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ANALYTİCAL METHOD DEVELOPMENT AND VALİDATİON OF STABİLİTY INDİCATİNG RP-HPLC METHOD FOR THE SİMULTANEOUS ESTİMATİON OF SOFOSBUVİR AND DACLATASVİR İN BULK AND PHARMACEUTİCAL DOSAGE FORM.

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Abstract

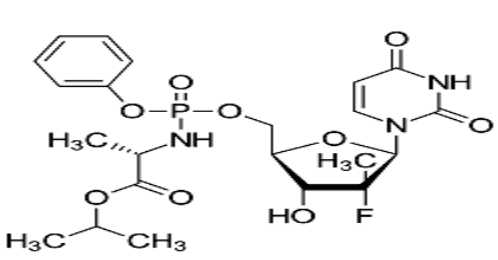
Special, effective high pressure liquid chromatography method has been developed for the simultaneous quantification of Sofosbuvir and Daclatasvir. By using Waters HPLC e-2695 quaternary pump with a PDA detector of 2998 instrument the chromatographic separation of Sofosbuvir and Daclatasvir was achieved on the column of Waters X-Bridge C18 150X4.6mm, 3.5µ using an isocratic elution with a buffer containing 0.1percent ortho phosphoric acid and acetonitrile at a rate of 70:30 as a mobile phase with a flow rate of 1 ml/min at ambient temperature. A detector wavelength of 265 nm utilizing the PDA detector were given in the instrumental settings. The linearity was studied between the concentration range of 40-600 µg/ml of Sofosbuvir and 6-90 µg/ml of Daclatasvir were injected. The plotted calibration curves were linear with a regression coefficient of R^2> 0.999, indicates that the linearity was with in the limit. As a part of method validation the parameters like specificity, linearity, accuracy, ruggedness, robustness were determined and the results were found to be within the allowable limit. The method developed was found to be applicable to routine analysis and to be used for the measurement of both active pharmaceutical ingredients (i.e, Sofosbuvir and Daclatasvir). Validation of the proposed method was carried out according to an International Conference on Harmonization (ICH) guidelines. Since, there is HPLC method reported in the literature for the estimation of Sofosbuvir and Daclatasvir, there is a need to develop quantitative methods under different conditions to achieve improvement in specificity, selecivity etc.

Key words: Sofosbuvir, Daclatasvir, HPLC, Development, Validation.

INTRODUCTION

Sofosbuvir, sold under the brand name Sovaldi among others, is a medication used to treat hepatitis C [1, 2]. It is only recommended with some combination of ribavirin[3], peginterferon-alfa, simeprevir, ledipasvir, daclatasvir, or velpatasvir. Safety during pregnancy is unclear; some of the medications used in combination may result in harm to the baby. It is taken by mouth. Common side effects include feeling tired, headache [4], nausea [5], and trouble sleeping [6]. Side effects are generally more common in interferon-containing regimens. Sofosbuvir may reactivate hepatitis B [7] in those who have been previously infected. In combination with ledipasvir, daclatasvir or simeprevir it is not recommended with amiodarone [8, 9] due to the risk of an abnormally slow heartbeat. Sofosbuvir is in the nucleotide analog family of medication and works by blocking the hepatitis C NS5B protein [10, 11]. Sofosbuvir may reactivate hepatitis B in those who have been previously infected.

Daclatasvir, sold under the trade name Daklinza, is a medication [12, 13] used in combination with other medications to treat hepatitis C (HCV). The other medications used in combination include sofosbuvir, ribavirin, and interferon, vary depending on the virus type and whether the person has cirrhosis [14, 15]. In significant difference to previous HCV therapies. With daclatasvir, sofusbivir, and ribavirin the most common side effects are headache, feeling tired, nausea, and red blood cell breakdown [16-18]. It should not be used with St. John's wort, rifampin, or carbamazepine. It works by inhibiting the HCV protein NS5A. There is a serious risk of bradycardia [19, 20] when daclatasvir is used with sofosbuvir and amiodarone.



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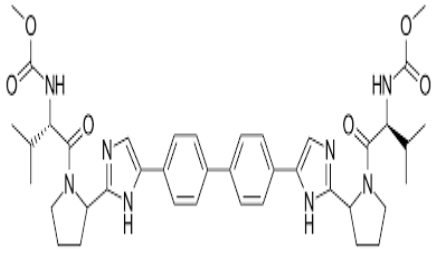

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FIG. 1: CHEMICAL STRUCTURE OF (A) SOFOSBUVIR (B) DACLATASVIR

MATERIALS AND METHOD

Chemicals: Acetonitrile, HPLC-grade ortho phosphoric acid, water were purchased from Merck India Ltd, Mumbai, India. APIs of Sofosbuvir and Daclatasvir standards were procured from Glenmark, Mumbai.

The Instrumentation: Waters alliance liquid chromatography (model e-2695) monitored with empower 2.0 data handling system and a detector of photo diode array (model 2998) was used for this study.

Preparation of buffer: 1 ml of ortho phosphoric acid is dissolved in 1 lt of HPLC grade water and filter through 0.45 µ filter paper.

Chromatographic conditions: The HPLC analysis was performed on reverse phase HPLC system with isocratic elution mode using a mobile phase of acetonitrile and 0.1% ortho phosphoric acid and Waters X-Bridge C18 150X4.6mm, 3.5µ with a flow rate of 1 ml /min.

Diluent: 0.1% OPA and Acetonitrile in the ratio (70:30) is used as diluent.

Preparation of the standard stock solution: For standard stock solution preparation, add 70ml of diluents to 400mg of Sofosbuvir and 60 mg of Daclatasvir taken in a 100 ml volumetric flask and sonicate for 10 minutes to fully dissolve the contents and then make up to the mark with diluent.

Preparation of Standard solution: 5 ml of solution is drawn from the above normal stock solution into a 50ml volumetric flask and diluted up to the level.

Preparation of Sample solution: For sample stock solution preparation, add 70ml of diluents to 674 mg of sample (equivalent to 400mg of Sofosbuvir and 60 mg of Daclatasvir) taken in a 100 ml volumetric flask and sonicate for 10 minutes to fully dissolve the contents and then make up to the mark with diluent.

Preparation of Sample solution: 5 ml of sample solution is drawn from the above sample stock solution into a 50ml volumetric flask and diluted up to the level.

RESULTS AND DISCUSSION

The main analytical challenge during development of a new method was to separate active Pharma ingredients. In order to provide a good performance the chromatographic conditions were optimized.

Method optimization: To optimize the chromatographic conditions, different ratios of phosphate buffer and the acetonitrile in the mobile phase with isocratic mode was tested. However the mobile phase composition was modified at each trial to enhance the resolution and also to achieve acceptable retention times. Finally 0.1% Ortho phosphoric acid buffer and acetonitrile with isocractic elution was selected because it results in a greater response of active pharmacy ingredients. During the optimization of the method various stationary phases such as C_8, C_{18} phenyl and amino, luna phenyl columns were tested. From these trials the peak shapes were relatively good with a Waters X-Bridge C18 150X4.6mm, 3.5µ with a PDA detector. The mobile phase flow rate has been done at 265 nm in order to obtain enough sensitivity. By using above conditions we get retention times of Sofosbuvir and Daclatasvir were about 2.770 and 5.118 min with a tailing factor of 1.05 & 0.99. The number of theoretical plates for Sofosbuvir and Daclatasvir were in acceptable limit which indicate the column's successful output the % RSD for six replicate injections was around 0.84% and 0.62%, the proposed approach suggests that it is extremely precise. According to ICH guidelines, the established method was validated.

Method validation

The optimized RP-HPLC validated method according to ICH guidelines in terms of system suitability, linearity, accuracy, precision and robustness.

System suitability: Device suitability was performed by injecting standard solution containing 400 µg/ml of Sofosbuvir and 60 µg/ml of Daclatasvir in six replicates. The results show that the machine fitness parameter is within the limit provided by ICH. The results were shown below table 1. Figure 2 represents the chromatogram of standard.

TABLE 1: RESULTS OF SYSTEM SUITABILITY

System suitability parameter	Acceptance criteria	Drug name

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		Sofosbuvir	Daclatasvir
USP Plate count	NLT 2000	3661	9074
USP Tailing	NMT 2.0	0.98	1.44
USP Resolution	NLT 2.0	-	11.62
% RSD	NMT 2.0	0.28	0.42
Retention Time	NLT 2.0	2.770	5.118

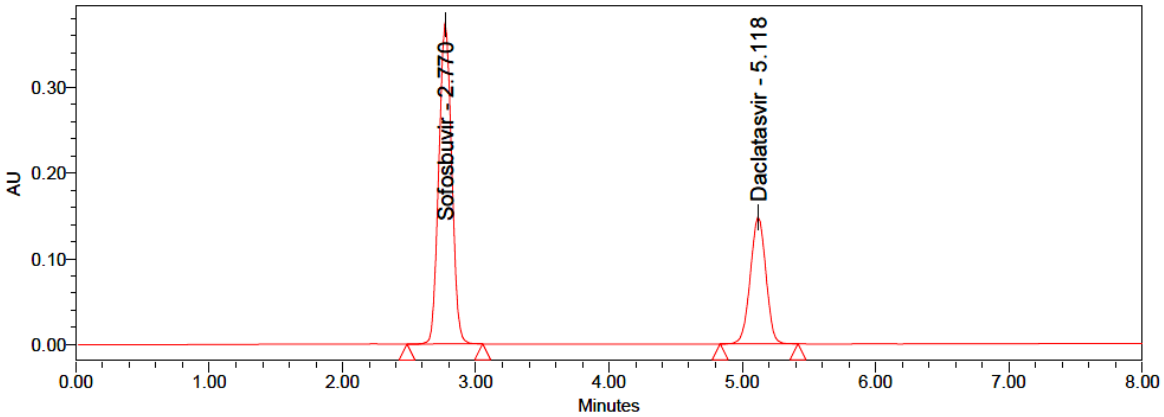

FIG. 2: CHROMATOGRAM OF STANDARD

Specificity: There was no interference from blank at the retention time of Sofosbuvir and Daclatasvir. This proves the technique is specific. Figure 3 shows the chromatogram of blank.

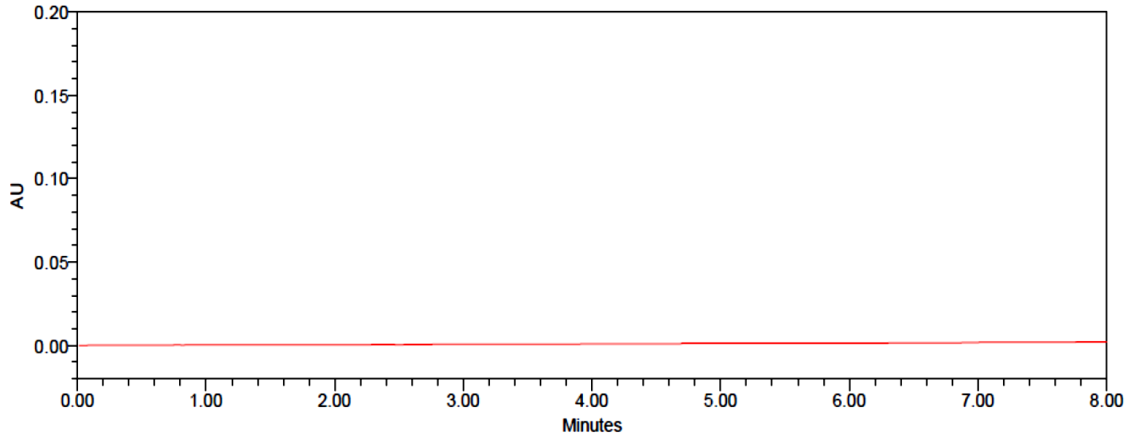

FIG. 3: CHROMATOGRAM OF BLANK

Linearity: Linearity was calculated by plotting a calibration curve of the peak area against its respective concentration, linearity was determined. From this calibration curve, it was noticed that the curve was linear between the range of 40-600µg/ml of Sofosbuvir and 6-90µg/ml of Daclatasvir. The regression equations for calibration curve was y = 5456.43x +30080.71 (R^2=0.9993) for Sofosbuvir and y =10217.65x + 1881.5 (R^2=0.9992) for Daclatasvir respectively and the results of linearity were shown in table 2. Calibration plots were shown in figure 4.

TABLE 2: LINEARITY RESULTS

Linearity	Sofosbuvir		Daclatasvir	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
Linearity-1	40.00	255890	6.00	62716
Linearity-2	100.00	621356	15.00	171818
Linearity-3	200.00	1105500	30.00	308822

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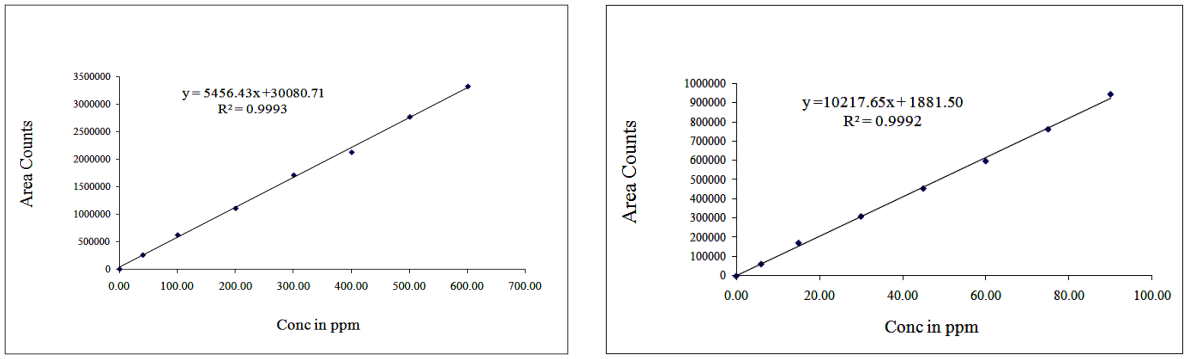
Linearity-4	300.00	1711265	45.00	453374
Linearity-5	400.00	2126456	60.00	595546
Linearity-6	500.00	2771404	75.00	760828
Linearity-7	600.00	3325525	90.00	941815
Slope	5456.43		1881.5	
Intercept	30080.71		10217.65	
CC	0.9993		0.9992	



A **B**

FIG. 4: CALIBRATION PLOTS OF (A) SOFOSBUVIR (B) DACLATASVIR

Accuracy: The accuracy of the system was achieved by measuring the recovery experiments at three stages (50 percent, 100 percent and 150 percent). APIs with concentrations of 200, 400 and 600µg/ml of Sofosbuvir and 30, 60 and 90µg/ml of Daclatasvir were prepared. For each spike stage, the test solution was injected three times and the test was performed according to the test process. The recovery results were similar to 100% and also the RSD values were less than ±2%. The percentage recovery, mean and relative standard deviations were determined. Recovery values shown within the desired range were correct. The results are summarized below. Accuracy findings have been shown in table 3.

TABLE 3: RESULTS OF ACCURACY

S. No.	% Level	Sofosbuvir % Recovery	Daclatasvir % Recovery
1	50	100.3	99.7
2	100	99.8	99.8
3	150	99.9	99.7

Precision: The precision of the analytical technique is the degree of proximity of the sequence of measurements obtained from multiple homogeneous mixture samplings. The accuracy of the process of the drugs were calculated by injection of six individual determinations of Sofosbuvir (400 µg/ml) and Daclatasvir (60µg/ml). Method precision results were shown in table 4 and sample chromatogram was shown in figure 5.

TABLE 4: RESULTS OF INTRADAY PRECISION

S. No.	Sofosbuvir			Daclatasvir		
	Conc. (µg/ml)	Area	% Assay	Conc. (µg/ml)	Area	% Assay
1	400	2001286	99.3	60	595977	100.3
2		2020871	100.3		597574	100.6
3		2012783	99.9		597354	100.5
4		2006874	99.6		594856	100.1
5		2017136	100.1		595669	100.3
6		2021784	100.4		597983	100.6

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% RSD	0.42	0.20

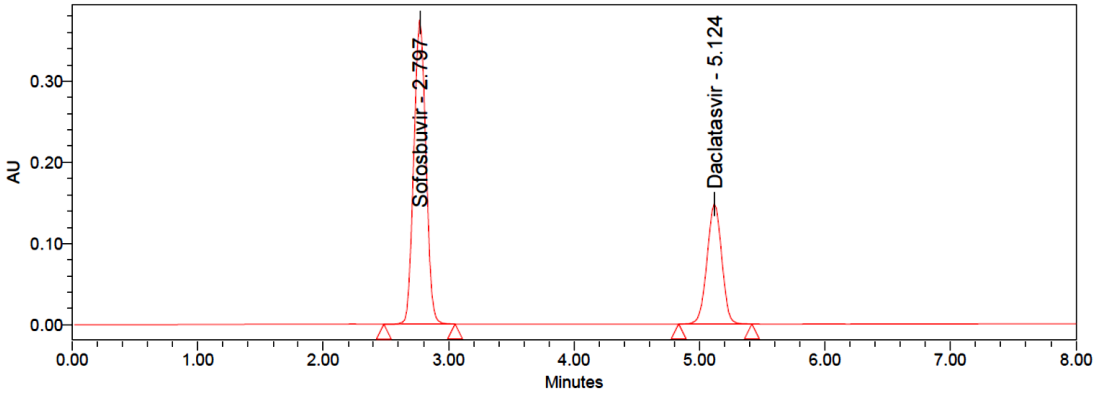

FIG. 5: CHROMATOGRAM OF SAMPLE

Intermediate Precision: Six replicates of the sample solution were analyzed by different researchers and different tools were checked on separate days. The peak regions used to assess the average percent of RSD values have been determined. The findings are shown in the table 5.

TABLE 5: INTER-DAY PRECISION RESULTS

S. No.	Sofosbuvir			Daclatasvir		
	Conc. (µg/ml)	Area	% Assay	Conc. (µg/ml)	Area	% Assay
1	400	2013125	99.9	60	595379	100.2
2		2015871	100.1		595559	100.2
3		2027154	100.6		584358	98.3
4		2016245	100.1		594652	100.0
5		2016871	100.1		594768	100.1
6		2027652	100.7		595883	100.2
%CV	0.32			0.76		

LOD and LOQ: LOD and LOQ were determined separately using the calibration curve technique. The LOD and LOQ of the compound were measured using the developed RP-HPLC method by injecting lower and lower concentrations of the standard solution. The LOD and LOQ concentrations and their s/n values of Sofosbuvir and Daclatasvir were represented in the following table 6 and the chromatograms of LOD and LOQ were shown in figure 6.

TABLE 6: LOD AND LOQ RESULTS

Sofosbuvir				Daclatasvir			
LOD		LOQ		LOD		LOQ	
Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n
0.505	8	1.666	27	0.075	4	0.246	24

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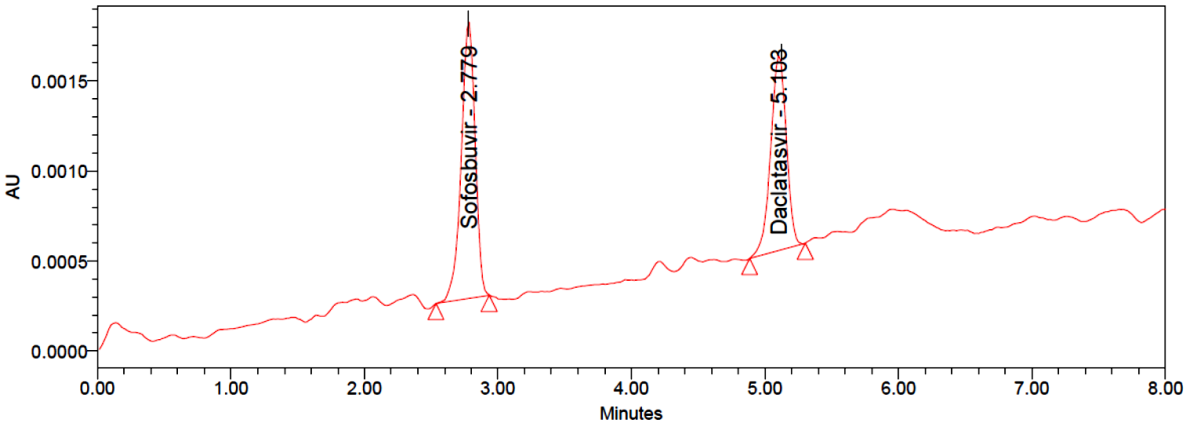

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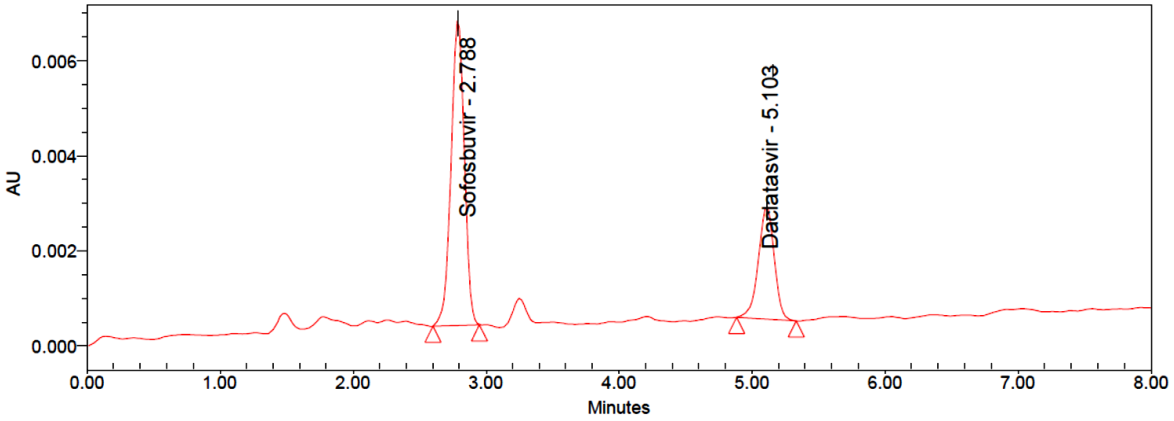

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FIG. 6: CHROMATOGRAM OF (A) LOD (B) LOQ

Robustness: The conditions of the experiment was designed to measure the robustness of the intentionally changed conditions such as flow rate, organic percentage in mobile phase. Results of robustness were shown in table 7.

TABLE 7: ROBUSTNESS RESULTS

Parameter name	% RSD	
	Sofosbuvir	Daclatasvir
Flow rate (0.8 ml/min)	0.45	0.12
Flow rate (1.2 ml/min)	0.87	0.21
Org Plus (33:67)	0.64	0.50
Org Minus (27:73)	0.23	0.47

Degradation studies: Sofosbuvir and Daclatasvir standard was subjected to various conditions of forced degradation in order to induce partial degradation of the compound. Forced degradation experiments have been performed to establish that the process is acceptable for degradation materials. In addition the studies include information on the condition under which the drug is unstable, such that the steps are also taken during formulation to prevent possible instabilities. Forced degradation results were shown in table 8.

Acid degradation: 5 ml of sample stock solution was moved to a volumetric flask of 50 ml, add 1 ml of 1N HCl and left it for 15 min. After 15 min add 1 ml of 1N NaOH and make up to the diluent mark.

Alkali degradation: 5 ml of sample stock solution was moved to a volumetric flask of 50 ml, add 1 ml of 1N NaOH and left it for 15 min. After 15 min add 1 ml of 1N HCl and make up to the mark.

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Peroxide degradation: 5 ml of sample stock solution was moved to a volumetric flask of 50 ml, add 1 ml of 30% hydrogen peroxide solution and make upto the mark with diluents.

Reduction degradation: 5 ml of sample stock solution was moved to a volumetric flask of 50 ml and add 1 ml of 30% sodium bi sulphate solution and make upto the mark with diluents.

Thermal degradation: The sample solution was set in an oven at 110°C for 24 hrs. The resultant solution was injected into HPLC system.

Photolytic degradation: The sample solution was placed in sun light for 24 hrs. The resultant solution was injected into HPLC system.

TABLE 8: FORCED DEGRADATION RESULTS

Degradation condition	Sofosbuvir		Daclatasvir	
	% Assay	% deg	% Assay	% deg
Control	100.0	0.0	99.8	0.2
Acid deg	86.4	13.6	84.6	15.4
Alkali deg	87.1	12.9	85.4	14.6
Peroxide deg	83.7	16.3	86.1	13.9
Reduction deg	85.2	14.8	88.0	12.0
Thermal deg	87.9	12.1	88.4	11.6
Photolytic deg	88.6	11.4	87.5	12.5

CONCLUSION

This method described the quantification of Sofosbuvir and Daclatasvir in bulk and pharmaceutical formulation as per ICH guidelines. The evolved technique was found to be accurate, precise, linear and reliable. The advantage lies in the simplicity of sample preparation and reproducibility data are satisfactory. The evolved chromatographic method can be effectively applied for regular investigation in drug research.

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CONFLICTS OF INTEREST

Author declares that there have been no conflicts of interest.

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