CHAPTER - 4
DACLATASVIR AND SOFOSBUVIR

DRUG PROFILE

SOFOSBUVIR:

Structure:

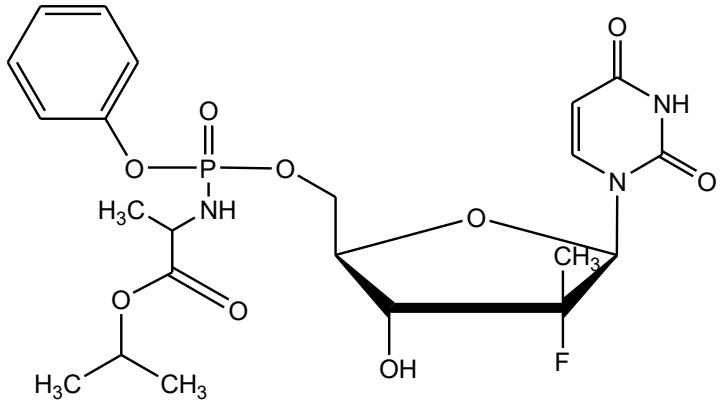

Fig. 4.1: Structure of Sofosbuvir

IUPAC Name	**:**	Isopropyl (2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4- fluoro-3-hydroxy-4-methyl-tetrahydrofuran-2-yl]methoxy-phenoxy-phosphoryl]amino] propanoate
Molecular formula	**:**	$C_{22}H_{29}FN_3O_9$
Molecular Weight	**:**	529.453 g/mol.
Solubility	**:**	Soluble in Methanol and water
P^{ka}	**:**	9.3
Category	**:**	Inhibitor of the hepatitis C NS5B protein. It acts as a high barrier to the development of resistance

DACLATASVIR:

Structure:

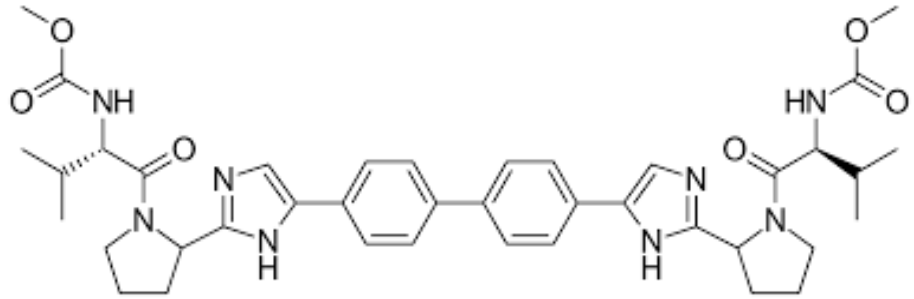

Fig. 4.2: Structure of Daclatasvir

IUPAC Name	**:**	Dimethyl N,N'-([1,1'-biphenyl]-4,4'-diylbis{1H-imidazole-5,2-diyl-[(2S)-pyrrolidine-2,1-diyl][(2S)-3-methyl-1-oxobutane-1,2-diyl]})dicarbamate
Molecular formula	**:**	$C_{40}H_{50}N_8O_6$
Molecular Weight	**:**	738.89 g/mol.
Solubility	**:**	Soluble in Water, Methanol, and Acetonitrile
P^{ka}	**:**	3.74
Category	**:**	Anti-viral

MATERIALS AND METHODS

Instrumentation

Chromatographic separation was performed on a Agilent chromatographic system equipped with 1200 series isocratic pump; Rheodyne injector with 20 µL fixed volume loop, variable wavelength programmable UV detector and the output signal was monitored and integrated by EZICHROME ELITE Chromatographic Software. Double beam UV-Visible spectrophotometer (Labindia-3120) was used to carry out spectral analysis and the data was recorded by UVWIN-5 software. Ultrasonicator (1.5L) was used for degasification of mobile phase and samples. Standard and sample drugs were weighed by using shimadzu electronic analytical balance (AX-220) and pH of the mobile phase was adjusted by using systronics digital pH meter.

Chemicals and solvents

All chemicals and reagents used were of HPLC grade. Pure standards of Daclatasvir and (DCV) Sofosbuvir (SFV) employed in the study were obtained as gift sample from Micro labs, Bangalore. The other reagents used were methanol and acetonitrile from qualigens Ltd. Mumbai, India, triethylamine from Hi-media, Mumbai, India, and HPLC grade water from Merck chemicals, Mumbai, India.

Preparation of standard stock solution

Standard stock solution of Daclatasvir and Sofosbuvir (1mg/mL) were prepared by accurately weighing about 100 mg pure drug and transferring in to 100 mL volumetric flask and dissolved in methanol. The standard stock solution was further diluted with mobile phase to get calibration curve standard concentrations of 5, 10, 15, 20 and 25 µg/mL of Daclatasvir and 2, 4, 6, 8 and 10 µg/mL of Sofosbuvir.

Preparation of 0.1% triethylamine buffer (pH - 3.0)

Buffer solution was prepared by taking accurately a quantity of 0.1 mL of triethylamine dissolved in 100 mL HPLC grade water. The pH of the solution was adjusted to 3.0 with ortho- phosphoric acid and degassed for about 30 min in a ultra-bath sonicator.

Preparation of mobile phase

The mobile phase used was acetonitrile, methanol and freshly prepared 0.1% triethylamine buffer solution (pH- 3.0) in the ratio of 25:35:40 (v/v/v) and the mobile phase was filtered through 0.45 µ membrane filter and sonicated before use.

Preparation of sample solution: Accurately weighed twenty tablets were ground to obtain fine powder equivalent to 400 µg of Sofosbuvir and 60 µg of Daclatasvir sample were weighed and transferred to 100 ml of volumetric flask and dissolved in diluents. The flask was shaken and volume was made up to mark with diluent to give a primary stock solution. From the above solution 4 ml of solution is pipette out into a 100 ml volumetric flask and volume was made up to mark with diluent to give a solution containing 160 µg/ml of Sofosbuvir and 24 µg/ml of Daclatasvir.

METHOD DEVELOPMENT

Method development involves in the evaluation and optimization of the various stages of sample preparation, chromatographic separation, qualification and quantification. Optimization of various parameters was performed in order to develop a selective and sensitive method for analysis on HPLC using UV detection.

Trail -1:

Chromatographic conditions	:	
Mobile Phase	:	Water : ACN(50:50%V/V)
Column		Discovery C18 (250mm x 4.6 mm, 5µ).
Detection Wavelength		245 nm

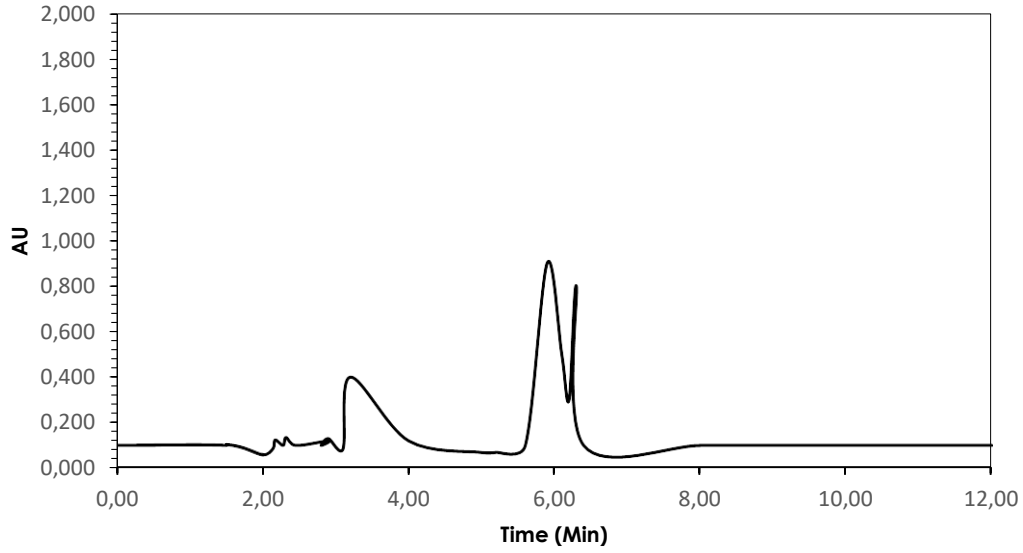

Fig. 4.3: Chromatogram Showing Trial – 1

Conclusion: The baseline and peak shape is not good.

Trail -2:

Chromatographic conditions	:	
Mobile Phase	:	Water: ACN(60:40%V/V)
Column		Chromolith Speed ROD RP- 18,4.6 X 50mm,5µm
Detection Wavelength		250 nm.

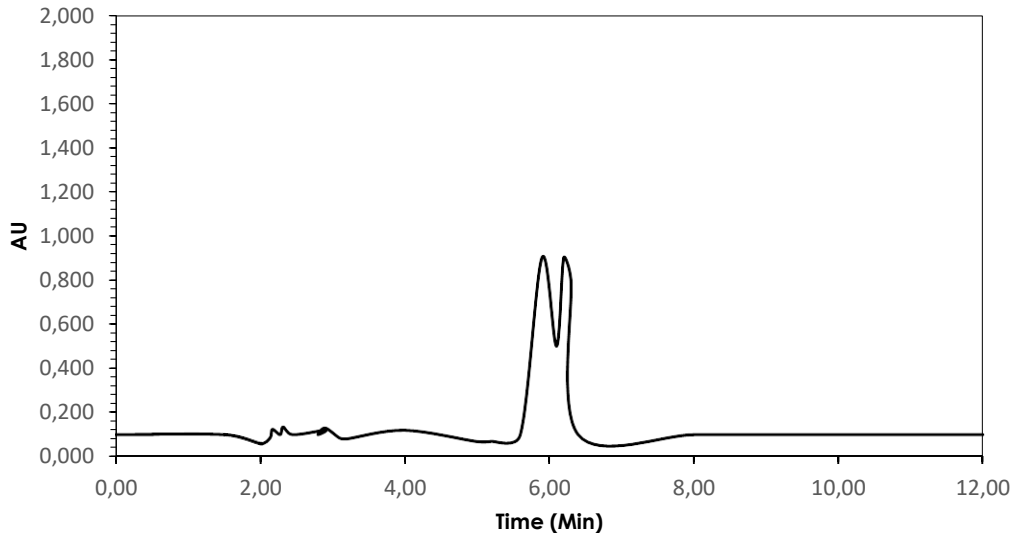

Fig. 4.4: Chromatogram Showing Trial – 2

Selection of Wavelength (λ max): 10 mg of the Sofosbuvir and Daclatasvir standard drug is taken in a 10 ml volumetric flask and dissolved in Acetonitrile and volume made up to the mark, from this solution 0.1ml is pipetted into 10 ml volumetric flask and made up to the mark with the Acetonitrile to give a concentration of 10 µg/ml. The above prepared solution is scanned in UV between 200-400 nm using Acetonitrile as blank. The λmax was found to be 250nm.

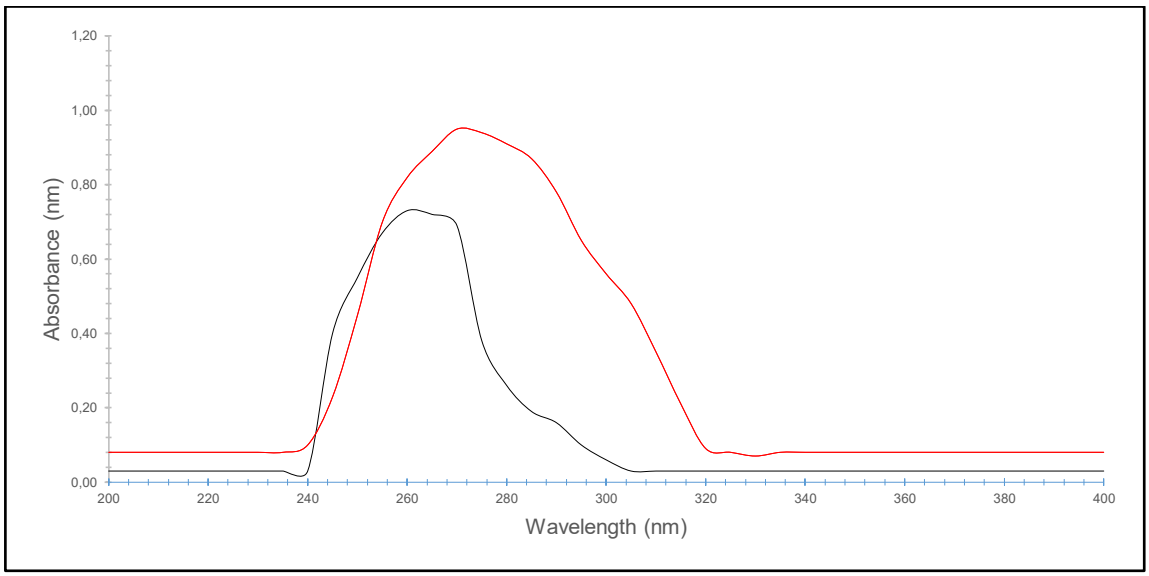

Fig: 4.5: UV spectrum of standard Daclatasvir and Sofosbuvir

Choice of stationary phase: Initially the separation was tried with different columns having different dimensions like diameter and length and pore size. Finally good separation with finest peak shape was achieved with the analytical column Inertsil ODS-C_{18}; 5µm (4.6 X 250mm).

Selection of mobile phase: Several systematic test plans were performed to optimize the mobile phase. Different solvents like methanol, water and acetonitrile in different ratios and different pH values of the mobile phase ratios, by using different buffer solutions in order to get sharp peak and base line separation of the components and without interference of the excipients. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase Acetonitrile: Methanol: 0.1% Triethylamine buffer (pH-3.0) 25:35:40 (v/v/v).

Selection of the mobile phase flow rate: Flow rates of the mobile phase were changed from 0.5-1.0 ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution of the analyte.

Optimized Chromatographic conditions: After series of trials, the chromatographic conditions was accomplished with following:

Buffer	:	0.1% TFA in water
Mobile Phase	:	Acetonitrile: Methanol: 0.1% Triethylamine buffer (pH-3.0) 25:35:40 (v/v/v)
Column	:	Inertsil ODS-C_{18} column (250 x 4.6 mm, 5µ)
Flow Rate	:	1.0 ml/min
Temperature	:	Ambient
Volume	:	20 µl
Detector	:	250 nm
Diluent	:	Water: Acetonitrile (50:50) column with a mixture of as mobile phase. UV detection was performed at 250 nm

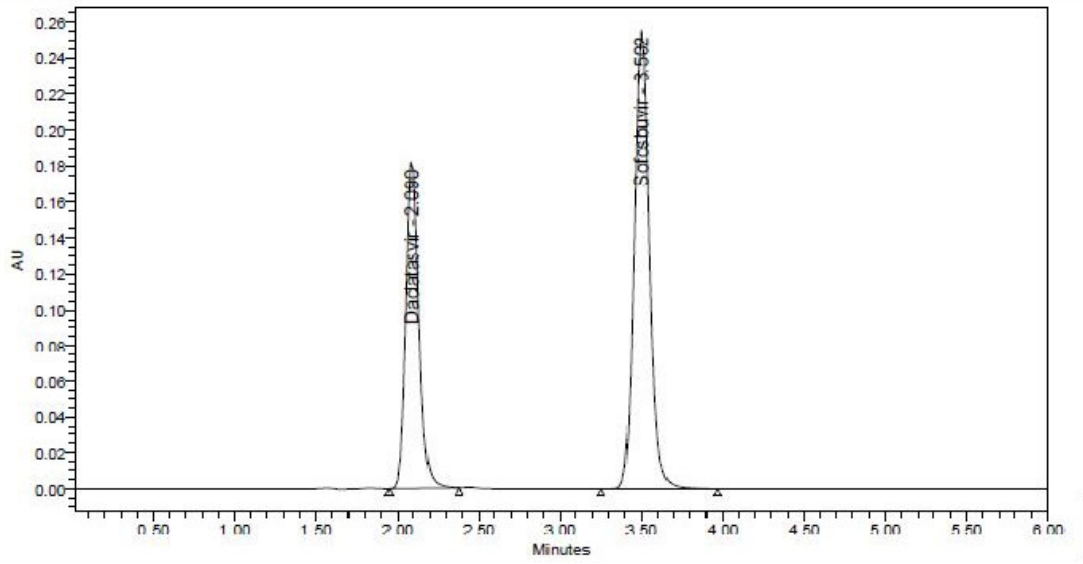

Fig. 4.6: Chromatogram of Daclatasvir and Sofosbuvir

METHOD VALIDATION

Specificity: The chromatogram of standard and sample are identical with nearly same retention time, shown in the Figures 4.25 and 4.26. No interference due to placebo and sample at the retention time of analyte which shows that the method was specific. It was shown in Figures 4.27 and 4.28.

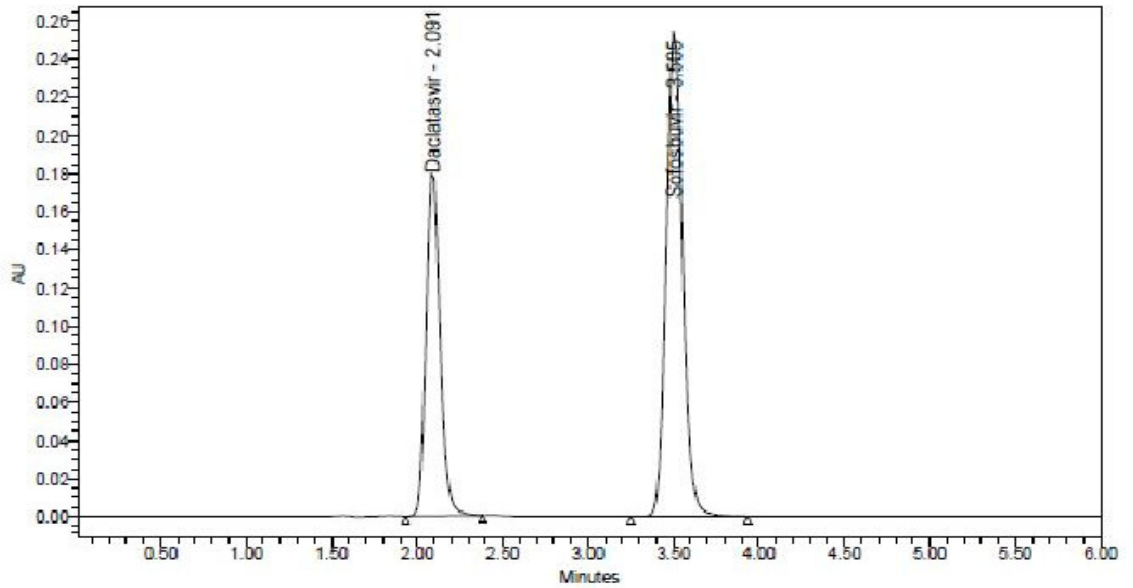

Fig. 4.7: Chromatogram representing specificity of standard

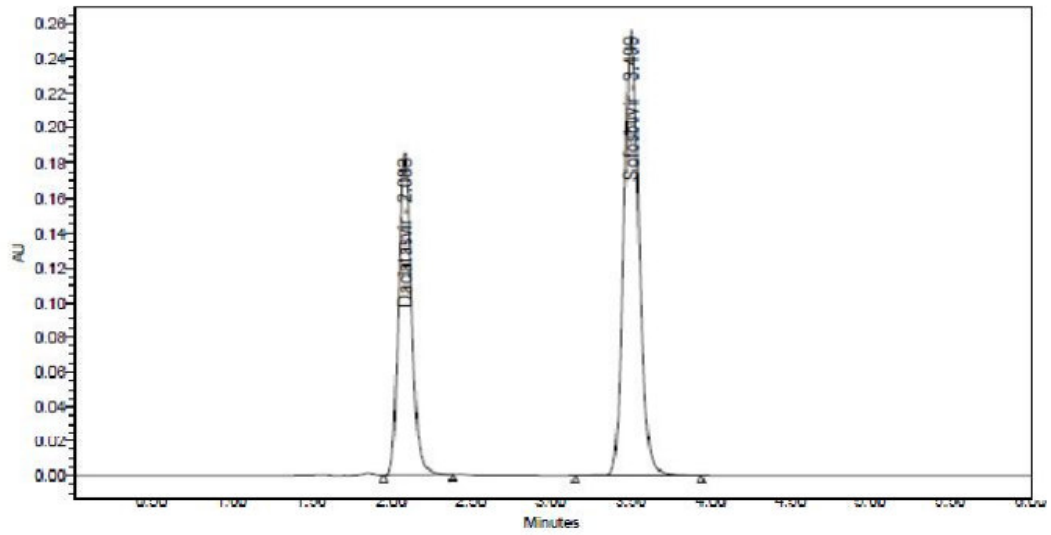

Fig. 4.8: Chromatogram representing specificity of sample

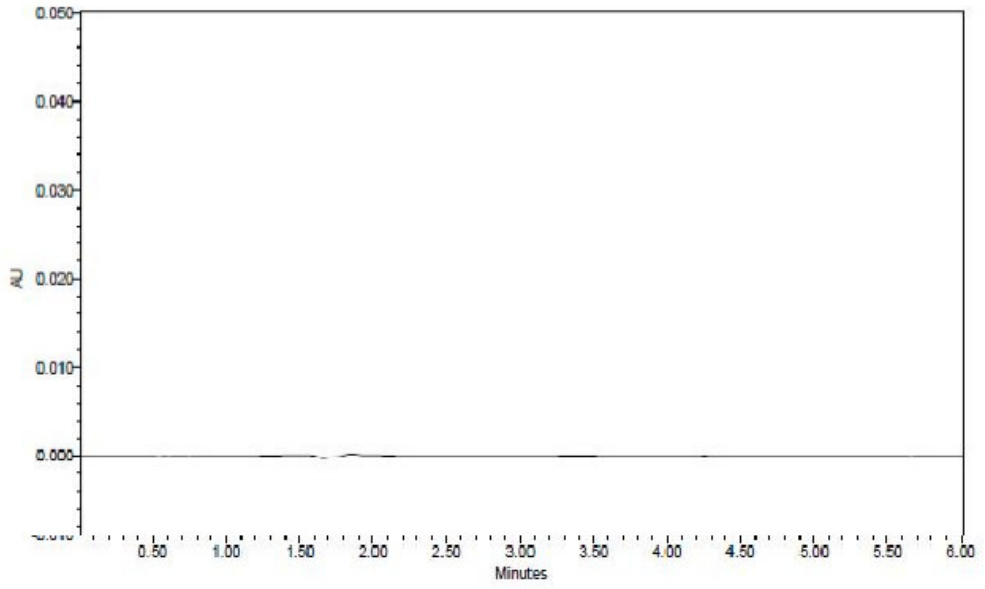

Fig. 4.9: Typical chromatogram of the blank

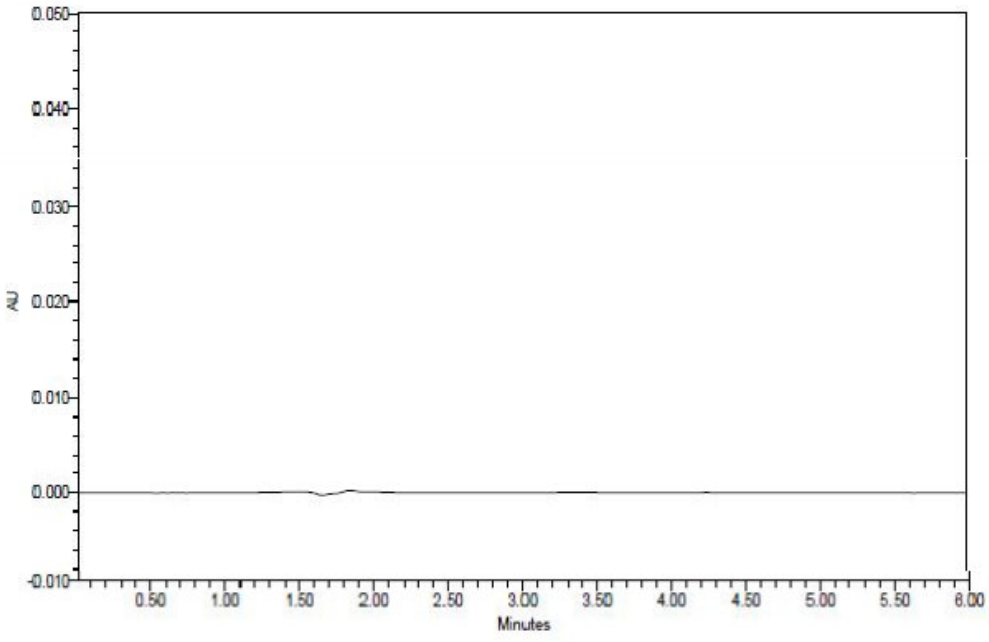

Fig. 4.10: Typical chromatogram of the Placebo

Table 4.1: Specificity data for Sofosbuvir and Daclatasvir

Sr. No.	Sample Name	Rt (min) Sofosbuvir	Rt (Min) Daclatasvir
1	Standard	3.505	2.091
2	Sample	3.499	2.083
3	Blank	-	-
4	Placebo	-	-

Result:

Chromatograms explain that retention time for standard, sample and commercial product of Daclatasvir and Sofosbuvir are same (Table 4.1). This proved that, Excipients have no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So, the method is highly selective.

System suitability:

The system suitability parameters such as US tailing factor, US theoretical Plates and resolution was achieved by injecting the prepared solution five times individually into the chromatographic system separately (Figures 4.9 to 4.14).

Table 4.2: System suitability data of Sofosbuvir and Daclatasvir

Parameter	Sofosbuvir	Daclatasvir	Acceptance Criteria
Retention time (min)	3.502	2.089	$\pm$10
Theoretical plates	6613	3226	>3000
Tailing factor	1.11	1.15	<2.00
% RSD	0.08	0.27	<2.00

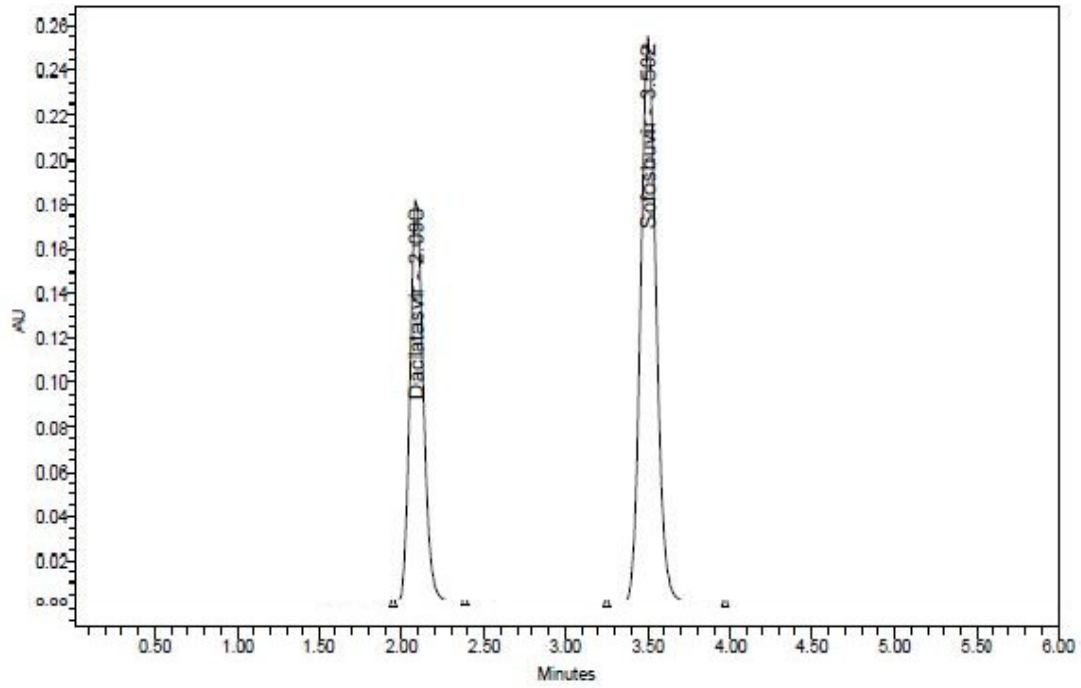

Fig. 4.11: Typical Chromatogram of Standard Injection-1

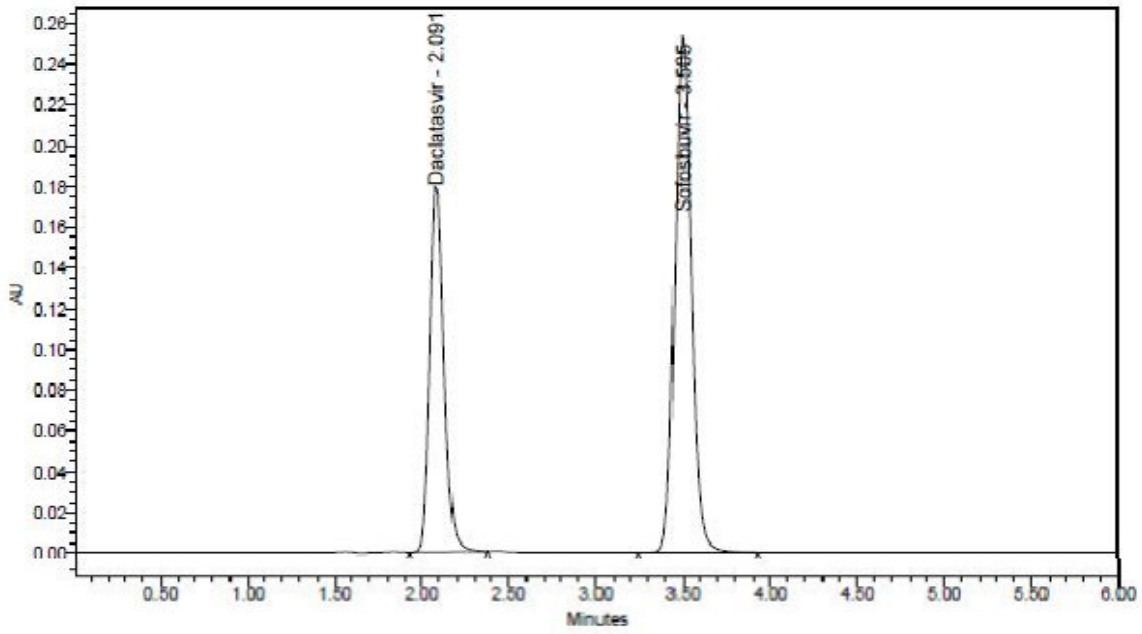

Fig. 4.12: Typical Chromatogram of Standard Injection-2

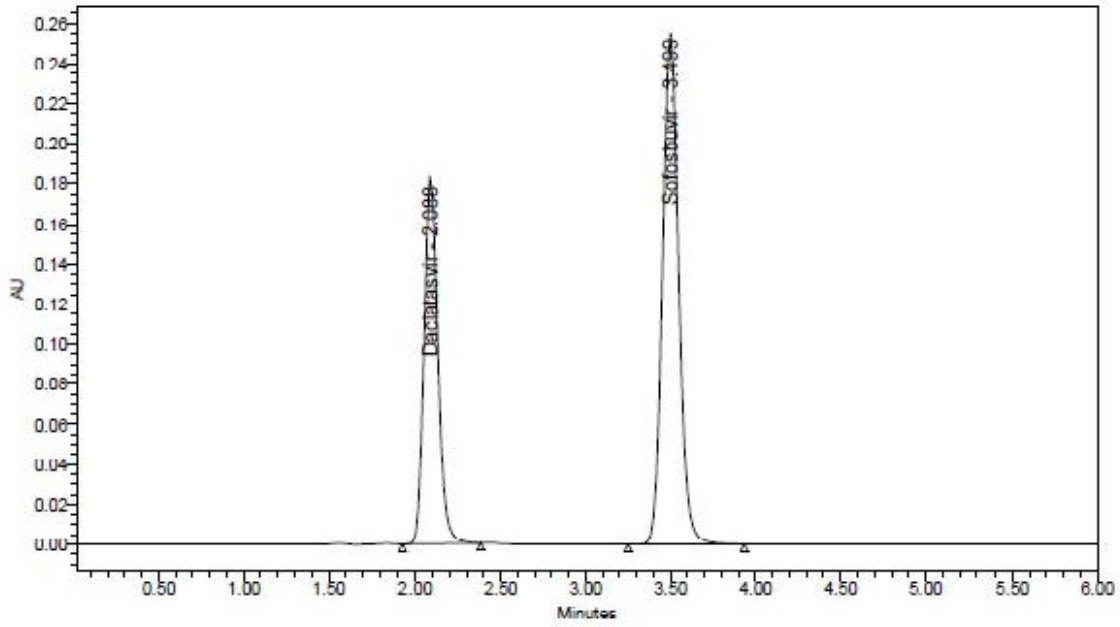

Fig. 4.13: Typical Chromatogram of Standard Injection-3

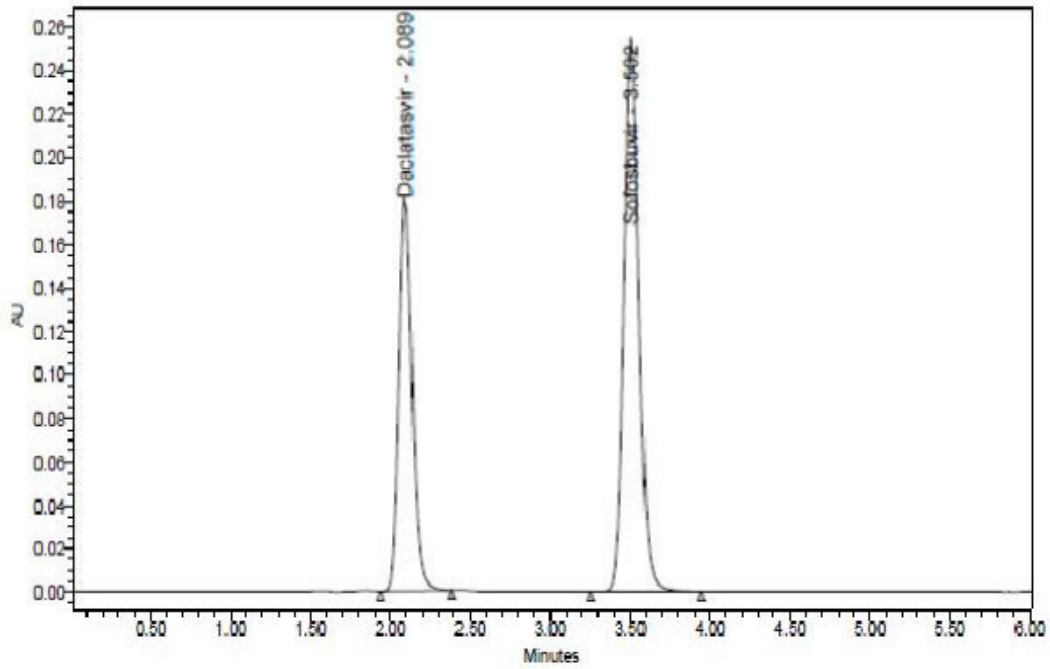

Fig. 4.14: Typical Chromatogram of Standard Injection-4

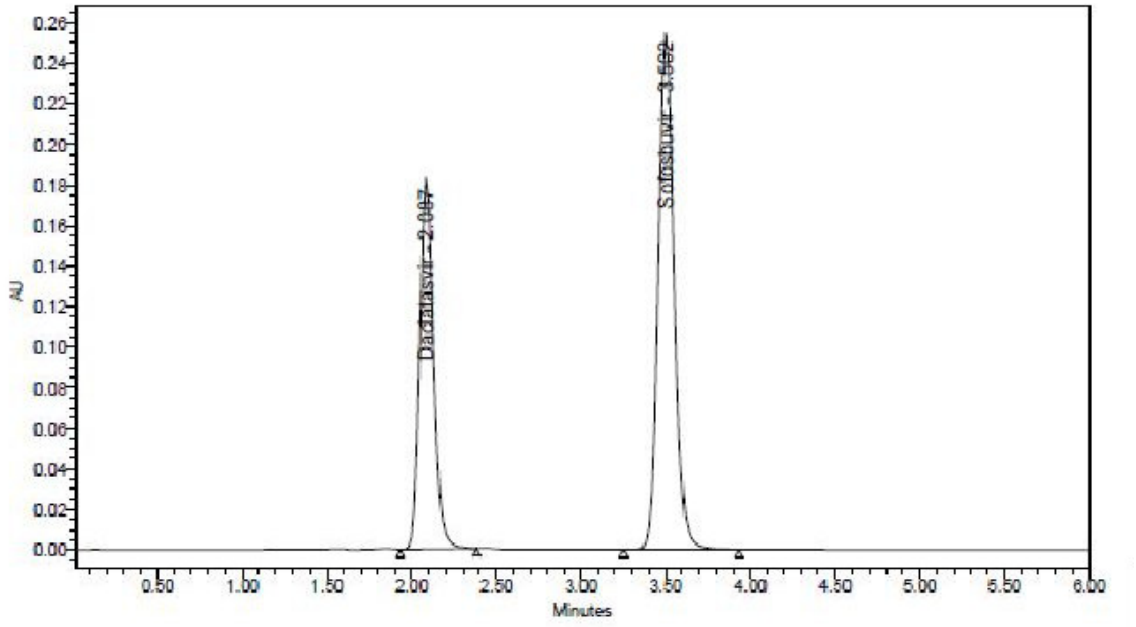

Fig. 4.15: Typical Chromatogram of Standard Injection 5

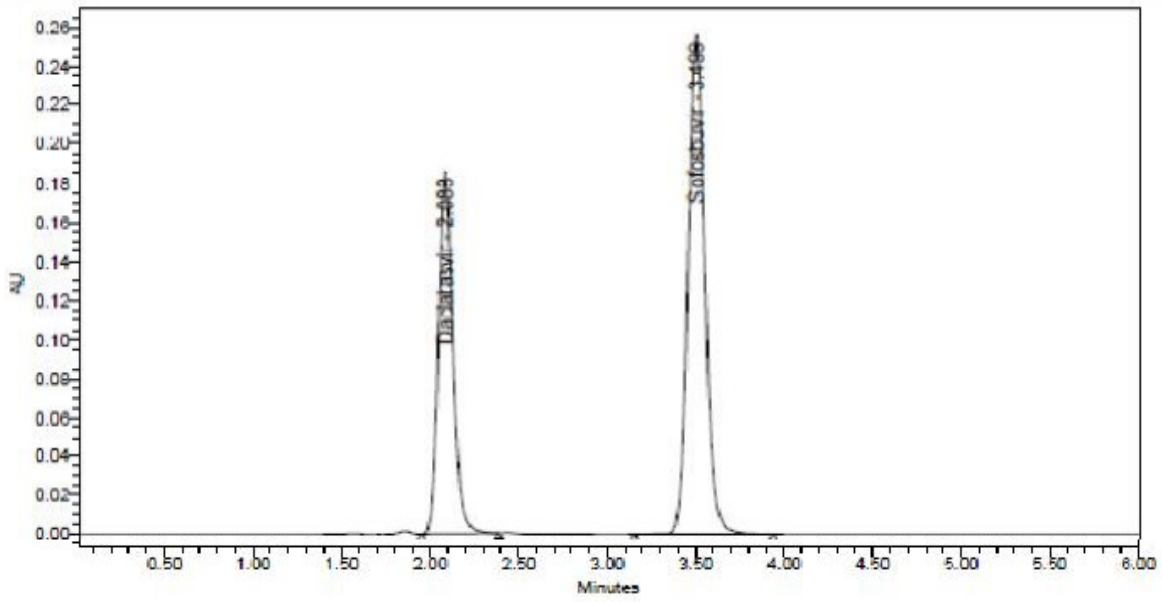

Fig. 4.16: Typical Chromatogram of Sample

Table 4.3: Standard Results of Sofosbuvir and Daclatasvir

S. No.	Sample Name	RT (Min)		Area		USP plate		USP tailing	
		SFB	DCT	SFB	DCT	SFB	DCT	SFB	DCT
1	Injection1	3.502	2.090	1682706	1035661	6647	3257	1.11	1.15
2	Injection 2	3.505	2.091	1682880	1034398	6527	3215	1.11	1.15
3	Injection 3	3.499	2.088	1680431	1030858	6609	3238	1.11	1.15
4	Injection 4	3.502	2.089	1679825	1029640	6642	3237	1.11	1.15
5	Injection 5	3.502	2.087	1680710	1029757	6638	3185	1.11	1.16

Result: Results of system suitability study are summarized in the Table 4.2. Five consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicate a good system for analysis, which was shown in Table 4.3.

Linearity: A series of standard concentrations were prepared from 50 % to 150 % of the target concentration of DCV and SFV. Linearity was assessed by performing single measurement at several analyte concentration varying quantities of stock standard solution diluted with the mobile phase to give a concentration of 5, 10, 15, 20 and 25 µg/mL of DCV and 2, 4, 6, 8 and 10 µg/mL of SFV. Injection was made at intervals of 10.0 min. Linearity of DCV was found to be exist between 5-25 µg/mL and for SFV was 2-10 µg/mL. The chromatograms were recorded and linearity graph was plotted by using peak area of drug against respective concentrations to obtain the linearity range. Calibration curve with concentration verses peak areas was plotted by injecting the above prepared

solutions, shown in figures 4.6 and 4.7 and the obtained data were subjected to regression analysis using the least squares method, shown in **Table 4.3.**

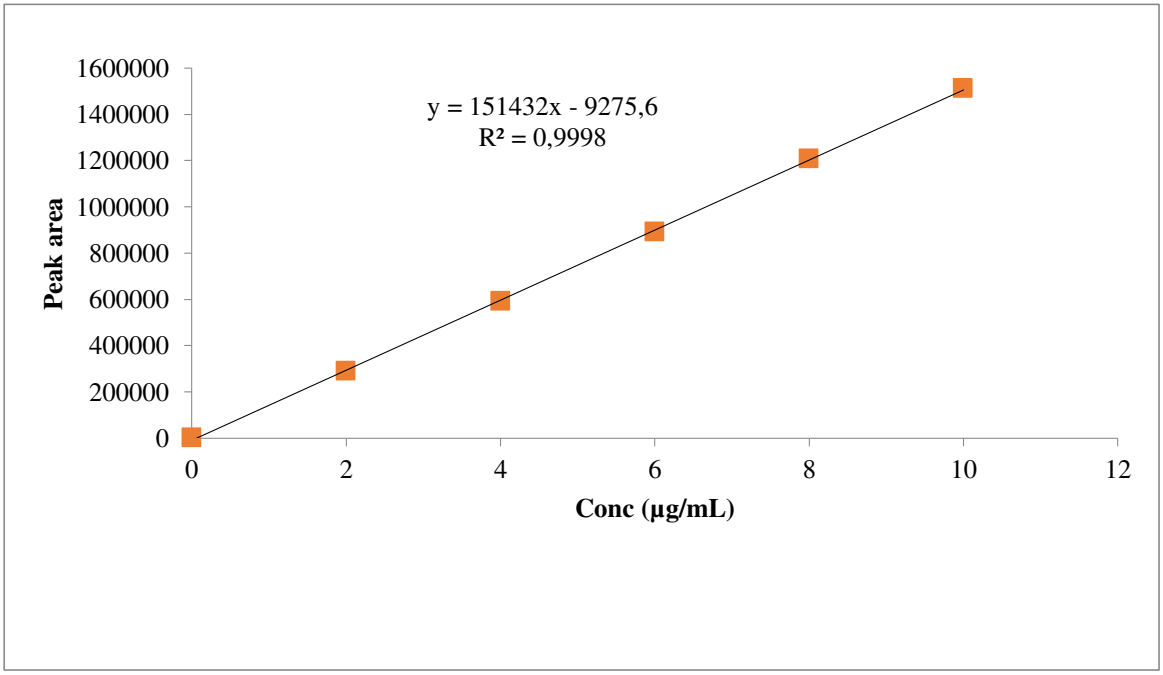

Fig. 4.17: Linearity plot of Sofosbuvir

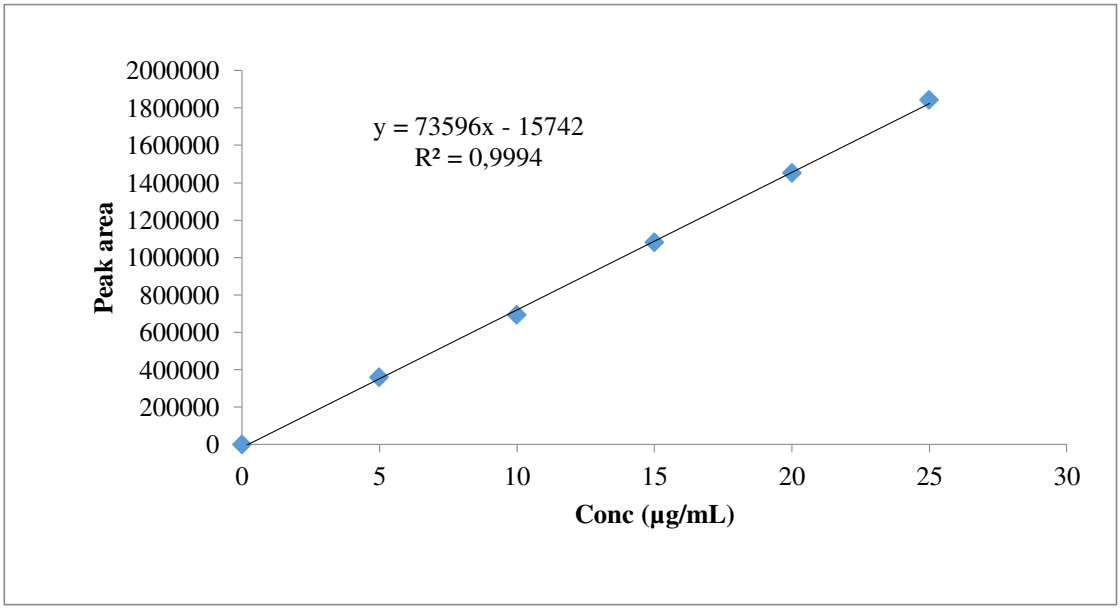

Fig. 4.18: Linearity plot of Daclatasvir

Table 4.4: Linearity data for Sofosbuvir and Daclatasvir

%Level	Concentration µg/mL	Area of Daclatasvirin	Concentration µg/mL	Area of Sofosbuvir
50%	5	358575	2	289124
75%	10	691556	4	591568
100%	15	1081521	6	890541
125%	20	1451468	8	1205846
150%	25	1842135	10	1510214
Concentration range	5-25µg/mL		2-10 µg/mL	
Slope (m)	73596		151432	
Correlation coefficient (r^2)	0.9992		0.9997	

Precision:

Method precision (Repeatability):

The precision of the instrument was checked by repeated injections and measurement of peak areas and retention times of solutions (n = 6) for 160 µg/ml of Sofosbuvir and 24µg/ml Daclatasvir without changing the parameter of the proposed chromatographic method, shown in Figures 4.17 to 4.20.

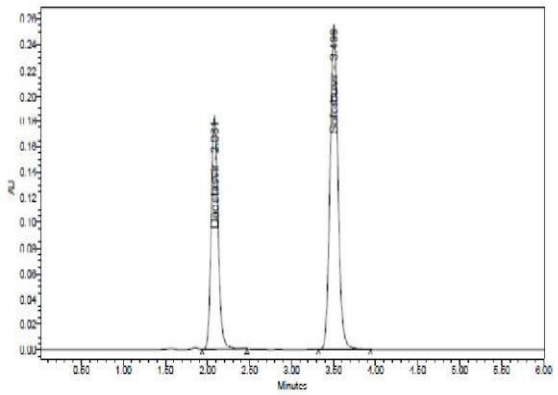

Fig 4.19: Chromatogram for injection 1

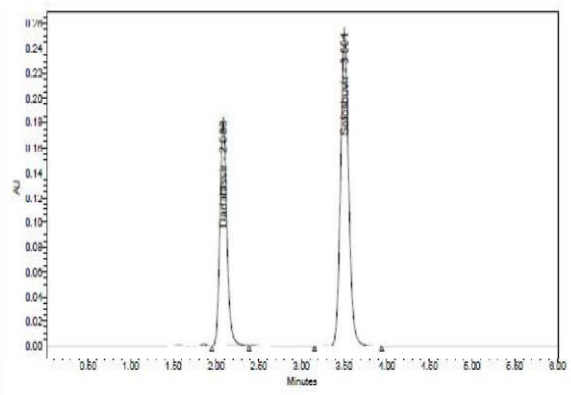

Fig 4.20: Chromatogram for injection 2

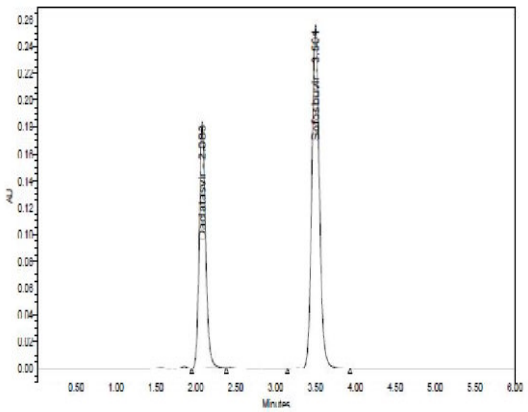

Fig. 4.21: Chromatogram for injection 3

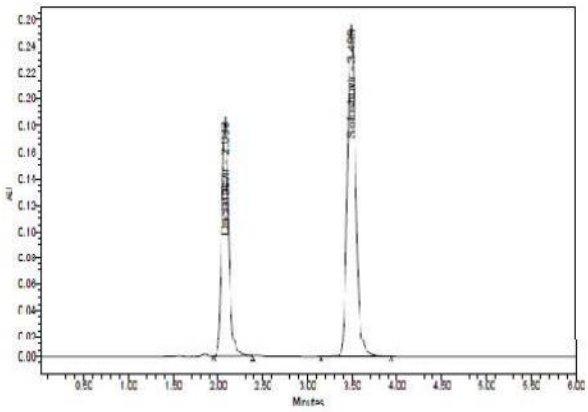

Fig. 4.22: Chromatogram for injection 4

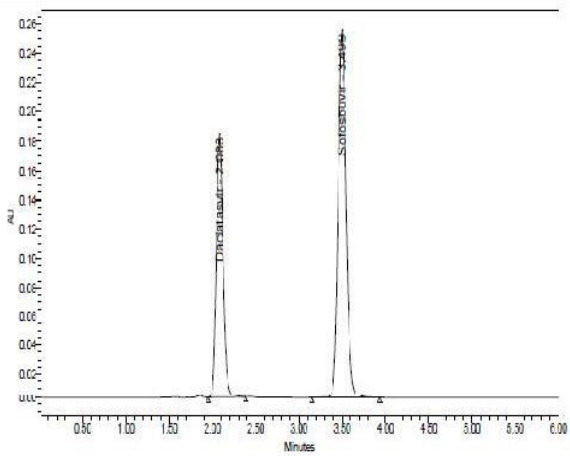

Fig. 4.23: Chromatogram for injection 5

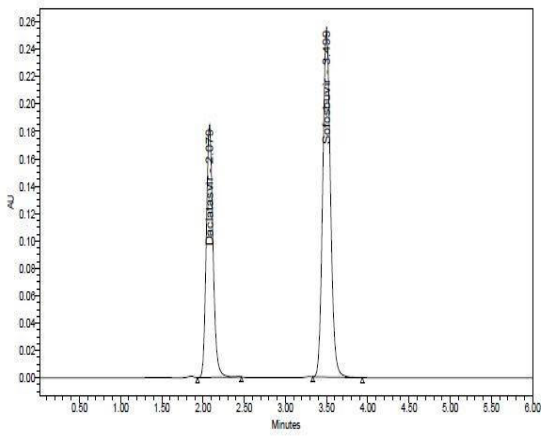

Fig. 4.24: Chromatogram for injection 6

Table 4.5: Summary of peak areas for method precision for Sofosbuvir and daclatasvir

Sample No.	Sofosbuvir			Daclatasvir		
	Retention time (min)	Peak area	% Assay	Retention time (min)	Peak area	% Assay
1	3.499	1686766	99.9	2.083	1035922	99.2
2	3.499	1677924	99.2	2.081	1034144	100.0
3	3.501	1687828	99.2	2.083	1022382	98.9
4	3.501	1679039	99.7	2.082	1038818	99.9
5	3.499	1674492	99.2	2.079	1046773	100.0
6	3.499	1682158	99.3	2.080	1048278	99.6
Mean			99.4			99.6
%RSD			0.31			0.48

Result: Results of variability were summarized in the above table 4.5. Percentage relative standard deviation (%RSD) was found to be less than 2% which proves that method is precise.

Accuracy (Recovery study):

The accuracy of the method was determined by calculating the recoveries of Sofosbuvir and Daclatasvir by analyzing solutions containing approximately 50%, 100% and 150% of the working strength of Sofosbuvir and Daclatasvir (Figures 4.21 to 4.23) and the percentage recovery results obtained are listed in Table 4.6.

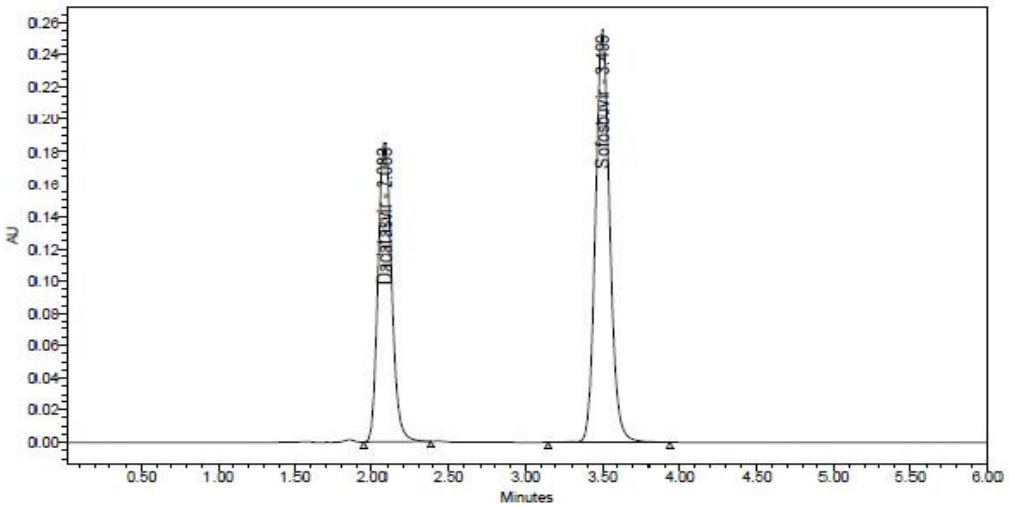

Fig. 4.25: Typical chromatogram for Accuracy 50 %

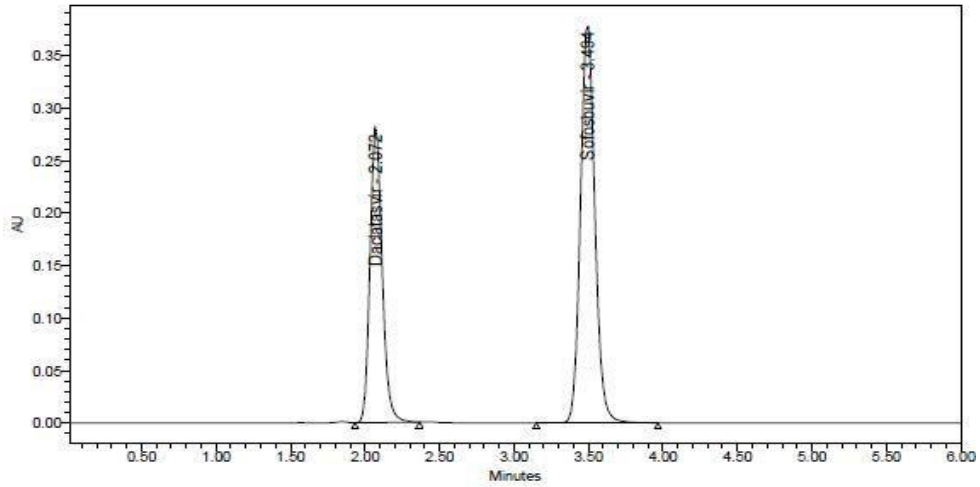

Fig. 4.26: Typical chromatogram for Accuracy 100 %

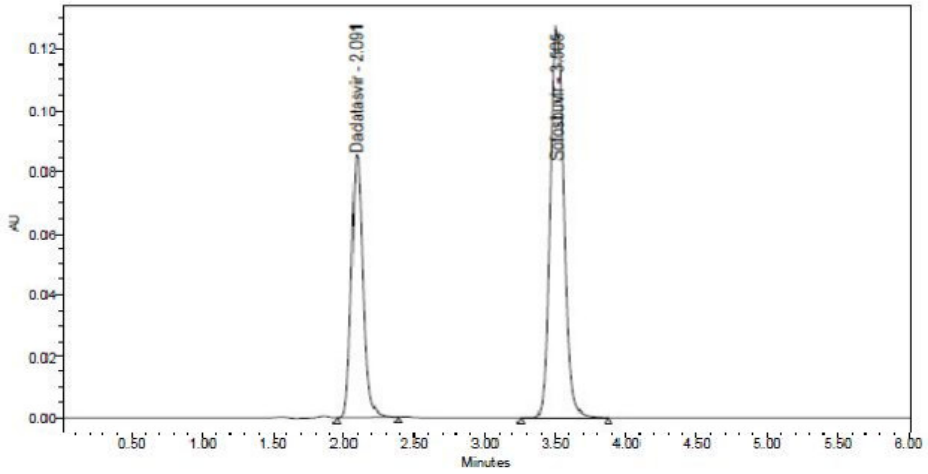

Fig. 4.27: Typical chromatogram for Accuracy 150 %

Table 4.6: Recovery data for Sofosbuvir and Daclatasvir

S. No.	Accuracy level	Injection	Sofosbuvir		Daclatasvir	
			% Recovery	Average	% Recovery	Average
1	50%	1	99.4	99.3%	99.7	99.7%
		2	99.2		99.5	
		3	99.3		99.9	
2	100%	1	99.9	99.4%	99.2	99.4%
		2	99.2		100.0	
		3	99.2		98.9	
3	150%	1	99.1	99.0%	99.0	99.2%
		2	99.3		98.9	
		3	98.8		99.5	

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 and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied ±2 nm and flow rate was varied ±0.2 ml/min. The chromatograms were shown in Figures 4.24 and 4.25 and the results were shown in Table 4.7.

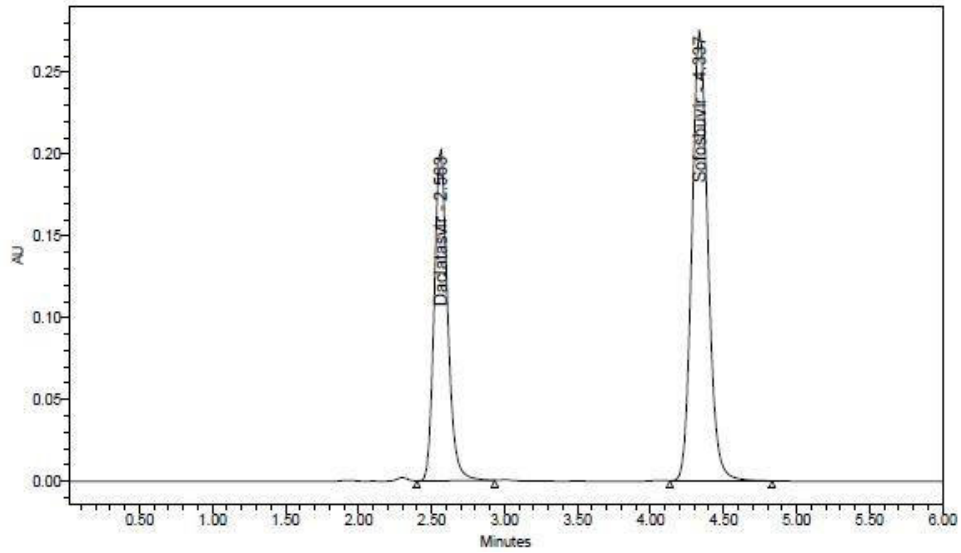

Fig. 4.28: Chromatogram for decreased flow rate

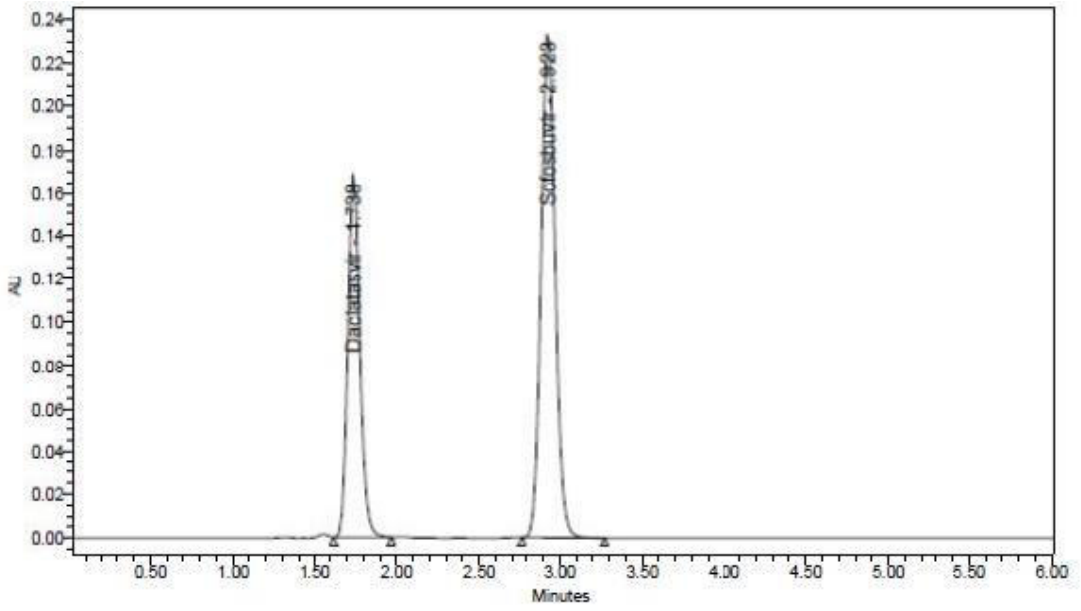

Fig. 4.29: Chromatogram for increased flow rate

Table 4.7: Results of Robustness data for Sofosbuvir and Daclatasvir

parameter	RT (min)		Area	
	Sofosbuvir	**Daclatasvir**	**Sofosbuvir**	**Daclatasvir**
Decreased flow rate (0.9ml/min)	4.337	2.563	2098358	1290171
Increased flow rate (1.1ml/min)	2.923	1.738	1383875	852316

Result: The results of Robustness of the present method had shown that changes made in the Flow and wavelength did not produce significant changes in analytical results which were presented in the above table. As the changes are not significant we can say that the method is Robust.

Limit of detection and limit of quantification: The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve by using the equations (1) and (2), respectively. The data obtained was shown in Table 4.6.

Where,

LOD = 3.3 σ /S ……. (1) LOQ =10 σ /S …….. (2)

σ = the standard deviation of the response

S = the slope of the calibration curve

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

Table 4.8: LOD and LOQ

	SOFOSBUVIR (µg)	DACLATASVIR (µg)
LOD	0.005	0.001
LOQ	0.014	0.003

Analysis of Marketed Formulation

Preparation test solution: A total of 20 tablets were accurately weighed and powdered in a mortar. An amount equivalent to one tablet (Containing 400 mg of SFV and 60 mg of DCV) was transferred to 25 mL volumetric flask, 10 mL of methanol was added and content of the flask were ultra-sonicated for 10 min. The solution was filtered through whatmann filter paper No. 41. The sample solution thus prepared was diluted with mobile phase to get the solution containing SFV and DCV in 15 & 6 µg/mL proportion, respectively. The test solution was injected in to HPLC and % assay was calculated.

The validated method was applied for the assay of commercial tablets of daclatasvir and sofosbuvir (400 mg of Sofosbuvir and 60 mg of Daclatasvir). Peak area of the detector response was used to calculate % assay. The % assay was found to be 99.57 % for Daclatasvir and 99.38 % for Sofosbuvir. The results presented good agreement with the labelled content.

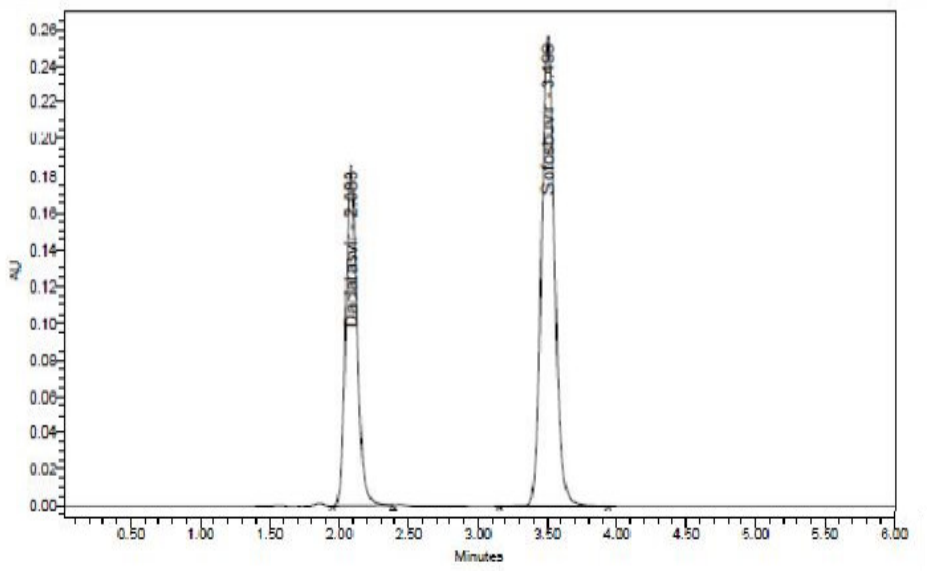

Fig. 4.30: Typical Chromatogram of Sample

Table 4.9: Assay of test sample

Test formulation (Tablet)	**Label claimed (mg/tab)**	
Sofosbuvir and Daclatasvir	**SFV**	**DCV**
	400	60
	Conc found (mg)	
	15	6
	%Assay	
	99.57	99.38