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DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD AND HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHICMETHOD FOR ESTIMATION OF SOFOSBUVIR IN PHARMACEUTICAL FORMULATIONS

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ABSTRACT

Simple, rapid, precise, economical and specific UV spectrophotometric and high- performance thin-layer chromatographic (HPTLC) methods have been developed for quantitative analysis of Sofosbuvir (SFR) in pharmaceutical formulation. For UV spectrophotometric method, a mixture of methanol and water was used as a solvent. Initial stock solution of SFR was prepared in methanol and subsequent dilutions were done in water. The standard solution of SFR in water showed absorption maxima at 261nm. The drug obeyed Beer–Lambert's law in the concentration range of 10–50 µg/mL with coefficient of correlation (R^2) of 0.999. The recovery was obtained with values close to the 99.41% at three different concentrations, which reflect that the method is free from interference of the impurities and other additives used in tablet formulation. For HPTLC method, aluminum plates precoated with silica gel G60F254 was used as the stationary phase. The solvent system consisted of toluene: chloroform: ethanol in the proportion of 4:4:1, v/v/v. This solvent system was found to give compact spots for Sofosbuvir with *RF*value 0.30 ± 0.01. Densitometric analysis of Sofosbuvir was carried out in the absorbance mode at 261 nm. Linear regression analysis showed good linearity (r^2 =0.998) with respect to peak area in the concentration range of 100–800 ng/spot. The both methods were validated for precision, limit of detection (LOD), limit of quantitation (LOQ), accuracy and specificity. Statistical analysis proved that the methods are repeatable and

specific for the estimation of the said drug.

KEYWORDS: Sofosbuvir, Spectrophotometry, HPTLC, Method Validation.

INTRODUCTION

Sofosbuvir is a new drug candidate for hepatitis C treatment, with the chemical name L•Ala nine, N-[[P(S),2′R]-2′-deoxy-2′-fluoro2′-methyl-P-phenyl-5′-uridylyl]-, 1•methylethyl ester and a molecular formula of C22H29FN3O9P.[1-2] Previously known as PS•7977or GS•7977, It is a nucleotide analogthat is a highly potentinhibitor of the NS5B polymerase in HCV. This drug has shown high efficacy in combination with several other drugs with and without PEG•INF, against HCV Sofosbuvir is of special interest among the directly acting antiviral drugs under development, due to its high potency, low side effects, oral administration, and high barrier to resistance.[3]

The literature review revealed Development of a sensitive UPLC-ESI-MS/MS method for quantification of sofosbuvir and its metabolite, GS-331007, in human plasma Application toa bioequivalence study is carried out and Characterization of forced degradation products and in silico toxicity prediction of Sofosbuvir. Further, no official or draft monograph of Sofosbuvir was published in any of the pharmacopoeia for compendia applications.

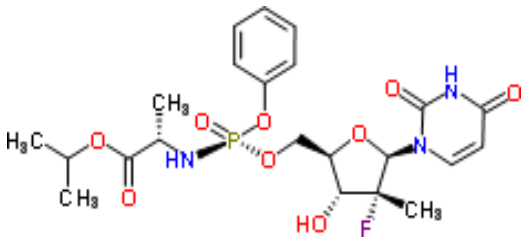

Figure 1: Chemical Structure of Sofosbuvir.

The present work deals with the development of UV spectrophotometric method and high-performance thin-layer chromatographic (HPTLC) method and their validation as per International Conference on Harmonisation (ICH) guidelines.[4-5] The developed method can be adopted in routine analysis of Sofosbuvir in bulk and tablet dosage form and it involves relatively low cost solvents and no complex extraction techniques.

MATERIALS

SFR bulk drug was obtained from Gilead science, methanol (HPLC grade) from Merck Fine Chemicals (Mumbai, India), and Whatman filter paper number 1 from Qualigens Fine

Chemicals (Glaxo, Mumbai, India). The commercially tablets of sofosbuvir are not available in the indian market; hence we have manufactured sofosbuvir immediate release tablets containing 25mg sofosbuvir. Milli-Q water was used throughout the work. Other chemicals used analytical or HPLC-grade and glassware used Class A grade.

METHOD-A: UV SPECTROPHOTOMETRY

Instruments

Shimadzu UV - 1700 UV/VISIBLE spectrophotometer with UV probe 2.10 software and 1 cm matched quartz cells were used for absorbance measurements. Analytical balance used for weighing standard and sample was Make-Mettler Toledo, Model XP 105.

EXPERIMENTAL AND RESULTS

Preparation of standard stock solution

Accurately weighed 50 mg of SFR working standard was transferred into a 50 mL volumetric flask and dissolved in 5 mL of methanol. The volume was made up to 50 mL with water to give the solution containing 1000 µg/mL of SFR.

Selection of Maximum Wavelength for Analysis

The standard stock solution was further diluted with Mili-Q water to get a 10 µg/mL of concentration. The solution was scanned between 200 and 400 nm using water as blank. The UV spectrum of SFR in water had shown λmax, at 261 nm. Hence, it was selected for the analysis of SFR (Figure 2).

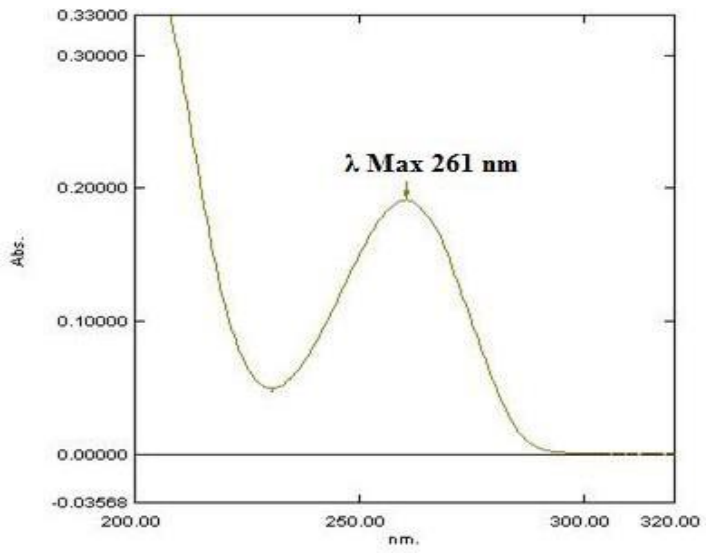

Figure 2: UV spectrum of standard Sofosbuvir (10µg/mL)Preparation of the calibration curve.

Aliquots of standard stock solution were further diluted with Milli-Q water to get the solutions of concentration 10–50 µg/mL. The absorbances were measured at 261nm against Milli-Q water as blank. All measurements were repeated three times for each concentration.

The calibration curve was constructed by plotting mean of absorbance against corresponding concentration (Figure 3).

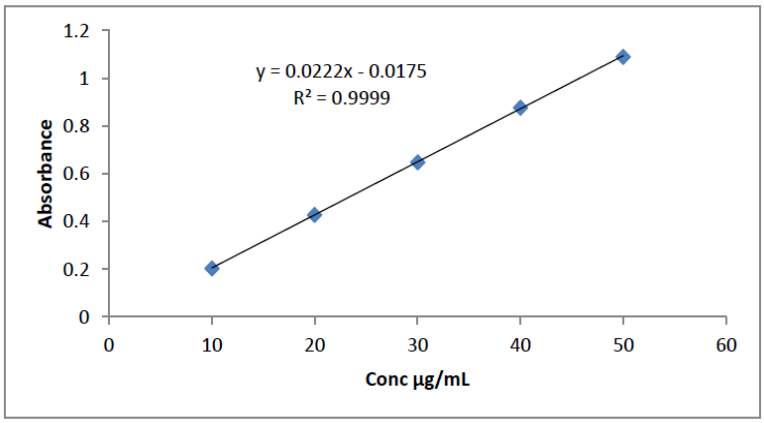

Figure 3: Calibration Plot for Sofosbuvir by UV methodAssay of Sofosbuvir in Tablet.

The tablets of SFR are not available in Indian market; hence tablets manufactured in laboratory were assayed. These were labeled to contain 25 mg of SFR as an active substance per tablet. Twenty tablets were accurately weighed and powdered. The powder equivalent to 50 mg of SFR was weighed and transferred to a 50 mL volumetric flask; 5 mL methanol was added and sonicated for 5 min. to this solution 30 ml water was added and sonicated for 5 min, The volume was adjusted to 50 mL with water. The solution was filtered through Whatman filter paper No. 01. From this filtrate, 1mL was transferred to a 100 mL volumetric flask and diluted with water to 100 mL. The absorbance was measured at 261 nmusing water as blank. This procedure was repeated for six times. The amount of SFR present in formulation was calculated by comparing it with standard absorbance. Content of Sofosbuvir in tablet formulation determined by developed method was in good agreement with the labeled claim. The results obtained are shown in Table1.

Table 1: Assay of Tablet Formulation by UV method.

Labeled claim (mg)	25mg
Amount found* ± SD (mg)	24.80 ± 0.04
% Labeled claim	99.21
% RSD	0.075

*Mean of six determinations

Method Validation[4-5]

The developed method was validated as per ICH guidelines for following parameters.

Linearity

Aliquots of standard stock solution were further diluted with water to get the solutions of concentration within range from 10 to 50µg/mL. The absorbance was measured at wavelength 261 nm. Linear calibration graph was obtained by plotting the absorbance value versus concentration of Sofosbuvir. The drug obeyed Beer–Lambert's law in the concentration range of 10–50 µg/mL with coefficient of correlation (r^2) of 0.999. The results are shown in Table 2.

Table 2: Linear regression data by UV method.

Parameter	Result
Linearityrange	10-50 µg/ml
Regression equation	y = 0.022x - 0.017
Correlation coefficient (r2)	0.999
Slope	0.022
Y-Intercept	- 0.017

Specificity

The spectra obtained from tablet solutions were identical with that obtained from standard solution containing an equivalent concentration of SFR. This showed that there was no any interference from excipients. Therefore, it could be said that developed method is highly selective.

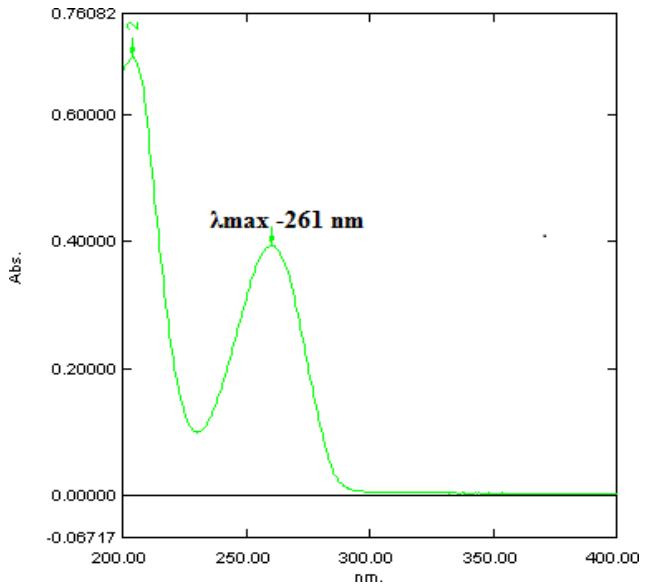

Figure 4: Specificity for Sofosbuvir by UV method.

Recovery studies

To ensure accuracy of the method, recovery studies were performed by standard addition method at 80%, 100%, and 120% level to preanalyzed samples and subsequent solutions were reanalyzed. At each level, three determinations were performed. The absorbances were measured at 261 nm using Milli-Q water as blank and the amount of drug recovered from the formulation were calculated, and the results obtained are shown in Table 3.

Table 3: Result of Recovery studies by UV method.

Level ofaddition (%)	Amount of stddrug added (µg/mL)	Amount recovered* ± SD (µg/mL)	% Recovery	% RSD
80	8	7.94 ± 0.39	99.25	0.052
100	10	9.93 ± 044	99.31	0.47
120	12	11.96 ± 0.48	99.67	0.311

*Mean of three determinations, SD- Standard Deviation

Precision

Precision of the method was determined in terms of repeatability and intraday and interday precisions.

Repeatability

Repeatability of the method was determined by analyzing six samples of same concentrations of drug. The absorbances were measured; the amount of SFR was calculated by comparing it with standard absorbance. The results of this determination are reported in Table 4.

Table 4: Results of repeatability studies of UV method.

Concentration applied (µg/mL)	10
Concentration found* ± SD (µg/mL)	9.73 ± 0.012
% RSD	0.54

*Mean of six determinations

Intraday and Interday precision

The three concentrations of sample solution were analysed in triplicate on same day to determine intraday precision. The procedure was repeated for three consecutive days to determine the interdy precision. The results of analysis are shown in Table 5.

Table 5: Results of Intermediate Precision Studies of UV method.

Concentration Applied (µg/mL)	Intra-day precision		Inter-day precision	
	Concentration found* ± SD(µg/mL)	% RSD	Concentration found* ± SD(µg/mL)	% RSD
10	10.07 ± 0.002	1.13	10.11 ± 0.001	0.68
20	19.28 ± 0.002	0.49	19.31 ± 0.001	0.23
30	29.86 ± 0.002	0.40	29.72 ± 0.001	0.15

*Mean of three determinations

Robustness

To determine the robustness of the method, the experimental conditions were deliberately altered and assay was evaluated. The effect of detection wavelength was studied at ±2 nm. For changes of conditions, the sample was assayed in triplicate. Assay of SFR for deliberate change in detection wavelength was in the range of 99.24 – 99.42%. The results are shownin Table 6.

Table 6: Result of Robustness Studies of UV method.

Wavelength (nm)	% Assay* ± SD	%RSD
259	99.24 % ± 0.002	0.8
263	99.42 % ± 0.003	1.4

*Average of three determination

METHODS-B: HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

HPTLC instrumentation and Chromatographic Conditions

The HPTLC plates were prewashed with methanol and activated at 110°C for 5 min prior to chromatography. The samples were spotted in the form of bands 8 mm width with a Camag 100 microlitre sample syringe (Hamilton, Bonaduz, Switzerland) on silica gel precoated HPTLC aluminum plate G60 F254, [(20 ×10cm) with 250 μm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai] using a Camag Linomat V applicator (Switzer-land). A constant application rate of 0.2μL/s was used and the space between two bands was16 mm. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag , Muttenz , Switzerland) saturated with the mobile phase. The mobile phase was consisted of toluene: chloroform: ethanol (4:4:1,v/v/v) and 20 mL were used per chromatography run. The optimized chamber saturation time for mobile phase was 10 min using saturation pads at room temperature. The length of chromatogram run was 8 cm. Densitometric scanning was performed using a CAMAG TLC operated by

CATS software (V 3.15, Camag). The slit dimension was kept at6 mm × 0.45 mm and the scanning speed was 100 nm/s. The source of radiation used was a deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. All determinations were performed at detection wavelength of 267 nm.

Preparation of Standard Solution

Accurately weighed 100 mg of Sofosbuvir was transferred to a 100 mL volumetric flask and dissolved in and diluted up to the mark with methanol to obtain a standard solution of Sofosbuvir (1000 μg/mL). The aliquot of 10 mL from this solution was diluted to 100 mL with methanol to obtain standard solution of 100µg/mL.

Selection of Analytical Wavelength

The UV absorption spectrum of Sofosbuvir showed maximum absorbane at 267nm so it was selected as detection wavelength (Figure 5).

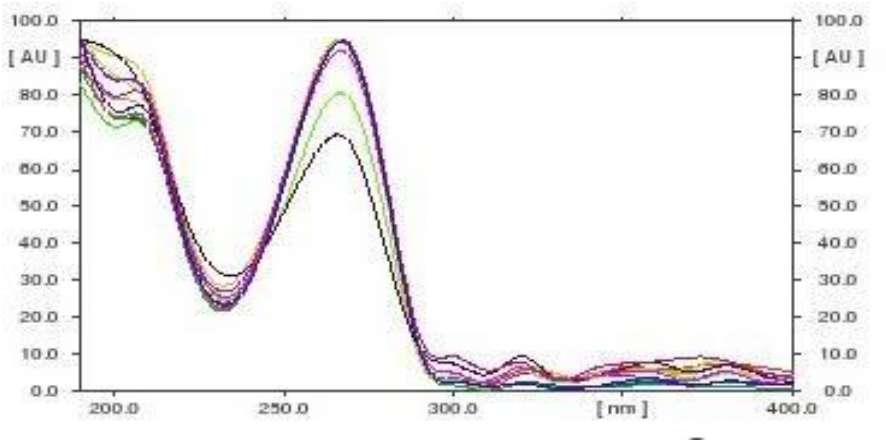

Figure 5: UV spectrum of Sofosbuvir by HPTLC Optimization of the Chromatographic Conditions.

The HPTLC procedure was optimized with a view to develop simple HPTLC method. The pure drug was spotted on HPTLC plates and run in different solvent systems. Initially, toluene: chloroform: ethanol: diethyl amine was tried in different ratio. The optimum mobile phase was found to be consisted of toluene: chloroform: ethanol (4:4:1, v/v/v). The sharp peak was obtained with *Rf* value of 0.29 ± 0.01 (Figure 6). In order to reduce the neck less effect, the TLC chamber was saturated for 10 minute using saturation pads. The mobile phase was run upto distance of 8cm, which takes approximately 20 minute for development of HPTLC plate.

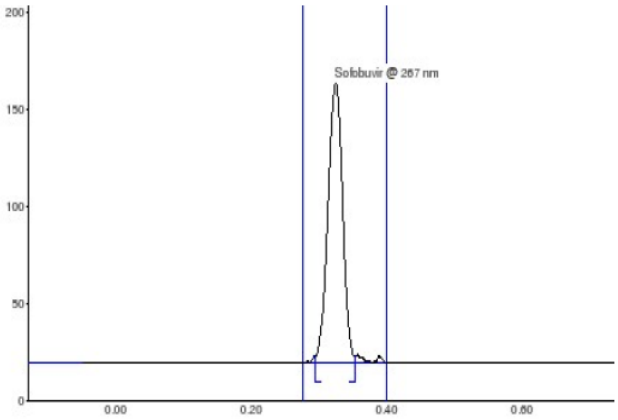

Figure 6: Densitogram of standard Sofosbuvir (*Rf*: 0.29 ± 0.01)Method Validation.

The HPTLC method was validated as per the ICH guidelines.

Linearity

The standard solution was spotted on the HPTLC plate (1µL to 8 µL) to obtain the spots in the concentration range of 100–800 ng/spot. Each concentration was spotted six times on the HPTLC plate. The plate was developed using the previously described mobile phase and scanned. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph. Sofosbuvir showed linear response in the concentration range of 100–800 ng/spot (Figure 7). Linear regression data is shown in table 7.

Table 7: Linear regression data for Sofosbuvir by HPTLC method.

Parameter	Result
Linearity range	100-800 ng/spot
Regression equation	Y = 3.761x + 343.5
Correlation coefficient (r^2)	0.998
Slope	3.761
Y-Intercept	343.5

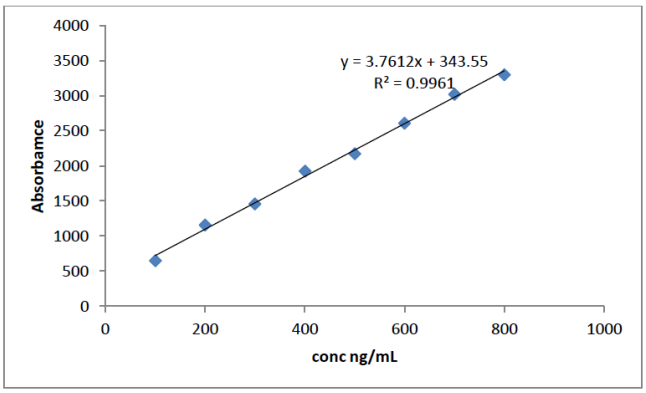

Figure 7: Plot of Concentration versus Peak area of Sofosbuvir by HPTLC Method.

Precision

Precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analyses of the drug (500 ng/spot) in hexaplicate on the same day. The % RSD of six determinations was calculated. Intermediate precision of the method was checked by repeating studies on two different days. The % RSD of twelve determinations was calculated. The results of the repeatability and inter-mediate precision experiments are shown in Table 8. The developed method was found to be precise as the % RSD values for repeatability and intermediate precision studies were less than 2 %.

Table 8: Results of precision studies of Sofosbuvir by HPTLC method.

Concentration applied (ng/spot)	Repeatability		Intermediate precision (Interday)	
	Concentration found* ± SD (ng/spot)	%RSD (n=6)	Concentration found ± SD (ng/spot)	%RSD (n=12)
500	6.23 ± 0.002	0.025	6.42 ± 0.001	0.02

n=number of determinations

Limit of Detection and Limit of Quantitation

The sensitivity of the method was determined in terms of limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were calculated by using the formula, LOD = 3.3 × σ/S and LOQ = 10 × σ/S, where σ is residual standard deviation of regression line and S is slope of corresponding regression line. The LOD and LOQ were found to be136 ng/spot and 408 ng/spot respectively.

Accuracy

Accuracy of the method was determined by standard addition method in which the known amount of standard Sofosbuvir solutions were added to pre-analyzed sample solution. These amounts corresponded to 80, 100, and 120 % of the sample concentration. The amount of Sofosbuvir was estimated by comparing the peak area of sample with that of standard. Accuracy study was performed in triplicate, and % recovery of Sofosbuvir was calculated.

The developed method showed high and consistent recoveries at all studied levels. The results obtained from recovery studies are presented in Table 9.

Table 9: Results of recovery studies of Sofosbuvir by HPTLC method.

Level of Addition	Standard Drug Added (ng/spot)	Drug Recovered* ± SD(ng/spot)	% Recovery	% RSD
80 %	400	398.4 ± 15.4	99.6 %	0.004
100 %	500	496.55 ± 14.8	99.31 %	0.005
120 %	600	595.8 ± 5.4	99.3%	0.015

*Mean of three determinations.

Specificity

Specificity of the method was determined by comparing the chromatogram of sample with the chromatograms of standard. A single peak of Sofosbuvir in tablet solution was observedat Rf 0.29 (Figure 8). No interference of excipients with the Sofosbuvir peak was observed.

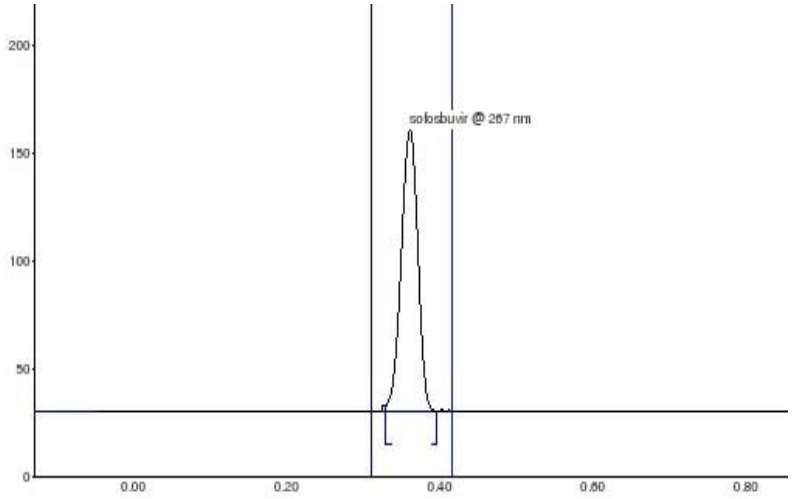

Figure 8: Chromatogram of tablet solution.

The data of summary of validation parameters are listed in Table11.

Table 10: Summary of validation parameter.

Parameters	Results
Linearity	100-800 ng/spot
LOD	136 ng/spot
LOQ	408 ng/spot
Precision	Repeatability: % RSD = 0.025 Intermediate: % RSD =0.02
Recovery	99.3 % to 99.6 %

CONCLUSION

Simple and reliable UV Spectrophotometric and HPTLC methods have been developed and successfully validated for estimation of Sofosbuvir in tablet dosage form. The results of the

validation tests indicated that the developed methods were accurate, precise, robust and reproducible. Hence, the developed methods are suitable for routine determination of Sofosbuvir in pharmaceutical formulation.

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