Development of a High-Performance Thin-Layer Chromatographic Method for the Simultaneous Determination of Newly Co-formulated Antiviral Drugs Sofosbuvir and Velpatasvir in Their Pure Forms and Tablet Dosage Form

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Key Words:

High-performance thin-layer chromatography
Sofosbuvir
Velpatasvir
Hepatitis C

Summary

The discovery of new direct-acting antiviral drugs gave rise to a leap forward in the treatment of hepatitis C viral infections. For the first time since 1998, the Food and Drug Administration (FDA) approved interferon-free oral treatment paradigms. Among the new treatment regimens, the combinations of sofosbuvir/daclatasvir and sofosbuvir/velpatasvir became ideal treatment regimens for being potent, highly tolerated, and used once daily. For that purpose, a new sensitive high-performance thin-layer chromatography (HPTLC) method was developed and validated for the simultaneous determination of sofosbuvir and velpatasvir. The proposed method used Merck HPTLC silica gel 60 F_{254} aluminum plates as the stationary phase. The mobile phase was a combination of methylene chloride–methanol–ethyl acetate–ammonia (25%) at a ratio of 5:1:3:1 (*v*/*v*). The calibration curves were linear over a wide range of 100–2000 ng per spot for the studied drugs. The limits of detection were 30.02 and 27.60 ng per spot, and the limits of quantification were 90.06 and 82.80 ng per spot for sofosbuvir and velpatasvir, respectively. The suggested method was successfully applied for analysis of the studied drugs in their pharmaceutical dosage forms, and excellent recovery results were obtained. Being simple, fast, robust, and economic, this method is eligible for use in the routine work in pharmaceutical quality control laboratories.

1 Introduction

Chronic hepatitis C virus (HCV) infected more than 170 million people worldwide, which leads to a yearly death of more than 350,000 people with liver diseases caused by this infection [1]. HCV is a worldwide epidemic infection, and its regional prevalence data are about 11% in Europe, 52.2% in Asia, 17.5% in Africa, 10.2% in middle east, 8.9% in Americas, and 0.3% in Australia [2]. China, India, and Egypt were the highest countries with the epidemic HCV infection. About 14.1% of Egypt's populations suffer from liver diseases caused by HCV infection; this percent represents about 12-million Egyptians [3]. The previous data give us information about the marketing size of the new anti-viral drugs used in the treatment of this epidemic infection.

Epclusa® is a co-formulated antiviral drug containing velpatasvir and sofosbuvir which was first approved in June 2016. Epclusa was the first HCV therapy proven to effectively treat all the 6 main HCV genotypes [4].

Sofosbuvir (SFS; **Figure 1**) is 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. 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 [5].

Many analytical methods were reported for analyses of SFS whether alone or in a combination with other new antiviral drugs. These methods include spectrophotometry [6], high-performance liquid chromatography (HPLC) connected to a UV detector [6–9], HPLC with a mass spectrophotometer [10, 11], ultra-performance liquid chromatography (UPLC) connected to a mass detector [12], high-performance thin-layer chromatography (HPTLC) [13, 14], HPLC with a diode array detector [15], and chemometry [16].

Velpatasvir (VLP; Figure 1) is methyl((S)-1-((S)-2-(5-(6-(2-((S)-1-((methoxycarbonyl)-L-valyl)pyrrolidin-2-yl)-1H-imidazol-4-yl)naphthalen-2-yl)-1H-benzo[d]imidazol-2-yl)pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-yl)carbamate. VLP is a direct-acting antiviral drug which has been recently approved by the Food and Drug Administration (FDA) in 2016, as part of a combination therapy with SFS for the treatment of HCV infections. VLP has direct strong antiviral activity, as it has an

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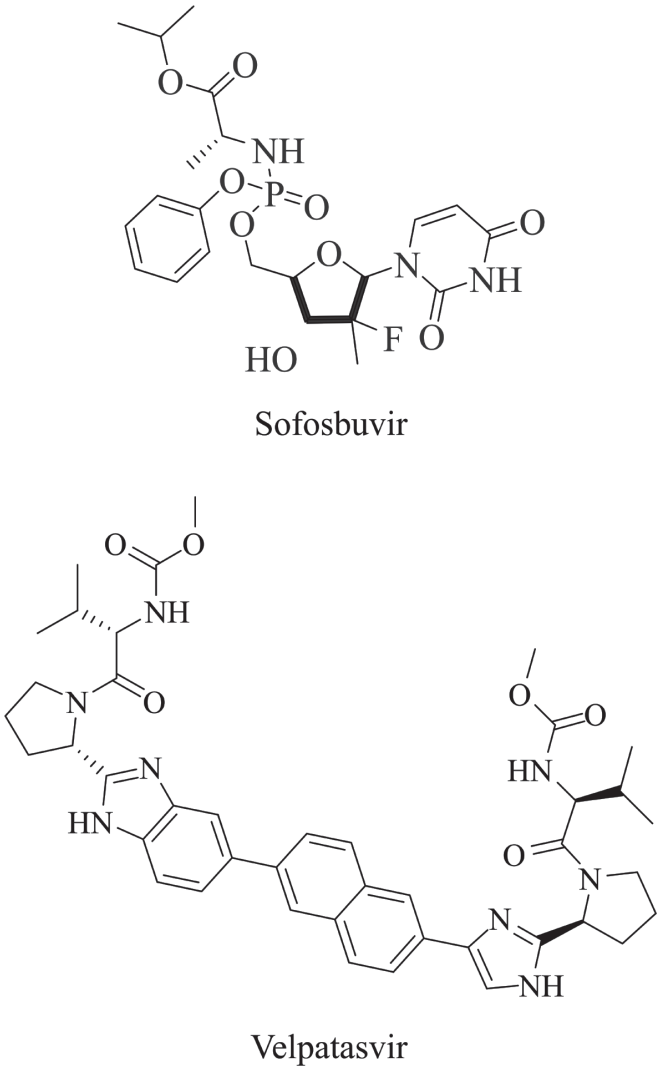

Figure 1

The chemical structures of the studied drugs.

inhibitory result on nonstructural protein 5A (NS5A), which is an essential protein for normal HCV replication complex [5].

VLP is now available on the market in combination with SFS in a fixed dosage form, Epclusa®. Only a few analytical methods were reported for determinations of VLP in combination with SFS using HPLC attached to a UV detector [7–9]. To the best of our knowledge, to date, no HPTLC method has been reported for the determination of a co-formulated mixture of SFS and VLP. Thus, the need to develop a sensitive, rapid, and valid HPTLC method for the analysis of SFS and VLP with accepted R_F values and small LOQ motivated us to develop the proposed method. HPTLC was used as an analytical method for the determination of many pharmaceutical drugs mixtures [17, 18] and also as a stability-indicating method [19, 20]. 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.

2 Experimental

2.1 Instrumentation

A CAMAG (Muttenz, Switzerland) Linomat HPTLC system was attached to a semi-automatic sample application system and equipped with one dosing syringe 100 µL. All sample spottings were done under a nitrogen stream.

A CAMAG TLC Scanner 3 was used for densitometric scanning operated by HPTLC software version, CATS Basic (version 1.4.4.6337). The system is equipped with a halogen lamp and a deuterium lamp as radiation sources for drug scanning.

Merck HPTLC aluminum sheets pre-coated with silica gel 60 F_{254} (20 cm × 20 cm with 250-µm thickness) were purchased from Merck (Darmstadt, Germany).

2.2 Materials and Reagents

SFS (as dihydrochloride salt) with 99.25% purity was kindly supplied by Amoun Pharmaceutical Company (El Obour City, Cairo, Egypt). VLP with 99.7% purity was kindly supplied by EIPICO Pharmaceutical Company (Tenth of Ramadan City, Egypt).

Ammonia (25%), ethyl acetate, methanol, and methylene chloride were of analytical grade and were purchased from El Nasr Chemical Co. (Cairo, Egypt).

Epclusa® tablets (400 mg SFS and 100 mg VLP/tablet, batch No. UHFDD) produced by Gilead Sciences, Inc. (Foster City, CA, USA) were purchased from a local pharmacy in the Egyptian market.

2.3 Chromatographic Conditions

The stationary phase used throughout the whole method was Merck aluminum plates 60 F_{254} (20 cm × 20 cm with a 250-µm thickness), and each plate was divided into smaller plates of 20 cm × 10 cm diameter before use. The mobile phase consisted of methylene chloride–methanol–ethyl acetate–ammonia (25%) (5:1:3:1; *v*/*v*). All HPTLC plates were activated at 60°C for 10 min directly before sample application. Samples were applied as bands of 3 mm long and 5 mm intervals under a nitrogen stream. Linear ascending chromatogram development to a distance of 9 cm was performed in a 20 cm × 20 cm twin-trough tightly closed TLC jar (CAMAG) at room temperature, and before chromatogram development, the chamber was saturated by the mobile phase for 30 min; the elution time was about 7 min. The TLC plates were dried well after samples application in a current of air using air drier. The plates were scanned by using a CAMAG TLC Scanner 3 at 275 nm at the absorbance mode using a deuterium lamp as a radiation source.

2.4 Standard Solutions

Standard solutions of SFS and VLP were prepared by careful transferring exactly weighed SFS and VLP powder equivalent to 10 mg SFS and VLP to two different 10-mL volumetric flasks, and the powder was dissolved well in about 3 mL methanol; then the volume was completed to the mark with methanol to give final stock standard solutions with concentration 100 µg mL^{-1} of SFS and VLP. The standard solutions were stored in a refrigerator (0 to −8°C) and allowed to take room temperature each time before spotting to the plates.

2.5 Calibration Curves

Different volumes of the standard stock solutions range from 1 to 20 µL for SFS and VLP were spotted on the TLC plates, to give final spot concentrations ranges from 100 ng per spot to 2000 ng per spot for SFS and VLP. The calibration curves were obtained by using 6 different points with 6 different concentrations. The calibration curves were obtained by plotting area under the peak against the corresponding concentration of each drug.

2.6 Analytical Solution Stability

In order to avoid any unexpected changes in the stock solutions during analysis due to delay in the analysis, we must have detailed information about the stability of the prepared stock solutions. It was found that methanolic solutions of SFS and VLP were stable for at least 48 h at room temperature when protected from light and for 7 days when stored refrigerated at 5ºC, as it showed no chromatographic or absorbance changes.

2.7 Procedure for Pharmaceutical Dosage Forms

Ten Epclusa® tablets were weighed accurately, finely powdered, and mixed thoroughly. An accurate amount equivalent to the content of 1 tablet (400 mg SFS and 100 mg VLP) was accurately weighed and carefully transferred to a 100-mL volumetric flask and extracted three times with 25 mL methanol. The contents of the flask were swirled, sonicated for 5 min each time, and filtered to a 100-mL volumetric flask, and then the volume was completed to 100-mL with methanol, to obtain a final solution with concentration (4 mg mL^{-1} SFS and 1 mg mL^{-1} VLP). The obtained solution was spotted to the TLC plates, in different volumes (0.1, 0.2, 0.3, 0.4, and 0.5 µL) to obtain a final concentration within the calibration range. The general analytical procedure was repeated with tablet extraction. Each concentration was repeated five times, and the data represented as %Recovery ± SD.

3 Results and Discussion

3.1 High-Performance Thin-Layer Chromatography

The proposed analytical method can successfully separate the studied drugs with high accuracy and sensitivity **(Figure 2).** Selection of the optimum mobile phase gives excellent separation between the studied drugs with a sharp symmetric non-tailed peak, and several trials and errors were done among the studied mobile phases, chloroform–ethyl acetate–water, acetonitrile–methanol–triethylamine, chloroform–water–ethanol, and methylene chloride–ethyl acetate–methanol in different ratios. It was found that all tried mobile phases resulted in imperfect separation of the studied drugs with broad band, except for the mobile phase consisting of methylene chloride–ethyl acetate–methanol which can separate the studied drugs but with tailed separated peaks. Thus, an amount of ammonia (25%) was added to the mobile phase system to avoid tailing. Different ratios of this mobile phase were tried until sharp, symmetric, and untailed peaks were obtained, which results from using mobile phase consisting of methylene chloride–methanol–ethyl acetate–ammonia (25%) with a ratio of 5:1:3:1 (*v*/*v*). The R_F values were 0.28 ± 0.01 and 0.84 ± 0.02 for SFS and VLP, respectively. The densitometric scan of the HPTLC plates was tested at different wavelengths, and among the tested wavelengths, namely, 210 nm, 275 nm, and 300 nm, it was found that scanning plates at 275 nm gave the best sensitivity for the studied drugs, so the densitometric scanner was set at 275 nm for quantitative analysis using a deuterium lamp as the source of radiation. It was found that activation of the TLC plates for 10 min at 60°C before spotting of the sample leads to excellent improvement in the peak sharpness.

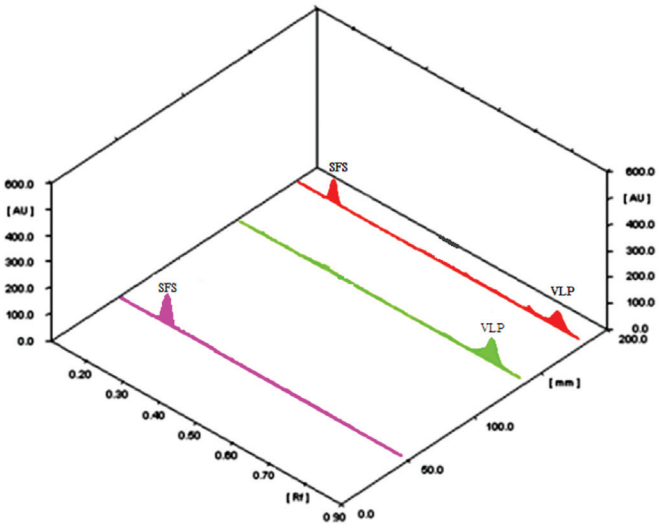

Figure 2

Atypical 3D (HPTLC) chromatogram of a synthetic mixture of SFS (100 ng per spot) and VLP (100 ng per spot).

3.2 Validation of the Proposed Analytical Method

The proposed analytical method was implemented for validation according to the International Council for Harmonization (ICH) guidelines [21] regarding linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), selectivity, and robustness.

3.2.1 Linearity and Range

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 (3 replicates for each concentration). The proposed analytical method was found to be linear over the range of 100–2000 ng per spot for both SFS and VLP.

Table 1

Analytical parameters for the analysis of SFS and VLP by the proposed HPTLC method.

Parameter	SFS	VLP
Concentration range [ng per spot]	100–2000	100–2000
Correlation coefficient (r)	0.9998	0.9998
Determination coefficient (r^2)	0.9996	0.9997
Slope	1.76	2.79
Intercept	868.64	235.10
Linearity equation	$y = 1.7618x + 668.64$	$y = 2.7862x + 235.1$
SD of the intercept (Sa)	16.01	23.25
SD of slope (Sb)	0.02	0.02
RSD of the slope [%]	1.14	0.72
LOD [ng mL^{-1}]	30.02	27.60
LOQ [ng mL^{-1}]	90.06	82.80

LOD: limit of detection; LOQ: limit of quantitation

The calibration curves of the analyzed drugs were obtained by plotting the peak area against its corresponding concentration.

The statistical treatment of the data was carried out using linear regression analysis, and the analytical parameters were calculated **(Table 1).** The correlation coefficients (r) for the analyzed drugs were 0.9998 for both SFS and VLP.

3.2.2 Accuracy and Precision

The accuracy of the proposed analytical method was assessed at 5 concentrations within the specific analytical range of the SFS and VLP. Each concentration was replicated three times. The mean of the 3 measurements was calculated as the found concentration. The %Recovery ± standard deviation was calculated for each concentration **(Table 2).** 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.

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 (3 replicates for each concentration), and the results represents the method's repeatability. The inter-day precision was evaluated at 3 different days using three concentration levels of each drug (3 replicates for each concentration), and the obtained result represents the method's intermediate precision. The obtained results of both intra-day and inter-day precision are plotted in **Table 3,** indicating high precision of the analytical method.

3.2.3 Selectivity

The selectivity of the method was determined by analyzing the studied drugs in different laboratory-prepared mixtures of the studied drugs within the analytical concentration range. Excellent results were obtained, presented in **Table 4,** indicating the high selectivity of the analytical method.

3.2.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

In order to determine the limit of detection and limit of quantification, both the standard deviation of response and the slope of the calibration curve were used, using the equations: $LOD = 3.3\sigma / S$ and $LOQ = 10\sigma / S$, where σ is the standard deviation of intercept and S is the slope of the calibration curve;

Table 2

Evaluation of the accuracy of the proposed HPTLC procedure for the determination of SFS and VLP at 5 concentration levels within the specified range.

Sample number	Taken [ng per spot]	SFS		VLP	
		Found [ng per spot]	%Recovery[a)] ± SD	Found [ng per spot]	%Recovery[a)] ± SD
1	100	100.0	100.0 ± 0.4	99.2	99.2 ± 0.7
2	150	150.6	100.4 ± 0.2	151.3	100.9 ± 0.3
3	250	249.0	99.6 ± 0.9	251.0	100.4 ± 0.7
4	400	398.0	99.5 ± 1.1	402.5	100.6 ± 0.15
5	500	502.5	100.5 ± 0.5	497.7	99.5 ± 1.2
Mean		100.00		100.12	
SD		0.45		0.73	
RSD		0.45		0.73	
RE		0.55		0.27	

[a)]Mean of 3 replicate measurements
SD: standard deviation; RSD: relative standard deviation; RE: relative error

Table 3

Evaluation of the intra-day and inter-day precisions of the proposed HPTLC method for the determination of SFS and VLP in the pure form.

Precision level	Conc. [ng per spot]	SFS			VLP		
		%Recovery[a)]	± SD	RSD	%Recovery[a)]	± SD	RSD
Intra-day	100	100.1	± 0.30	0.30	99.7	± 0.21	0.21
	250	100.5	± 0.70	0.70	99.6	± 0.38	0.38
	500	99.8	± 0.22	0.22	100.3	± 0.50	0.50
Inter-day	100	100.0	± 0.40	0.40	100.3	± 0.55	0.55
	250	100.6	± 0.87	0.87	99.7	± 0.25	0.25
	500	99.9	± 1.29	1.29	99.9	± 0.36	0.36

[a)]Mean of 3 replicate measurement
SD: standard deviation; RSD: relative standard deviation

Table 4

Determination of SFS and VLP in laboratory prepared mixtures using the proposed HPTLC method.

Mix no	Conc. [ng per spot]		%Recovery[a)] ± SD	
	SFS	VLP	SFS	VLP
1	150	150	100.1 ± 0.25	100.1 ± 0.15
2	200	200	99.7 ± 0.54	99.6 ± 1.25
3	600	150	99.6 ± 0.76	99.6 ± 0.95
4	800	200	99.5 ± 1.15	99.5 ± 0.45
5	250	500	100.1 ± 0.90	99.9 ± 0.70
6	500	200	99.9 ± 0.20	100.3 ± 0.64
7	1000	750	99.5 ± 0.32	100.4 ± 0.24
8	1250	500	100.4 ± 0.45	99.3 ± 0.38
	Mean		99.85	99.84
	SD		0.33	0.40

[a)]Mean of 3 replicate measurements
SD: standard deviation

the obtained results are summarized in Table 1. The limits of detection were 30.02 and 27.60 ng per spot, and the limits of quantitation were 90.06 and 82.80 for SFS and VLP, which indicates the high sensitivity of the proposed analytical method when compared with other analytical methods.

3.2.5 Robustness

To check the robustness of the method, different mobile phases with different ratios of composition were used; it was found that a change in the ratio of the mobile phase leads to a change in the R_F value of the separated drugs, but with an insignificant change in the percent of recovery **(Table 5).**

3.3 Application to Pharmaceutical Dosage Forms

The proposed analytical method was successfully applied for the determination of the studied drugs in its pharmaceutical dosage forms **(Figure 3).** The selectivity of the method was studied by observing any effect resulted from tablet excipients. The results showed no interference results from tablet excipients with the accuracy of the proposed method. Each point was repeated five times, and the data were represented as %Recovery ± SD. The obtained results were compared with those obtained from reported methods [7] using the Student's *t*-test and *F*-test with respect to accuracy and precision. From the obtained results presented in **Table 6,** it is clear that there is no significant difference

Table 5

Robustness study of the proposed HPTLC method for the determination of SFS and VLP (100 and 250 ng per spot) in the pure form.

Variation Effect of mobile phase composition	Conc.(ng per spot)	SFS	VLP
		%Recovery[a)] ± SD	
Methylene chloride–methanol–ethyl acetate–ammonia (25%) (5.5:1:2.5:1, *v/v*)	100	99.70 ± 0.30	98.03 ± 1.19
	250	99.13 ± 0.45	99.34 ± 0.48
Methylene chloride–methanol–ethyl acetate–ammonia (25%) (4.5:1:3.5:1, *v/v*)	100	99.00 ± 0.72	99.95 ± 0.74
	250	98.30 ± 0.98	98.99 ± 1.02

[a)]Mean of 3 replicate measurements
SD: standard deviation

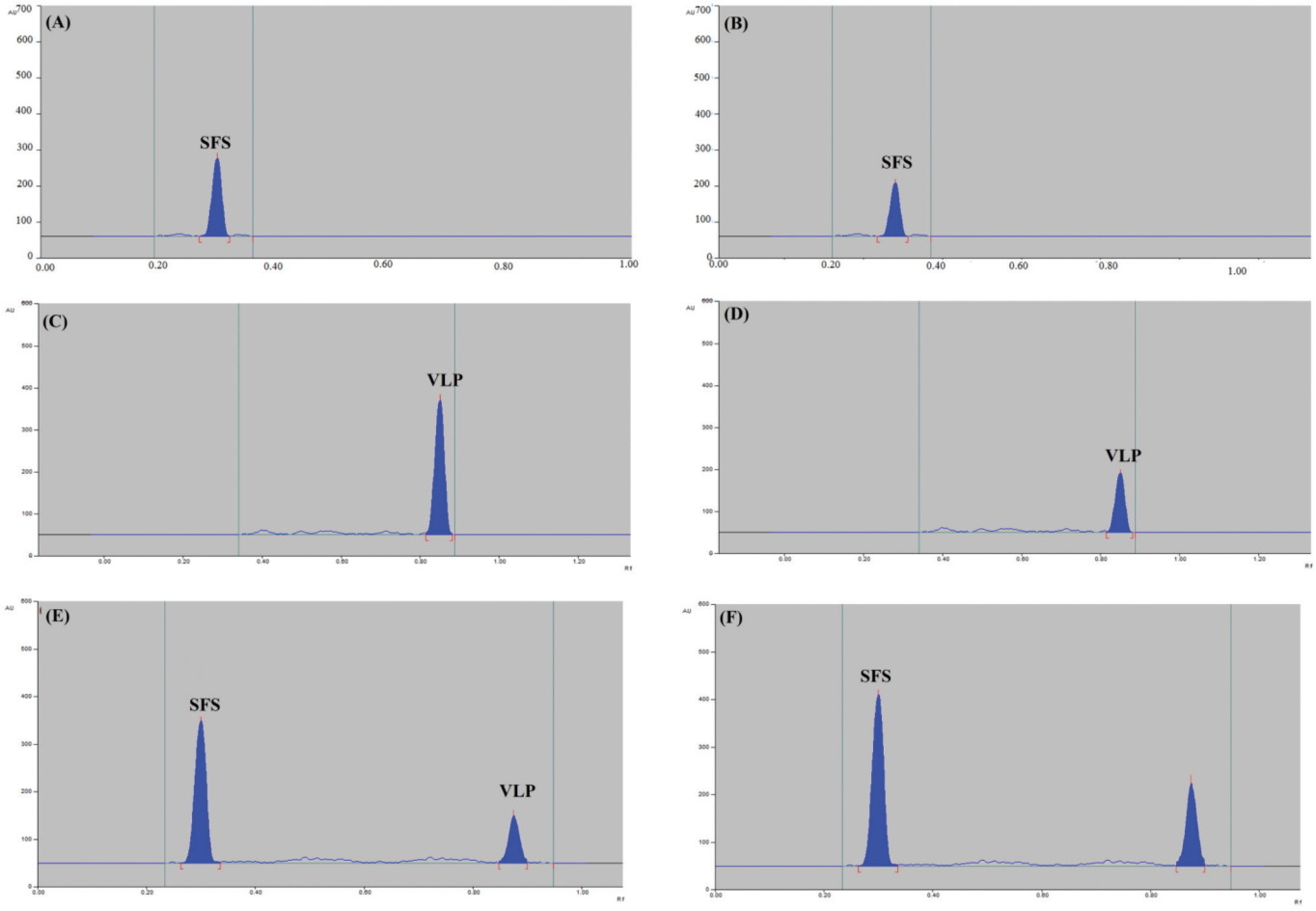

Figure 3

Atypical 2D chromatogram of (A) SFS (250 ng per spot), (B) SFS (100 ng per spot), (C) VLP (250 ng per spot), (D) VLP (100 ng per spot), (E) Epclusa® tablet (SFS 400 ng and VLP 100 ng), and (F) Epclusa® tablet (SFS 600 ng and VLP 200 ng).

Table 6

Comparison between the proposed HPTLC and reported methods for the determination of SFS and VLP in its pharmaceutical dosage forms.

Dosage form		%Recovery[a] ± SD		*t*-Value[b]	*F*-value[b]
		Proposed	Reported[c]		
Epclusa® tablet	400 mg of SFS per tablet	99.97 ± 0.27	99.75 ± 0.45	1.21	1.59
	100 mg VLP per tablet	100.03 ± 0.37	99.70 ± 0.30	1.72	1.46

[a]The values are the mean of 5 determinations
[b]The tabulated *t*- and *F*-values at 95% confidence limit are 2.78 and 6.39, respectively
[c]Reported methods [6]

between the results obtained from both methods, as indicated by the Student's *t*-test and *F*-test, as the calculated values did not exceed the theoretical values at 95% confidence level. This indicates high accuracy and precision of the proposed method.

4 Conclusion

The proposed method has advantages of being a very simple, rapid, accurate, and precise tool for the routine analysis of sofosbuvir and velpatasvir. Our proposed method has high sensitivity and can analyze the drugs under study in nanograms per spot. 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. Moreover, due to the very low concentration of the used organic solvents, our proposed method is considered environment-friendly. The mentioned advantages make it more preferable to be applied in the routine analysis of the studied drugs in quality control and research laboratories than the used HPLC methods.

Acknowledgment

The authors are thankful for EIPICO and Amoun Pharmaceutical Company for the continued support and for supplying authentic powders during the study.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Ms received: October 2, 2018
Accepted: January 1, 2019