

## Validated simultaneous spectrophotometric quantification of a new antiviral combination

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### ARTICLE INFORMATION



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### ABSTRACT

Simple, selective and precise spectrophotometric methods were applied for simultaneous quantification of sofosbuvir (SFV) and ledipasvir (LDI) both in their raw and dosage forms. In the first method, the two cited drugs were determined simultaneously using first derivative ( $D^1$ ) method. It was accomplished by measuring peak heights at 275 nm and 344 nm, for SFV and LDI, respectively, in concentration ranges of 5 - 80  $\mu\text{g/mL}$  and 3 - 50  $\mu\text{g/mL}$ , for SFV and LDI, respectively. In the second one, a first derivative of ratio spectra ( $D^1$ DD) method was adopted to quantify SFV in concentration range of 5 - 80  $\mu\text{g/mL}$ . It was adopted by measuring the peak amplitudes (valley and peak) at 259 nm and 280 nm, using 25  $\mu\text{g/mL}$  LDI as a divisor. The proposed method was also used to determine LDI in concentration range of 3 - 50  $\mu\text{g/mL}$  by recording the peak amplitudes (valley and peak) at 319 nm and 375 nm, using 80  $\mu\text{g/mL}$  SFV as a divisor. The developed methods were validated with respect to linearity, accuracy, precision, selectivity, robustness and limits of detection and quantification (LOD and LOQ), as per the guidelines of International Conference on Harmonization (ICH)-Q2B.

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### 1. Introduction

Hepatitis C virus (HCV) is considered as one of the most dangerous pathogens that hindered the medical care all over the world. Unlike other types of hepatitis, more than 80 percent of HCV infections become chronic and lead to liver disease. It can be considered as the major cause of cirrhosis and liver cancer that in turn lead to liver transplantation [1]. Sofosbuvir (SFV) is a nucleotide prodrug analog that can be used for the treatment of HCV, either alone or in combination with other drugs like ribavirin and ledipasvir [2]. Ledipasvir (LDI) is an antiviral agent, which can inhibit an important viral phosphoprotein (NS5A) involved in the viral replication [3]. A fixed-dose combination of SFV and LDI was approved by food and drug administration (FDA) for the treatment of patients infected with genotype 1HCV [4]. Referring to the literature, SFV was determined using chromatographic and spectrophotometric techniques [5,6]. Also, SFV forced degradation was studied using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [7]. LDI was quantified alone using high performance liquid chromatography (HPLC) [8]. On the other hand, the studied mixture was quantified using HPLC [9] and LC-MS/MS methods [10-13].

There is no doubt that simplicity and ease applicability are important features that should characterize the analytical method used for the routine analysis of pharmaceutical dosage forms in quality control laboratories. It is obvious from the literature that, most of the published analytical methods dealing with the quantification of SFV and LDI mixture use the LC-MS/MS technique which is complicated and expensive if compared with spectrophotometry. The aim of this work is to develop and validate simple and economic spectrophotometric methods for simultaneous quantification of SFV and LDI, either in pure or tablet forms. These methods can be easily applied for the routine analysis work in quality control laboratories.

### 2. Experimental

#### 2.1. Chemicals and reagents

Pure SFV (PSI-7977) and LDI (L320100) were purchased from Cayman Chemical Company, Ann Arbor, United States of America (USA); their purity was certified to be 99.9 %. Methanol (HPLC grade) was supplied by Sigma Aldrich, St. Louis, USA.

## 2.2. Pharmaceutical formulations

Harvoni® tablets labeled to contain 400 mg SFV and 90 mg LDI, batch no. 14SFC013UD. It was manufactured by Gilead Sciences, Limited IDA Business & Technology, Ireland, manufactured for Gilead Sciences International, Cambridge, UK.

## 2.3. Instrumentation

Double beam spectrophotometer (JASCO, Japan) with 1 cm path length matched quartz cuvettes. It is connected to IBM compatible computer with HP 680 inkjet printer (Hewlett Packard, USA).

## 2.4. Standard solutions

SFV and LDI stock standard solutions (1 mg/mL) were prepared by accurate weighing and transferring of 50 mg of pure SFV or LDI into two separate 50 mL volumetric flasks. The drugs were dissolved by aid of a vortex mixer in 20 mL methanol then the volume was completed to the mark using the same solvent.

SFV and LDI working standard solutions (100 µg/mL) were prepared by accurate dilution of 5 mL SFV or LDI stock standard solution (1 mg/mL) into two separate 50 mL volumetric flasks using methanol as a diluting solvent.

## 2.5. Method validation

The developed analytical methods were fully validated according to ICH-Q2B guidelines [14].

### 2.5.1. Linearity

Aliquots of SFV and LDI working standard solutions (100 µg/mL) equivalent to 50-800 µg SFV and 30-500 µg LDI were transferred separately into two groups of 10 mL volumetric flasks then the volume of each flask was completed to the mark with methanol and mixed well. The absorption spectrum of each solution was scanned using methanol as a blank.

For the D<sup>1</sup>-Method, the peak heights were measured at 275 and 344 nm, for SFV and LDI, respectively, in concentration ranges of 5-80 µg/mL and 3-50 µg/mL, for SFV and LDI, respectively.

On the other hand, the D<sup>1</sup>DD-Method was applied to determine SFV in concentration range of 5-80 µg/mL by measuring the peak amplitudes (valley and peak) at 259 nm and 280 nm, using 25 µg/mL LDI as a divisor. The proposed method was also used to determine LDI in concentration range of 3-50 µg/mL by recording the peak amplitudes (valley and peak) at 319 nm and 375 nm, using 80 µg/mL SFV as a divisor.

### 2.5.2. Accuracy

Accuracy can be defined as the percent of the recovered analyte from a known added quantity [14]. It was carried out by analyzing three different concentrations of pure SFV (10, 30 and 50 µg/mL) and LDI (5, 10 and 20 µg/mL). The concentrations were calculated from the corresponding regression equations.

### 2.5.3. Precision

Precision was defined as the degree of repeatability under normal operational conditions. It can be expressed as repeatability (intra-day) and intermediate precision (inter-day) as % relative standard deviation (%RSD) [14]. So, three concentrations of SFV (10, 30 and 50 µg/mL) and LDI (5, 10 and 20 µg/mL) were analyzed three times within the same day (intra-day) or on three successive days (inter-day) using the

two proposed methods, then the results were documented as %RSD.

### 2.5.4. Specificity and selectivity

Specificity of the proposed methods can be assured by comparing the UV-scan obtained for a mixture of SFV, LDI together with the commonly used excipients, with that obtained from the blank (excipients solution in methanol without drugs) [14]. The chosen excipients were colloidal silicon dioxide, copovidone, croscarmellose sodium, lactose monohydrate, magnesium stearate, and microcrystalline cellulose. These additives were used in the manufacture of Harvoni® tablets as mentioned in its monograph. The drug to excipient ratio was similar to that used in the market product. Selectivity was checked by analyzing laboratory prepared mixtures containing different ratios of the analytes.

### 2.5.5. Limits of detection and quantification (LOD and LOQ)

LOD is the lowest concentration of the analyte that the analytical method can reliably differentiate from the background. On the other hand, LOQ can be defined as the lowest concentration that can be quantified with acceptable accuracy and precision [14]. The LOD and LOQ were calculated as

$$\text{LOD} = 3.3 \sigma / S \quad (1)$$

$$\text{LOQ} = 10 \sigma / S \quad (2)$$

where,  $\sigma$  is the standard deviation of the lowest standard level and  $S$  is the slope of the standard curve.

### 2.5.6. Robustness

Robustness can be checked by evaluating the effect of minute changes in assay conditions on method validity. It was accomplished by performing minute changes in solvent composition (adding 1% acetonitrile to methanol) and in the degree of smoothing concerning the derivative spectra.

## 2.6. Analysis of pharmaceutical formulation

Ten Harvoni® film coated tablets were weighed to get the average weight of a tablet then crushed, finely powdered and mixed well. Tablet powder equivalent to 40 mg SFV / 9 mg LDI was transferred to a beaker of 250 mL capacity then; a suitable volume of methanol (40 mL) was added and stirred for about 20 minutes. Filtration was carried out into 100 mL volumetric flask. Washing of the residue was done using 15 mL methanol (three times) then the same solvent was used to complete the volume to 100 mL, then 1 mL of the prepared solution was diluted to 10 mL in a volumetric flask, then the procedure was completed as under linearity. Moreover, standard addition procedure was applied by spiking different known quantities of pure SFV and LDI to the tablet formulation, and then the procedures were followed as mentioned before.

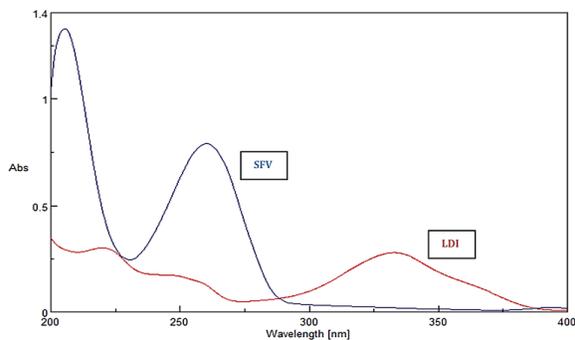
## 2.7. Comparison with reference published methods

The results obtained by applying the proposed procedures for determination of pure SFV and LDI samples were statistically compared to those obtained by reference reported methods [6,8] to ensure accuracy and precision of the suggested methods.

## 3. Results and discussion

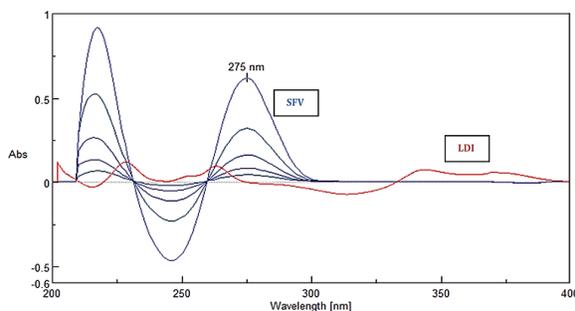
Absorption spectrophotometric methods were applied to quantify active pharmaceutical ingredients (API) in pharma-

ceutical preparations but this technique had the problem that most drugs absorb in the ultra violet region and so a strong spectra overlapping was noticed [15]. Severe overlapping was observed between the zero order absorption spectra ( $D^0$ ) of SFV and LDI in the same proportion used in tablet manufacture (Figure 1).

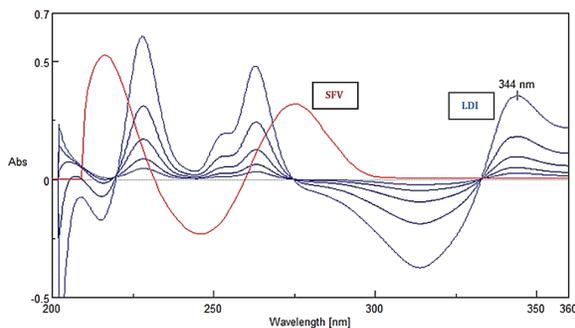


**Figure 1.** Absorption spectra of 40 µg/mL SFV and 9 µg/mL LDI using methanol as blank.

A simple first derivative spectrophotometric method was applied to resolve the interference shown between SFV and LDI in their  $D^0$  spectrum (Figures 2 and 3). All instrumental parameters affecting the derivative spectra were adjusted to get optimum peak resolution.



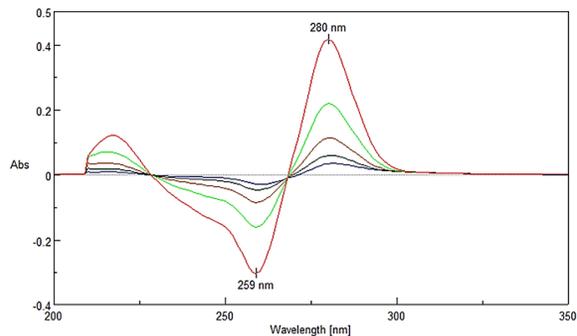
**Figure 2.**  $D^1$ -Spectra of SFV in concentration range of 5-80 µg/mL and LDI in concentration of 9 µg/mL.



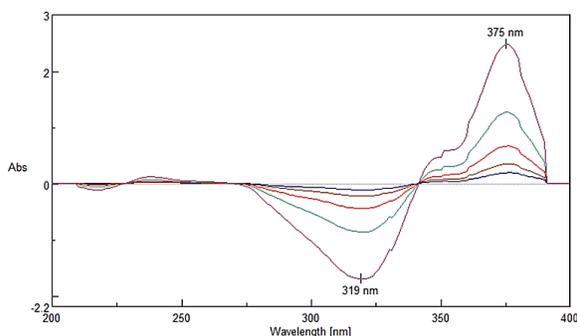
**Figure 3.**  $D^1$ -Spectra of LDI in concentration range of 3-50 µg/mL and SFV in concentration of 40 µg/mL.

Also, the overlap between SFV and LDI was resolved by applying the first derivative of ratio spectra ( $D^1DD$ ) method (Figures 4 and 5). This method is based on the derivatization of the ratio spectra to resolve the binary mixtures [16]. The major advantages of such method are the ability to measure peak amplitudes that has a wonderful effect on the sensitivity of the assay. Also, the availability of many maxima and minima to do the measurements at them is another advantage. All

parameters affecting ratio spectra shape were carefully tested to get the best results with respect to average recovery percent of the analytes, either in bulk powder or in laboratory prepared mixtures [17].



**Figure 4.**  $D^1DD$ -Spectra of SFV in concentration range of 5-80 µg/mL.



**Figure 5.**  $D^1DD$ -Spectra of LDI in concentration range of 3-50 µg/mL.

### 3.1. Method validation

#### 3.1.1. Linearity

For the  $D^1$ -method, linearity relationships were constructed between the peak heights at 275 and 344 nm, for SFV and LDI, respectively, against the corresponding concentrations over the ranges of 5-80 µg/mL and 3-50 µg/mL for SFV and LDI, respectively (Figures 2 and 3), from which the linear regression equations were computed and found to be:

$${}^1D_{SFV} = 0.0077 C + 0.0057 \text{ for SFV } (r^2 = 0.9998) \quad (3)$$

$${}^1D_{LDI} = 0.0069 C + 0.0054 \text{ for LDI } (r^2 = 0.9999) \quad (4)$$

where,  ${}^1D$  is the peak heights of the spectra,  $C$  is the corresponding concentration and  $r$  is the correlation coefficient.

For the  $D^1DD$ -method, linearity relationship was obtained between the peak amplitudes (valley and peak) for the  $DD^1$  spectra at 259 and 280 nm, using 25 µg/mL LDI as a divisor, to get SFV concentrations in the range of 5-80 µg/mL (Figure 4). Also, the peak amplitudes (valley and peak) for the  $DD^1$  spectra at 319 and 375 nm were recorded, using 80 µg/mL SFV as a divisor, to get LDI concentrations in the range of 3-50 µg/mL (Figure 5). The linear regression equations were computed and found to be:

$${}^1DD_{SFV} = 0.0088 C + 0.0215 \text{ for SFV } (r^2 = 0.9997) \quad (5)$$

$${}^1DD_{LDI} = 0.0826 C + 0.0583 \text{ for LDI } (r^2 = 0.9999) \quad (6)$$

where,  ${}^1DD$  is the peak amplitudes (valley and peak) of the spectra,  $C$  is the corresponding concentration and  $r$  is the correlation coefficient.

**Table 1.** Method validation parameters for determination of SFV and LDI by the adopted methods.

	SFV		LDI	
	D <sup>1</sup> -Method	<sup>1</sup> DD-Method	D <sup>1</sup> -Method	<sup>1</sup> DD-Method
<b>Linearity</b>				
Range (µg/mL)	5 - 80	5 - 80	3 - 50	3 - 50
Slope	0.0077	0.0088	0.0069	0.0826
Intercept	0.0057	0.0215	0.0054	0.0583
r <sup>2</sup>	0.9998	0.9997	0.9999	0.9999
<b>Accuracy</b>				
Mean±SD *	100.88±1.018	100.34±1.116	100.44±0.597	99.92±0.501
Variance	1.036	1.245	0.356	0.251
% RSD	1.009	1.112	0.594	0.501
<b>Precision</b>				
Intraday precision	98.88±0.992	99.22±1.041	99.89±0.948	98.29±0.321
Interday precision	99.18±1.243	102.78±1.179	100.54±1.541	102.87±0.529
<b>Robustness</b>				
Mean±SD *	99.51±0.841	101.58±0.984	99.64±0.894	101.34±0.684
% RSD	0.845	0.969	0.897	0.675
<b>LOD</b> (µg/mL)	2.87	2.49	0.89	1.00
<b>LOQ</b> (µg/mL)	4.97	4.90	2.97	2.89

\* Average of three determinations.

**Table 2.** Determination of SFV and LDI in laboratory prepared mixtures by the proposed methods.

Claimed concentration of SFV/LDI mixture (µg/mL)	SFV		LDI	
	D <sup>1</sup> -Method	<sup>1</sup> DD-Method	D <sup>1</sup> -Method	<sup>1</sup> DD-Method
40/9				
Mean±SD *	101.24±0.544	98.54±0.741	100.57±0.321	99.18±0.992
% RSD	0.537	0.752	0.319	1.000
40/20				
Mean±SD *	102.54±0.781	102.85±0.873	99.59±0.297	98.99±1.128
% RSD	0.762	0.849	0.298	
20/9				
Mean±SD *	99.56±0.854	99.11±1.021	100.39±0.991	100.87±0.941
% RSD	0.858	1.030	0.987	0.933

\* Average of three determinations.

### 3.1.2. Accuracy

The accuracy of the proposed methods was validated by analyzing nine quality control samples representing three concentration levels of SFV and LDI, covering the specified linearity range for each analyte. The recovery and % RSD were calculated and found to be satisfactory for both methods, which confirm the accuracy of the developed methods (Table 1).

### 3.1.3. Precision

The intraday and inter-day precisions were checked by analyzing three different concentrations of SFV or LDI by adopting the proposed methods, either in the same day or during three successive days. The % RSD values for intraday and inter-day precisions were less than 2% for the proposed methods, which confirm the good precision of both methods (Table 1).

### 3.1.4. Specificity and selectivity

The proposed methods were specific as none of the tried excipients interfered with the analytes, so the proposed methods can be efficiently applied for the quantification of the studied drugs in their dosage form without matrix interference. Laboratory prepared mixtures containing variable amounts of the studied drugs were analyzed to validate the selectivity of the suggested methods. The results are shown in Table 2.

### 3.1.5. Limits of detection and quantification (LOD and LOQ)

The obtained values of LOD and LOQ confirmed the sufficient and acceptable sensitivity of the proposed methods (Table 1).

### 3.1.6. Robustness

It is a measure of the method capability to maintain unaffected by slight changes in its parameters. The studied

parameters were the minute changes in solvent composition and in the degree of smoothing concerning the derivative curves. The proposed methods were not affected by the slight changes in their conditions as the % RSD values were less than 1% and so this confirms the robustness of the methods (Table 1).

### 3.2. Analysis of pharmaceutical formulation

The proposed methods were successfully applied for the quantification of SFV and LDI in Harvoni® tablets to ensure content uniformity (Table 3). The commercial dosage form showed acceptable recoveries by applying the proposed methods which were within the acceptable limits of content uniformity. Also, the standard addition procedure was applied by spiking different known quantities of pure SFV and LDI to the tablet formulation to ensure the applicability and reliability of the proposed methods. The results showed satisfactory recoveries of the pure added drug by the proposed methods (Table 3).

### 3.3. Comparison with reference published methods

The results obtained by adopting the proposed methods for determination of pure SFV and LDI samples were statistically compared to those obtained by reference reported methods [6, 8] as shown in Table 4. The comparison declares that, there is no significant difference between the proposed methods and the reference reported methods which confirm the accuracy and precision of the proposed methods.

## 4. Conclusions

The suggested methods are simple; as they are implemented via a simple instrument (spectrophotometer), which is available in all quality control laboratories. Also, the proposed methods do not require any complicated or sophisticated software to be applied. The cited methods are capable of the simultaneous quantification of the studied drugs without previous separation.

**Table 3.** Determination of SFV and LDI in Harvoni® tablets and application of standard addition procedure by the proposed methods.

Harvoni® tablets labeled to contain 400 mg SFV and 90 mg LDI (Batch no. 14SFC013UD)	Content uniformity		Standard addition	
	SFV		LDI	
	D <sup>1</sup> -Method	<sup>1</sup> DD-Method	D <sup>1</sup> -Method	<sup>1</sup> DD-Method
Mean±SD *	101.31±0.927	99.67±0.641	99.24±0.694	102.21±1.436
% RSD	0.915	0.643	0.699	1.405

\* Average of three determinations.

**Table 4.** Statistical comparison between the results obtained by applying the proposed methods and that obtained by the reference reported methods for determination of pure SFV and LDI.

Item	SFV			LDI		
	D <sup>1</sup> -Method	<sup>1</sup> DD-Method	Reference method [6]	D <sup>1</sup> -Method	<sup>1</sup> DD-Method	Reference method [8]
Mean±SD	100.88±1.018	100.34±1.116	100.85±1.130	100.44±0.597	99.92±0.501	100.17±0.686
% RSD	1.009	1.112	1.120	0.594	0.501	0.685
n	9	9	5	9	9	5
Variance	1.036	1.245	1.277	0.356	0.251	0.471
F-value (3.84) *	1.233	1.026	-	1.323	1.876	-
Student's t-test (2.179)	0.042	0.755	-	0.714	0.735	-

\* Values in parenthesis are the theoretical values of t and F at  $p = 0.05$ .

The proposed methods offer a cost effective alternative to the published liquid chromatographic methods of analysis with acceptable accuracy, precision and selectivity.

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