

Assay and Dissolution Methods Development and Validation for Simultaneous Determination of Sofosbuvir and Ledipasvir by RP-HPLC Method in Tablet Dosage Forms

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Abstract

Specific, accurate, simple, selective and stability-indicating RP-HPLC method is developed and validated for simultaneous determination of Sofosbuvir and Ledipasvir in tablet dosage form for assay and dissolution methods. RP-HPLC method was performed on the Eclipse XDB C18 column (250 mm X 4.6 mm, 5 μ m particle size, using buffer solution of pH 3.0 containing 0.02 M potassium dihydrogen phosphate and 5.7 mM hexane sulfonate:acetonitrile (50:50 v/v) as the mobile phase at a flow rate of 1.5 ml/min, injection volume 10 μ L and UV detection at 254 nm. This method is validated according to BP, USP and ICH requirements for new methods, which include accuracy, precision, selectivity, robustness, ruggedness, LOD, LOQ, linearity and range. Linear relationships were obtained in the ranges of 40-500 μ g/mL and 30-300 μ g/mL with correlation coefficients of 0.9998, 0.9996, 0.9996 and 0.9993 at R_t value of 2.429 min and 4.529 min for Sofosbuvir and Ledipasvir respectively for assay content and dissolution rate. The forced degradation studies as acidity, alkalinity, oxidation, heat, thermal, humidity and photo degradation were performed according to ICH guidelines. The accurate determination of both drugs is very important for Forensic and Criminal Investigations from the point of view of Forensic pharmacy.

Keywords: Sofosbuvir; Ledipasvir; Forced degradation; Tablet dosage form; Assay; Dissolution; HPLC; Forensic pharmacy

Abbreviations: EDHS: Egyptian Demographic Health Survey; SOF: Sofosbuvir; FDC: Fixed-Dose Combination; LDV: Ledipasvir; IS: Internal Standard; DAC: Daclatasvir; AD: Alcohol Dependence; HCV: Hepatitis C virus; HIV: Human Immuno deficiency Virus; EPCI :Egyptian Pharmaceutical and Chemical Industry

Introduction

Globally, between 130-150 millions of people have chronic hepatitis C infection. A significant number of those who are chronically infected will develop liver cirrhosis or liver cancer. Approximately 700 000 people die each year from hepatitis C-related liver diseases [1]. Egypt has the largest epidemic of HCV in the world according to the released Egyptian Demographic Health Survey [EDHS] and the overall prevalence (percentage of people) positive for antibody to HCV in Egypt was 14.7% [2]. The current population in Egypt is about 90 millions, thus \approx 13, 2millions of persons who have been infected with this virus. Gilead Sciences overcome most common related liver diseases by its Great invention (Harvoni). Harvoni (90 mg ledipasvir/400 mg sofosbuvir) approved by United States FDA. It is indicated for the treatment of chronic HCV genotypes 1, 4, 5, and 6 in adults and also indicated for the treatment of

chronic HCV in patients co-infected with HIV. Sofosbuvir (SOF); is chemically known as (*S*)-Isopropyl 2-(((*S*)-(((2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)-(phenoxy)phosphorylamino)propanoate. It has a molecular formula of $C_{22}H_{29}FN_3O_9P$ and a molecular weight of 529.45 (Figure 1).

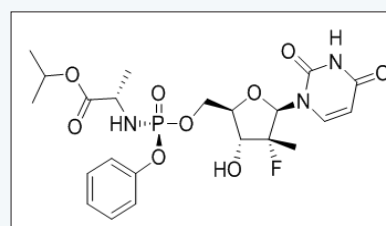


Figure 1: Chemical structure of Sofosbuvir.

Sofosbuvir is a white to off-white powder with a solubility of ≥ 2 mg/mL across the pH range of 2-7.7 at 37°C. The partition coefficient (log P) for Sofosbuvir is 1.62 and the pKa is 9.3 [3]. Sofosbuvir is a pangenotypic inhibitor of the HCV NS5B RNA-dependent RNA polymerase, which is essential for viral replication [4]. Sofosbuvir is a nucleotide prodrug that undergoes intracellular activation to form GS-461203 (active triphosphate, not detected in plasma), and ultimately the inactive, renally eliminated metabolite GS-331007 [5]. The pharmacologically active uridine analog triphosphate (GS-461203) can be incorporated by HCV NS5B and acts as a chain terminator. In a biochemical assay, GS-461203 inhibits the polymerase activity of the recombinant NS5B from HCV genotype 1b, 2a, 3a and 4a with an IC₅₀ value ranging from 0.7 to 2.6 μ M. GS-461203 is neither an inhibitor of human DNA and RNA polymerases nor an inhibitor of mitochondrial RNA polymerase [6]. Sofosbuvir/Ledipasvir is a fixed-dose combination (FDC) tablet containing Sofosbuvir (a previously approved NS5B polymerase inhibitor) and ledipasvir, a new NS5A-inhibitor [3]. Ledipasvir (LDV); is chemically known as *Methyl [(2S)-1-((6S)-6-[5-(9,9-difluoro-7-{2-[(1R,3S,4S)-2-((2S)-2-[(methoxycarbonyl)amino]-3-methylbutanoyl]-2-azabicyclo[2.2.1]hept-3-yl]-1H-benzimidazol-6-yl)-9H-fluoren-2-yl]-1H-imidazol-2-yl]-5-azaspiro[2.4]hept-5-yl]-3-methyl-1-oxobutan-2-yl]carbamate*.

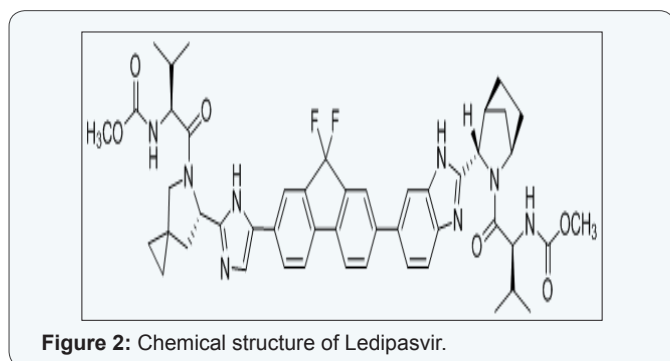


Figure 2: Chemical structure of Ledipasvir.

It has a molecular formula of C₄₉H₅₄F₂N₈O₆ and a molecular weight of 889.00 (Figure 2). Ledipasvir is a white to tinted (off-white, tan, yellow, orange, or pink), slightly hygroscopic crystalline solid. Ledipasvir is practically insoluble (<0.1 mg/mL) across the pH range of 3.0-7.5 and is slightly soluble below pH 2.3 (1.1 mg/mL). The partition coefficient (log P) for ledipasvir is 3.8 and the pKa₁ is 4.0 and pKa₂ is 5.0 [3]. Ledipasvir is an HCV inhibitor targeting the HCV NS5A protein, which is essential for both RNA replication and the assembly of HCV virions. Biochemical confirmation of NS5A inhibition of ledipasvir is not currently possible as NS5A has no enzymatic function. In vitro resistance selection and cross-resistance studies indicate ledipasvir targets NS5A as its mode of action [6]. The combination of these two drugs is not official in any pharmacopoeia [7, 8]. Very recently, a limited number of methods have been developed for the individual and simultaneous determination of both drugs. The degradation products of SOF under several stress conditions have been determined by HPLC [9,10]. SOF's disposition was

characterized into various in vivo cell types [11].

Sofosbuvir in human plasma was determined by UPLC-MS/MS method [12]. Quantification of Sofosbuvir and its metabolite, GS-331007, in human plasma has been determined by UPLC-ESI-MS/MS method [13]. Simultaneous quantification of ribavirin, sofosbuvir and its metabolite in rat plasma by UPLC-MS/MS has been reported [14]. SOF in pure form [15], in bulk and tablet dosage form was determined by RP-HPLC [16]. Finally, sofosbuvir (SOF) was used as an internal standard (IS) in an UPLC-MS/MS method for the determination of daclatasvir (DAC) in human plasma [17]. While for LDV, only two methods have been published for its individual determination in bulk drug form by simple UV spectrophotometry [18] and by RP-HPLC [19]. Both sofosbuvir and ledipasvir in human plasma were determined by UPLC-MS/MS method [20] and besides some antiviral agents [21]. Ledipasvir, sofosbuvir and its metabolite in rat plasma were also, determined by UPLC-MS/MS [22].

According to the best of our knowledge, only one HPLC method [23] has been published, during the preparation of the present work for publishing. The present study aims to develop a simple, sensitive, short retention time and accurate RP-HPLC method for the simultaneous determination of both SOF and LDV together in pure and tablet dosage forms with high sensitivity, selectivity that are required to forensic pharmacy which is an application of the sciences of drugs to legal issues. Forensic pharmacists engage in work relating to litigation, the regulatory process, and the criminal justice system. Forensic pharmacy overlaps with many other forensic fields. A forensic pharmacist can be a valuable resource in legal cases relating to malpractice, adverse drug reactions, drunk and drugged driving, health care fraud, poisoning, and numerous other types of civil and criminal cases [24]. Forensic science can e.g., reveal old alcohol dependence (AD), the internal comorbidities and the presence of any psychiatric comorbidity. Liver problems are one of the most common causes of alcohol-related liver damage. 45% of deaths from cirrhosis are alcohol-related [25]. Validation of the developed methods was performed according to ICH guidelines [26].

Experimental

Chemicals and reagents

- i. Pure samples: Pure samples of sofosbuvir and ledipasvir were kindly supplied by the Egyptian Pharmaceutical and Chemical Industry (EPCI); EPCI pharmaceutical company which is a part of Hikma group, Beni-Suef, Egypt with claimed purity of 99.8% according to manufacturer certificates of analysis.
- ii. Pharmaceutical dosage form: Heterosofir Plus (Harvoni®) 400 mg/90 mg 28 FCT (Batch No. 141610A) were manufactured by Pharmed Healthcare, 7th industrial zone, Al Sadat city, Al Menufia-Egypt. Each tablet is claimed to contain 400 mg of sofosbuvir & 90 mg ledipasvir.

iii. Chemicals: Acetonitrile, Methanol, water HPLC-grades, potassium dihydrogen orthophosphate analytical grade and 1-sodium hexane sulfonate were procured from (scharlau, Spain).

Instrumentation

i. HPLC system (Agilent 1260 Infinity, Germany) instrument was equipped with an Agilent 1260 Infinity preparative pump (G1361A), Agilent 1260 Infinity DAD detector VL (G131SD), Agilent 1260 Infinity Thermostated column compartment (G1316A) and Agilent 1260 Infinity preparative Auto sampler (G2260A). Separation and quantitation was performed on Agilent Eclipse XDB- C18 (250 X 4.6 mm i.d, 5 μ m particle size).

ii. HPLC system (Shimadzu LC SPD 20 A) with a detector (dual wavelength), equipped with a binary pump, Auto sampler, oven CTO-20A/20AC with temperature range (10-85°C), LC Solution software.

iii. The UV- 1800 double beam UV-Visible spectrophotometer (Shimadzu-Japan) with highest resolution which spectral bandwidth is (1 nm from 190-1100 nm range) was used for all absorbance measurements. Matched with 1cm quartz cells. Perform data analysis by software (UV-Probe 2.5.2).

iv. Dissolution apparatus (DIS 6000).

v. Stability chamber (VOSTCH VP 1300, Germany).

A. Mobile phase preparation: Buffer: Acetonitrile (50:50): Phosphate buffer was prepared by dissolving 2.72 gm of potassium dihydrogen phosphate and 1.167 gm of Hexanesulphonic acid of sodium salt monohydrate in 700 ml HPLC grade water and sonicated to dissolve, adjust pH to 3.0 by orthophosphoric acid solution. Make up to 1000 ml with HPLC grade water, filtered and degassed mixtures of buffer and acetonitrile (50:50) through 0.45 μ m membrane filter under vacuum pump.

i. Diluent: Methanol HPLC-grade

B. HPLC Chromatographic Conditions: Chromatographic separation was performed on column Agilent Eclipse XDB- C8 (250 X 4.6 mm i.d, 5 μ m particle size). Using a mobile phase mixture of phosphate buffer and acetonitrile in the ratio of 50:50% v/v at ambient temperature, flow rate of 1.5 mL/min, UV detection was performed at 254 nm, injection volume was 10 μ L and run time was 8.0 min.

i. Preparation of Standard Solution and Test solution for Assay: Stock standard solutions of Sofosbuvir and Ledipasvir: 40 mg of SOF and 9.0 mg of LDV working standards were weighed accurately, transferred into 20 ml volumetric flask, add 10 ml of diluent and sonicate to dissolve. Make up to the mark with the same diluent and mix (Conc. of SOF 2000 μ g/

mL and LDV 450 μ g /mL).

ii. Authentic Prepared Mixture: Accurately transfer 1.0 ml of each of SOF and LDV from their stock solutions into 10 ml volumetric flask, add diluent and sonicate to dissolve. Make up to the mark with the same diluent and mix. Inject into the chromatographic system (Conc. of SOF 200 μ g/mL and LDV 45 μ g/mL). The chromatogram obtained was shown in (Figure 3).

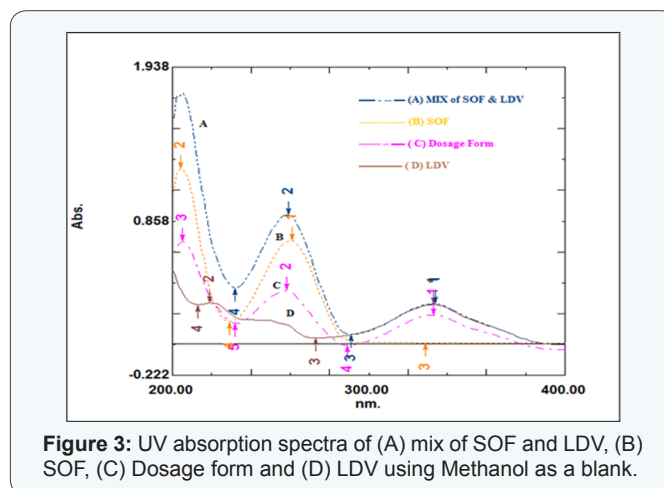


Figure 3: UV absorption spectra of (A) mix of SOF and LDV, (B) SOF, (C) Dosage form and (D) LDV using Methanol as a blank.

iii. Application to pharmaceutical formulation (Heterosofir Plus 400 mg/90 mg 28 FCT): Weigh not less than 10 tablets and determine the average weight of one tablet. Grind to fine powder. Transfer an accurately weighed weight from powdered tablets equivalent to the average weight of one tablet into a 200-ml volumetric flask. Add about 100 ml diluent, sonicate till dissolve and complete to volume with the same diluent and mix well. Take, from the prepared flask, 10 ml into 100 ml volumetric flask and complete volume with diluent. Filter through 0.45 μ m syringe filter and inject into the chromatographic system. The chromatogram obtained was shown in (Figure 4). Also the standard addition technique has been carried out to assess the validity of the method by spiking the pharmaceutical formulation with known amount of standard solution of SOF and LDV. The recovery of the added standards was then calculated after applying the proposed methods.

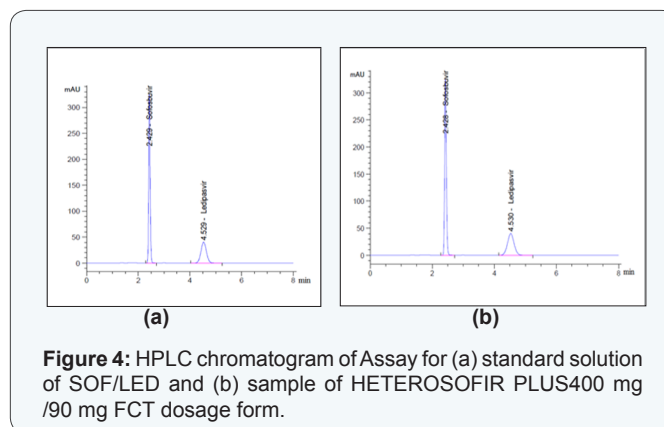


Figure 4: HPLC chromatogram of Assay for (a) standard solution of SOF/LED and (b) sample of HETEROSOFIR PLUS400 mg /90 mg FCT dosage form.

iv. Preparation of dissolution media, standard solution and test solution

a) Dissolution parameters

a. Medium: Phosphate buffer pH 6.8, Volume: 1000 ml, Apparatus: USP Type-II (Paddle), RPM: 75 at temperature $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$

b. Time: 10, 15, 25, 35, 45 minutes

b) Dissolution media: Weigh 6.8 g of potassium dihydrogen phosphate and 0.89 g of sodium hydroxide into 1000 ml volumetric flask then adjust the pH to 6.8 ± 0.1 using phosphoric acid or sodium hydroxide solutions.

v. Stock standard solution of sofosbuvir and ledipasvir:

40 mg of SOF and 9.0 mg of LDV working standards were weighed accurately, transferred into 20 ml volumetric flask, add 10 ml of diluent and sonicate to dissolve. Make up to the mark with the same diluent and mix (Conc. of SOF 2000 $\mu\text{g}/\text{mL}$ and LDV 450 $\mu\text{g}/\text{mL}$).

vi. Lab prepared mixture:

From the stock solution, transfer 1.0 ml into 10 ml volumetric flask then complete to volume using dissolution medium and inject into the chromatographic system. The obtained chromatogram was shown in (Figure 5).

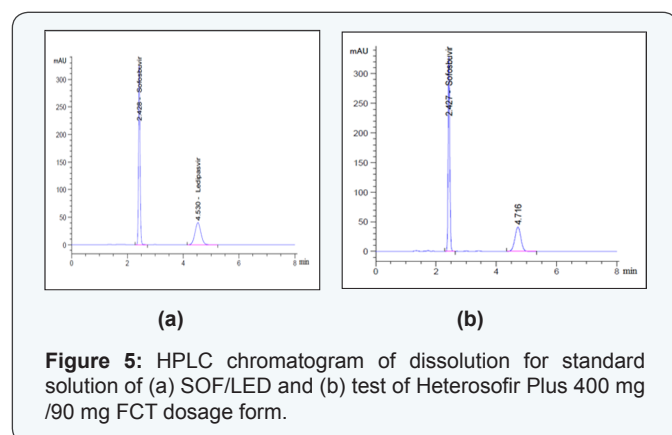


Figure 5: HPLC chromatogram of dissolution for standard solution of (a) SOF/LED and (b) test of Heterosofir Plus 400 mg /90 mg FCT dosage form.

vii. Test solutions: Fill the vessels with 1000 ml dissolution medium at $37 \pm 0.5^{\circ}\text{C}$. Place one tablet in each vessel, apply the conditions specified. After the specified time withdraw a portion of 50 ml of the dissolution medium, cool to ambient temperature. Filter through 0.45 μm syringe filter, discard the first 25 ml of filtrate and transfer 10 ml of filtrate into 20 ml volumetric flask then complete to volume using the dissolution medium and inject into the chromatographic system. The obtained chromatogram was shown in (Figure 5).

viii. Construction of calibration curves: Different concentrations of SOF and LDV equivalent to (40-500) $\mu\text{g}/\text{mL}$ and (30-300) $\mu\text{g}/\text{mL}$ for SOF and LDV, respectively, were separately weighed from their respective working standards

into separate series of 100 mL volumetric flasks, and the volumes were made up to volume with the diluent. Duplicate 10 μL injections were made for each concentration maintaining the flow rate at 1.5 mL/min and the effluent was UV- scanned at 254 nm. The chromatographic separation was performed following the procedure under chromatographic conditions. The chromatograms were recorded, peak areas of SOF and LDV were determined and the calibration curves relating the obtained integrated peak areas to the corresponding concentrations were constructed and the regression equations were performed.

Results

Chromatograms were obtained at retention times 2.429 min for SOF and 4.529 min for LDV. No occurrences of interfering peaks have been recorded.

Discussion

Very recently, while our work is in the course of publication, a publication [23] that related the simultaneous determination of both SOF and LDV by RP-HPLC method in Tablet dosage form has been published in 29/09/2016; this was performed on Luna analytical column octyl silica packing ($\text{Si}-[\text{CH}_2]_7-\text{CH}_3$) C_8 , using ammonium acetate buffer solution at pH 7.0 and acetonitrile 35:65% v/v as mobile phase at a flow rate of 0.7 mL min^{-1} for isocratic elution using a UV detector at 245 nm. The retention times of sofosbuvir and ledipasvir were 4.468 ± 0.013 min and 8.242 ± 0.012 min, respectively, and the total run time was 20min. The present method has been performed on Eclipse XDB C8 column (250 mm X 4.6 mm, 5 μm particle size, using buffer solution of pH 3.0 containing 0.02 M potassium dihydrogen phosphate and 5.7 mM hexane sulfonate:acetonitrile (50:50 v/v) as the mobile phase at a much slower flow rate of 1.5 ml/min, injection volume 10 μL and UV detection at 254 nm.

Advantages of the present method is that less time is consumed for analysis, where the retention times of sofosbuvir and ledipasvir were 2.429 min and 4.529 min, respectively, and the total run time was 8.0 min. compared with 20min. for the contemporaneous method. The conditions of the present method succeeded in achieving linear relationships in the ranges of 40-500 $\mu\text{g}/\text{mL}$ and 30-300 $\mu\text{g}/\text{mL}$, respectively, compared with corresponding ranges of 8.0-96.0 $\mu\text{g}/\text{mL}$ for SOF and 1.8 -21.6 $\mu\text{g}/\text{mL}$ for LDV [23]. The dynamic range of LDV is quite limited [23]. Also, significantly different and short R_t values of 2.429 min and 4.529 min. for SOF and LDV were achieved with correlation coefficients of 0.9999 and 0.9997, respectively compared to corresponding R_t values of 4.468 and 8.242 min with correlation coefficients of 0.9999 and 0.9999, respectively [23]. In the present work, heat stress studies and photo degradation have been performed. A comparison between the two methods was recorded in (Table 1).

Table 1: Regression and validation parameters of the proposed HPLC method for determination of SOF and LDV for assay and dissolution.

Parameter	Present Method		Zaman Method	
	SOF	LDV	SOF	LDV
Mobile phase	0.02 M potassium dihydrogen phosphate and 5.7 mM hexane sulfonate:Acetonitrile (50:50 v/v)		Ammonium acetate buffer solution pH 7.0 and acetonitrile 35:65% v/v	
Column Used	Eclipse XDB C8 column (250 mm X 4.6 mm, 5 µm		Luna analytical column octyl silica packing (Si-[CH ₂] ₇ -CH ₃) C ₈	
Flow rate	1.5 ml/min		0.7 ml/min	
Wave length	254 nm		245 nm	
Retention time	2.429 min	4.529 min	4.468 min	8.242 min
Run time	8.0 min		20 min	
Ranges (µg/mL)	40-500	30-300	8.0-96.0	1.8-21.6
Correlation coefficient (Assay)	0.9999	0.9999	0.9997	0.9999
Correlation coefficient (Dissolution)	Not Done	Not Done	0.9996	0.9997
Accuracy (Mean±RSD) (Assay)	99.33±0.42	99.51±0.38	99.03±1.38	99.60±0.74
Accuracy (Mean±RSD) (Dissolution)	Not Done	Not Done	100.00±1.31	99.44±0.72
Purity factor	Not Done	Not Done	999.955	999.993
Standard addition	Not Done	Not Done	Done	Done
Forced degradation	Not Done	Not Done	Done	Done
Dissolution profile	Done	Done	Done	Done
Comparative dissolution	Done	Done	Not Done	Not Done

Methods development and optimization

Different developing systems of different compositions and ratios were tried including: methanol: water (70:30, v/v), Acetonitrile: water (70:30, v/v). Sodium Acetate pH (5.0): ACN (50:50, v/v) and Sodium dihydrogen phosphate pH (4.0): ACN (50:50, v/v) no peak was observed for LDV, phosphate buffer pH (3.0) + hexane sulfonate: ACN (50:45, v/v). It was found that presence of hexane sulfonate in the developing system is essential for separation and improving tailing of peaks, but still eluted late. Finally, using mixture of buffer phosphate pH (3.0):ACN (50:50), it was found that this mixture is the best for the separation of both drugs and their degradation products. Different flow rates were tried (0.8, 1.2, 1.4 and 1.5 ml/min), scanning wavelengths (200-400 nm) were also tried for both drugs in pure form (Conc. of SOF 40 µg/mL and LDV 9 µg/mL), laboratory prepared mixture and dosage form, 254 nm was the best suitable absorbance for both drugs (Figure 3). Preliminary studies involved trying C18, C8 reversed-phase columns. The best developing system was phosphate buffer pH (3.0) + hexane sulfonate: ACN (50:50, v/v) at flow rate of 1.5 ml/min and at

wavelength of 254.0 nm using column Agilent Eclipse XDB- C8 (250 mm X 4.6 mm i.d., 5 µm). This selected developing system allows good separation between the drug and its degradation with good R_t values without tailing of the separated bands and good theoretical plates.

Method validation

The method was validated, in accordance with ICH guidelines (ICH Q2R1), for system suitability, precision, accuracy, linearity, specificity, ruggedness, robustness, LOD and LOQ [26].

Linearity and range

The linearity of the proposed methods was obtained in the concentration range (40.0 to 500.0 µg/ml) for sofosbuvir and (30.0 to 300.0 µg/ml) for ledipasvir for assay content and dissolution rate. Calibration graphs were plotted on the basis of analysis of each calibration solution. The coefficient of regression obtained was 0.9999 for SOF and 0.9997 for LDV for assay content and 0.9997 for SOF and 0.9996 for LDV for dissolution rate. Linearity results were shown in (Table 2).

Table 2: Regression and validation parameters of the proposed HPLC method for determination of SOF and LDV for assay and dissolution.

Parameter	Assay		Dissolution	
	SOF	LDV	SOF	LDV
Linear range(µg/mL)	40-500	30-300	40-500	30-300
Slope	6.3006	6.4685	6.3441	6.3586
Intercept	0.8149	12.0601	5.6967	10.2569
Correlation coefficient	0.9998	0.9996	0.9996	0.9993
LOD ^a (µg/mL)	4.14	3.64	7.11	4.62
LOQ ^a (µg/mL)	12.54	11.03	21.55	14.00
Repeatability ^b	0.85511	0.68562	0.889	1.204

^aLimit of detection ($3.3 \times \sigma$ /Slope) and limit of quantitation ($10 \times \sigma$ /Slope).

^bRepeatability for $n \geq 5$, RSD ≤ 2 .

Repeatability

Repeatability of the method was evaluated by calculating the RSD of the peak areas of six replicate injections for the standard concentration (200.0 µg/ml) of SOF and (45.0 µg/ml) of LDV. Results were examined as %RSD values of the concentrations of determined drugs. Low values of %RSD (less than 2) indicate high precision of the method as shown in (Table 2).

Detection and quantitation limits

These approaches are based on the Standard Deviation of the Response and the Slope. A specific calibration curve should be studied using samples containing an analyte in the range of LOD and LOQ. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation. $LOD = 3.3 \times \sigma$ /slope and $LOQ = 10 \times \sigma$ /slope, where σ = the standard deviation of the

response (Table 2).

Accuracy and recovery

Accuracy of the proposed methods was calculated as the percentage recoveries of pure samples of the studied drugs. Accuracy is assessed using three different concentrations covering the specified range from (40.0 to 500.0 µg/ml) for SOF and (30.0 to 300.0 µg/ml) for LDV (i.e. three concentrations and three replicates). Concentrations were calculated from the corresponding regression equations. The mean % recoveries for SOF and LDV were between 98.0% to 102% for assay content and dissolution rate. These data are shown in (Tables 3 & 4). Accuracy was further assessed by applying the standard addition technique to Heterosofir Plus, where good recoveries were obtained revealing that there was no interference from excipients (Table 5).

Table 3: Data of Accuracy for SOF and LDV for Assay.

Sofosbuvir Standard (µg/ml)	SOF			Ledipasvir Standard (µg/ml)	LDV		
	µg/ml (Injected)	µg/ml (found)	Recovery%		µg/ml (Injected)	µg/ml (found)	Recovery%
80	80	80.35	100.44%	30	30	29.66	98.87%
	80	79.16	98.96%		30	29.68	98.91%
	80	79.22	99.03%		30	29.62	98.74%
200	200	198.57	99.29%	50	50	48.95	97.90%
	200	198.38	99.19%		50	48.89	97.77%
	200	197.07	98.53%		50	48.61	97.22%
350	350	351.02	100.29%	90	90	90.57	100.63%
	350	351.35	100.38%		90	91.32	101.46%
	350	351.05	100.300%		90	89.78	99.75%
Accuracy (Mean)			99.44	Accuracy (Mean)			99.03

Table 4: Determination of SOF and LDV in pharmaceutical formulation by the proposed HPLC method and application of standard addition technique.

Sofosbuvir Standard (µg/ml)	SOF			Ledipasvir Standard (µg/ml)	LDV		
	µg/ml (Injected)	µg/ml (found)	Recovery%		µg/ml (Injected)	µg/ml (found)	Recovery%
80	80	80.57	100.70%	30	30	29.89	99.63%
	80	79.39	99.23%		30	29.90	99.66%
	80	79.45	99.31%		30	29.85	99.50%
200	200	197.98	98.99%	50	50	49.52	99.04%
	200	197.79	98.89%		50	49.45	98.90%
	200	196.49	98.24%		50	49.17	98.34%
350	350	349.39	99.82%	90	90	91.80	102.00%
	350	349.71	99.91%		90	91.61	101.78%
	350	349.42	99.83%		90	91.05	101.16%
Accuracy (Mean)			99.44	Accuracy (Mean)			100.00

Table 5: Determination of SOF and LDV in pharmaceutical formulation by the proposed HPLC method and application of standard addition technique.

Pharmaceutical formulation	Added(µg/mL)		Recovery %	
	SOF	LDV	SOF	LDV
Heterosofir Plus 400mg/90MG FCT	80	100.17	98.22	90
SOF, 400 mg(claimed)	160	99.15	99.76	100
LDV, 90 mg(claimed)	200	101.09	100.69	180
Mean ± RSD			100.14±0.97	99.56±1.25

Formulation assay

The validated method was applied on commercially available Heterosofir Plus. The results of the assay undertaken yielded 99.84% and 97.06% of the label claim for SOF and LDV.

Results of the assay indicated that the method is selective for the analysis of Heterosofir Plus ® FCT without interference from the excipients used to formulate and produce these tablets. The results were displayed in (Tables 6 & 7).

Table 6: Assay result for the determination of Sofosbuvir and Ledipasvir in pharmaceutical formulation by the proposed HPLC method.

Pharmaceutical formulation	Conc.(µg/mL)		Recovery %		Recovery %	
	SOF	LDV	SOF	LDV	SOF	LDV
Heterosofir Plus 400mg/90MG FCT SOF, 400 mg(claimed) LDV,90 mg(claimed)	400	90	97.0%	96.68%	(90-110)	(90-110)
			96.68%	96.34%		
			97.33%	96.98%		
			98.65%	98.27%		
			97.44%	97.087%		
			97.39%	97.03%		
Mean ± RSD			97.42±0.68	97.06 ± 0.76		

Table 7: Dissolution profile results for the determination of Sofosbuvir and Ledipasvir in pharmaceutical formulation by the proposed HPLC method.

	SOF						LDV					
	10	15	20	25	35	45	10	15	20	25	35	45
I.	87.34%	93.13%	97.31%	100.47%	102.25%	102.36%	90.60%	95.15%	101.20%	98.74%	100.77%	98.33%
II.	87.24%	93.08%	97.31%	100.55%	102.18%	102.36%	90.31%	95.41%	102.57%	98.87%	101.0%	98.74%
III.	87.18%	93.37%	97.28%	100.41%	102.02%	102.34%	90.87%	96.38%	101.93%	102.12%	100.90%	98.87%
IV.	91.86%	93.38%	97.32%	100.45%	102.04%	102.48%	92.26%	101.20%	101.94%	102.22%	100.89%	102.12%
V.	91.83%	93.21%	98.48%	100.35%	99.47%	103.06%	90.07%	102.57%	96.31%	100.76%	100.81%	102.23%
VI.	92.03%	93.23%	98.46%	100.47%	102.28%	101.37%	92.26%	101.93%	96.29%	101.00%	101.02%	100.77%
Mean±	89.5±	93.24	97.69	100.44	101.70	102.3±	91.06	96.28	101.73	99.96	100.89	100.91
RSD	2.84	±0.13	±0.61	±0.06	±1.0	0.53	±1.0	±1.0	±0.54	±0.91	±0.09	±1.45

Intermediate precision (ruggedness)

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipments, etc. Good results were obtained and presented in (Table 8).

Table 8: Ruggedness of the method.

Parameter (%RSD)	SOF	LDV
Intraday	1.93	1.95
Interday	0.685	0.649
Analyst to Analyst	1.42	0.464
Column to Column	0.829	1.88

Robustness

The robustness of the proposed methods was evaluated in the development phase where the effects of different factors on the method were studied to obtain the optimum parameters for complete separation. Robustness of the method was studied by deliberately varying parameters like flow rate (±0.1 mL/min) and studying the effect of changing mobile phase pH by (±0.2), acetonitrile composition (±5%) and column temperature changed (35° & 25°). The low values of the %RSD, as given in (Table 9), indicate the robustness of the proposed methods.

Table 9: Table 9: Robustness of the method.

Parameter (%RSD)	SOF	LDV
Flow rate change (±0.1mL/min)	0.828	2.624
pH change of mobile phase (±0.2)	0.869	1.204
Wave length change (260 nm, 254nm)	1.966	2.618
Column temperature change(35, 25°C)	0.741	1.620

System suitability

Tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability was checked by calculating tailing factor (T), column efficiency (N), resolution (Rs) factors. All calculated parameters were within the acceptable limits indicating good selectivity of the methods and ensuring system performance, (Table 10).

Table 10: Result of stability of analytical solution.

Item	Obtained Value		Reference values
	SOF	LDV	
Tailing factor	1.104	1.012	T ≤ 2
Resolution	-	7.116	R _s > 2
Selectivity	-	3.387	k' > 2
Injection precision	0.855	0.685	RSD ≤ 1%
Retention time(R _t)	0.027	0.183	RSD ≤ 1%
Number of theoretical plates(N)	7067.70493	2885.63326	N > 2000

Stability of analytical solution

To demonstrate the stability of standard solution during analysis, solution was analyzed over a period of 24h at room temperature and refrigerator. The results showed that for all the solutions, the retention times and peak areas of SOF and LDV remained almost unchanged (RSD<2.0%) indicating that no significant degradation occurred within this period, i.e. both solutions were stable for at least 24h, which was sufficient to complete the whole analytical process. The results were displayed in (Table 11).

Table 11: Result of stability of analytical solution.

Condition	SOF	LDV
Fridge	100.74%	97.97%
Room temperature (25°C)	99.70%	97.43%

Specificity

a. Placebo interference: Specificity was tested against standard compounds and against potential interferences in the presence of placebo. No interference was detected.

b. Forced degradation: The Forced degradation of API was carried out as per ICH guidelines (ICH, Q2B) in acid, base, humidity, oxidation, photo, heat and thermal. The results were displayed in (Table 12). Also, peak purity was applied for SOF and LDV in (Figure 6) and data was shown in (Table 13).

Table 12: Results of peak purity of SOF and LDV.

Name	Effect	Sofosbuvir			Ledipasvir		
		Observed t _R	Peak Area	Degradation %	Observed t _R	Peak Area	Degradation %
Test	Without Effect(control)	2.428	1267.50415	-	4.714	290.3852	-
	Oxidation Effect	2.427	1191.23193	5.40	4.784	274.33533	1.60
	Alkali Effect	2.371	1100.41870	12.60	4.414	249.75481	10.05
	Acid Effect	2.369	1102.47620	12.44	4.414	248.97804	10.32
	Light Effect (Sun light)	2.522	1179.10083	6.36	4.845	265.52496	4.63
	Heat Effect	2.521	1159.76709	7.89	4.849	261.19614	6.12
	Humidity Effect	2.370	1101.21057	12.54	4.402	248.14626	10.60
	Placebo	No peak observed	No area observed	-	No peak observed	No area observed	-

Table 13: Results of peak purity of SOF and LDV.

Item	SOF	LDV
Purity factor	999.993	999.955
Threshold	999.973	999.865
Spectra	5	5
Noise Threshold	0.017	0.050

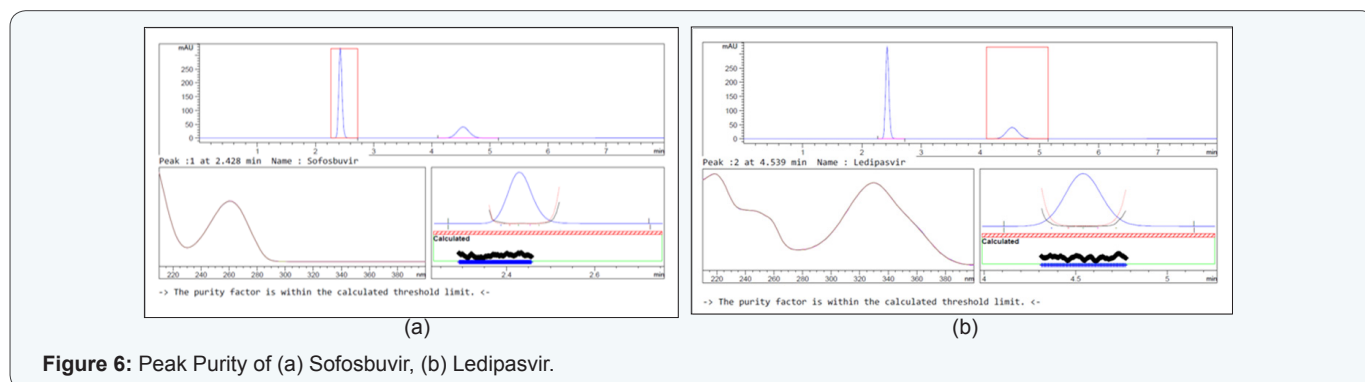


Figure 6: Peak Purity of (a) Sofosbuvir, (b) Ledipasvir.

c. Acid degradation: Weigh accurately 20.0 mg of SOF and 4.5 mg of LDV into 100 mL volumetric flask, dissolve in 70 mL of diluent and 5 ml of 1.0 N aqueous HCl solution and close the volumetric flask by stopper. Keep the solution at room temperature up to 48 hr. Neutralize with 1.0 N aqueous NaOH solution and make up to the mark with the diluent, inject into the chromatographic system and calculate the percent of degradation (Table 12).

d. Base degradation: Weigh accurately 20.0 mg of SOF and 4.5 mg of LDV into 100 mL volumetric flask, dissolve in 70 mL of the diluent and 5ml of 1.0N aqueous NaOH solution and close the volumetric flask by stopper. Keep the solution at room temperature up to 48 hr. Neutralize with 1.0 N aqueous HCl solution and make up to the mark with the diluent, inject into the chromatographic system and calculate the percent of degradation (Table 12).

e. Peroxide degradation: Weigh accurately 20.0 mg of SOF and 4.5 mg of LDV into 100 mL volumetric flask, dissolve in 70 mL of the diluent, add 3 ml of 3.0% aqueous H₂O₂ solution and close the volumetric flask by stopper. Keep the solution at room temperature up to 48 hr. Inject into the chromatographic system and calculate the percent of degradation. No interference was found, although there was some degradation products appeared after H₂O₂ treatment due to the peak of hydrogen peroxide. And the degradation percent was calculated as shown in (Table 12).

f. Photo degradation: Photo degradation is carried out by keeping the powders of SOF and LDV under sun light for 48 hours then accurately transfers 20.0 mg of SOF and 4.5 mg of LDV into 100 mL volumetric flask, dissolve in 70 mL of the diluent and dilute up to the mark with the same solvent. Inject into the chromatographic system and calculate the percent of degradation. No degradation products appeared after light degradation. But it has been noted that peak areas of SOF and LDV are affected after exposure to sunlight, data was shown in (Table 12).

g. Heat degradation: Heat stress studies were carried out by keeping the powders of SOF and LDV in drying oven at 80°C for 8 hr then accurately transfers 20.0mg of SOF and 4.5 mg of LDV into 100 mL volumetric flask, dissolved in 70 mL of diluent and up to the mark. Inject into the chromatographic system and calculate the percent of degradation. No degradation products appeared after heat degradation. But it has been noted that peak areas of SOF and LDV are affected after exposure to heat, therefore, it is recommended to avoid heating during sonication; data was shown in (Table 12).

h. Thermal degradation: Thermal stress studies were carried out by keeping the powders of SOF and LDV in climatic chamber at storage condition 40°C ± 2 & 75% ± 5% RH for one week then accurately transfer 20.0 mg of SOF and 4.5 mg of LDV into 100 mL volumetric flask, dissolve in 70 mL of diluent and complete to the mark with the same solvent. Inject into the chromatographic system, and calculate the percent of degradation. No degradation products appeared after thermal degradation. Data was shown in (Table 12).

Conclusion

The proposed RP-HPLC method for simultaneous estimation of sofosbuvir and ledipasvir in their dosage form is simple, precise, specific, highly accurate, selective and less time consuming for analysis. The analytical method is valid, fit for use and can be used for regular routine analysis, stability studies and forensic sciences & criminal investigation.

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Research involving human participants and/or animals

This article does not contain any studies with human participants or animals performed by any of the authors.

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