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Chemometrically Assisted RP-HPLC and Spectroscopic Method Development for Simultaneous Multi-Component Analysis of Ledipasvir and Sofosbuvir in Pure and Pharmaceutical Formulation

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ABSTRACT

***Objective:** A strategic quantitative analysis of Ledipasvir (LEDI) and Sofosbuvir (SOF) has been developed in Bulk and pharmaceutical (Tablet) dosage form with a reliable, Precise and sensitive First-Order Derivative spectrophotometric and RP-HPLC methods.*

***Methods:** Derivative method was developed firstly using methanol as a solvent, obeying the Beer's Law in a concentration range from 2-12 µg/ml and 8-48 µg/ml for LEDI and SOF showing the ZCP at 284 nm and 257 nm respectively. Secondly, better chromatographic separation and estimation was attained with a sample Pre-treated automated and analysed using a BDS-C18 (250 mm × 4.6 mm, 5µm) column and with mobile phase composition of Methanol: Acetonitrile: (1%) Ammonium acetate (50:20:30 % v/v/v) at 1 ml/min flow rate with an UV detection at 281 nm. Developed methods were validated rendering to ICH Q2 (R1) guidelines.*

Results: *Linear Calibration curve was obtained covering the whole of the concentration range with regression coefficient value 0.998 and 0.999 for LEDI and SOF in the Spectrophotometric method. Rectilinear relationship with acceptable regression coefficient 0.9987 and 0.997 was found over the concentration ranging from 1-11 µg/ml for LEDI and 4-44 µg/ml for SOF respectively in the RP-HPLC method. SOF and LEDI were detected at retention time of 5.388 and 8.085 min respectively. The mean percentage recoveries were in the range of 98.51 – 101.6 % and 99.11 – 101.22 % for LEDI and SOF.*

Conclusion: *The results indicate that the developed RP-HPLC and UV spectrophotometric methods are suitable for the routine quality control testing of marketed tablet formulations.*

Keywords: Ledipasvir, Sofosbuvir, RP-HPLC, First order derivative, Validation.

INTRODUCTION

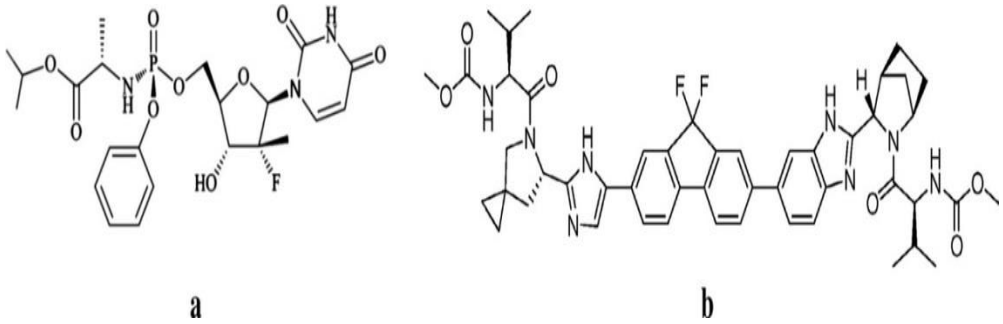
Hepatitis C virus (HCV) is an RNA virus that chronically infects about 71 million individuals worldwide [1]. Approximately 80 % of acutely infected HCV patients progress to chronic infection, 20 % of whom develop cirrhosis within 25 years, with 25 % of patients with cirrhosis developing hepatocellular carcinoma and/or decompensated liver disease. There are six major HCV genotypes with many subtypes based on genomic sequence heterogeneity. Among the people who have chronic HCV infection, approximately 60 % have the genotype 1 strain of the virus. The treatment of patients infected with HCV genotype 1 is rapidly evolving. HCV therapy has been recently revolutionized by the development and approval of direct-acting antiviral agents (DAAs), borne out of the intense study of the viral life cycle and the elucidation of the crystal structure of several critical viral proteins [2,3].

Ledipasvir and Sofosbuvir in combination are directly acting antivirals attacking at the former stage of replication, reducing the disease leading to its chronicity. SOF is chemically notorious as (S)-Isopropyl 2-((S)-(2R, 3R, 4R, 5R)5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3hydroxy-4-methyl tetrahydrofuran-2-yl)methoxy)-(phenoxy) phosphorylamino) propanoate (Figure 1a), with an molecular formula of $C_{22}H_{29}FN_3O_9P$ and a molecular weight of 529.54 g/mol. The partition coefficient-log P is 1.62 and pKa is 9.3 for SOF. It is a pangenotypic inhibitor of the HCV NS5B RNA-dependent RNA polymerase, which is vital for viral replication [4,5].

LEDI is chemically notorious as methyl N-[(2S)-1-[(6S)-6-[5-[9,9-difluoro-7-[2-[(1S,2S,4R)-3-[(2S)-2-(methoxy carbonylamino)-3-methylbutanoyl]-3-azabicyclo[2.2.1]heptan-2-yl]-3H-benzimidazol-5-yl]fluoren-2-yl]-1H-imidazol-2-yl]-5-azaspiro[2.4]heptan-5-yl]-3-methyl-1-oxobutan-2-yl]carbamate (Figure 1b), with molecular formula of $C_{49}H_{54}F_2N_8O_6$ and a molecular weight of 889.00 g/mol. The partition coefficient-log P is 3.8 and pKa1 is 4.0 and pKa2 is 5.0, respectively for LEDI. It is an HCV inhibitor targeting the HCV NS5A protein, which is necessary for RNA replication and the assembly of HCV virions [4,6].

The combination of SOF and LEDI or it individually is not official in any of the Pharmacopeia. Recently, a few methods have been developed and reported for individual and simultaneous determination of both drugs. The degradation study of SOF under several stress conditions by HPLC has been determined [7,8]. Deposition study of SOF into various *in vivo* cell [9], UPLC-MS/MS for SOF in human Plasma [10], UPLC-ESI-MS/MS method for quantification of Sofosbuvir and its metabolite, GS-331007, in human plasma [11], Simultaneous quantification of ribavirin, Sofosbuvir and its metabolite in rat plasma by UPLC-MS/MS [12], RP-HPLC method for SOF in pure form [13], RP-HPLC for SOF in bulk and tablet dosage form [14],

Determination of Daclatasvir (DAC) in human plasma using SOF as an internal standard (IS) by UPLC-MS/MS [15]. While for LEDI, UV spectrophotometry [16] and RP-HPLC [17] methods are reported.



a b

Figure 1: Chemical structure of (a) SOF and (b) LEDI.

Both SOF and LEDI were estimated by UPLC-MS/MS [18] and LC-MS/MS method [19] and Chromatographic analysis in Human plasma [20]. UPLC-MS/MS for Ledipasvir, Sofosbuvir and its metabolite in rat plasma [21]. LEDI, SOF, and various directly acting antivirals quantification by A UHPLC-MS/MS [22].

According to the best of our acquaintance, only two HPLC methods [23,24] has been published, while current work was prepared for publishing. The present study targets to develop a highly resolved, precise and accurate RP-HPLC method and First-Order derivative UV-Spectroscopic method for the simultaneous estimation of both SOF and LEDI in pure and tablet dosage forms with high sensitivity, selectivity. Developed methods were validated in accordance with ICH Q2 (R1) guideline [25].

MATERIALS AND METHODS

Reagents and materials

LEDI and SOF were acquired as a gratis sample from NATCO Pharma (Hyderabad) and MSN LABS (Hyderabad) and the marketed formulation used was HEPCINET-LP manufactured by NATCO Pharma. All employed Chemicals were AR and HPLC grade.

Instruments

The instruments used were double beam UV-Visible spectrophotometer (Shimadzu 1800), HPLC (Analytical Technologies), Rheodyne injector (20 μl), S1122 series pump and 2203 UV-visible detector. Analytical Balance of Sartorius Gottingen AG BP 211 D, Japan.

Chromatographic conditions

A BDS-C_{18} (250 mm × 4.6 mm, 5 µm) chromatographic column and a mobile phase composition of Methanol: Acetonitrile: (1 %) Ammonium acetate (50:20:30 % v/v/v) were used. 1 ml/min of the flow rate was maintained and effluents were monitored at 281 nm. The sample was injected using Rheodyne injector (20 μL). Freshly prepared samples were used at the time of analysis.

Test and standard solution preparation for first-order derivative and RP-HPLC method

Preparation of standard stock solutions

Accurately weighed 10 mg of individual SOF and LEDI was dissolved in 100 ml of volumetric flask, diluted up to the mark with methanol to get a standard stock solution (100 µg/ml).

Preparation of combined standard solution of LEDI and SOF

Accurately weighed LEDI (9 mg) and SOF (40 mg) was transferred into 100 ml volumetric flask. Dissolved and diluted up to the mark with Methanol to get a combined stock solution (90 µg/ml) of LEDI and (400 µg/ml) of SOF. Stock solution (10 ml) was transferred in 100 ml volumetric flask and diluted up to mark with Methanol to obtain combined working standard solution (9 µg/ml) of LEDI and (40 µg/ml) of SOF. This solution was used to prepare a standard solution for linearity in RP-HPLC.

Preparation of tablet sample solution

Twenty tablets were weighed accurately using sensitized balance and powdered. The powder equivalent to 9 mg of LEDI or 40 mg of SOF was transferred to a 100 ml volumetric flask, dissolved and diluted up to mark with Methanol to get strength 90 µg/ml Ledipasvir or 400 µg/ml Sofosbuvir (Stock solution). The solution was filtered through Whatman filter paper no.41 and first few ml of filtrate were discarded. From stock solution, 10 ml solution was transferred to 100 ml volumetric flask and volume were adjusted to the mark with Methanol to get strength 9 µg/ml Ledipasvir and 40 µg/ml Sofosbuvir (Working solution).

Validation parameters

First-order derivative and RP-HPLC method were validated in terms of Specificity, linearity, accuracy, precision, LOD, and LOQ, robustness in accordance with ICH Q2 (R1) guideline and system suitability test as per USP [26].

System suitability test RP-HPLC

System suitability is performed to prove that reproducibility and suitability of the chromatographic system are adequate to perform a quantitative analysis. A single set of the mixed standard solution was prepared as mentioned in the test method and six replicate injection of mixed standard preparation was injected and chromatogram was taken. Various parameters like peak area, tailing factor, Theoretical plates, Resolution and Retention time were determined.

Specificity

Chromatograms of standard and sample solutions of LEDI and SOF were compared for detection of any extra peak.

Linearity and Range

The linearity of an analytical method is its ability, within an established range, to provide results that are directly proportional to the concentration of the analyte in the sample. The linearity response was determined by analyzing 6 independent levels of the calibration curve in the selected interval between upper and lower quantitation level of LEDI and SOF, respectively. Plot the calibration curve of absorbance versus concentration and determine Regression co-efficient and Regression equations for Ledipasvir and Sofosbuvir.

Accuracy

The accuracy of the method was determined in terms of % Recovery of the standard using standard addition method. Recovery studies were performed by spiking standard drug solution at the level of 80%, 100% and 120 % to the pre-analyzed sample. In this method, the known concentration of drug was added to the assay sample. Recovery studies were performed in triplicate by calculating the % Recovery and % RSD for both the drugs.

Precision

The precision of an analytical method was expressed as the percent relative standard deviation and standard error of the mean of the series of measurement. It was ascertained by the replicate estimation of standard drugs. It involves Repeatability, Intraday, and Interday precision. In repeatability study, one concentration of both drugs was analyzed by 6 times replication. Intra-day precision was carried out by performing three replicates of three different concentrations on the same day Interday precision, three replicates of three concentrations were analyzed at three consecutive days and peak area measured was expressed in terms of percent relative standard deviation (% RSD).

LOD and LOQ

Limit of Detection (LOD) and Limit of Quantification (LOQ) of the developed method were calculated from the standard deviation of the response and slope of the calibration curve of drugs using the formula as per ICH guidelines.

$$LOD = 3.3 \times (SD/Slope)$$

$$LOQ = 10 \times (SD/Slope)$$

Where SD = Standard Deviation of *Y*-intercept of 5 calibration curves.

Slope = Mean slope of the 5 calibration curves.

Robustness (For RP-HPLC)

The robustness of an analytical method is the measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability throughout the normal usage. The determination of robustness requires that method characteristics are assessed when single or more operating parameter is varied.

RESULT AND DISCUSSION

Spectroscopic experimental condition

Selection of analytical wavelength

LEDI 10 µg/ml solution was prepared by diluting 1.0 ml of Standard stock solution (100 µg/ml) in 10 ml volumetric flask and Sofosbuvir 40 µg/ml prepared by diluting 4.0 ml of standard Stock solution (100 µg/ml) in 10 ml volumetric flask – and both were diluted up to mark with Methanol. Both solutions were scanned separately in the range of 200-400 nm. Convert these spectra into first order derivative spectra. It was observed that LEDI showed dA/dλ zero at 284.0 nm in contrast to SOF that has considerable dA/dλ at this wavelength. Further, SOF has zero dA/dλ at 257.0 nm while at this wavelength LEDI has significant dA/dλ. Therefore wavelength 284.0 nm and 257.0 nm were employed for the determination of LEDI and SOF, respectively without any interference.

Construction of calibration curve

The solution of LEDI ranging from 2-12 µg/ml and SOF 8-48 µg/ml were prepared by pipetting out 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 ml and 0.8, 1.6, 2.4, 3.2, 4.0 and 4.8 ml of the working standard solution of LEDI (100 µg/ml) and SOF (100 µg/ml) into series of 10 ml of volumetric flask and the volume was adjusted upto the mark with Methanol. An absorbance of each solution was measured at 257.0 nm for LEDI and 284.0 nm for SOF using first-order derivative spectrophotometry as depicted in Figures 2-6. The graph of absorbance at an individual wavelength of 257.0 nm and 284.0 nm were plotted against their respective concentration.

Analysis of tablet formulation (Assay)

The tablet sample solution of the final concentration of LEDI 9 µg/ml and SOF 40 µg/ml was analyzed by the first-order derivative spectroscopic method, and absorbance was measured at 257.0 nm for determination of LEDI and 284.0 nm for SOF respectively. The procedure was repeated five times for sample analysis. The concentrations of LEDI and SOF were calculated from the calibration graph.

Chromatographic experimental conditions

Method development and optimization of chromatographic conditions

The optimization of chromatographic conditions was done with a view to develop HPLC method for the simultaneous determination of SOF and LEDI in bulk and in a Pharmaceutical dosage form. Various mobile phases comprising different ratios of water, acetonitrile, methanol, and ammonium acetate were tried. Finally, mobile phase comprising of Methanol: Acetonitrile: (1%) Ammonium acetate (50:20:30 % v/v/v) was found to be satisfactory and gave two symmetric and well-resolved peaks at an acceptable retention time of LEDI (5.381 ± 0.447 min) and SOF (8.078 min; 0.3951 min) at 281.0 nm and 1.0 ml/min flow rate (Figure 2). The injection volume to carry out chromatography was set at 20 µl.

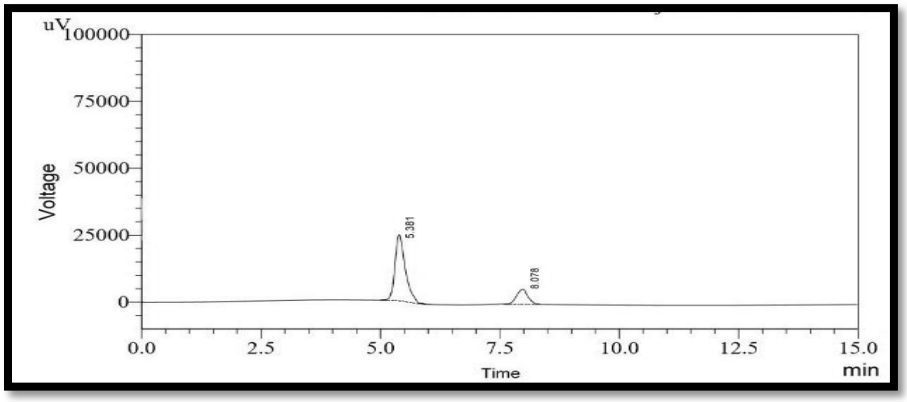


Figure 2: Chromatogram of LEDI (10 µg/ml) and SOF (µg/ml) in methanol: acetonitrile: ammonium acetate (1%) (50:20:30 %v/v/v)

Calibration curve for ledipasvir and sofosbuvir

The combined solution of LEDI and SOF ranging from 1-11 µg/ml and 4–44 µg/ml were prepared by pipetting out series of samples within the range from the combined working standard solution of Ledipasvir (90 µg/ml) and Sofosbuvir (400 µg/ml) into

10 ml volumetric flasks and the volume was adjusted to mark with Methanol. Chromatogram of each solution was recorded and linearity was developed as depicted in Figures 2-6. The graph of area versus respective concentration was plotted.

Analysis of marketed formulation

The tablet sample solution of the final concentration of LEDI 9 µg/ml and SOF 40 µg/ml was analyzed by taking the Chromatogram of this solution at a detection wavelength of 281 nm. The procedure was repeated five times for sample analysis. The concentrations of LEDI and SOF were calculated using the regression equation.

Method validation

The developed and optimized method was validated for system suitability parameters as per USP, specificity, sensitivity, Limit of detection (LOD) and limit of quantitation (LOQ), linearity, precision (repeatability, Intraday precision, Interday precision), accuracy and robustness as per ICH Q2 (R1) guidelines.

System suitability parameters (For RP-HPLC)

The system suitability testing was carried at a 9 µg/ml and 40 µg/ml standard solutions of LEDI and SOF respectively by five replicate injections. Various parameters like peak area, tailing factor, theoretical plates, and resolution and retention time were evaluated as depicted in Table 1.

Table 1: System suitability test parameters for SOF and LEDI by RP-HPLC method.

Parameters	DRUGS	
	SOF	**LEDI**
Retention time[a]	5.388	8.085
Tailing factor[a]	1.446	1.336
Theoretical plates[a]	3147.926	4516.577
Resolution factor[a]	-	6.233
Peak area (%RSD)[a]	375676	29122
Note: [a] Mean of 6 determination		

Specificity (For RP-HPLC)

The specificity of the method was determined by comparing the chromatogram of the standard and sample solutions of LEDI and SOF. For HPLC peak purity index was determined of each drug in the sample solution which was found to be nearer to 1. Result obtained under optimized conditions has shown no interference from common Tablet excipients and impurities. Result demonstrates the specificity of the method (Figure 3).

Linearity

Linearity was checked by diluting standard stock solution at six different concentrations. The linear regression analysis obtained by plotting the absorbance (for UV) and peak area (for RP-HPLC) of analyte vs. concentration shown correlation coefficients (r^2) greater than 0.995. The statistical results such as correlation coefficient (r^2), slope and intercept are reported in Table 2.

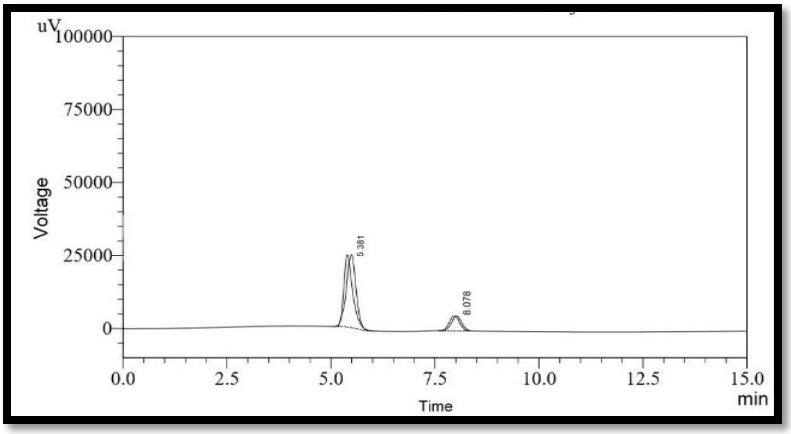


Figure 3: Overlay chromatogram of blank, standard, and sample solutions of LEDI and SOF.

Table 2: Linear regression data for calibration curve.

Parameters	UV		RP-HPLC	
	LEDI	**SOF**	**LEDI**	**SOF**
Wavelength	257.0 nm	284.0 nm	281.0 nm	
Concentration range(µg/ml)	2-12 µg/ml	8-48 µg/ml	1-11 µg/ml	4-44 µg/ml
correlation coefficient(r^2)	0.9984	0.9994	0.9987	0.997
Intercept	0.0425	0.008	27939	229904
Slope	0.0517	0.0018	1135.8	5323.4
Note: [a] Mean of 6 determination				

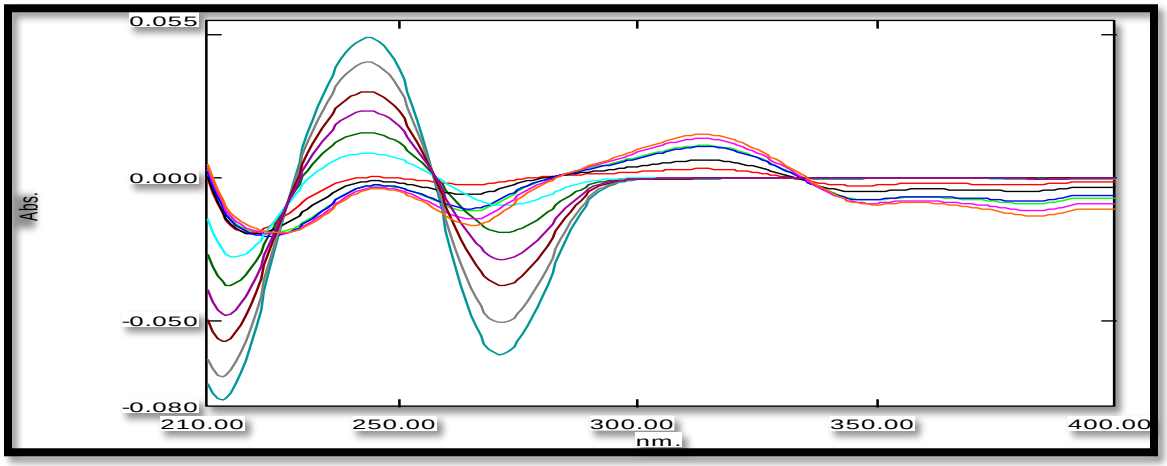


Figure 4: Overlain spectra of Ledipasvir (2-12 µg/ml) and SOF (8-48 µg/ml).

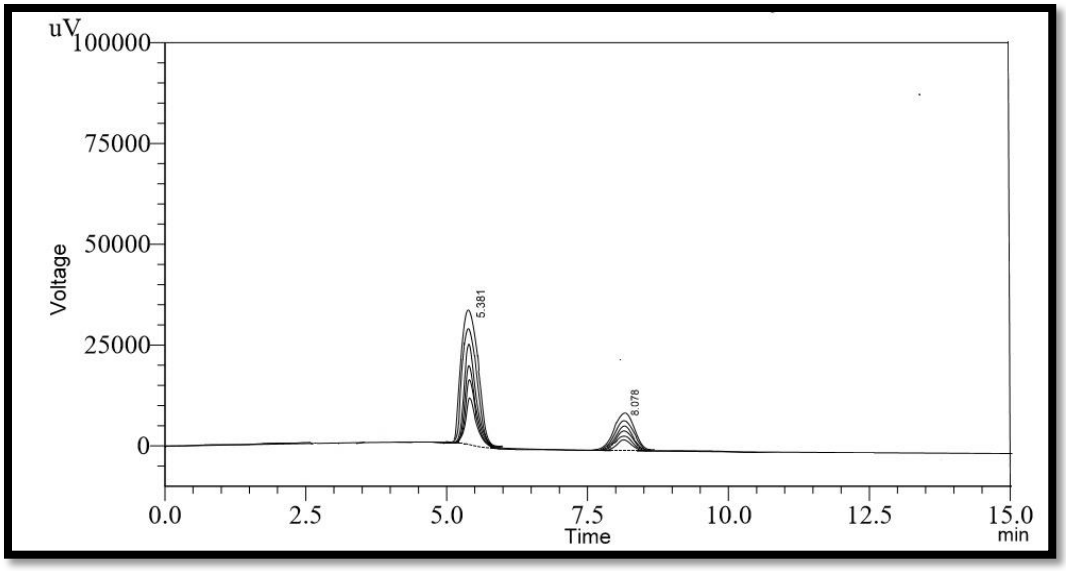


Figure 5: First-order derivative overlay spectra of LEDI (2-12 µg/ml) and SOF (8-48 µg/ml).

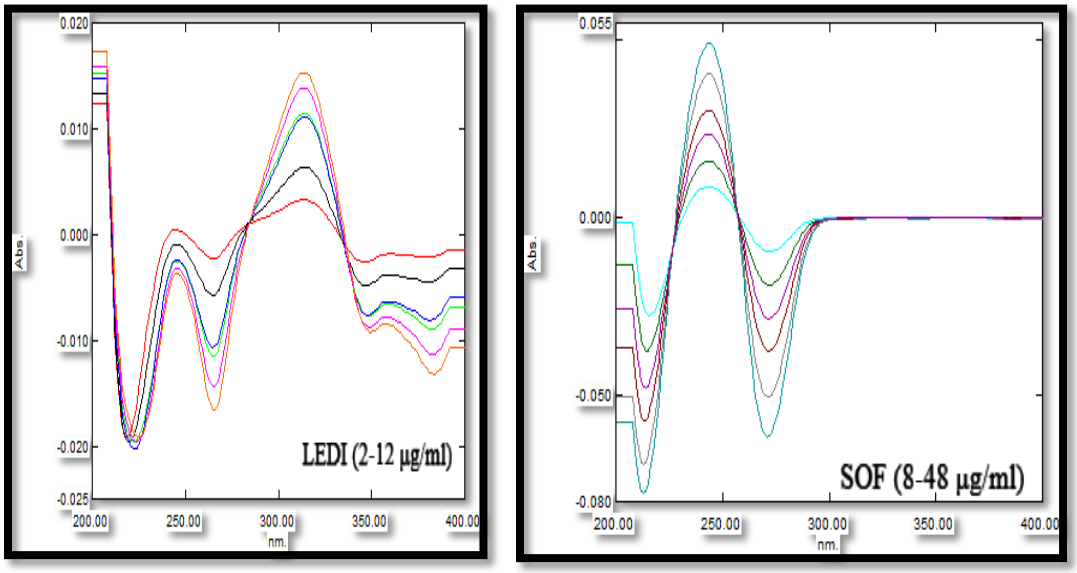


Figure 6: Overlain chromatogram of Ledipasvir (1-11 µg/ml) and Sofosbuvir (4-44 µg/ml).

Accuracy

The accuracy was determined by performing recovery study using standard addition method at 80%, 100%, and 120% level for LEDI and SOF. The recovery study was done by adding pure drug solution to the pre-analyzed tablet formulation, and concentrations of LEDI and SOF were determined by using the calibration graph. The values of % RSD and recovery studies were showing satisfactory accuracy as depicted in Tables 3 and 4.

Table 3: Result of recovery studies of LEDI.

Parameters	Ledipasvir					
	UV			RP-HPLC		
Level (%)	80	100	120	80	100	120
Sample concentration (µg/ml)	3	3	3	3	3	3
Amount of standard added (µg/ml)	2.4	3	3.6	2.4	3	3.6
Total concentration (µg/ml)	5.4	6	6.6	5.4	6	6.6
Found concentration (µg/ml)[a]	5.41	6.017	6.62	5.38	6.057	6.23
% Recovery (mean ± sd)[a]	100.31 ± 0.7511	100.27 ± 1.2339	100.31 ± 1.1348	99.75 ± 1.3046	100.29 ± 1.2307	100.39 ± 1.0292
% RSD[a]	0.7487	1.2305	1.1313	1.3079	1.2271	1.0252
Note: [a] mean of 3 determination						

Table 4: Result of recovery studies of SOF.

Parameters	SOFOSBUVIR					
	UV			RP-HPLC		
Level (%)	80	100	120	80	100	120
Sample concentration (µg/ml)	20	20	20	12	12	12
Amount of standard added (µg/ml)	16	20	24	9.6	12	14.4
Total concentration (µg/ml)	36	40	44	21.6	24	26.4
Found concentration (µg/ml)[a]	36.06	40.14	44.24	21.66	24.07	26.29
% Recovery (mean ± sd)[a]	100.16 ± 0.7636	100.36 ± 1.0641	100.58 ± 0.6568	100.29 ± 0.7304	100.32 ± 0.8464	99.66 ± 0.7015
% RSD[a]	0.7623	1.0603	0.6532	0.7272	0.8437	0.7039
Note: [a] Mean of 3 determination						

Precision

The precision of both the method was confirmed by repeatability and intermediate precision. Repeatability expresses the precision under the same operating conditions over a short interval of time. The repeatability was performed by the analysis of the formulation was repeated for six times with the same concentration.

The intermediate precision of the method was confirmed by intraday (variation of results within the same day) and interday (variation of results between days) analysis with three different concentrations repeated for three times. The results were reported in terms of percentage of relative standard deviation (% RSD). The precision studies comparison data are represented in Tables 5, 6 and 7 for LEDI and SOF, respectively.

Table 5: Result of repeatability studies of LEDI and SOF.

Parameters	UV		RP-HPLC	
	LEDI	SOF	LEDI	SOF
Concentration (µg/ml)	6	24	5	20
Mean (Absorbance, Area)	0.2674	0.0355	33585	331942
SD[a]	0.0011	0.0003	185.813	230.493
% RSD[a]	0.3969	0.8046	0.553	0.069
Note: [a] mean of 6 determination				

Table 6: Results of intraday precision and Interday precision studies of LEDI.

Parameters		UV			RP-HPLC		
Concentration (µg/ml)	**Variables**	**2**	**8**	**12**	**1**	**7**	**11**
Intra-day precision	Mean (Absorbance/ Area ± SD)[a]	0.0572 ± 0.0005	0.3596 ± 0.0013	0.5775 ± 0.0015	29122 ± 147.85	35695 ± 120.20	40655 ± 195.59
	% RSD[a]	0.874	0.374	0.262	0.51	0.34	0.48
Inter-day Precision	Mean (Absorbance/ Area ± SD)[a]	0.0577 ± 0.0009	0.3601 ± 0.0021	0.5785 ± 0.0027	29124 ± 234.16	35696 ± 211.14	40653 ± 290.50
	% RSD[a]	1.475	0.599	0.471	0.80	0.59	0.71
Note: [a] Mean of 3 determination							

Table 7: Results of Intraday precision and inter-day precision studies of SOF

Parameters		UV			RP-HPLC		
Concentration (µg/ml)	**Variables**	8	32	48	4	28	40
Intra-day precision	Mean (Absorbance/ Area ± SD)[a]	0.0711± 0.0006	0.0493± 0.0004	0.0792± 0.0005	252376 ± 145.93	375676 ± 188.74	448538 ± 199.27
	% RSD[a]	0.847	0.819	0.636	0.06	0.05	0.04
Inter-day Precision	Mean (Absorbance/ Area ± SD)[a]	0.0715± 0.0010	0.0490± 0.0009	0.0795± 0.0010	252374 ± 226.24	375676 ± 252.04	448532 ± 275.27
	% RSD[a]	1.343	1.704	1.181	0.09	0.07	0.06
Note: [a] mean of 3 determination							

Sensitivity

The sensitivity of UV and RP-HPLC method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were calculated on the basis of the standard deviation of the y-intercept and slope: DL = 3.3 * ***σ/S*** and QL = 10 * *σ/S*, where ***σ*** is the standard deviation of the response and S is the slope of the calibration curve of analyte as reported in Table 8.

Table 8: LOD and LOQ of LEDI and SOF for the proposed methods

Parameters	UV		RP-HPLC	
	LEDI	**SOF**	**LEDI**	**SOF**
LOD (µg/ml)	0.0710	0.209	0.328	0.0707
LOQ (µg/ml)	0.2153	0.633	0.995	0.2144

Robustness

Robustness of the method was optimized by Preparing the Combined standard solutions of LEDI (9 *µ*g/ml) and SOF (40 *µ*g/ml) and analyzed by changing mobile phase ratio, flow rate, and wavelength and measuring the corresponding response 3 times. The flow rate was changed to 1.0 ± 0.02 ml/min. The mobile phase ratio was changed to ± 2 % for both the components. The wavelength of detection was changed to 281 ± 2 nm. The method was found to be robust with respect to variability in applied conditions. Result of robustness was shown in Table 9.

Table 9: Result of Robustness study of LEDI and SOF (n=3)

Chromatographic parameters	Actual condition	Change condition	%RSD	
			LEDI	SOF
Flow rate ± 0.02 ml/min	1.0	0.98	0.349	0.029
		1.02	0.406	0.035
Wavelength ± 2 nm	281	279	0.330	0.027
		283	0.374	0.031
Change in the mobile phase ratio ± 2 %	50:20:30	52:19:29	0.325	0.028
		48:21:31	0.393	0.032
Note: [a] mean of 3 determination				

Analysis of marketed formulation

The validated UV spectrophotometric and RP-HPLC methods applied in the analysis of the marketed formulation HEPCINET-LP with a label claim of 90 mg of LEDI and 400 mg of SOF per Tablet. The results of the assay show good agreement with the label claims. The result of the assay was shown in Table 10.

Table 10: Results of assay in commercial sample

Parameters	UV		RP-HPLC	
	LEDI	SOF	LEDI	SOF
Labelled claim (mg)	90	400	90	400
Amount found[a] (mg)	89.10	397.2	88.95	397.6
% Assay ± SD[a]	99.19 ± 0.50	99.30 ± 0.87	98.83 ± 1.41	99.40 ± 1.68
Note: [a] Mean of 6 determination				

Comparison of the UV Spectrophotometric and RP-HPLC Methods

The comparison of the developed UV spectrophotometric and RP-HPLC methods was carried out by applying t-test to the assay results of both the drugs obtained by developed methods. It was found that t_{stat} value was less than $t_{critical}$ value for both the drugs. Hence there was no significant difference between the developed methods. So both the developed methods can be successfully applied for quality control analysis of this drug in the combined pharmaceutical formulation. The result of statistical analytical comparison was shown in Table 11.

Table 11: Result of t-test for LEDI and SOF.

Parameters	LEDI		SOF	
	UV Method	RP-HPLC Method	UV Method	RP-HPLC Method
Mean[a]	99.56333	99.75	99.7633	99.86667
Variance[a]	0.768133	1.0317	1.382033	1.265833
Observations	6	6	6	6
Hypothesized Mean Difference	0		0	
d_f	4		4	
t_{stat}	-0.241		-1.0999	
P(T<=t) two-tail	0.821407		0.917715	
$t_{critical}$ two-tail	2.776445		2.776445	
Note: [a]Mean of 6 assay determinations				

CONCLUSION

UV Spectrophotometric (First-order derivative method) and RP-HPLC methods were successfully developed and validated for the simultaneous determination of LEDI and SOF. The developed methods were found to be sensitive, accurate, precise, and robust. The results of the assay of the commercial formulation obtained from the UV and HPLC methods were not significantly different as per statistical analysis. This implies that the proposed UV and HPLC methods can be used for quality control analysis of LEDI and SOF in the combined pharmaceutical formulation.

REFERENCES

1. World Health Organization, WHO Global Hepatitis c report. Geneva, Switzerland: WHO, **2017**.
2. Mauss, Berg, Rockstroh, Sarrazin, Wedemeyer; Hepatology – A clinical textbook, **2017**, (8th edn), p: 55-385.
3. Kirby, BJ., et al. Pharmacokinetic, Pharmacodynamic and Drug-Interaction Profile of the Hepatitis C Virus NS5B Polymerase Inhibitor Sofosbuvir. *Clin Pharmacokinet*, **2015**, 54(7): 677-690.
4. Gilead Sciences, Inc. Harvoni (Ledipasvir and Sofosbuvir) tablets prescribing information, **2016,** Foster City, CA.
5. http://www.chemspider.com/Chemical-Structure.26286922.html. (Accessed june **2017**).
6. http://www.chemspider.com/Chemical-Structure.29271894.html?rid=6d1fd6b0-7b6c-42c9-95f2-6e5905933ee5. (Accessed june **2017**).
7. Swain, D., et al. Characterisation of forced degradation products and in silico toxicity prediction of Sofosbuvir: a novel HCV NS5B polymerase inhibitor. *J Pharm Biomed Anal*, **2016.** 120: 352-363.
8. Nebsen, M., and Elzanfaly, ES., Stability-indicating method and LC-MSMS characterization of forced degradation products of sofosbuvir. *J Chromatogr Sci*, **2016.** 54(9): 1631-1640.
9. Joseph, ER., et al. Validation and application of an LC-MS/MS method to determine the concentrations of Sofosbuvir. *Antimicrobial Agents and Chemotherapy*, **2015.** 59(12): 7671-7679.
10. Rezk, MR., Basalious, EB., and Amin, ME., Novel and sensitive UPLCMS/MS method for quantification of Sofosbuvir in human plasma: application to a bioequivalence study. *Biomed Chromatogr*, **2016**. 30: 13541362.
11. Rezk, MR., Basalious, EB., and Karim, IA., Development of a sensitive UPLC-ESI-MS/MS method for quantification of sofosbuvir and its metabolite, GS-331007, in human plasma: Application to a bioequivalence study. *J Pharm Biomed Anal*, **2015.** 114: 97-104.
12. Shi, X, et al. Evaluation of a rapid method for the simultaneous quantification of ribavirin, sofosbuvir and its metabolite in rat plasma by UPLC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci*, **2015.** 1002: 353-357.
13. Vikas, PM., et al. Development and validation of new RP-HPLC method for the determination of sofosbuvirin pure form. *World Journal of Pharmacy and Pharmaceutical Sciences*, **2016.** 5(5): 775-781.
14. Ravikumar, V., Subramanyam, CVS., and Veerabhadram, G., Estimation and validation of sofosbuvir in bulk and tablet dosage form by RP-HPLC. *International Journal of Pharmacy*, **2016.** 6(2): 121-127.
15. Rezk, MR., et al. Development and validation of sensitive and rapid UPLC-MS/MS method for quantitative determination of daclatasvir in human plasma: Application to a bio equivalence study. *J Pharm biomed Anal*, **2016.** 128: 61-66.
16. Ranjana, S., et al. Development and validation of simple UV spectrophotometric method for the determination of ledipasvir in bulk form and stress degradation studies. *Inventi Rapid: Pharm Analysis & Quality Assurance*, **2016**. 3: 1-5.

17. Devilal, J., et al. New method development and validation for the determination of ledipasvir in bulk drug form by using reverse phase HPLC technique. *World Journal of pharmacy and pharmaceutical Sciences*, **2016**. 5(8): 13121321.
18. Rezk, MR., et al. Quantification of Sofosbuvir and ledipasvir in human plasma by UPLC-MS/MS method: Application to fasting and fed bioequivalence study. *J Chromatogr B Analyt Technol Biomed Life Sci*, **2016.** 1028: 63-70.
19. Ehab, F., et al. A rapid and optimized LC-MS/MS method for the simultaneous extraction and determination of Sofosbuvir and Ledipasvir in Human Plasma. *Journal of AOAC International*, **2016**. 99(5).
20. Nehal, F., et al. Chromatographic analysis of ledipasvir and sofosbuvir: New treatment for chronic hepatitis C infection with application to human plasma. *Journal of Liquid Chromatography & Related Technologies*, **2017**.
21. Ariaudo, A., et al. A UHPLC-MS/MS method for the quantification of direct antiviral agents simeprevir, daclatasvir, ledipasvir, sofosbuvir/GS-331007, dasabuvir, ombitasvir and paritaprevir, together with ritonavir in human plasma. *J Pharm biomed Anal*, **2016**. 125: 369-375.
22. Pan, C., et al. Simultaneous determination of ledipasvir, sofosbuvir and its metabolite in rat plasma by UPLC-MS/MS and its application to a pharmacokinetic study. *J Chromatogr B Analyt Technol Biomed Life Sci,* **2016**. 1008: 255-259.
23. Zaman, B., Siddique, F., and Hassan, W., RP-HPLC method for simultaneous determination of sofosbuvir and ledipasvir in tablet dosage form and its application to *in vitro* dissolution studies. *Chromatographia,* **2016.** 79(23): 1605-1613.
24. El-Kassem, M., et al. Assay and dissolution methods development and validation for simultaneous determination of sofosbuvir and ledipasvir by RP-HPLC method in tablet dosage forms. *J Forensic Sci & Criminal Inves*, **2017.** 1(3).
25. International Conference on Harmonization, ICH Q2 (R1). Validation of analytical procedures: text and methodology, ICH Secretariat, Geneva, **2005.**
26. United States Pharmacopeia 27, National Formulary 22, The United States Pharmacopoeial Convention, Rockville, **2003**.2: 2281.