Simultaneous chromatographic analysis of Sofosbuvir/Ledipasvir in their combined dosage form: an application to green analytical chemistry

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Abstract

Application of green solvents for developing green analytical methodologies has grown dramatically in the past few years. The "more hazardous reagents" are replaced by more "environment-friendly solvents" without affecting method performance. In the present study, two simple and accurate chromatographic methods were developed and validated for determination of the new antiviral combination sofosbuvir (SBR) and ledipasvir (LPV). The first adopted method is high-performance thin-layer chromatography coupled to densitometric determination where silica gel 60 F_{254} plates were used as the stationary phase. Whereas, the running mobile phase used was toluene: ethanol: ammonia (4:1:0.2, v/v/v). Reversed-phase high-performance liquid chromatography with ultraviolet detection was the second method developed. The column used was Inertsil C_{18} column (150 × 4.6 mm, 5 μm) and the mobile phase was 20 mM potassium dihydrogen orthophosphate (adjusted to pH = 3 using acetic acid): ethanol (60:40, v/v) with a flow rate of 1.0 mL/min. The detection wavelength for both methods was 265 nm. The validation of both methods was done according to ICH guidelines where both methods were found to be accurate, reproducible, and selective. The linearity range for HPTLC and RP-HPLC methods were 0.8–25.6 and 0.4–12.8 μg/band and 6.0–100.0 and 4.0–80.0 μg/mL for sofosbuvir and ledipasvir, respectively. Comparison of the developed methods was done with reported HPLC method where no significant difference was found.

Keywords: Sofosbuvir/ledipasvir, HPTLC-densitometry, RP-HPLC-UV, Green analytical chemistry, Green solvents

Introduction

Sofosbuvir (SBR) is a new drug used for treating hepatitis C viral infection. Hepatitis C virus (HCV) infects the liver and has many complications which might need liver transplantation (Jacobson et al. 2010; Lavanchy 2011). The mode of action of the prodrug SBR is a nucleotide analog inhibitor of HCV nonstructural protein 5B (NS5B) (Keating and Vaidya 2014; Gorman et al. 2015). Ledipasvir (LPV) also is a new antiviral drug for the treatment of HCV. The combination of SBR and LPV for the treatment of HCV is approved by FDA in 2014 (Afdhal et al. 2014; Link et al. 2014; Pollack 2014). After literature survey, we found many chromatographic methods for the determination of the

combination such as LC-MS/MS (Rezk et al. 2016; Pan et al. 2016; Elkady and Aboelwafa 2016), RP-HPLC-DAD (Farid and Abdelwahab 2017), and dissolution studies using RP-HPLC (Zaman et al. 2016; Hassouna et al. 2017) and in dosage forms (Rote et al. 2017). Also, densitometric methods were reported (Salama et al. 2017; Baker et al. 2018). But we noticed that all the published methods utilize organic solvents which are hazardous or toxic to the environment.

In green analytical chemistry, hazardous chemicals are replaced with more eco-friendly solvents without affecting the overall performance of the method (Welch et al. 2010). To the best of our knowledge, no previous researches had been developed for the simultaneous determination of the suggested two-drug combination: SBR/LPV using alternative green solvents. So, our main challenge was to simultaneously

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determine both analytes using more eco-friendly solvents without affecting method performance. Therefore, two accurate and reproducible chromatographic methods for the simultaneous estimation of SBR/LPV in their binary laboratory-prepared mixtures and in their combined tablet dosage form using green solvents were first introduced in this work. ICH guidelines were followed for the complete validation of the developed methods (ICH 2005). Statistical comparison was applied with reported RP-HPLC method and no significant difference was found.

Methods
Instruments

- Linomat IV auto-sampler (Camag, Muttenz, Switzerland), Camag microsyringe 100 μL (Hamilton, Bonaduz, Switzerland), Pre-coated silica gel aluminum plates 60 F_{254} 250 μm thickness 20 cm × 10 cm (E. Merck, Darmstadt, Germany), Twin-trough Automatic Developing Chamber ADC 2 chamber 20 cm × 10 cm (Camag, Muttenz, Switzerland), Camag TLC scanner III S/N 130319 (Camag, Muttenz, Switzerland) operated using Win-CATS software version 3.15.
- The HPLC System: Jasco (JASCO, Tokyo, Japan) model LC-Net II/ADC and a UV detector UV-2070 plus equipped with an isocratic pump PU-2080 plus and 4-line degasser DG-2080-54. The stationary phase was Inertsil C_{18} column (150 × 4.6 mm, 5 μm). Data acquisition was performed by the use of ChromNAV software.
- pH meter (Jenway 3310, UK)

Chemicals and solvents
Ethanol (Sigma-Aldrich, Germany), toluene (Merck, Darmstadt, Germany), acetic acid (Riedel-de Haen, Seelze, Germany), ammonia (Merck, Darmstadt, Germany), and potassium dihydrogen phosphate (Merck, Darmstadt, Germany).

Samples

(a) Pure standard of SBR (with certified purity of 99.89 ± 0.691)
(b) Pure standard of LPV (with certified purity of 99.79 ± 0.461)

Both standards were kindly supplied by Memphis Co. for Pharmaceutical and Chemical Industries, Cairo, Egypt. Their chemical structures are presented in Fig. 1.

(c) Heterosofir Plus® tablet dosage form—each tablet contains SBR (400 mg) and LPV (90 mg), manufactured by PHARMED Healthcare Co., Sadat City, Egypt.

Standard solutions
For HPTLC-densitometric method: stock standard solutions of SBR and LPV were prepared in ethanol to obtain concentrations of 2.0 mg/mL and 1.0 mg/mL, respectively. For RP HPLC-UV method, the two previously prepared stock standard solutions for HPTLC-densitometric method for each drug were then subjected to further dilutions using the HPLC mobile phase to obtain two working standard solutions having concentrations of 200.0 μg/mL for SBR and 100.0 μg/mL for LPV.
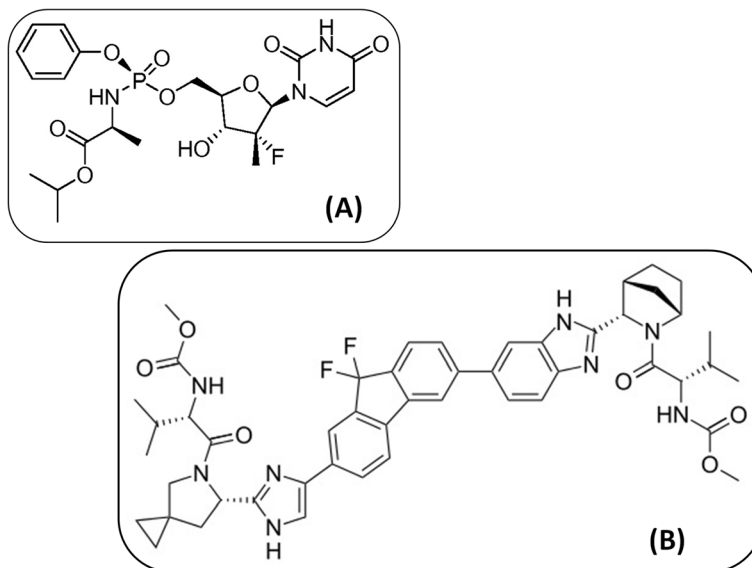

Fig. 1 Chemical structure of SBR (a) and LPV (b)

All experiments were conducted away from direct sunlight and stock standard solutions were refrigerated up to 1 week.

Chromatographic conditions
HPTLC-densitometric method
On a pre-coated silica gel aluminum plate 60 F_{254} 20 cm × 10 cm, samples from the prepared stock standard solutions were applied in the form of bands (6 mm width) by the Camag microsyringe 100 μL using Camag Linomat IV autosampler. After chamber saturation, linear ascending development was carried out in 20 cm × 10 cm twin-trough Automatic Developing Chamber ADC 2 chamber by the use of a mobile phase system consisted of toluene: ethanol: ammonia (4:1:0.2, v/v/v). As soon as the solvent front reached a pre-defined position, the plate was removed and was subjected to flow-optimized conditions to dry. The experimental wavelength selected for detection and quantification was 265 nm.

RP HPLC-UV method
The stationary phase was Inertsil C_{18} column (150 × 4.6 mm, 5 μm) and the mobile phase was 20 mM potassium dihydrogen orthophosphate (adjusted to pH = 3 using acetic acid): ethanol (60:40, v/v). Injection volume was 10 μL and the flow rate was adjusted at 1.0 mL/min and the UV detection was performed at 265 nm. Data manipulation was carried out using ChromNAV software.

Construction of calibration curves
HPTLC-densitometric method
Onto the pre-coated silica gel aluminum plates 60 F_{254} 20 cm × 10 cm, accurate volumes were transferred from the stock standard solution of SBR (2.0 mg/mL) and LPV (1.0 mg/mL) and applied separately in triplicate in the form of bands 6 mm width to reach to a concentration range of 0.8–25.6 for SBR and 0.4–12.8 μg/band for LPV.

The chromatographic procedure described under "Chromatographic Conditions" was then followed. The average of the integrated peak area ratio (peak area of the drug/peak area of an external standard (1.6 μg/band for SBR and 0.8 μg/band for LPV)) was then computed for each concentration of each drug. Two calibration curves were then constructed for SBR and LPV, respectively using the integrated peak area ratios against their corresponding concentrations. The calculation of polynomial regression equations was then performed.

RP HPLC-UV method
The working standard solutions were used to prepare serial dilutions of both drugs. The linearity range was 6.0–100.0 μg/mL for SBR and 4.0–80.0 μg/mL for LPV.

Then, 10 μL were injected in triplicate into the system. The relative peak area ratio for each concentration to that of an external standard (20.0 μg/mL for SBR and 10.0 μg/mL for LPV) was used to construct the calibration graph. Then, the regression equation for each drug was computed.

Laboratory-prepared mixtures assay
For HPTLC-densitometric method, different aliquots of SBR and LPV were transferred from their respective stock standard solutions and completed to volume with ethanol to prepare a series of laboratory-prepared solutions containing different ratios of both drugs. For RP HPLC-UV, the working standard solutions were used to prepare different concentrations of laboratory prepared mixtures. The concentration of each drug in the laboratory prepared mixtures was calculated by the corresponding regression equation.

Application to their combined pharmaceutical preparation
Twenty tablets of Heterosofir Plus® were grounded and then weighed. A portion of the equivalent to one tablet (400 mg SBR and 90 mg LPV) was accurately weighed and transferred to a 100-mL volumetric flask, sonicated for 30 min with 50 mL of ethanol, and then the volume was completed with the same solvent and filtered to prepare a stock solution, possessing a concentration of 4.00 mg/mL SBR + 0.90 mg/mL LPV.

Accurate aliquots from the stock solution already prepared were applied three times on the TLC plates in the form of bands for the HPTLC-densitometric method. As for RP HPLC-UV method, further dilutions were done using the HPLC mobile phase then injected in triplicate. The concentrations and recoveries were then calculated.

Application of standard addition technique
The validity and accuracy of the proposed chromatographic methods were also checked by applying the standard addition technique. Three accurately weighed portions of the previously powdered tablets, each claimed to contain 400 mg SBR/90 mg LPV, were mixed with pure standards of SBR and LPV as follows: 200 mg SBR/45 mg LPV, 400 mg SBR/90 mg LPV and 600 mg SBR/135 mg LPV, respectively. Each spiked sample was then transferred to a 100-mL volumetric flask, sonicated for 20 min in the ultrasonic bath with 20 mL methanol then the volume was adjusted with the same solvent and filtered to prepare three stock solutions of concentrations: 6.00 mg/mL SBR + 1.35 mg/mL LPV, 8.00 mg/mL SBR + 1.80 mg/mL LPV, and 10.00 mg/mL SBR + 2.25 mg/mL LPV.

For HPTLC-densitometric method, 1 μL from each spiked sample was then applied onto TLC plates in triplicate. The procedure described above was followed to

determine the concentration of each drug using their respective regression equations.

For RP HPLC-UV method, the three stock solutions were then further diluted with the mobile phase to reach concentrations within the linear range for each drug. Then, the general procedure described before for RP HPLC-UV method was followed to determine the concentration of both SBR and LPV.

Results and discussion
The basic target of this research work was to adopt green analytical chemistry through the development of smart chromatographic methods using green solvents, e.g., ethanol instead of the commonly used toxic acetonitrile and toluene instead of the carcinogenic benzene for the simultaneous estimation of SBR and LPV in their co-formulated tablets.

Development and optimization of the proposed chromatographic methods
The adopted chromatographic procedures were developed and fully optimized with a view to develop eco-friendly reversed-phase HPLC-UV and HPTLC-densitometric methods. The principles, concepts, and fundamentals of green analytical chemistry were considered during trying different solvent systems as mobile phases for the development and optimization of the proposed environmentally green chromatographic methods (Clark and Tavener 2007).

HPTLC-densitometric method
The separation and quantitative determination of several mixtures could be done by the well-established and highly applied HPTLC accompanied by densitometric detection. In this work, the adopted method depends on the difference in the value of retardation factor (R_f) between SBR and LPV.

In the HPTLC technique, it is common and better to replace hexane with heptane and replace the extremely carcinogenic benzene with toluene (Alfonsi et al. 2008). In order to obtain the best separation with sharp symmetric peaks, various mobile phase systems with different ratios were concisely tried as follows: ethyl acetate: heptane (2:8, v/v), acetone: heptane (4:6, v/v), toluene: acetone (4:1, v/v). On using the first mobile phase system, both drugs fail to be eluted from their places on the plate. Using the second system, the separation among the two drugs was not enhanced to a great extent giving two tailed peaks for both of them. When using toluene, the acetone (4:1, v/v), SBR, and LPV were greatly eluted but had very close R_f values. Replacing acetone with ethanol enhanced to high extent the separation between the two drugs but with tailed peaks for SBR. On the addition of ammonia (0.2 by volume) to the mobile phase, symmetric, and sharp peaks were obtained with no effect on the separation of the two drugs under

this study. Complete and optimum separation of SBR and LPV was achieved by using the mobile phase of toluene: ethanol: ammonia (4:1:0.2, v/v/v) as a developing system.

In the adopted HPTLC-densitometric method, toluene proves to be a satisfactory and the best substitute for the carcinogenic benzene (Alfonsi et al. 2008). Scanning wavelength effect on the detection sensitivity of the separation method was also ascertained by testing different scanning wavelengths (254, 260, and 265 nm). Scanning at 265 nm gave the optimum and best detection sensitivity with minimum noise for the drugs under the study. After the developed method had been fully optimized, compact, sharp, and symmetric peaks were obtained for SBR and LPV with R_f values = 0.38 and 0.61, respectively as presented in the densitograms in Figs. 2 and 3.

RP HPLC-UV method
RP-HPLC is the most common separation technique with extensive application used in pharmaceutical industries for drug analysis and quality control. Till today acetonitrile is the most preferred and common organic solvent used in RP-HPLC, and this is due to the optimum physical properties of this organic solvent that are greatly appropriate for HPLC separations (Snyder et al. 1997). Pertaining to the toxic properties of acetonitrile and considering its aqueous waste streams that are typically discarded as chemical waste, it may be the best time to take into account greener replacements for acetonitrile in RP-HPLC. Although methanol is less toxic than acetonitrile, methanol containing streams are also treated as chemical waste. A more easily renewable solvent and ecofriendly such as ethanol (Ribeiro et al. 2004; Capello et al. 2007) could permit for the decrease in the environmental impact of waste solvent removal. Ethanol has been recently adopted for the RP-HPLC analysis of cosmetics as an eco-friendly co-solvent (Salvador and Chisvert 2005). Due to the cost of acetonitrile itself and the resulting cost in its waste elimination continue to increase; the switch to ethanol as a greener alternative (with a reduced cost relative to acetonitrile) becomes greatly compulsory. Another important advantage of the use of ethanol as an HPLC solvent is the worldwide availability of this solvent. The recent increase in the use of ethanol as fuel proposes that the cost and quality of ethanol must continue to get improved in the upcoming years. Finally, the reduced environmental effect of ethanol vs. acetonitrile waste streams is another important consideration arguing in favor of the use of this greener solvent for different HPLC procedures (Welch et al. 2009).

In the present research work, ethanol as a greener solvent was adopted for use in this chromatographic
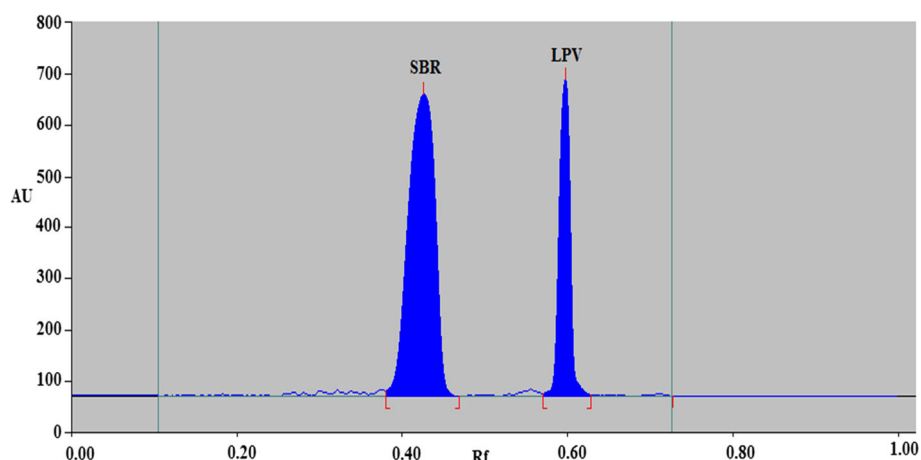
Fig. 2 TLC chromatogram of SBR (12.8 μg/band, $R_f = 0.38$) and LPV (6.4 μg/band, $R_f = 0.61$)

separation which perfectly replaced acetonitrile. Factors which influence the separation had been concisely studied and fully optimized. Different mobile phases as developing systems had been tried several times to reach the best chromatographic separation for the drugs under the study such as potassium dihydrogen orthophosphate (20 mM; pH = 3.5 adjusted by o-phosphoric acid): ethanol (60:40, v/v). This system separated LPV as a very broad peak. When trying potassium dihydrogen orthophosphate (20 mM; pH = 3.5 adjusted by o-phosphoric acid): ethanol (50:50, v/v) as a mobile phase system, it separated SBR peak with tailing. A satisfactory chromatographic separation and resolution was achieved upon using a mobile phase of potassium dihydrogen orthophosphate (20 mM; pH = 3 adjusted by acetic acid):

ethanol (60:40, v/v) which had produced symmetric sharp peaks without peak broadening or tailing for both drugs. Detection wavelength effect on method sensitivity was also evaluated by trying different scanning wavelengths (254, 260, and 265 nm). Detection wavelength at 265 nm was found to produce LOD and LOQ of the lowest values. The best chromatographic separation had been developed on Inertsil C_{18} (4.6 × 150 mm with 5 μm particle size) using 20 mM potassium dihydrogen orthophosphate (adjusted to pH = 3 using acetic acid): ethanol (60:40, v/v) as mobile phase system at a flow rate of 1.0 mL min^{-1} and the effluent was monitored at 265 nm. Two peaks were produced at R_t: 3.03 and 4.07 min for SBR and LPV, respectively, as illustrated in Fig. 4.
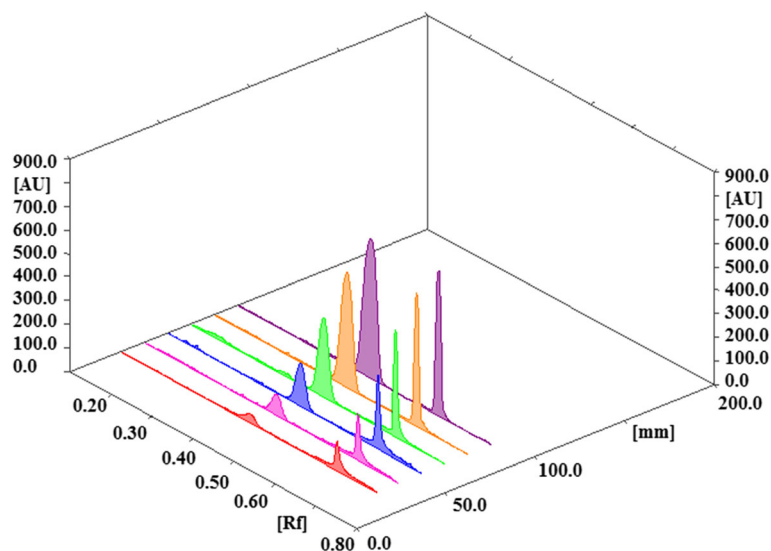

Fig. 3 3D densitogram of SBR ($R_f = 0.38$) in the concentration range of 0.80–25.6 μg/band and LPV ($R_f = 0.61$) in the concentration range of 0.4–12.8 μg/band at 265 nm using toluene: ethanol: ammonia (4:1:0.2, v/v/v) as a developing system
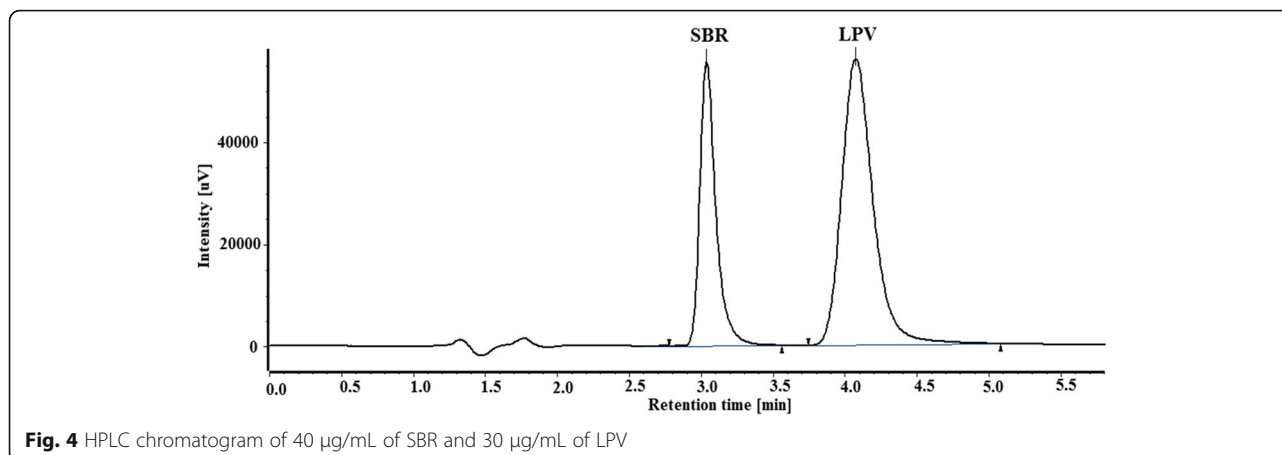
Fig. 4 HPLC chromatogram of 40 μg/mL of SBR and 30 μg/mL of LPV

Validation of the developed chromatographic methods

ICH guidelines for method validation (ICH 2005) were followed for the adopted methods.

System suitability testing

System suitability testing was conducted to confirm that the whole operating system performed in a proper way during routine analysis. Each chromatographic procedure was repeated six times, and then the average for each result parameter was calculated. Satisfactory results for the chromatographic methods' performance including capacity factor, resolution, tailing factor, and selectivity were obtained as presented in Table 1.

Linearity and range

For HPTLC-densitometric method, the relationship between the integrated peak area ratios (using 1.6 μg/band and 0.8 μg/band for SBR and LPV, respectively, as external standard solutions) and different concentrations of each of SBR and LPV was studied with linear and polynomial regression functions. Fitting with polynomial function gave the best correlation with the lowest standard deviation values and was therefore used for the quantitative estimation of SBR and LPV in the range between 0.8–25.6 and 0.4–12.8 μg/band for SBR and LPV, respectively. The polynomial regression of second-order was computed then the regression equations were calculated and found to be:

Table 1 Parameters of system suitability testing of the adopted chromatographic methods for the simultaneous determination of SBR and LPV

Parameters	SBR	LPV	Reference value
HPTLC-densitometric method			
Retardation factor (R_f)	0.38	0.61	
Resolution[a] (R_s)	4.09 [a]	–	$R_s \geq 2$
Tailing factor (T)	0.79	0.56	$T \leq 2$
Capacity Factor (K′)	6.25	7.62	$1 < K' < 10$
Selectivity (a)	3.61[a]	–	$a > 1$
RP HPLC-UV method			
Retention time ($R_{t;\ min}$)	3.03	4.07	
Resolution[a] (R_s)	3.58		$R_s \geq 2$
Tailing factor (T)	1.49	1.26	$T \leq 2$
Asymmetry factor (A_f)	1.04	1.00	$0.9 < A_f < 1.1$
Capacity Factor (K′)	1.32	2.12	$1 < K' < 10$
Selectivity (a)	1.60[a]	–	$a > 1$
Injection repeatability[b]	0.83	0.75	$RSD \leq 1\%$ for $n \geq 6$
Theoretical plates (N)	3936	2108	$N > 2000$
Height equivalent to theoretical plate (HETP; cm plate^{-1})	0.0038	0.0071	The smaller the value, the higher the column efficiency

[a]To LPV
[b]RSD% for six injections

$$A_1 = -0.0223\ C_1{}^2 + 1.0976\ C_1 + 0.3402 \quad r_1 = 0.9998 \quad \text{For SBR}$$

$$A_2 = -0.0248\ C_2{}^2 + 0.6093\ C_2 + 0.5385 \quad r_2 = 0.9998 \quad \text{For LPV}$$

Where A_1 and A_2 are the integrated peak area ratios, C_1 and C_2 are the concentrations in μg/band, and r_1, r_2 are the correlation coefficients of SBR and LPV, respectively.

For RP HPLC-UV method, calibration graphs were constructed by plotting the integrating peak area ratios (using 20.0 μg/mL for SBR and 10.0 μg/mL for LPV as external standard solutions) versus their corresponding concentrations in the range of 10.0–100.0 μg/mL for SBR and 5.0–80.0 μg/mL for LPV. Then the linear regression equations were computed for each of SBR and LPV and were found to be:

$$A_1 = 0.0454\ C_1 + 0.0283 \quad r_1 = 0.9996 \quad \text{For SBR}$$

$$A_2 = 0.1031\ C_2 - 0.0257 \quad r_2 = 0.9998 \quad \text{For LPV}$$

Where A_1 and A_2 are the integrated peak area ratios, C_1 and C_2 are the concentrations in μg/mL, and r_1 and r_2 are the correlation coefficients of SBR and LPV, respectively.

Regression data analysis was computed for both HPTLC-densitometric and RP HPLC-UV methods data sets of SBR and LPV using residual data plots, line fit data plots and normal probability plots as shown in Figs. 5 and 6. The regression equations characteristic parameters of the adopted chromatographic methods are presented in Table 2.

Limit of detection and limit of quantitation

As per ICH recommendations, various approaches for determining the lower limits of detection and quantitation are applicable. The calculation approach using standard deviation value of the intercept and the slope value was conducted to compute limits of detection and quantitation, in which:

$$\text{LOD} = 3.3 \times \text{SD of intercept/slope coefficient}$$

$$\text{LOQ} = 10 \times \text{SD of intercept/slope coefficient}$$

Low values for limit of detection (LOD) and limit of quantitation (LOQ) presented in Table 2 indicated the high sensitivity of the adopted chromatographic methods.

Accuracy

Accuracy of the proposed chromatographic methods was checked by testing different samples of pure SBR and LPV. The respective regression equation computed in each of the adopted methods for each drug was used to calculate their concentrations and the data results are presented in Table 2.
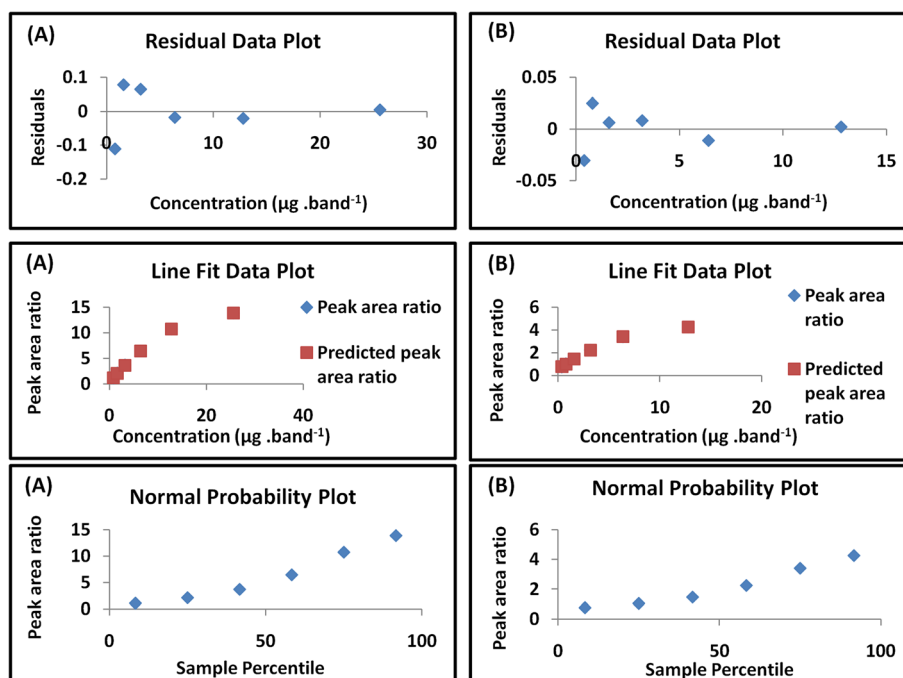

Fig. 5 Residual data plot, line fit data plot, and normal probability plot for SBR (a) and LPV (b) in HPTLC-densitometric method
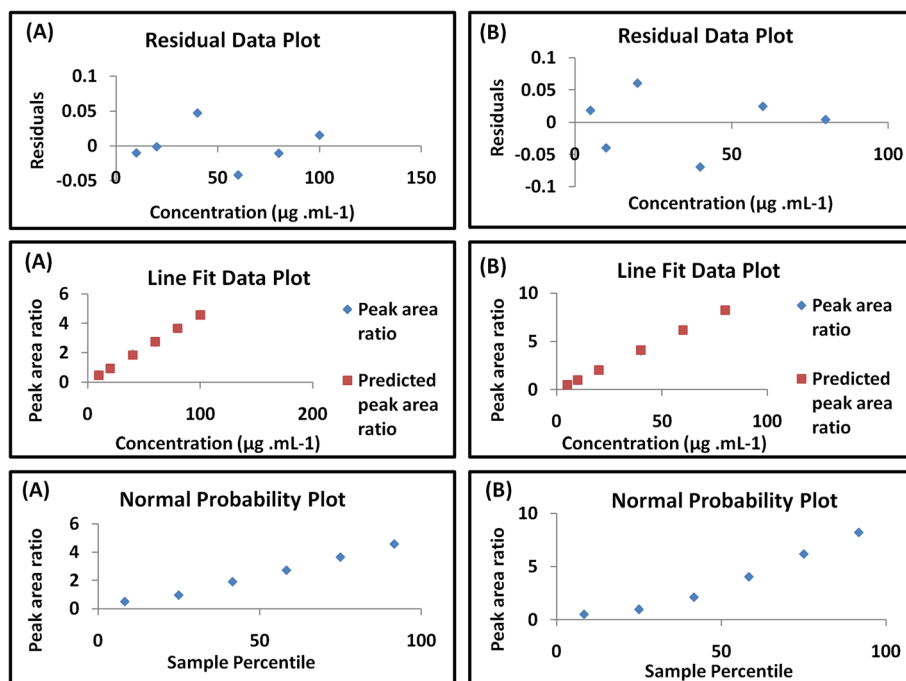
Fig. 6 Residual data plot, line fit data plot, and normal probability plot for SBR (a) and LPV (b) in RP HPLC-UV method

Precision

Intra-day Three concentrations (1.6, 6.4, and 12.8 μg/band) of SBR and (0.8, 3.2, and 6.4 μg/band) of LPV for HPTLC-densitometric method and (20.0, 40.0, and 80.0 μg/mL) of SBR and (10.0, 20.0, and 60.0 μg/mL) of LPV for RP HPLC-UV method were tested in the same day three times using the developed chromatographic methods. Satisfactory % RSD values were obtained which confirmed the repeatability of the adopted methods as presented in Table 2.

Table 2 Validation parameters of the regression equations and assay results of the proposed chromatographic methods for the simultaneous quantitation of SBR and LPV

Validation parameters	HPTLC-densitometric method		RP HPLC-UV method	
	SBR	LPV	SBR	LPV
Linearity range	0.8–25.6 (μg band^{-1})	0.4–12.8 (μg band^{-1})	6.00–100.00 (μg ml^{-1})	4.0–80.0 (μg ml^{-1})
Slope (X^2 coefficient)	− 0.0223	− 0.0248	–	–
SE of Slope (X^2 coefficient)	0.0006	0.0007	–	–
Slope (X coefficient)	1.0976	0.6093	0.0454	0.1031
SE of slope (X coefficient)	0.0170	0.0094	0.0004	0.0008
Intercept	0.3402	0.5385	0.0283	− 0.0257
SE of intercept	0.0688	0.0191	0.0258	0.0353
Correlation coefficient (r)	0.9998	0.9998	0.9996	0.9998
LOD	0.21 (μg band^{-1})	0.10 (μg band^{-1})	1.88 (μg ml^{-1})	1.13 (μg ml^{-1})
LOQ	0.63 (μg band^{-1})	0.31 (μg band^{-1})	5.69 (μg ml^{-1})	3.42 (μg ml^{-1})
Accuracy [a]	99.78 ± 0.970	100.04 ± 1.090	99.87 ± 1.270	99.64 ± 1.190
Precision [b]				
Intra-day	0.575	0.862	0.613	1.276
Inter-day	1.274	1.543	1.628	1.903

[a]Mean ± SD
[b]RSD%, *n* = 9

Inter-day The chromatographic procedures mentioned above were conducted on three different days for the quantification of the three chosen concentrations of SBR and LPV. Satisfactory values for % RSD were obtained as illustrated in Table 2.

Specificity

Method specificity was tested by how accurately, precisely, and specifically the drug of interest is estimated in presence of other components (e.g., co-formulated drugs, impurities, related substances, or possible degradation products). This is assured from HPTLC 2D and 3D densitograms presented in Figs. 2 and 3, respectively, and HPLC chromatogram shown in Fig. 4 which gives the evidence of the optimum specificity of the adopted methods. Accepted results presented in Table 3 indicate the good specificity of the adopted chromatographic methods for the simultaneous determination of SBR and LPV in different proportions.

Robustness

Analytical method robustness is its capability to remain uninfluenced with small deliberate changes in method parameters which gives an adequate indication of the reliability of the proposed chromatographic method during routine work, e.g., changing mobile phase ratio ($\pm$ 0.2 mL) for HPTLC-densitometric method and changing mobile phase ratio ($\pm$ 2.0 mL) and pH of the mobile phase ($\pm$ 0.1 unit) in RP HPLC-UV method. The low % RSD values show that the adopted chromatographic methods are robust and that the deliberate minor changes in the chromatographic factors mentioned above produced no significant changes in R_t or R_f values, T, N, and Rs of the chromatographed peaks as presented in Table 4.

Application to their combined pharmaceutical preparation

The adopted chromatographic methods were then applied for the determination of SBR and LPV in their combined tablet dosage form. The obtained results were acceptable and satisfactory with low values of % RSD as shown in Table 5.

Moreover, validity and accuracy of the proposed chromatographic methods were also confirmed by standard addition technique application where satisfactory recoveries were obtained which assured that there was not any interference due to added excipients in the tablet matrix, as illustrated in Table 6.

Statistical comparison to an in-house method

The statistical comparison of the data results obtained from the application of the suggested chromatographic methods with those obtained from the application of a

Table 3 Simultaneous determination of SBR and LPV in laboratory prepared mixtures by the developed chromatographic methods

HPTLC-densitometric method			
Laboratory-prepared mixture		% Recovery[a]	
SBR (μg band^{-1})	LPV (μg band^{-1})	SBR	LPV
8.00	0.90	98.53	100.43
4.00	1.80	99.72	99.92
4.00	0.90	100.99	98.99
4.00	0.45	99.81	101.01
2.00	0.90	101.11	98.81
Mean $\pm$ SD%		100.03 $\pm$ 1.059	99.83 $\pm$ 0.936
RP HPLC-UV method			
Laboratory-prepared mixture		% Recovery[a]	
SBR (μg ml^{-1})	LPV (μg ml^{-1})	SBR	LPV
80.00	9.00	98.14	98.17
40.00	18.00	99.32	98.92
40.00	9.00	99.72	99.57
40.00	4.50	100.38	98.72
20.00	9.00	98.61	98.61
Mean $\pm$ SD %		99.23 $\pm$ 0.886	98.80 $\pm$ 0.512

[a]Average of 3 experiments

reported RP-HPLC method (Rote et al. 2017) presented no significant statistical difference with confidence limit of 95% in concern of both accuracy and precision as described in Table 7.

Conclusion

The plan for the replacement of the traditional widely-used toxic solvents and chemicals with less toxic and inert ones presents environmentally benign alternatives to the most toxic ones in the pharmaceutical analysis field. This study demonstrates that the developed

Table 4 Robustness testing[a] of the adopted chromatographic methods for the simultaneous estimation of SBR and LPV

HPTLC-densitometric method					
Condition	SBR			LPV	
	R_f	T	R_s[b]	R_f	T
Mobile phase ratio ($\pm$ 0.2 mL)	1.501	1.315	1.513	1.412	1.543

RP HPLC-UV method							
Condition	SBR				LPV		
	R_t	T	N	R_s[b]	R_t	T	N
Mobile phase ratio ($\pm$ 2.0 mL)	0.796	0.317	0.398	1.573	0.843	0.486	1.059
pH of of the mobile phase ($\pm$ 0.1 unit)	0.264	0.341	0.486	1.583	0.386	0.563	1.251

[a]RSD% (*n* = 6)
[b]Resolution to LPV

Table 5 Simultaneous estimation of SBR and LPV in Heterosofir Plus® tablets using the adopted chromatographic methods

HPTLC-densitometric method

Pharmaceutical preparation	Sample no.	Taken amount (μg band^{-1})		Found[a] amount (μg band^{-1})		% Recovery	
		SBR	LPV	SBR	LPV	SBR	LPV
Heterosofir Plus® tablets labeled to contain 400 mg SRB + 90 mg LPV per tablet	1	4.00	0.90	4.02	0.90	100.50	100.00
	2	8.00	1.80	8.10	1.79	101.25	99.44
	3	16.00	3.60	15.92	3.62	99.50	100.56
	Mean ± % RSD					100.42 ± 0.878	100.00 ± 0.556

RP HPLC-UV method

Pharmaceutical preparation	Sample no.	Taken amount (μg.ml^{-1})		Found[a] amount (μg.ml^{-1})		% Recovery	
		SBR	LPV	SBR	LPV	SBR	LPV
Heterosofir Plus® tablets labeled to contain 400 mg SRB + 90 mg LPV per tablet	1	20.00	4.50	20.14	4.51	100.70	100.22
	2	40.00	9.00	40.03	8.83	100.08	98.11
	3	80.00	18.00	79.52	17.92	99.40	99.56
	Mean ± % RSD					100.06 ± 0.650	99.30 ± 1.079

[a]Mean of three determinations

chromatographic methods using green solvents are found to be quite specific, sensitive, accurate, reproducible, and precise. Satisfactory results for method validation parameters can encourage the use of greener analytical approaches in quality control field. The proposed HPTLC-densitometric and RP HPLC-UV methods can be used in quality control laboratories for the routine analysis and the simultaneous quantitative determination of SBR and LPV in their combined dosage form (Hetersofir Plus®). The well-known advantages of the HPTLC-densitometric method is that different samples can be run simultaneously using a small volume of the mobile phase system with short run time while RP HPLC-UV method offers optimum resolution, good specificity, higher sensitivity and wider range of quantitation over previously published and reported

Table 6 Results of standard addition technique application on Heterosofir Plus® tablets to the simultaneous analysis of SBR and LPV using the proposed chromatographic methods

HPTLC-densitometric method

Sample no.	Claimed conc. (μg band^{-1})		Pure added conc. (μg band^{-1})		Pure found conc. (μg band^{-1})		% Recovery[a] of the added conc.	
	SBR	LPV	SBR	LPV	SBR	LPV	SBR	LPV
1	4.00	0.90	2.00	0.45	1.98	0.45	99.00	100.00
2	4.00	0.90	4.00	0.90	4.04	0.89	101.00	98.89
3	4.00	0.90	6.00	1.35	6.03	1.33	100.50	98.52
Mean ± SD %							100.17 ± 1.041	99.14 ± 0.771

RP HPLC-UV method

Sample no.	Claimed conc. (μg ml^{-1})		Pure added conc. (μg ml^{-1})		Pure found conc. (μg ml^{-1})		% Recovery[a] of the added conc.	
	SBR	LPV	SBR	LPV	SBR	LPV	SBR	LPV
1	40.00	9.00	20.00	4.50	19.78	4.52	98.90	100.44
2	40.00	9.00	40.00	9.00	40.02	9.11	100.05	101.22
3	40.00	9.00	60.00	13.50	60.90	13.26	101.50	98.22
Mean ± RSD %							100.15 ± 1.303	99.96 ± 1.557

[a]Mean of three determinations

Table 7 Results of statistical comparison[a] between the developed chromatographic methods and reported HPLC method (Rote et al. 2017) used for the simultaneous determination of SBR and LPV

Parameters	Proposed HPTLC-densitometric method		Proposed RP HPLC-UV method		In-house HPLC method[b]	
	SBR	LPV	SBR	LPV	SBR	LPV
Mean	99.78	100.04	99.87	99.64	100.90	100.08
SD	0.970	1.090	1.270	1.190	0.835	0.553
Variance	0.941	1.188	1.613	1.416	0.697	0.306
t test[a]	1.957	0.073	1.515	0.750	–	
F-test[a]	1.349	3.885	2.313	4.631	–	

[a]The theoretical values of t and F at $P = 0.05$ are (2.306) and (6.388), respectively where $n = 5$
[b]RP-HPLC method was performed on the Systronics isocratic HPLC system equipped with SP930 D HPLC pump and dual wavelength UV-VIS detector and C18 column (250 mm × 4.6 mm, 5 μm), using the mobile phase (Methanol: Water 83:17 v/v) pH 3.0 with 0.05% acidic acid at a flow rate of 1.0 ml/min, injection volume 20 μl and UV detection at 245 nm

HPLC methods with the desired accuracy and reproducibility through reasonable analysis time. An important conclusion of this study is that these green eluents have the ability to be used for different analyses in chromatographic science and thus making the separation process more ecofriendly to the surrounding environment. Safer alternatives should be studied on the basis of their safety, health, life cycle, and environmental assessment to replace the traditional hazardous solvent adopting green separation science because developing green analytical methods to replace the traditional ones becomes a very important requirement. The use of a greener solvent such as ethanol would be strongly preferred for such applications.

Abbreviations
DAD: Diode array; HPLC: High-performance liquid chromatography; HPTLC: High-performance thin layer chromatography; ICH: International Conference on Harmonization; LOD: Limit of detection; LOQ: Limit of quantification; LPV: Ledipasvir; MS/MS: Tandem mass; R_f: Retardation factor; RP: Reversed phase; RSD: Relative standard deviation; R_t: Retention time; SBR: Sofosbuvir; SD: Standard deviation; UV: Ultraviolet

Acknowledgements
Not applicable.

Authors' contributions
Both AH and MSE participated in the design of the study. AH carried out HPLC-UV method, performed the validation parameters and the statistical analysis for the method and helped to draft the manuscript while MSE carried out HPTLC-densitometry method, performed the validation parameters and the statistical analysis for the method and put the manuscript in the final form for journal submission. All authors read and approved the final manuscript.

Funding
The authors receive no fund to develop this study.

Availability of data and materials
Not applicable.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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