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EXPLORATION OF HPTLC METHOD FOR ESTIMATION OF NUCLEOTIDE ANALOG INHIBITOR OF HEPATITIS C: SOFOSBUVIR

Bhupendra L. Deore*and Rajendra D. Wagh
Department of Pharmaceutical Quality Assurance, DCS's, A.R.A. College of Pharmacy, Nagaon, Dist: Dhule (MS) India 424 005

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Abstract:
Sofosbuvir was a breakthrough new medication for the treatment of patients with chronic hepatitis C. Sofosbuvir has a number of ideal properties, including once daily dosing, no meal restrictions, few adverse effects, minimal drug-drug interactions, high genetic barrier to resistance, and relatively good safety and efficacy in patients with advanced liver disease. For that purpose, a simple, specific, precise High-Performance Thin-Layer Chromatographic (HPTLC) method for analysis of Sofosbuvir, both as a bulk drug and in tablets was developed and validated. The method employed TLC aluminum plates pre-coated with silica gel 60 F_{254} as the stationary phase. The solvent system consisted of dichloromethane: methanol: ammonia (3:2:0.5 v/v/v). This system was found to give compact spots for Sofosbuvir (R_f value–0.59). Densitometric analysis of Sofosbuvir was carried out in the absorbance mode at 261 nm. The linear regression analysis data for the calibration spots showed good relationship with (regression) r^2 = 0.9992 in the range of 300–1800 ng per band. The LOD and LOQ were 6.71 ng/band and 20.34 ng/band, respectively. The method was validated with respect to linearity, accuracy, precision, robustness and ruggedness. The proposed HPTLC method has been validated according to ICH guidelines and show high sensitivity, accuracy and precision.

Key words: *Sofosbuvir; HPTLC; Method Validation*

Corresponding author:
Bhupendra L. Deore*,
DCS's, A.R.A. College of Pharmacy, Mumbai Agra Highway, Nagaon,
Dist: Dhule (MS) India 424 005
Contact: (02562)261021
Mobile: +918275563822
Email: bhupendradeore@gmail.com

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INTRODUCTION:

Hepatitis C virus (HCV) infection is the main cause of severe liver disease, cirrhosis, and liver cancer. It is estimated that 170 million people are suffering from chronic HCV infection worldwide [1], that of these, 10–20% will develop complications of chronic liver disease and 1–5% will develop liver cancer [2]. Previous standard treatment of chronic HCV included pegylated interferon, ribavirin, and a direct-acting antiviral (DAA) for up to 1 year. This regimen was challenging due to a high pill burden and the associated adverse effects [3]. Recently, treatment options for chronic HCV infection have been significantly improved with the introduction of more potent DAAs, such as the antiviral nucleoside analog [4, 5]. Sofosbuvir (SFS; Figure 1) is isopropyl (2S)-2-[[[(2R, 3R, 4R, 5R)-5-(2, 4 dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran2yl] methoxyphenoxyphosphoryl] amino] propanoate. SFS is a newly approved nucleotide prodrug, which is rapidly activated after intracellular metabolism *via* its conversion to active uridine analog triphosphate (GS-461203). SFS is a direct acting medication used in the treatment of HCV infection by its direct inhibition action on HCV-NS5B RNA-dependent RNA polymerase [6].

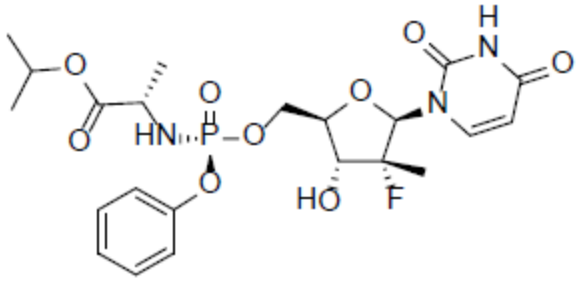

Figure 1. Structure of Sofosbuvir

HPTLC is one of the important chromatographic techniques. Its main advantages are low cost and the possibility of analyzing a large number of samples simultaneously. Thin-layer chromatography (TLC) is a very useful, rapid and inexpensive chromatographic method. It is especially suitable for screening tests, in which pretreatment of the analytes can be avoided, even with dirty samples. The thin-layer format provides a better arrangement for high sample throughput, flexible detection strategies and a greater tolerance of samples with a high-matrix burden. Saving time and money is regarded as the most important features of HPTLC methods; in addition, HPTLC methods use very small volumes of organic solvents, when compared with other analytical methods, so HPTLC not only is an economical analytical method but also has a great advantage as a green-chemistry analytical method. [7] Hence, the aim of this work was to develop an accurate, selective and precise TLC-densitometric method for simultaneous determination of SFS in their dosage form. The developed TLC-densitometric procedure has a lot of advantages such advantages include; accuracy, selectivity, sensitivity.

MATERIALS AND METHODS:

Chemicals and reagents:

Pure standard Sofosbuvir was kindly supplied from Hetero Pharmaceutical Industries Ltd, Andheri(E), Mumbai (Maharashtra), India. Sofovir (400 mg SFS per tablet) were purchased from local pharmacy. All the other reagents and solvents used were of analytical grade.

Selection and optimization of mobile phase:

Initially, different ratio of methanol, chloroform, dichloromethane and toluene were tried but tailing of spots were observed. Finally, the mobile phase consisted of dichloromethane: methanol: ammonia (3:2:0.5 *v/v/v*) gives excellent and sharp symmetric non tailed peak, and several trials and errors were done among the studied mobile phases having good resolution. Few optimization trials for mobile phase are shown in Table 1.

Table 1: Optimization of mobile phase

Sr. no.	Solvents	Proportion (mL)	R_f of SFS
1	n hexane: Methanol	4:1	Tailing
2	n- propanol	5.0	0.90
3	Carbon tetrachloride	5.0	0.25
4	Toluene: n-propanol	3.0: 2.0	0.70
5	Toluene: Methanol	3.5: 1.5	0.75
6	Dichloromethane: methanol	3.0: 2.0	0.57 with tailing
7	Dichloromethane: Methanol: Ammonia	3: 2: 0.5	0.59

Instrumentation and chromatographic conditions:
The TLC plates used throughout the whole procedure are precoated silica gel aluminium plate 60 F_{254} (20cm ×10cm with 0.2mm thickness), supplied by Anchrom technologists, (Mumbai) using a CAMAG Linomat 5 sample applicator (Switzerland). The bands were separated by a distance of 15.4 mm apart and constant application rate of 200 nl/sec was employed. The slit dimension was kept 6 mm x 0.45 mm. The mobile phase consisted of dichloromethane: methanol: ammonia (3:2:0.5 *v/v/v*) was selected which gave sharp and symmetrical peak with R_f 0.59 as shown in Figure 2 Densitometric scanning was performed in the absorbance–reflectance mode at 261 nm using a deuterium lamp emitting a continuous UV spectrum in the range of 400 - 200 nm. The optimized chamber saturation time for mobile phase was 20 min at room temp (25ºC ± 2 ºC) and relative humidity 60% ± 5%. The length of chromatogram run was approximately 8 cm. Each track was scanned three times, and baseline correction was used. The winCATS software version 4.0.5(CAMAG) was used to control the operating parameters during the entire experiment.

Preparation of standard stock solution:
An accurately weighed quantity of 10 mg SFS was transferred to 10 ml volumetric flasks, dissolved in methanol followed by sonication for 10 min and volume was made up to mark with the same solvent. The resulting solution was filtered through Whatmann filter paper (No.1) to obtain a working standard having concentration 1000 ng/µl.

Validation of method:
The method was validated as per the ICH guidelines in terms of its linearity, accuracy, intra-day and inter-day precision, robustness, ruggedness, LOD & LOQ.

Linearity (Calibration curve):
In order to determine the linearity of the proposed analytical method, 6 different volumes of each drug were transferred to a series of 10-mL volumetric flasks and diluted to the mark with methanol, and the general analytical procedure was applied. The proposed analytical method was found to be linear over the range of 300-1800 ng per band for Sofosbuvir aliquots of 0.3, 0.6, 0.9, 1.2, 1.5 and 1.8 µl of Sofosbuvir from standard stock solution was applied on TLC plate with the help of microlitre syringe, using Linomat 5 sample applicator to obtain the concentration of 300, 600, 900, 1200, 1500 and 1800 ng/band. TLC plates were developed under above established conditions. Three dimensional overlay of HPTLC densitograms of standard plot are shown in Figure 3. Area under peak was recorded and plotted against concentration. The calibration curve is as shown in Figure 4.

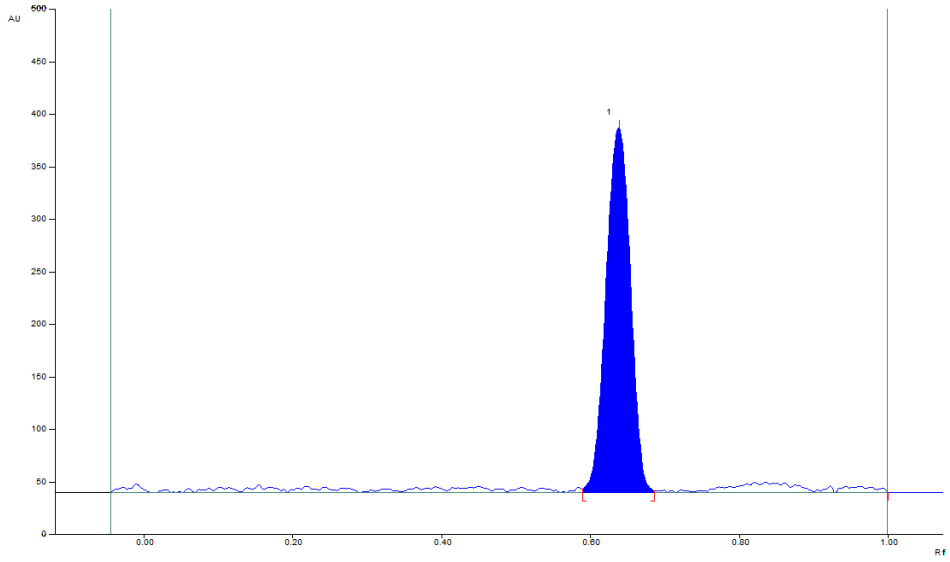

Figure 2. Chromatogram of Standard SFS (R_f: 0.59)

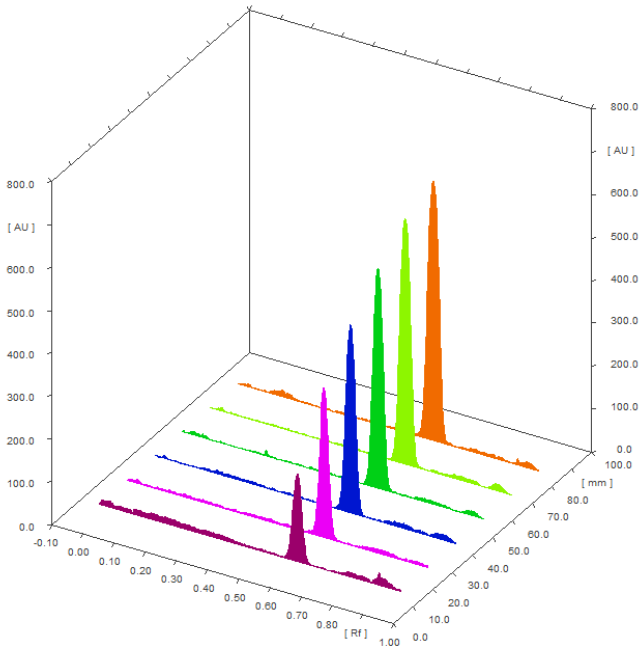

Figure 3. 3D Linearity Graph for SFS

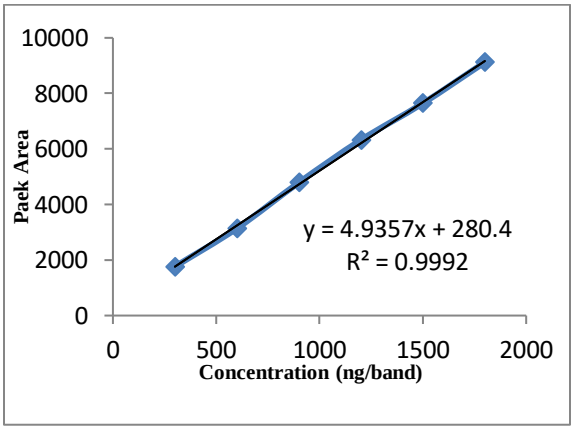

Figure 4. Calibration Curve for SFS

Accuracy:

The pre-analyzed samples were over spotted with extra 80, 100 and 120 % of the Sofosbuvir from tablets on TLC plate. The total concentrations of the drug was determined (n=3), to check for the recovery of the drug at different levels in formulation. The results showed a close agreement between the found concentration and the taken concentration. From the results, the accuracy of the proposed method was established.

Precision:

The precision of the analytical method was assessed by calculation of both intra-day and inter day precisions. The intra-day precision was evaluated on the same day at 3 concentration levels of each drug by spotting 600, 900, 1200 ng/band of SFS on TLC plate.

Robustness:
To check the robustness the method was performed by spotting 900ng of drug on TLC plate by making small deliberate changes in chromatographic conditions like mobile phase composition and the effects on the results were examined. Mobile phases having different composition of dichloromethane: methanol: ammonia (2.8:1.2:0.5 and 3.2:1.8:0.2 v/v/v) were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ±5%. The plates were prewashed by methanol and activated at 60 ± 5°C for 2, 5 and 7 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20 and 40 min.

Ruggedness:
Ruggedness of the method was performed by spotting 900ng of Sofosbuvir by two different analyst keeping same experimental and environmental conditions.

Limit of detection (LOD) and limit of quantification (LOQ):
For HPTLC, the LODs and LOQs were experimentally estimated by analyzing different concentration of Sofosbuvir solutions of 300, 350, 400, 450, 500 and 550 ng/µl were prepared and applied in triplicate. LOD and LOQ were calculated using the formulas LOD = 3.3S/b and LOQ = 10S/b, where S = SD of blank determinations and b = slope of the calibration curve. The tabulated values showed that the methods were sufficiently sensitive.

Results and Discussion:

HPTLC method development:
Selection of suitable mobile phase is carried out by controlled trials and errors. These trials failed to resolve the mixture with suitable R_f values. Different ratios of this mobile phase were tried until sharp, symmetric, and untailed peaks were obtained, which results from using mobile phase consisting with dichloromethane: methanol: ammonia with the ratio of 3:2:0.5 (v/v/v) having R_f value of 0.59 ± 0.2. The densitometric scan of the HPTLC plates was tested at different wavelengths, and among the tested wavelengths, it was found that scanning plates at 261 nm gave the best sensitivity for the studied drugs, so the densitometric scanner was set at 261 nm for quantitative analysis using a deuterium lamp as the source of radiation. It was found that activation of the TLC plates for 15 minutes before spotting of the sample leads to excellent improvement in the good reproducibility and peak sharpness.

Method Validation:
Linearity:
Six concentration levels of SFS were selected to construct the calibration curves. Linearity of the proposed method was evaluated and found to be in the concentration range of 300-1800ng/band for SFS. The regression plots were found to be linear over the mentioned range, the linear regression equation was found to be $y = 4.9357x + 280.4$ ($r^2 = 0.9992$).

Accuracy:
SFS reference standards were accurately weighed and added to a commercial formulation of tablet powder, at three different concentration levels 80, 100 and 120 % for SFS. At each level, samples were prepared in triplicate, and the recovery percentage was determined. Accuracy as percent recovery was calculated, the calculated values confirm afforded accuracy as shown in table 2.

Table 2: Results of recovery studies

Label claim (mg/tab)	Amount of standard drug added (%)	% Drug recovery*	% R.S.D.
400	80	99.94	0.57
	100	99.65	0.53
	120	99.76	0.41

*mean of three estimations at each level

Precision:
The precision of the developed HPTLC method was expressed in terms of % relative standard deviation (% RSD). The results depicted revealed high precision of the method is presented in Table 3.

Table 3: Intra-day and Inter-day Precision

Conc. (ng/µl)	Intra-day		Inter-day	
	% Amount found*	% R.S.D.	% Amount found*	% R.S. D
600	99.86	0.33	99.73	0.23
900	99.70	0.12	99.58	0.15
1200	99.98	0.22	100.15	0.21

*mean of three estimations at each level

Robustness:
The effect of small deliberate variations on the method parameters was evaluated. The standard deviation of peak areas was calculated for each parameter and % R.S.D. was found to be less than 2 %. The results are shown in Table 4.

Table 4: Robustness of the method

Parameters	S.D. of peak area	% R.S.D.*
Mobile phase composition	46.65	0.87
Mobile phase volume	39.43	0.98
Development distance	37.48	0.79
Duration of saturation	31.82	0.83

*mean of three estimations at each level

Ruggedness:
Ruggedness of the method was performed by applying 900 ng by two different analyst keeping same experimental and environmental conditions. The results summarized in Table 5.

Table 5: Results of Ruggedness study

Analyst	%Amount found of SFS (Mean ± S.D.)	%R.S.D.*
I	99.54 ± 0.43	0.43
II	99.65 ± 0.45	0.45

*mean of three estimations at each level

LOD and LOQ:
Detection limit and quantification limit for Sofosbuvir was found to be 6.71ng and 20.34ng respectively. This indicates adequate sensitivity of the method.

CONCLUSION:

The proposed method has advantages of being a very simple, rapid, accurate, and precise tool for the routine analysis of Sofosbuvir. Our proposed method has high sensitivity and can analyze the drugs under study in nanograms per band. No special pretreatment or extraction is required for the drugs in bulk powders or in their pharmaceutical dosage forms, indicating a simple technique. The proposed method is economical and rapid as it does not depend on expensive or critical reagents or expensive instrumentation. The mentioned advantages make it more preferable to be applied in the routine analysis of the studied drugs in quality control and research laboratories

Abbreviations:

Sofosbuvir (SFS), Thin layer chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), Limit of Detection (LOD), Limit of Quantification (LOD)

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