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THE NOVEL USE OF PENTABROMOBENZYL COLUMN FOR SIMULTANEOUS DETERMINATION OF THREE ANTIVIRAL DRUGS, SOFOSBUVIR WITH DACLATASVIR OR LEDIPASVIR IN THEIR TABLET DOSAGE FORM AND SPIKED HUMAN PLASMA

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ABSTRACT

New sensitive, selective and specific reversed phase high performance liquid chromatographic methods for the simultaneous determination of two antiviral mixtures, sofosbuvir (SOF) with ledipasvir (LED) or with daclatasvir (DAC) has been developed. The proposed methods were optimized and validated on Pentabromobenzyl (PBr) analytical column (5 µm; 4.6 mm × 150 mm). The analysis of the studied drug combinations was determined within 8 min for SOF and LED mixture and 5 min for SOF and DAC mixture, using photodiode array detector (PDA). The mobile phase consists of 30 mM phosphate buffer pH (3.0), methanol and acetonitrile (20:20:60, v/v) for SOF and LED mixture and 40 mM phosphate buffer (pH 3.6) and acetonitrile (50:50, v/v) for SOF and DAC mixture. A linear response was observed for all the studied compounds in the range of the concentration studied with mean percentage recoveries of 100.16% ± 1.48 and 100.07% ± 1.82 for SOF and LED, respectively and 100.05 % ± 1.38 and 100.81% ± 1.36 for SOF and DAC, respectively. The proposed methods were successfully applied for the determination of these drugs in their pharmaceutical dosage forms and in human plasma samples.

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INTRODUCTION

About 150-200 million people are infected with chronic hepatitis C virus (HCV). Moreover, nearly 3-4 million people are infected per year, and more than 350,000 people die from hepatitis C – related diseases every year. It was found that Egypt has the highest HCV prevalence in the world (14.7%). Thus, HCV infection and its complications are considered the most important public health challenges in Egypt today leading to serious liver problems, including cirrhosis or liver cancer Salama, et al.[1]. Evolution of a new antiviral generation to avoid the side effects of Interferon/ ribavirin therapy remains a high priority. The virus can be eradicated from most chronic hepatitis C patients with short courses of Direct acting antivirals (DAA) combination therapy, generally ranging from 8 to 24 weeks [2]. The development of DAA has made momentous changes in HCV therapy. These drugs reduce hepatitis C virus amount in the body preventing the virus from multiplying within the body [3].

Sofosbuvir (SOF) (Figure 1A)(propan-2-yl (2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl]methoxy-phenoxyphosphoryl]amino)propanoate is an oral nucleotide analogue inhibitor of the HCV-specific NS5B polymerase with in vitro activity against all HCV genotype[4]. SOF is taken orally once daily, and has a good safety profile [5].It has nearly no resistance till now , and limited drug–drug interactions[6].

Ledipasvir (LED), (Figure 1B), is methyl *N*-[(2*S*)-1-[(6*S*)-6-[5-[9,9-Difluoro-7-[2- [(1*S*,2*S*,4*R*)-3-[(2*S*)-2-(methoxycarbonylamino)-3-methylbutanoyl]- 3-azabicyclo[2.2.1]heptan-2-yl]-3*H*-benzimidazol-5-yl] fluoren-2-yl]-1*H*-imidazol-2-yl]-5-azaspiro[2.4]heptan-5-yl]- 3-methyl-1-oxobutan-2-yl] carbamate according to the IUPAC nomenclature [7].

Daclatasvir (DAC) (Figure 1C), is Methyl [(2*S*)-1-{(2*S*)-2-[4-(4'-{2-[(2*S*)-1-{(2*S*)-2-[(methoxycarbonyl)amino]-3-methylbutanoyl}-2-pyrrolidinyl]-1*H*-imidazol-4-yl}-4-biphenylyl)-1*H*-imidazol-2-yl]-1-pyrrolidinyl}-3-methyl-1-oxo-2-butanyl] carbamate; dihydrochloride. Both LED and DAC are powerful inhibitors of HCV NS5A which is a phosphoprotein essential in viral replication via blocking two distinct stages of the viral lifecycle, namely viral RNA synthesis and virion assembly secretion [7-8].

SOF and LED were co-formulated as a one-drug therapy, for the treatment of chronic (HCV) genotype 1, 4, 5 and 6 infection [7]. This combination allows high cure rates in people infected with genotype 1 (the most common subtype in the U.S., Japan, and much of Europe) without interferon. Due to safety profile, they substituted the injectable drug, interferon, that was the main treatment of HCV. SOF and DAC combination provides a high rate of Sustained viral response in difficult-to-treat patients infected with genotype 1 or 4 [9].

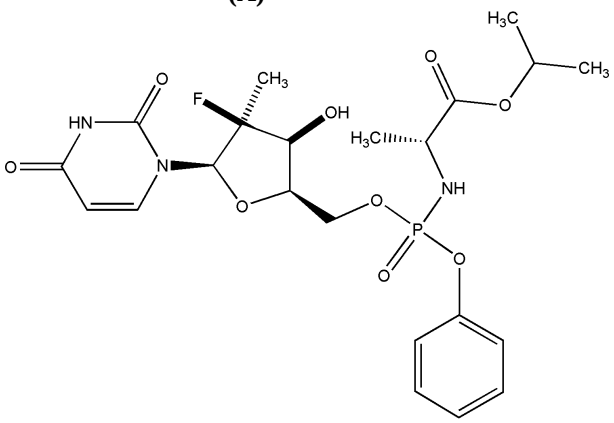
SOF, LED and DAC are non-pharmacopeial drugs. HCV being spread in the past years, it is a great priority to develop highly sensitive analytical techniques for the determination of the combined drugs in pure form, pharmaceutical dosage forms and biological fluids without interference from additives.

Limited methods were found in the literature for the simultaneous determination of SOF and LED including chemometry [10-11] high performance liquid chromatography [7, 12-14], spectrophotometry [13, 15].

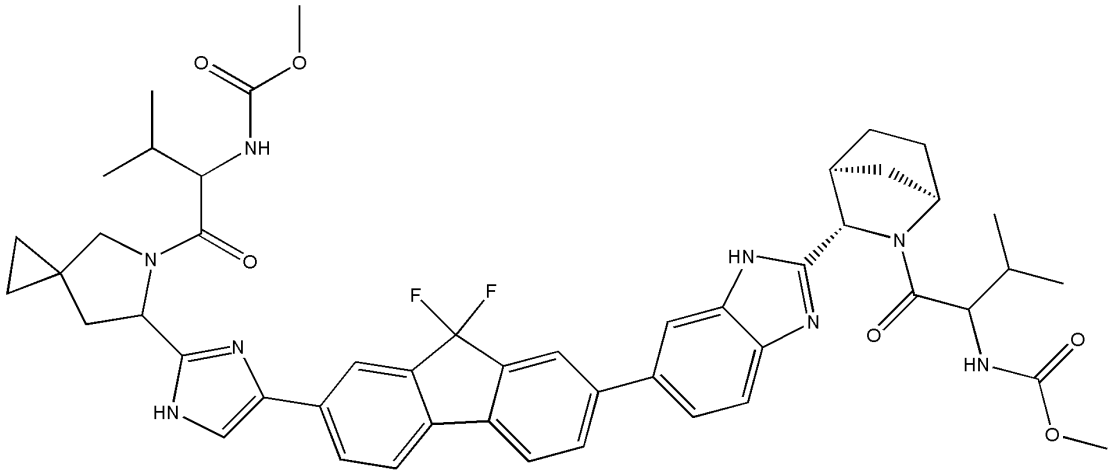
Few methods were reported in the literature for SOF and DAC combination including UV-VIS spectrophotometry [16-17] HPLC [3, 17-18] densitometry[19] and Ultra performance liquid chromatography mass spectrometry [18].

A new pentabromobenzyl column known as Cosmosil PBr was used for their determination under reversed phase mode offering unique selectivity for structurally similar compounds utilizing the dispersion force interaction known as 'hydrophobic' or 'lyophobic' interactions.

(A)



(B)



(C)

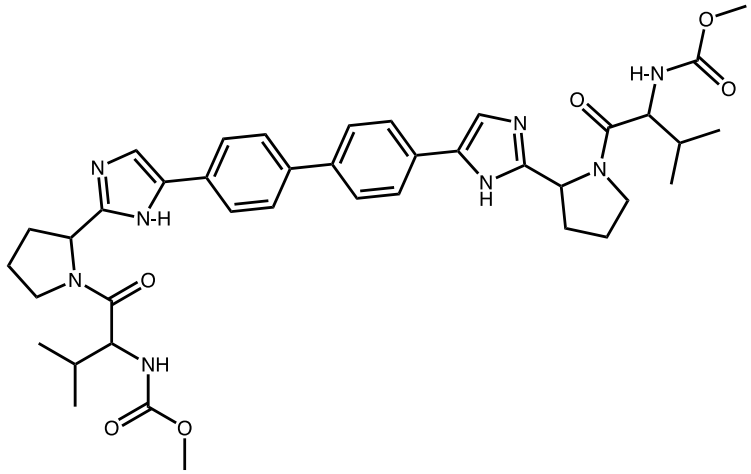


FIGURE 1: Chemical structure of (A) Sofosbuvir ($C_{22}H_{29}FN_3O_9P$), (B) Ledipasvir ($C_{49}H_{54}F_2N_8O_6$) and (C) Daclatasvir dihydrochloride ($C_{40}H_{52}Cl_2N_8O_6$).

This interaction makes it useful for separation of structural isomers differing only by a double bond. Moreover, it shows a high selectivity of the halogenated compounds. Also, the popularity of this stationary phase has been increasing due to their ability to separate closely related p-electron containing species. The bromine atoms on the aromatic ring of the Cosmosil PBr column have been found to affect dispersion interactions (which may be due to the increased surface contact with the solute) and charge transfer interactions (where the electron-withdrawing bromine atoms can create an electron deficient zone in the ring center), and therefore, this column favours charge interactions on the separation of polycyclic aromatic hydrocarbons [20].

The research aimed to provide an isocratic HPLC-DAD method for the simultaneous determination of SOF and LED in pharmaceutical dosage forms and in spiked human plasma with higher sensitivity. The purpose of the proposed method was to optimize a method for the simultaneous determination of SOF and DAC in pharmaceutical dosage forms and in spiked human plasma with better resolution, short analysis time and high sensitivity.

Herein, we offer a rapid, robust and sensitive isocratic HPLC-PDA for high throughput and accurate quantitation of the novel and most commonly prescribed HCV antiviral combination drugs SOF/ LED and SOF/DAC in bulk, pharmaceutical dosage form and in spiked human plasma.

EXPERIMENTAL

MATERIALS AND METHODS

Reagents

Sofosbuvir (SOF) purity (99.6%), ledipasvir (LED) purity (99.5%) and Daclatasvir (DAC) purity (99.2%), were kindly supplied by National Organization for Drug & Control Research (NODCAR) (Giza, Egypt). Pharmaceutical preparations containing the drugs were purchased from the local pharmacy. Sofocivir Plus® tab (Zeta pharma, Egypt), Batch no. 170543A, 400 mg Sofosbuvir and 90 mg Ledipasvir, Gratisovir® tab (Pharco, Egypt), Batch no. 7107040, 400 mg Sofosbuvir and Daklanork® tab (MSD, Egypt), Batch no. M1010417, 65.92 mg Daclatasvir dihydrochloride equivalent to 60 mg DAC/tab were purchased from the local market. , was purchased from the local market. All Reagents were of Analytical Reagent Grade and solvents were of HPLC grade. High purity water was obtained using Elga Lab water, prima 7 (High Wycombe, UK) and was used throughout the study. Methanol (HPLC grade) and acetonitrile (ACN) were purchased from Sigma-Aldrich (Germany). Orthophosphoric acid (85% w/v) was used for pH adjustment of potassium dihydrogen phosphate buffer and both were purchased from Riedel-deHaen (Germany). Sodium hydroxide was purchased from Sigma-Aldrich (Germany). Fresh human plasma samples were obtained from NODCAR (Giza, Egypt) and were kept frozen until use after gentle thawing.

Mobile phase Preparation

The isocratic elution consisting of 30 mM phosphate buffer pH 3.0, methanol and ACN (20:20:60, v/v) and flow rate 1.5 mL/min for SOF and LED mixture .For SOF and DAC mixture, 40 mM phosphate buffer (pH 3.6) and ACN (50:50, v/v) and flow rate 0.8 mL/min was used. The mobile phase was filtered through 0.2 µm Millipore micro filter and sonicated for 30 minutes before use. All determinations were performed at 25°C for SOF and LED mixture and 30°C for SOF and DAC mixture.

Sample and standard solutions preparation

Standard methanolic stock solutions of 200.00 µg/mL SOF , 38.00 µg/mL LED and 100.00 µg/mL DAC were prepared separately in 100 mL volumetric flasks . Then the solutions were sonicated in an ultrasonic bath for 30 min. They were found to be stable for 7 days in the refrigerator and LED had to be kept in the dark, Working solutions containing 20.00-100.00 and 7.60-30.40 µg/mL for SOF and LED, respectively in SOF/LED combination and 20.00- 200.00 and 3.00-80.00 µg/mL for SOF and DAC, respectively in SOF/DAC combination were prepared by serial dilution of the standard solutions with the mobile phase. The solutions were sonicated for 5 minutes and filtered through a disposable syringe filter (0.45 µm) before column injection. Aliquots of 10.00 µL were injected (triplicate) and eluted with the mobile phase under the optimum chromatographic conditions. The average peak area versus the final concentration of the drugs in µg/mL was plotted. Alternatively, the corresponding regression equations were derived. Calibration curves for plasma were prepared by spiking different quantities of the three antiviral drugs to give a final concentration range of 20.00-65.00 µg/mL and 3.30-16.40 µg/mL for SOF and LED, respectively and 15.00-50.00 µg/mL and 7.50-20.00 µg/mL for SOF and DAC, respectively. The nominal content of the antiviral drugs was calculated using the constructed calibration graph or from the corresponding regression equation.

Instrumentation

The chromatography system consisted of Agilent 1260 series (quaternary pump, auto sampler, vacuum degasser, diode array, and photodiode array detector (PDA) model (G4212B) connected to a computer equipped with Chemstation software and connected to the chromatographic system through Agilent system interface (Agilent Technologies, Santa Clara, CA, USA). Mobile phase was filtered with membrane filters (Millipore, Ireland) through Charles Austen pumps Ltd filter, and degassed with vacuum membrane degasser built in the accella pump. The pH was measured with Jenway pH meter, 3510, (Essex-UK). Ultrasonic bath used was Falc , (Treviglio-Italy).

Operating Conditions

The DAD wavelength was set at 254 nm for SOF, 306 nm for DAC and 330 nm for LED. The chromatographic separation of SOF, LED and DAC were accomplished using the Cosmosil PBr (150 mm × 4.6 mm I.D., particle size 5 µm) column (NACALAI TESQUE. Japan).

Analytical applications

Analysis of SOF-LED and SOF-DAC in their tablets:

SOF/LED mixture:

Ten tablets of Sofocivir Plus® were weighed and the average weight was determined, homogenized in a mortar, then an accurately weighed amount of the powder claimed to 20.00 mg SOF and 4.50 mg LED, were transferred into 100 mL volumetric flasks, sonicated for 30 min, then the solutions were completed to the volume with methanol, mixed well and filtered using a disposable syringe filter (0.45 µm). Aliquots of the filtrates were transferred into 10 mL volumetric flasks, diluted to volume with the mobile phase and mixed well. Complete as previously described under chromatographic conditions. The nominal content of the tablets was calculated from the calibration graph or from the corresponding regression equation.

SOF/DAC mixture:

Ten tablets of Daklanork® and Gratisovir® were weighed and the average weight for each product was determined, homogenized in a mortar, then an accurately weighed amount of the powder corresponding to 10 mg SOF and 5 mg DAC declared active principle was transferred into 50 mL volumetric flasks. About 50 mL of methanol was added followed by sonication in an ultrasonic bath for 30 min, then the solutions were completed to the volume with methanol, mixed well and filtered using a disposable syringe filter (0.45 µm). Aliquots of the filtrates were transferred into 10 mL volumetric flasks, diluted to volume with the mobile phase and mixed well. Complete as previously described under chromatographic conditions. The nominal contents of the tablets were calculated from the calibration graph or from the corresponding regression equation.

Analysis of SOF, LED and DAC in spiked human plasma:

Different quantities of the three antiviral drugs were spiked to 1 mL aliquots of thawed human plasma into a series of centrifugation tubes to give a final concentration range of 20.00-65.00 µg/mL and 3.30-16.40 µg/mL for SOF and LED, respectively and 15.00-50.00 µg/mL and 7.50-20.00 µg/mL for SOF and DAC, respectively. and then deproteinized with 2 mL ACN followed by vortex mixing. The solutions were centrifuged at 5000 rpm for 15 minutes. Then, the solutions were filtered through a disposable syringe filter (0.45µm). The experiment was carried out in triplicate. Each solution was injected in triplicate. The nominal contents of the three antiviral drugs were calculated using previously constructed calibration graphs or from the corresponding regression equations.

RESULTS AND DISCUSSION

The proposed HPLC method represents a rapid and sensitive stability-indicating assay method for the separation and simultaneous determination of most commonly prescribed antiviral drugs. By virtue of its high sensitivity, the proposed method was applied for the simultaneous determination of two antiviral mixtures either SOF with LED or SOF with DAC with good resolution in pharmaceutical dosage forms and in spiked human plasma with no need for tedious sample pre-treatment steps.

The experimental parameters influencing the chromatograms of the studied drugs were accurately considered and optimized. The parameters giving the highest number of theoretical plates and the best resolution in a reasonable time were selected. (Figure 2) shows a typical chromatogram for SOF with LED and SOF with DAC under the described chromatographic condition in pure forms and in their pharmaceutical preparations (Figure 3). The detection was performed at 254 nm for SOF, 306 nm for DAC and 330 nm for LED. The separation was performed within short retention time R_T=1.52 and 5.60 min for SOF and LED, respectively and R_T=3.30 and 3.95 min for DAC and SOF, respectively. The proposed method showed high sensitivity. The method also permitted the accurate analysis of SOF with LED and SOF with DAC in their tablet formulations as well as in spiked human plasma.

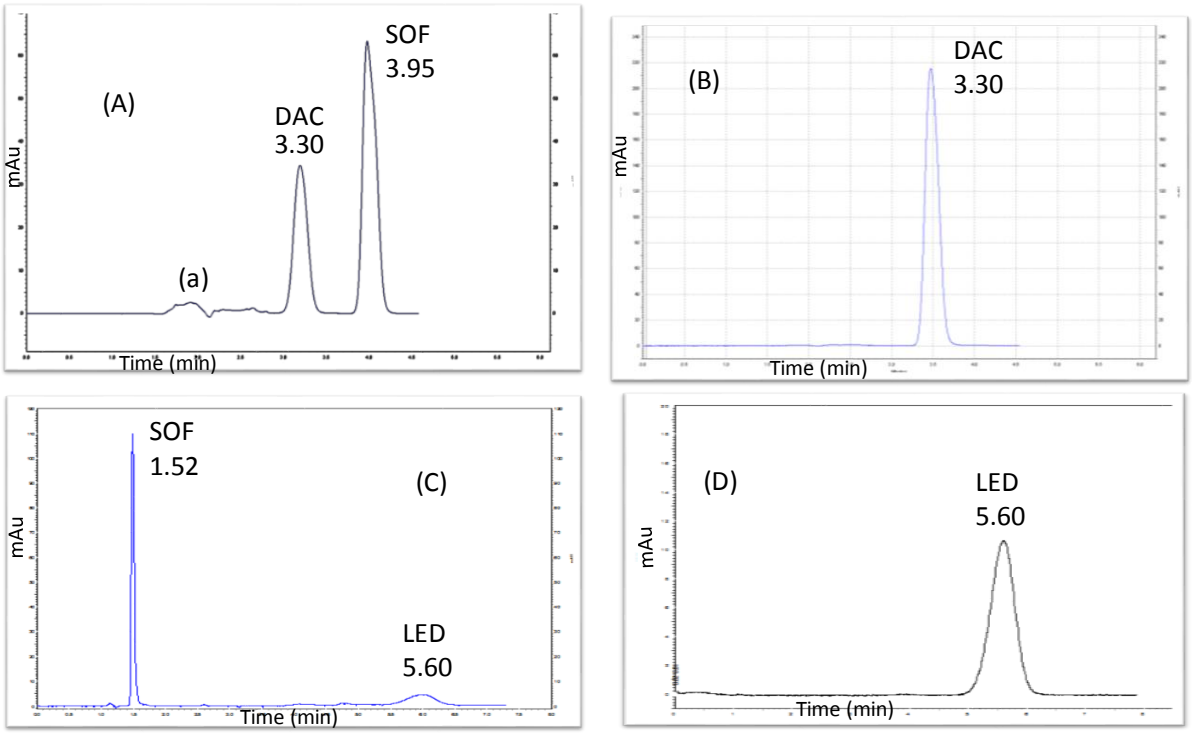


FIGURE 2: Typical chromatograms using the proposed HPLC method of (A) SOF and DAC mixture (a) solvent front at 254 nm, (B) SOF and DAC mixture at 306 nm, (C) SOF and LED mixture at 254 nm. (D) SOF and LED mixture at 330 nm.

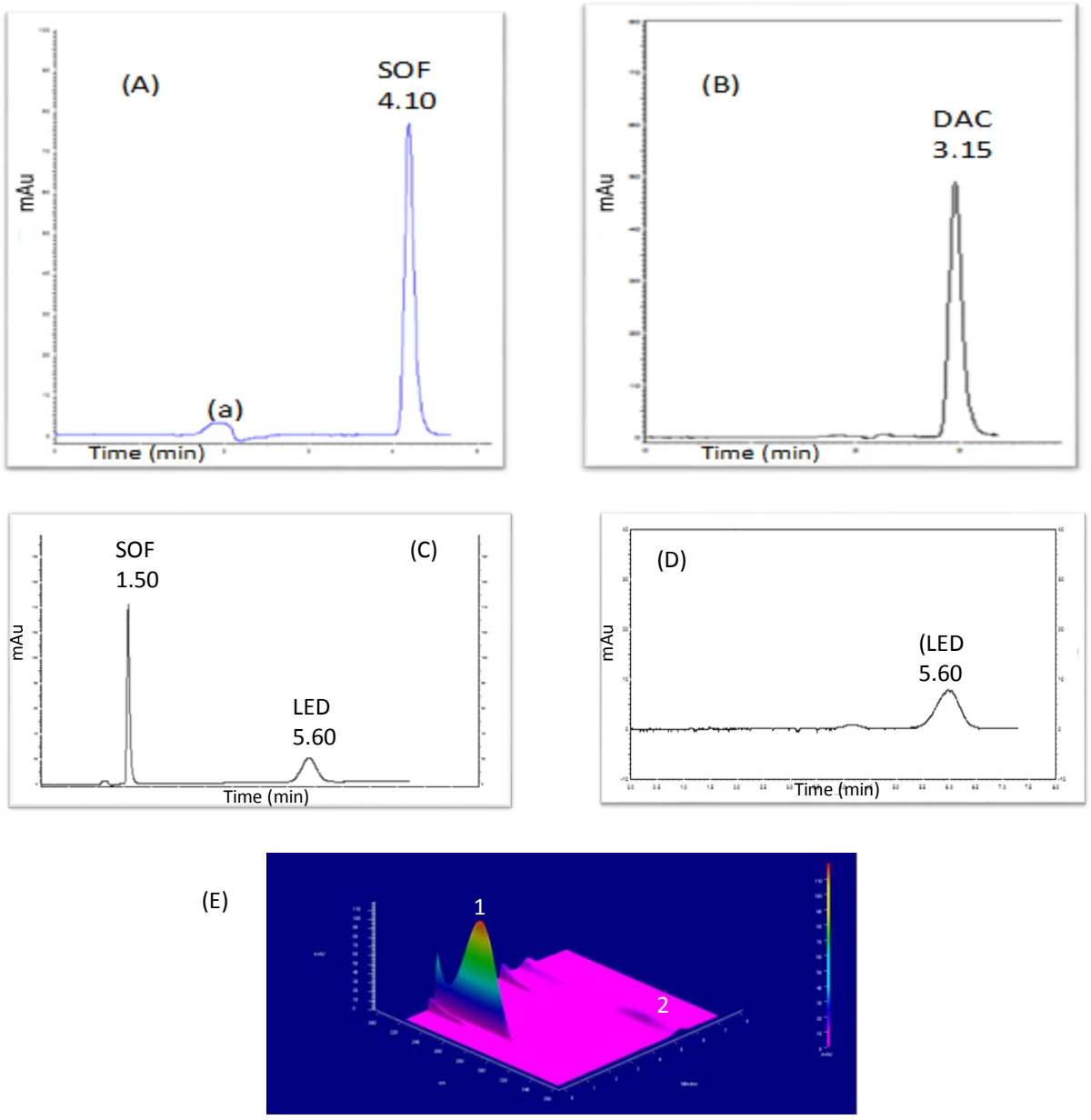


FIGURE 3: Typical chromatograms using the proposed HPLC method of (A) Gratisovir® tab dosage form (a)solvent front at 254 nm, (B) Daklanork® tab at 306 nm, (C) Sofocivir Plus® tab dosage form at 254 nm, (D) Sofocivir Plus® tab dosage form at 330 nm, (E) 3-D diagram showing the chromatogram of the SOF and LED dosage form using the proposed method, where 1. SOF and 2. LED.

Optimization of the chromatographic performance and system suitability:

PBr stationary phase offers high selectivity for separation of polarizable molecules containing aromatic functionality and/or heavy atoms through dispersive interactions (London's or instantaneous dipole-induced dipole interaction). While retention on alkyl columns (like C_8 or C_{18}) is directly related mainly to the solute hydrophobicity (or log P), dispersive interactions have a major contribution in retention on PBr stationary phases. Hence, PBr columns can be used to separate compounds that cannot be separated on C_{18} columns. PBr column will show an increased retention due to the increasing polarizability of halogen substituents. The presence of aromatic rings resulted in longer retention as demonstrated by LED. Different parameters affecting the chromatographic performance of the three antiviral drugs were carefully studied in order to achieve the most suitable chromatographic system. The results of the optimization study can be presented as follows:

Choice of detection wavelengths:

We use the wavelength that gives the maximum peak for each drug to enable us to determine both drugs simultaneously. We were able to measure the drugs simultaneously at their λ_{max}. So, 254 ,306 and 330 nm were selected for SOF, DAC and LED, respectively, as they are the optimum wavelengths for detection and determination of the three antiviral drugs with a reasonable sensitivity.

Mobile phase composition

The composition of the mobile phase is one of the most important parameters used to improve the performance of the chromatographic system concerning the type and % concentration of the organic modifier, the buffer strength and the pH. The effect of the strength of the mobile phase was investigated to describe retention changes of SOF/LED combination and SOF/DAC combination. (ACN and methanol) were tested and it was observed that ACN was preferred as methanol increases the analysis time and gives less sharp peaks.The conditions that gave the best resolution, symmetry and capacity factor were selected for the estimation. The results obtained are summarized in Table 1.

Concentration of the buffer

The molar concentration of phosphate buffer was studied over the range of 10-50 mM. Molar concentration below 30 mM provided low buffering capacity for the two combination drugs therefore poor peak symmetry and peak shape were obtained. Finally, 30 mM was selected as the optimum buffer concentration for SOF/LED combination and 40 mM was selected as the optimum buffer concentration for SOF/DAC combination yielding highest number of theoretical plates with good peak shape and lowest peak tailing with high resolution and highest peak area. At higher concentrations, however, there is a greater likelihood of salt crystals being formed.

pH

The effect of the mobile phase pH on the selectivity and retention time of the analytes was investigated using mobile phases of pH ranging from 2.50-4.00 for SOF and LED combination and 2.50-5.50 for SOF and DAC combination. For SOF/LED combination, when a buffer more than pH 3 was used, it causes peak broadening for LED. Finally, pH 3.0 was selected as the optimum pH value for this mixture yielding highest number of theoretical plates with better peak shape, lowest peak tailing and high resolution. It was found that changing the pH over 3.60 caused peak broadening for DAC and decrease the number of theoretical plates. Below pH 3.60, DAC peak was splitted and distorted. For SOF, Increasing the pH of the buffer caused increase in the retention time of SOF. Thus pH 3.60 was selected for this mixture.

Concentration of organic modifier (%)

For SOF/LED combination, when adding 20 % methanol to the mobile phase, this causes improving the peak shape of LED and decrease its retention time. Increasing methanol more than 20% leads to decreasing the retention factor, symmetry and resolution. For SOF/DAC combination, adding methanol to the mixture, leads to decreasing the resolution factor and peak purity is affected. As a result, 20% methanol and 50% ACN were chosen as the optimal organic modifier concentration for SOF/LED, and 50% ACN with 0% methanol was chosen as the optimal concentration for SOF/DAC where it offers a good combination of peak symmetry, resolution and analysis time.

Flow rate

We investigated the effect of flow rate over the range of 0.5-2.0 mL/min. For SOF/LED combination, Flow rate of 1.00 mL/min provides better peak shape but with a non-reasonable retention time for LED. Flow rate of 1.5 mL/min was chosen since it provides better peak shape within a reasonable retention time. For SOF/DAC combination, Flow rate of 0.8 mL/min was chosen since it provides better peak shape within a reasonable retention time and better resolution between peaks as shown in Table 1.

Temperature of the column

Different column temperature settings were studied over the range of 20°C-35°C, it was found that column temperature greatly affected the retention time and number of theoretical plates by affecting the interactions of solutes with the stationary phase and mobile phase and therefore, it effects the retention time and peak shape. Optimum Column temperature of 25 °C for SOF and LED mixture and 30 °C for SOF and DAC mixture was chosen for the analysis providing reasonable retention time and higher number of theoretical plates. We avoid higher temperature to reserve the column lifetime. The results obtained are listed in Table 1. After optimization of these variables, best peak shape, lowest peak tailing was achieved with well-defined peaks and good sensitivity within a reasonable analytical run time as shown in Figure 2.

TABLE 1 Optimization of the chromatographic conditions for (A) SOF and LED mixture, (B) SOF and DAC mixture by the proposed method.

(A) For SOF/LED mixture.

Parameters		No. of theoretical plates (N) per m		Retention factor (k)		Retention Time		Resolution (Rs)	Selectivity factor (α)	Tailing Factor	
		SOF	LED	SOF	LED	SOF	LED			SOF	LED
pH of the buffer	2.5	45428	11966	1.10	2.41	3.08	10.51	7.12	2.19	1.41	1.15
	3.0	47606	12542	1.28	2.63	3.19	11.79	8.47	2.05	1.29	0.98
	3.2	47003	13194	1.10	3.28	3.09	13.24	8.46	2.98	1.30	1.01
	3.5	46140	13210	1.10	3.31	3.07	13.22	9.31	3.01	1.31	1.12
	4.0	47512	12901	1.19	3.13	3.2	13.20	6.32	2.63	1.57	1.21
Buffer strength (mM)	10	44712	------	0.94	----	3.05	----	-----	------	1.31	---
	20	47625	12542	0.77	2.85	3.01	11.58	4.79	3.70	1.32	0.98
	30	45539	19511	1.16	2.74	3.15	11.79	9.34	2.36	1.31	1.04
	40	47607	19966	0.71	3.34	3.05	13.24	8.35	4.7	1.46	1.02
	50	45140	13194	1.01	3.41	3.01	13.28	8.67	3.38	1.57	1.01
Conc. of organic modifier (ACN:Methanol)	30 --	forked	-----	-----	----	---	-----	----	----	----	----
	40 20	19065	11260	-----	4.62	1.37	7.77	4.37	------	1.06	1.00
	50 20	42719	13989	0.54	2.59	2.7	9.7	8.64	4.80	1.29	1.09
	50 30	47447	12596	0.41	3.43	2.5	11.08	9.69	8.37	1.50	1.03
	60 20	52076	15568	0.28	2	2.1	6.3	6.51	7.14	1.58	1.01
	55 20	38633	11962	0.54	2.37	2.7	9.1	8.21	4.39	1.52	1.03
Flow rate (mL/min)	0.5	34519	9398	0.37	2.03	2.9	8.8	7.81	5.49	1.54	1.07
	1	50222	12809	0.44	2.12	2.45	7.64	7.69	4.81	1.60	1.03
	1.5	54470	15292	0.44	2.58	1.58	5.65	7.98	4.77	1.40	0.99
	2	38620	13210	-----	1.69	1.17	4.15	8.16	------	1.45	1.10
Column temp.	20	31723	11752	0.37	1.92	1.57	4.59	5.92	5.19	1.30	0.99
	25	52076	15180	0.42	2.06	1.52	5.65	6.73	4.9	1.12	0.99
	30	34415	13897	0.38	2.14	1.59	4.99	5.31	5.63	1.00	0.99

(B) For SOF/DAC mixture.

Parameters		No. of theoretical plates (N) per m		Retention factor (k)		Retention Time		Resolution (Rs)	Selectivity Factor(α)	Tailing Factor	
		SOF	DAC	SOF	DAC	SOF	DAC			SOF	DAC
pH of the buffer	2.5	45428	------	0.51	-----	3.08	-----	------	------	1.41	----
	3.0	47606	-----		-----	3.01	-----	-------	------	1.32	----
	3.2	47003	17258	0.44	0.51	3.05	2.12	1.06	0.86	1.43	0.96
	3.6	46140	19874	0.52	0.28	3.07	2.39	1.16	1.85	1.39	1.25
	4.0	47512	18559	0.26	0.1	3.3	2.63	1.15	2.60	1.39	1.34
	4.5	46941	18214	0.18	0.55	3.25	2.75	0.94	0.33	1.50	1.31
	5.5	47625	------	0.97	----	3.1	---	-------	----	1.48	----
Buffer strength (mM)	10	44712	------		-----	3.05	-----	-----	-----	1.67	----
	20	47625	-------		------	3.01	-----	-------	-------	1.55	----
	30	45539	forked	0.52	0.48	3.15	2.07	2.53	1.08	1.46	1.16
	40	53495	19874	0.59	0.36	3.05	1.92	2.76	1.64	1.30	1.01
	50	47506	5208	0.96	0.36	3.08	2.04	1.91	0.53	1.61	0.97
Conc. of organic modifier	30	forked	------	------	-------	-------	------				
	40	19125	forked	----	----	3.09	----	------	0.74	1.58	
	50	40108	11326	0.24	0.77	3.18	2.56	2.00	0.33	1.30	1.01
	55	38633	11154	0.18	0.82	2.79	2.37	0.82	0.22	1.44	1.21
	60	26784	10247	0.48	0.13	2.44	1.65	2.72	3.69	1.30	1.17
Flow rate (mL/min)	0.5	21278	13015	0.16	0.90	4.4	3.80	1.20	0.18	1.39	1.01
	0.8	20214	12511	0.26	0.68	3.9	3.10	1.68	0.38	1.30	1.00
	1	40108	11326	0.24	0.77	3.18	2.55	2.00	0.33	1.40	1.10
	1.5	9001	forked	0.60	-----	1.6	--	----	---	1.30	----
Column temperature	25	21663	9276	0.26	0.68	3.90	3.10	1.68	0.38	1.38	1.10
	30	38012	14222	0.19	0.85	3.95	3.33	1.72	0.22	1.30	1.01
	35	34415	11752	0.16	0.83	4.01	3.47	0.19	0.19	1.32	1.04

Where: (N/m)=Number of theoretical plates per meter , (N/m)

Capacity Factor (K)=t_r-$\mathring{t}$/ $\mathring{t}$
Selectivity Factor (α)=k_2/k_1
Resolution (R)=$2\Delta\ t_r\ /W_1+W_2$

Method Validation

The validity of the proposed method was assessed by studying the following parameters: linearity, range, LOD, LOQ, accuracy, precision, selectivity, sample solution stability, mobile phase stability, system suitability and robustness.

Linearity and range

Under the above described experimental conditions, a linear relationship was established by plotting the average peak area against the drug concentration in µg/mL. The calibration graphs were found to be rectilinear over the concentration range of 20.00-100.00 µg/mL and 7.60-30.40 µg/mL for SOF and LED, respectively and 20.00-200.00 µg/mL and 3.00-80.00 µg/mL for SOF and DAC, respectively. Linear regression analysis of the data gave the following equations:

For SOF/LED combination

P= 5952.7C - 13698 (r=0.9997) for SOF
P= 17689.28 C - 10378 (r=0.9996) for LED

For SOF/DAC combination

P= 9770.6C +62042 (r=0.9999) for SOF
P= 37568C+ 4276.3 (r=0.9999) for DAC

Where: P is the average peak area, C is the concentration of the drug in µg/mL and r is the correlation coefficient.
Statistical analysis of the data obtained by the proposed method, gave high value of the correlation coefficient (r) of the regression equation, accepted values of the standard deviation of residuals ($S_{y/x}$), standard deviation of intercept (S_a), and standard deviation of slope (S_b), and accepted value of the percentage relative standard deviation and the percentage relative error shown in Table 2. These data proved the linearity of the calibration curves and less scattering of the points around the calibration curves.

TABLE 2 Analytical performance data for the determination of the studied drugs in (A) their pure form (B) spiked human plasma ,by the proposed method.

(A) Pure form.

Parameters	SOF	LED	SOF	DAC
Concentration range (µg/mL)	20.00-100.00	7.60-30.40	20.00-200.00	3.00-80.00
Limit of detection	0.09	0.07	0.25	0.03
Limit of quantification	0.26	0.20	0.75	0.09
Correlation coefficient	0.9997	0.9996	0.9996	0.9999
Slope	5952.70	17689.28	9770.64	37568
Intercept	-13698	-10378.40	62042.41	4276.30
Standard deviation of residuals	154.92	349.94	733.90	329.64
S.D. of intercept (S_a)	3987.10	4297.69	5133.22	5793.65
S.D. of slope (S_b)	63.92	210.02	54.81	61.87
% RSD	1.49	1.22	1.38	1.36
% Error	0.56	0.46	0.49	0.51

Where RSD is the relative standard deviation .

(B) Spiked human plasma.

Parameters	SOF	LED	SOF	DAC
Concentration range (µg/mL)	20.00-65.00	3.30-16.40	15.00-50.00	7.50-20.00
Limit of detection	0.24	0.25	0.11	0.06
Limit of quantification	0.72	0.75	0.33	0.17
Correlation coefficient	0.9996	0.9997	0.9997	0.9998
Slope	6152.70	19463.68	8379.07	36328.57
Intercept	-28689	-16617.60	13031.04	12964.42
Standard deviation of residuals	443.59	1451.13	276.46	630.30
S.D. of intercept (S_a)	3643.47	3013.67	3135.60	5601.10
S.D. of slope (S_b)	90.38	301.04	91.20	429.02
% RSD	1.74	2.14	1.04	1.11
% Error	0.78	0.96	0.47	0.50

Where RSD is the relative standard deviation.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected. The limit of quantitation (LOQ) was determined by establishing the lowest concentration that can be measured according to ICH recommendations [21] below which the calibration graph is non-linear.

The values of LOD and LOQ were calculated according to the following equations:

$$\mathbf{LOD = 3.3\ \sigma / S} \qquad \mathbf{LOQ = 10\ \sigma / S}$$

Where σ = the residual standard deviation of the response

And S = slope of the calibration curve

Accuracy and precision

Accuracy of the proposed method was checked by comparing the results of the assay of the studied drugs with those obtained using the reported HPLC method for SOF/LED combination [7] and for SOF/DAC combination [3], where Student's t-test and variance ratio F-test showed no significant difference in the two methods performance regarding the accuracy and precision, respectively (Table 3) indicating high accuracy and precision of the proposed method. The reported method [7] depends on using reversed phase HPLC for determination of SOF and LED in pure form and in human plasma with UV detection at 260 nm (for SOF) and 330 nm (for LED). The proposed procedure offers additional advantages over the reported one in that the former allow shorter analysis time with higher sensitivity. The reported method [3] depends on using reversed phase HPLC for determination of SOF and DAC tablet dosage form with UV detection at 243 nm for SOF and DAC. The proposed procedure offers more advantages over the reported one in that the proposed method allows shorter analysis time of the drug with higher sensitivity in addition of being extended to the analysis of SOF and DAC in spiked human plasma.

Six replicate determinations of 50.00, /22.00 and 40 µg/mL of the pure drug SOF LED and DAC, respectively as well as the dosage forms were analysed in one day for evaluation of the intra-day precision of the proposed method and over three successive days for evaluation of the inter--day precision of the proposed method as shown in Table 4. The relative standard deviations were found to be very small indicating reasonable repeatability and intermediate precision of the proposed method (Table 4).

TABLE 3 Accuracy of the proposed method for the determination of the cited drugs in pure forms.

	Method	% found								Mean ± S.D	Student t-Test	Variance ratio F-test
SOF	Proposed Method* (µg/mL)	20.00 102.44	30.00 101.4	40.00 98.2	50.00 99.42	60.00 98.78	70.00 99.88	85.00 101.61	100.00 99.54	100.16 ±1.49	1.19 (2.20)	1.98 (6.09)
	Reported Method* [7](µg/mL)	10.00 101.95	20.00 100.23	25.00 100.98	30.00 102.33	40.00 99.88				101.07 ±1.06		
LED	Proposed Method* (µg/mL)	7.60 101.86	11.40 98.56	15.20 100.25	19.00 99.63	22.80 100.89	26.60 98.6	30.40 100.74		100.08 ±1.22	0.46 (2.23)	2.79 (6.16)
	Reported Method* [7](µg/mL)	20.00 99.75	30.00 100.54	40.00 100.23	50.00 99.75	60.00 101.52				100.36 ±0.73		
SOF	Proposed Method* (µg/mL)	20.00 98.91	40.00 98.75	50.00 103.23	60.00 100.34	100.00 101.06	120.00 100.02	200.00 99.44		100.25 ±1.54	0.10 (2.31)	16.26 (19.33)
	Reported Method* [3](µg/mL)		98.99 80.00	99.85 100.00	101.60 120.00					100.15 ±1.33		
DAC	Proposed Method* (µg/mL)	3.00 103.16	6.00 100.99	20.00 100.32	40.00 102.10	50.00 99.69	60.00 99.49	80.00 99.91		100.81 ±1.37	0.95 (2.31)	12.81 (19.33)
	Reported Method* [3](µg/mL)		6.00 99.71	20.00 100.45	50.00 99.91					100.02 ±0.38		

*Each result is the average of three separate determinations

Figures in parentheses are the tabulated t and F values, respectively at P = 0.05.

TABLE 4: Precision data for the determination of the proposed HPLC method for the determination of the drugs combinations in both pure and dosage forms.

	Intra-day precision*				Inter-day precision*			
	SOF/LED		SOF/DAC		SOF/LED		SOF/DAC	
	%Found ± SD		%Found ± SD		%Found ± SD		%Found ± SD	
Pure form	SOF	101.88+0.15	SOF	99.64+0.56	SOF	100.51+1.30	SOF	100.45+0.91
	LED	99.44 + 0.64	DAC	100.98 + 1.41	LED	99.92+1.78	DAC	101.67 + 1.25
Dosage form	SOF	103.17 + 1.02	SOF	100.99 + 0.73	SOF	102.72 + 1.08	SOF	101.47 + 1.41
	LED	102.38 + 1.18	DAC	101.41 + 1.28	LED	102.46 + 1.67	DAC	100.55 + 1.79

*Each result is the average of three separate determinations
Figures in parentheses are the tabulated t and F values, respectively at P = 0.05.

Robustness of the method

The robustness of the proposed method is a measure of the capacity to remain unaffected by small but deliberated variations in method parameters. The experimental parameters evaluated are pH of the mobile phase (± 0.2), flow rate (±0.05) mL/min and mobile phase composition (± 1%). These minor changes didn't affect the peak area of SOF, LED and DAC.

Selectivity

The interference from common excipients in pharmaceutical formulations was observed carefully through the analysis and It was clearly proved from the analysis results that these additives did not affect the results of the proposed method. Moreover, there was not any interference faced from human spiked plasma matrix. The high % recovery and high accuracy with low SD indicated that excipients and plasma matrix did not affect the results of the proposed method. Furthermore, to evaluate the selectivity of the method used in this study in human plasma , blank plasma was extracted as mentioned above and diluted with the mobile phase and injected under the recommended chromatographic conditions. No endogenous interference was observed at the retention times of the cited drugs, proving the selectivity of the method.

Sample solution stability and mobile phase stability

Evaluation of the stability of the studied drugs was achieved by quantification of the drugs on three successive days and comparison to freshly prepared dosage forms solution. Similarly, the stability of the mobile phase was checked. No significant changes were observed in standard solution or mobile phase responses, relative to freshly prepared ones except that LED had to be kept in the dark to avoid photodegradation which is noticed by changing the solution into yellow colour when being subjected to normal light. The results obtained in both cases proved that the sample solution and mobile phase used during the assay were stable up to 7 days in the refrigerator.

System Suitability Test (SST)

Evaluation of system suitability test parameters was performed during the development and optimization of the method. Moreover, to ascertain the effectiveness of the final operating system, it was subjected to suitability testing. The test was performed by injecting the standard sample in triplicate and the parameters were calculated as reported by USP [22]. SST parameters include capacity factor (k'), selectivity factor (α), Resolution factor (R_s), column efficiency (number of theoretical plates per meter, N/m). The final SST parameters under the optimum chromatographic conditions are abridged in Table 5.

TABLE 5. Chromatographic characteristics of system suitability solution.

	SOF/LED		SOF/DAC	
Parameter	**SOF**	**LED**	**SOF**	**DAC**
λ max	254	330	254	306
R_t ± %RSD	1.52±0.14	5.6±0.20	3.95±0.12	3.30±0.18
Capacity factor (K\)	0.42	2.06	0.19	0.85
Symmetry	1.12	0.99	1.30	1.01
Theoretical plates(N/m)	52076	15180	38012	14222
Resolution	6.73		1.72	
Selectivity	4.9		0.22	

Application

Application to pharmaceutical dosage form

The proposed method was successfully used to quantify SOF and LED in their pharmaceutical dosage form (Sofocivir Plus®tab) and SOF and DAC in their pharmaceutical dosage form Gratisovir ® tab and (Daklanork® tab), respectively,as shown in Table 5.Standard addition technique was used to assess the matrix effect of the tablet additives and its contribution in the deviation of the results obtained by the proposed method as shown in Table 6. The obtained results revealed no significant matrix effect.

TABLE 6. Results obtained by applying the proposed methods for the determination of (A) SOF and LED in combined tablet dosage form (B) SOF and DAC in tablet dosage forms, and by applying standard addition technique.

(A) SOF/LED combined dosage form.

		Assay	Amount taken (µg /mL)	Amount of st added (µg/mL)	% found
SOF	% Found	102.69		0.00	101.95
		104.73	60.00	10.00	102.35
		102.17		20.00	104.15
	Mean + SD	103.20 + 1.35	Mean + SD		102.82 + 1.17
	%RSD	1.31	%RSD		1.14
LED	% Found	101.86	20.00	0.00	101.32
		100.39		5.00	103.52
		103.59		10.00	103.85
	Mean + SD	101.95 ± 1.60	Mean + SD		102.90 ± 1.38
	%RSD	1.57	%RSD		1.34

(B) SOF and DAC dosage forms.

		Assay	Amount taken (µg /mL)	Amount of st added (µg/mL)	% found
SOF	% Found	101.52		0.00	98.85
		100.95	20.00	10.00	99.22
		102.25		20.00	10.66
	Mean ± SD	100.57 ± 0.65	Mean ± SD		99.58 + 0.96
	%RSD	0.64	%RSD		0.96
DAC	% Found	99.06	5.77	0.00	101.31
		101.81		5.00	99.18
		99.68		15.00	98.43
	Mean ± SD	100.18 ± 1.44	Mean ± SD		99.64 + 1.49
	%RSD	1.44	%RSD		1.50

*Each result is the average of three different separate determinations

Application to spiked human plasma

The proposed method after sample pre-treatment was successfully used to determine SOF, LED and DAC in spiked human plasma as shown in (Figure 4). Standard addition technique was used to assess the matrix effect of the plasma and its contribution in the deviation of the results obtained by the proposed methods, the obtained results revealed no significant matrix effect as shown in Table 7.

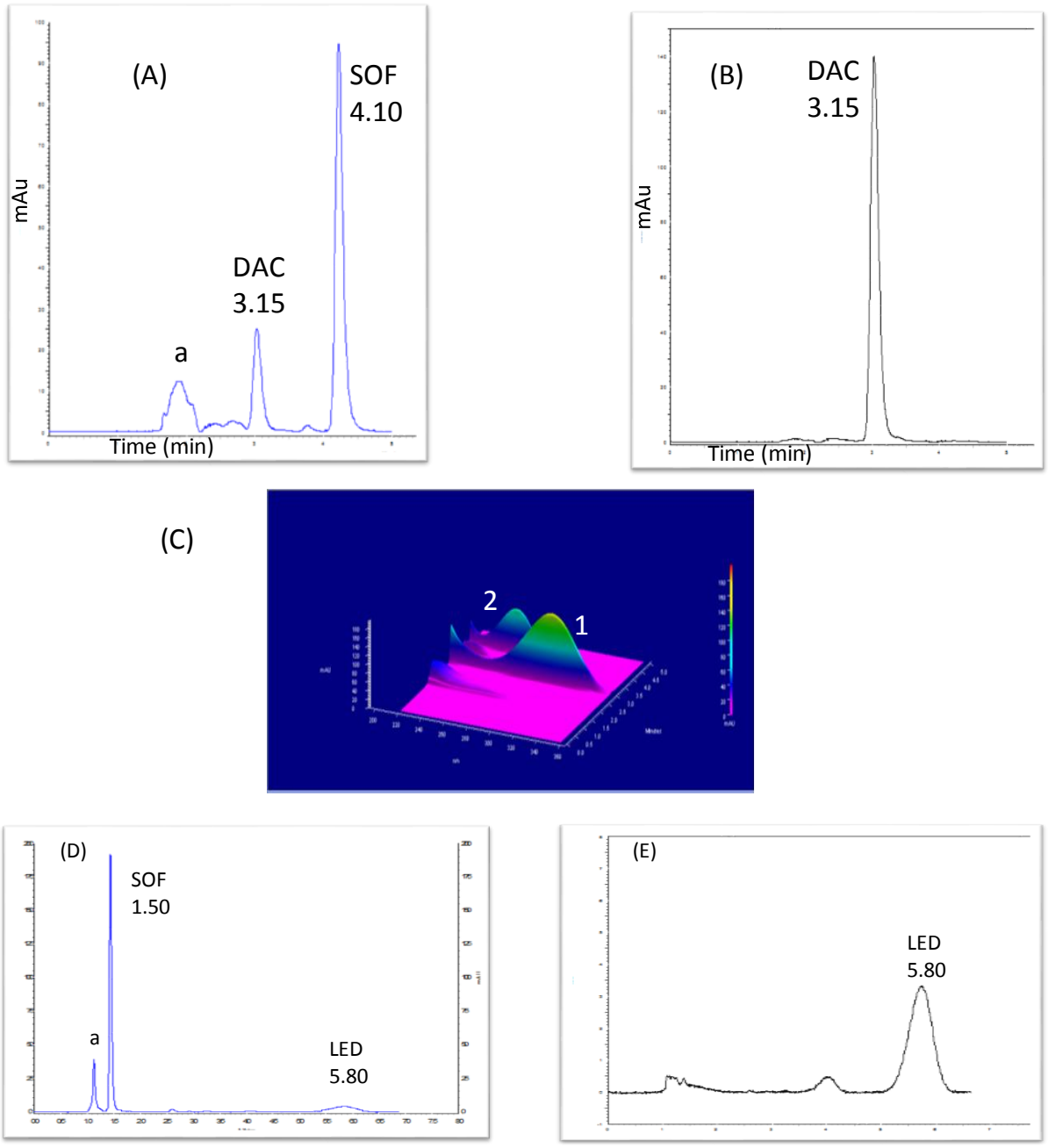


FIGURE 4: Typical chromatograms using the proposed HPLC method of (A) SOF and DAC mixture (a) plasma at 254 nm, (B) SOF and DAC mixture at 306 nm, (C) 3-D diagram showing the chromatogram of the DAC and SOF using the proposed method, where 1. DAC and 2. SOF, (D) SOF and LED mixture (a) plasma at 254 nm and (E) SOF and LED mixture at 330 nm.

TABLE 7. Results obtained by applying the proposed HPLC method for the determination of (A) SOF and LED, (B) SOF and DAC) in spiked human plasma and results obtained by applying standard addition technique.

(A) SOF and LED mixture.

		Assay	Amount taken (µg /mL)	Amount of st added (µg/mL)	% found
SOF	% Found	101.85	40.60	0.00	100.62
				10.80	103.70
				21.50	102.05
		98.91			
		101.85			
	Mean ± SD	100.31 + 1.48	Mean ± SD		102.12 + 1.54
	%RSD	1.47	%RSD		1.50
LED	% Found	102.69	7.60	0.00	101.05
		100.53		10.40	103.09
		99.27		17.20	102.15
	Mean ± SD	100.83 ± 1.73	Mean ± SD		102.10 ± 1.02
	%RSD	1.72	%RSD		1.00

*Each result is the average of three different separate determinations

(B) SOF and DAC mixture.

		Assay	Amount taken (µg /mL)	Amount of st added (µg/mL)	% found
SOF	% Found	100.07	20.00	0.00	101.22
				20.00	99.15
		99.47		30.00	102.25
		100.07			
	Mean ± SD	99.24 ± 0.96	Mean ± SD		100.87 ± 1.58
	%RSD	0.97	%RSD		1.57
DAC	% Found	100.07	8.00	0.00	101.66
		102.27		5.00	102.72
		101.23		10.00	101.33
	Mean ± SD	101.19±1.10	Mean ± SD		101.90 ± 0.72
	%RSD	1.09	%RSD		0.71

*Each result is the average of three different separate determinations

CONCLUSION

The proposed methods are based on the dispersion force interaction of the three cited drugs at Pentabromobenzyl column which causes an enhancement in the separation of these structurally related drugs through the RP-HPLC-PDA detection. The good validation criteria of the proposed methods allow their use in quality control laboratories. The method is simple, sensitive, rapid and could be applied easily for quick analysis of many samples in a short analysis time. We aim to use this column in the future work for separation of highly similar halogenated chemical structures of different analytes.

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ABBREVIATION LIST

hepatitis C virus	(HCV),
Pentabromobenzyl column	(Pbr)
Direct acting antivirals	(DAA),
Sofosbuvir	(SOF),
Ledipasvir	(LED),
Daclatasvir	(DAC),
Limit of Detection	(LOD),
Limit of Quantitation	(LOQ),
acetonitrile	(ACN),
System Suitability Test	(SST).

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