonitrile in 865: 135 ratio.**
****Buffer solution and Acetonitrile in 835:165 ratio.**

RESULT

The results of Robustness of the present method had shown that changes are not significant we can say that the method is Robust.

Ruggedness: Ruggedness was performed by analysing six test preparations different day, different analyst and different column as per the methodology, determined %RSD. The results were shown in Table 12.

Table 12: Results of Sofosbuvir, Velpatasvir and Voxilaprevir.

S. No.	% Assay		
	Sofosbuvir	Velpatasvir	Voxilaprevir
1	99.5	100.9	100.3
2	99.4	99.6	99.7
3	99.8	100.5	100.5
4	99.6	100.6	99.8
5	99.3	101.2	100.1
6	99.8	100.9	100.4
Average	99.6	100.6	100.1
% RSD	0.2	0.6	0.3

RESULT

The %RSD assay values between two analysts, Different days, Different columns was calculated and found that there was no variability in the test results, this indicates the method was rugged.

Solution Stability

The standard and sample solutions are prepared as per the methodology and analyzed these solutions after 24 hours at 25^{o} C. Calculated similarity factor for standard and % Difference for the test preparations. The results were shown in Table no.13 and 14.

Table 13: Stability of Standard Solution.

Interval	Similarity Factor		
	Sofosbuvir	Velpatasvir	Voxilaprevir
Initial	NA	NA	NA
After 24 hours	0.98	1.00	0.99

Table 14: Solution Stability of Sofosbuvir, Velpatasvir and Voxilaprevir.

Time	Sofosbuvir		Velpatasvir		Voxilaprevir	
	% Assay	Difference	% Assay	Difference	% Assay	Difference
Initial	99.6	- 0.4	99.4	-1.0	100.0	0.4
After 24 hours	100		100.4		99.6	

RESULT

The similarity factor for standard under stability against fresh standard was found within acceptable limits and % Assay not deviating more than ±2.0 from initial value.

Forced Degradation Studies

Forced degradation studies were performed to establish the stability, indicating property and specificity of the proposed method.

Table 15: Forced degradation results of Sofosbuvir, Velpatasvir and Voxilaprevir.

Nature of Degradation	Stress Condition	% Degradation for Sofosbuvir	% Degradation for Velpatasvir	% Degradation for Voxilaprevir	% Total Degradation
Acid	0.1 N HCl at 60°C for 6 Hours	4.2	9.90	4.40	18.5
Base/Alkali	0.1 N NaOH at 60°C for 6 Hours	3.4	8.90	3.70	16.0
Peroxide	3% (w/v) H_2O_2 at room temperature for 15 minutes	4.0	7.60	2.90	14.5
Dry heat	Drug substance heated at 110° C for 24 hours	4.0	6.90	3.30	14.2
Photolytic	UV Chambers at 200- watt hours/m2 for 24 hours and white light 1200 wh/sq meter	4.0	6.90	3.40	14.3

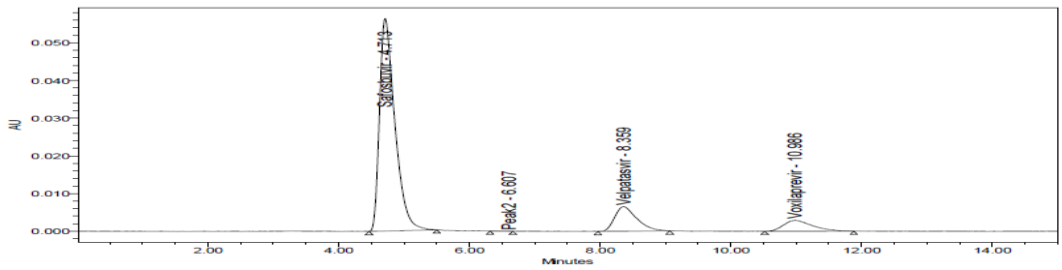

Fig. 9: Typical Chromatogram of Degradation sample.

Table 16: Summary of Sofosbuvir.

S. No	Parameter	Result	Acceptance criteria
1	**System suitability** Theoretical plates Asymmetry Retention time %RSD	 2229 1.4 4.47 0.1	 Not less than 2000 Not more than 2.0 Not more than 2.0
2	Specificity	Specific	Specific
3	Method precision(%RSD)	0.2	Not more than 2.0%
4	Linearity Range(ppm) Correlation coefficient(r)	100-600 0.999	 Not less than 0.990
5	Limit of Detection (S/N Ratio) Limit of Quantification (S/N Ratio)	3:1 10:1	S/N between 3 or 2:1 S/N is 10:1
6	Accuracy (Mean % recovery) 50% 100% 150%	 99.5 100 100.9	 97.0 – 103.0%
7	Robustness	All the system suitability parameters are within the limits.	All the system suitability parameters must be within the limits.
8	Solution stability Similarity Factor (For standard After 24hrs) %Assay Difference (For sample solution)	 0.98 -0.4	 0.98-1.02 ±2.0 from initial value
9	Ruggedness	0.2%	Not more than 2.0%

*RSD = Relative standard deviation

Table 17: Summary of Velpatasvir.

S. No	Parameter	Result	Acceptance criteria
1	System suitability Theoretical plates Asymmetry Retention time %RSD	 3102 1.4 7.93 0.3	 Not less than 2000 Not more than 2.0 Not more than 2.0
2	Specificity	Specific	Specific
3	Method precision(%RSD)	0.3	Not more than 2.0%
4	Linearity Range(ppm) Correlation coefficient (r)	25-150 0.999	 Not less than 0.990
5	Limit of Detection (S/N Ratio) Limit of Quantification (S/N Ratio)	3:1 10:1	S/N between 3 or 2:1 S/N is 10:1
6	Accuracy (Mean % recovery) 50% 100% 150%	 100.4 99.8 99.7	97.0 – 103.0%
7	Robustness	All the system suitability parameters are within the limits.	All the system suitability parameters must be within the limits
8	Solution stability Similarity Factor (For standard After 24hrs) % Assay Difference (For sample solution)	 1.00 -1.0	 0.98-1.02 ±2.0 from initial value
9	Ruggedness	0.6%	Not more than 2.0%

*RSD = Relative standard deviation

Table 18: Summary of Voxilaprevir.

S. No.	Parameter	Result	Acceptance criteria
1	System suitability Theoretical plates Asymmetry Retention time %RSD	 3331 1.4 10.46 0.3	 Not less than 2000 Not more than 2.0 Not more than 2.0
2	Specificity	Specific	Specific
3	Method precision(%RSD)	0.2	Not more than 2.0%
4	Linearity Range(ppm) Correlation coefficient (r)	25-150 0.999	 Not less than 0.990
5	Limit of Detection (S/N Ratio) Limit of Quantification (S/N Ratio)	3:1 10:1	S/N between 3 or 2:1 S/N is 10:1
6	Accuracy (Mean % recovery) 50% 100% 150%	 100.3 99.4 99.4	97.0 – 103.0%
7	Robustness	All the system suitability parameters are within the limits.	All the system suitability parameters must be within the limits
8	Solution stability Similarity Factor (For standard After 24hrs) % Assay Difference (For sample solution)	 0.99 0.4	 0.98-1.02 ±2.0 from initial value
9	Ruggedness	0.3%	Not more than 2.0%

*RSD = Relative standard deviation

CONCLUSION

From the above experimental results it was concluded that, this newly developed method for the simultaneous estimation of Sofosbuvir, Velpatasvir and Voxilaprevir was found to be simple, precise, accurate and high resolution and shorter retention time makes this method

more acceptable and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in meant in industries, approved testing laboratories.

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