INDO AMERICAN JOURNAL OF PHARMACEUTICAL RESEARCH

SIMULTANEOUS ESTIMATION OF SOFOSBUVIR AND LEDIPASVIR USING UV SPECTROPHOTOMETRIC AND RP-HPLC METHODS

Anjali Patel, Priya Shah, Sadhana Rajput*

Quality Assurance Laboratory, Centre of Relevance and Excellence in Novel Drug Delivery System, Pharmacy Department, The Maharaja Sayajirao University of Baroda, Fatehgunj, Vadodara – 390 002, Gujarat, India.

ARTICLE INFO

Article history
Received 01/04/2017
Available online
30/04/2017

Keywords
Sofosbuvir,
Ledipasvir,
Absorption Correction Method,
First Derivative Zero Crossing Point Method,
RP-HPLC.

ABSTRACT

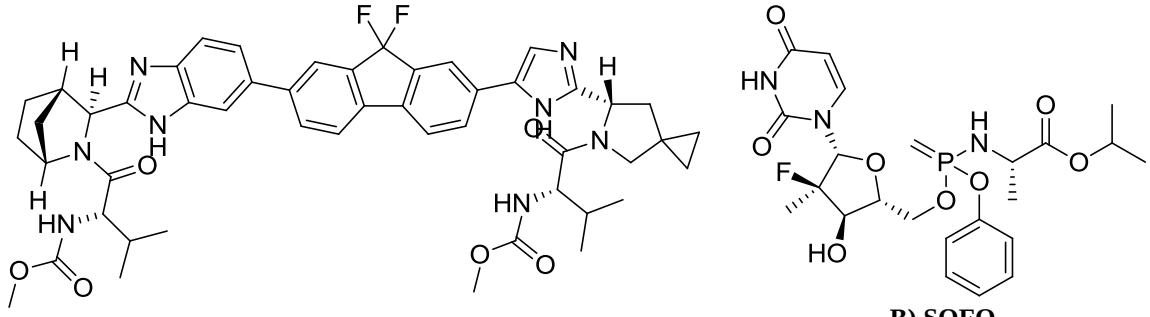
The present work includes two simple, inexpensive, rapid, accurate and precise UV spectrophotometric methods and another isocratic RP-HPLC method for estimation of Sofosbuvir (SOFO) and Ledipasvir (LEDI) in bulk and in synthetic mixture. The UV methods are based upon absorption correction (ACM) and first derivative zero crossing point method (FDZC) analysis approach. Chosen wavelength maxima were 260nm and 334nm for SOFO and LEDI respectively for ACM. Whereas for FDZC, estimation of SOFO and LEDI were carried out at 274.5 and 260.6 respectively. Selected linearity ranges for SOFO and LEDI were 24-40 µg/mL and 5.4-9 µg/mL respectively for UV methods. The simple, isocratic RP-HPLC method involved separation of SOFO and LEDI using reverse phase C18 CHROMBUDGET column (250 mm x 4.6 mm,5 µ) having mobile phase composition of acetonitrile and ammonium formate (pH 2.8)::55:45. The developed methods were validated successfully according to ICH Q2 (R1) guideline. Both spectrophotometric and chromatographic methods showed a linear response having r^2 values of 0.999. The percentage relative standard deviation (%RSD) was found to be less than two indicating that the methods were precise. The methods were successfully applied for analysis of SOFO and LEDI in laboratory mixture.The mean percentage accuracy values obtained for UV and RP-HPLC methods were between 99-102% and 99-101% respectively. SOFO and LEDI in its formulation could be accurately determined with assay values ranging from 99-101%. Thus it can be concluded that both the developed methods were specific, selective and robust.

Corresponding author
Prof. (Mrs.) Sadhana J. Rajput
Faculty of Pharmacy, Kalabhavan Campus
The Maharaja Sayajirao University of Baroda
+91-026-52434187; (M) +91-9998309662
sjrajput@gmail.com

*Please cite this article in press as **Anjali Patel** et al. Simultaneous Estimation of sofosbuvir and Ledipasvir Using UV Spectrophotometric and RP-HPLC Methods. Indo American Journal of Pharmaceutical Research.2017:7(04).*

INTRODUCTION

Ledipasvir (LEDI) is a hepatitis C virus NS5A inhibitor. The mechanism of action of LEDI is as a P-glycoprotein inhibitor and breast cancer resistance protein inhibitor. Chemically, LEDI is methyl N-[(2S)-1-[(6S)-6-[5-[9,9-difluoro-7-[2-[(1S,2S,4R)-3-[(2S)-2-(methoxycarbonylamino)-3-methylbutanoyl]-3-azabicyclo[2.2.1]heptan-2-yl]-3H-benzimidazol-5-yl]fluoren-2-yl]-1Himidazol-2-yl]-5-azaspiro[2.4]heptan-5-yl]-3-methyl-1-oxobutan-2 yl]carbamate having molecular formula of $C_{49}H_{54}F_2N_8O_6$ [1]. Another anti-HIV drug utilized was Sofosbuvir(SOFO) is a nucleotide analogue works by blocking hepatitis C NS5B protein. The mechanism of action of SOFO is as a RNA replicase inhibitor [2]. Chemical structure of LEDI and SOFO is shown in following figure 1.



A) LEDI **B) SOFO**

Figure-1. Chemical Structure of A) LEDI and B) SOFO.

In the present work two UV spectrophotometric methods based on absorption correction method (ACM) and first derivative zero crossing point method (FDZC) along with a simple and robust RP-HPLC chromatographic method were developed for simultaneous estimation of SOFO and LEDI in bulk and synthetic mixture. ACM is used when the sample contains two absorbing drugs (X and Y), one of which (X) absorbs at the λ_{max} of other (Y) but other (Y) shows zero absorbance at λ_{max} of first (X) then, it may be possible to determine both the drug by simplified simultaneous equation method using following formula [3].

$$A1 = aX1 * Cx + aY1 * Cy$$

$$A2 = aX2 * Cx + aY2 * Cy$$

Where, A1 and A2=Absorbance of the mixture containing two drugs X and Y at λ1 and λ2 respectively; aX1=absorptivity of X at λ1 nm; aX2=absorptivity of X at λ2 nm; aY1=absorptivity of Y at λ1 nm; aY2= absorptivity of Y at λ2 nm; Cx =concentration of X; Cy = concentration of Y.

FDZC method involves measurement of the absolute value of the total derivative spectrum at an abscissa value corresponding to the zero-crossing wavelength of the derivative spectra of individual components, which should be only a function of the concentration of other component. It is a useful means of resolving the overlapping spectra and eliminating the interference. It involves conversion of normal spectrum to first, second or higher order spectra where the amplitude in the derivative spectra is proportional to the concentration of analyte provided and the Beer's law is obeyed. This technique exploits the signal crossing through the abscissa axis, for a given component of a mixture, to assign the absorbance value to remaining components. This technique is particularly effective in the analysis of several complex mixtures, when wide overlapping peaks are present in the corresponding zero-order spectrum. However, suitable analytical signals are often placed on the peak shoulders or characterized by a too low absorbance. This could heavily limit the accuracy and precision of the method, as the low stability of such signals is well known [4].

High-performance liquid chromatography (HPLC), is a technique which involves solid stationary phase and a liquid mobile phase where separations is achieved by partition, adsorption, or ion-exchange processes, depending upon the type of stationary phase used. Compounds to be analyzed are dissolved in a suitable solvent, and most separations take place at room temperature. Thus, most drugs, being nonvolatile or thermally un-stable compounds, can be chromatographed without decomposition or the necessity of making volatile derivatives [5].

Various spectrophotometric and chromatographic methods are available for quantification of SOFO and LEDI in combination [6-10]. The purpose of present study was to develop simple, robust and sensitive and economic method performing UV and HPLC analysis.

MATERIALS AND METHOD

Apparatus and Software:

Shimadzu UV-1700 double beam spectrophotometer connected to a computer loaded with Shimadzu UV Probe 2.10 software was used for all the spectrophotometric measurements. The absorbance spectra of the reference and test solutions were carried out in 1cm quartz cells over the range of 200-400 nm. An electronic analytical balance A120, Shimadzu, Kyoto, Japan was used for weighing. Chromatographic separation was performed on Shimadzu, Kyoto, Japan. The LC system equipped with Shimadzu LC-20AT pump and Rheodyne 7725 injector with a fixed loop of 20 µL connected to Shimadzu SPD-20AV detector. Data acquisition and integration was performed using Spinchrome® software (Spincho biotech, Vadodara). Stationary phase used was CHROMBUDGET C18 RP-HPLC column (250 mm x 4.6 mm, 5 µ).

Chemicals and Reagents:

SOFO and LEDI were obtained as gift samples from Hetero Healthcare Ltd, Hyderabad and Mylan Laboratories Ltd, Nashik respectively. Analytical reagent (AR) grade methanol (MeOH) was purchased from Spectrochem Pvt. Ltd (Mumbai, India) and single distilled water was used as the solvent and diluents to perform UV spectrophotometric analysis. For chromatographic method, HPLC grade acetonitrile (ACN) was utilized and procured from Rankem Pvt. Ltd., (Mumbai, India). Other reagents like ammonium formate and formic acid were procured from Loba Chemie Pvt. Ltd., (Mumbai, India). HPLC grade water was used throughout the chromatographic analysis. Unless otherwise specified, all solutions were filtered through a 0.2 µm Ultipor® N66® Nylon 6, 6 membrane filter (Pall Life Sciences, USA) prior to use.

Preparation of Stock Solution and working solution:

UV spectrophotometer:

10mg of SOFO and LEDI were separately weighed accurately and transferred into a separate two 10mL volumetric flasks. Methanol was added into the volumetric flasks to dissolve the standards and finally volume was made up to the mark using same solvent to obtain standard solutions of 1000µg/mL concentration of SOFO and LEDI. From the prepared standard stock solution of SOFO and LEDI, working solutions of SOFO and LEDI having concentration of 100 µg/mL were prepared by transferring 2mL aliquot to two different 20 mL volumetric flasks individually and making up the volume with water and methanol in the ratio 50:50.

HPLC:

For chromatographic method, 40 mg of SOFO and 9 mg LEDI were separately weighed accurately and transferred into a 10 mL volumetric flasks. Methanol was added into the volumetric flasks to dissolve the standards and used as diluent. Working solutions of SOFO (400µg/mL) and LEDI (90 µg/mL) were prepared by transferring 2mL aliquot from standard stock solution to two 20 mL volumetric flasks individually and making up the volume with the mobile phase, ACN:ammonium formate in ratio of 55:45.

Preparation of Calibration Standards of SOFO and LEDI

UV methods A and B:

From working solution of SOFO (100µg/mL) aliquots of 2.4mL, 2.8mL, 3.2mL, 3.6mL and 4mL were withdrawn and transferred to 10mL volumetric flasks. Volume was made upto the mark with water to get concentration of 24µg/mL, 28µg/mL, 32µg/mL, 36µg/mL and 40µg/mL of SOFO respectively. From the working solution of LEDI(100µg/mL) aliquots of 0.54mL, 0.63mL, 0.72mL, 0.81mL and 0.9mL were withdrawn and transferred to 10 mL volumetric flasks. Volume was made upto the mark with water to produce 5.4µg/mL, 6.3µg/mL, 7.2µg/mL, 8.1µg/mL and 9µg/mLconcentrations of LEDI respectively. Mixed standard solutions of SOFO and LEDI were prepared in ratio of 4.4:1 as approximated in the marketed formulation.

Method A: Absorption correction method (ACM):

For this method overlapped absorption spectra of SOFO and LEDI were used to determine the analytical wavelengths then mixed standards were used to plot the calibration curves and lastly absorbances were noted at 260nm and 334nm for SOFO and LEDI respectively. Because of the unavailability of marketed formulation (MyHep LVIR™) in local market, a synthetic mixture of SOFO and LEDI in the ratio of 4.4:1 was prepared and absorbance spectra of this mixture were recorded onboth wavelength and absorbance were calculated.

Method B: first derivative zero crossing point method (FDZC):

The absorption spectra of the solutions of SOFO and LEDI were recorded in the range of 200 nm to 400 nm and were stored in the memory of the instrument and transformed to first derivative with $\Delta\lambda$ = 5nm and scaling factor = 20. Figure 5 shows that at 260.6 nm,SOFO shows zero crossing point and hence LEDI can be determined at this wavelength while at 274.5 nm, LEDI shows zero crossing point and hence SOFO can be determined. Calibration curves were constructed with five mixed standard solutions having different concentrations in the range between 24-40 µg/mL and 5.4-9 µg/mL for SOFO and LEDI respectively. Each concentration was analyzed in triplicate. The concentration of the drug present in the laboratory mixture was determined against the calibration curve. Figure 4 (a) and Figure 4 (b) show calibration graphs of SOFO and LEDI at 274.5nm and 260.6nm respectively.

HPLC method:

From the standard solution of SOFO and LEDI aliquots of 0.12 mL and 0.027mL were withdrawn and transferred to 10 mL volumetric flask, which was made upto the mark using solvent mixture(MeOH:Water :: 50:50). Same procedure was followed for other aliquots of 0.24mL, 0.48mL, 0.96mL, 1.92 mL, 3.84mL and 0.054mL, 0.108mL, 0.216mL,0.432mL, and 0.864mL of SOFO and LEDI respectively. Final solutions of mixture of SOFO and LEDI possessing both drug in ratio of 4.4:1 as approximated in the marketed formulation.

Selection of Detection Wavelength:

Standard solutions of SOFO and LEDI were scanned between 200-400 nm in UV spectrophotometer and a common wavelength at which both the drugs showed more or less absorbance was selected *i.e.*253 nm was selected for further analysis as shown in following figure 2.

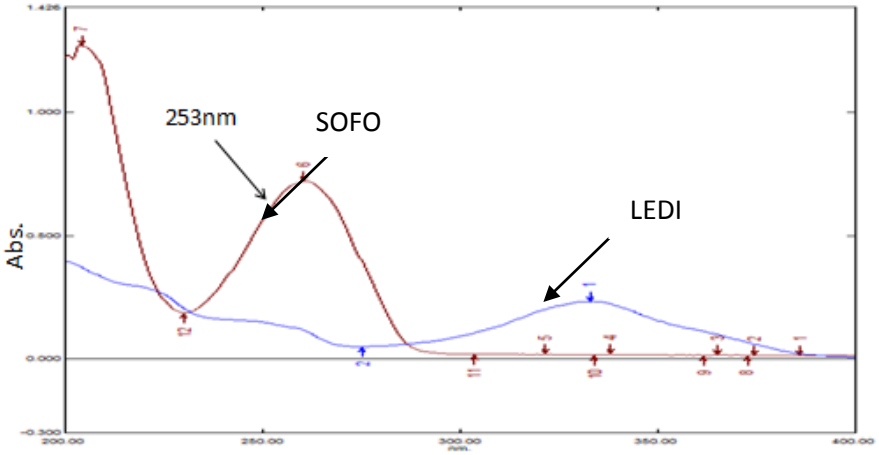


Figure-2. Common wavelength selection for SOFO and LEDI.

Chromatographic conditions:

HPLC analysis was carried out using CHROMOBUDGET C18 column (250 mm×4.6 mm, 5µ). Ammonium formate buffer (20mM) was prepared by dissolving 0.126gm of ammonium formate in 100 mL of double distilled water and adjusted to pH 2.8 using formic acid which was finally filtered with 0.2 µm Nylon membrane filter. The mobile phase consists of HPLC grade ACN and ammonium formate buffer (pH 2.8, 20mM) in ratio 55:45 which was degassed by ultrasonication for 5 minutes prior to use. HPLC analysis was performed at detection wavelength 253 nm keeping flow rate of 1mL/min with chromatographic run time of 12 min. Optimized chromatographic condition is listed in following table 1.

Table 1. Optimized RP-HPLC method parameters.

METHOD PARAMETER	OPTIMIZED CONDITION
Column	CHROMBUDGET 100-5-C18 column (250 mm × 4.6 mm, 5µ)
Mobile Phase	ACN:Ammonium Formate (20 mM, pH:2.8) :: 55:45
Flow Rate	1 mL/min
Retention Time	3.8min for SOFO and 9.92 min for LEDI
Injection Volume	10 µL
Detection Wavelength	253nm
Temperature	Ambient

Validation of developed UV method and HPLC method:

Developed spectrophotometric and chromatographic methods were validated for various parameters like accuracy (%recovery), intra-day and inter-day precision, linearity and range, LOD (limit of detection) and LOQ (limit of quantitation), robustness as per ICH Q2(R1) guideline. For HPLC method, besides this parameters, system suitability parameters were also measured

RESULT AND DISCUSSION:

Developed UV and HPLC methods were validated as per ICH Q2(R1) guideline for various parameters as discussed below.

Linearity

Linearity solutions ranging from concentration of 24-40 and 5.4-9 µg/mL (for UV method A and B) and 12-384µg/mLand 2.7-86.4 µg/mL(for HPLC method) for SOFO and LEDI respectively were prepared and analyzed in triplicate at a constant injection volume of 10µL. Calibration curve and r^2 value for UV and HPLC method were generated as shown in below figure 3 and 4 respectively. Overlain UV spectra and HPLC chromatogram for linearity is depicted in figure 5 and 6respectively (Table 2).

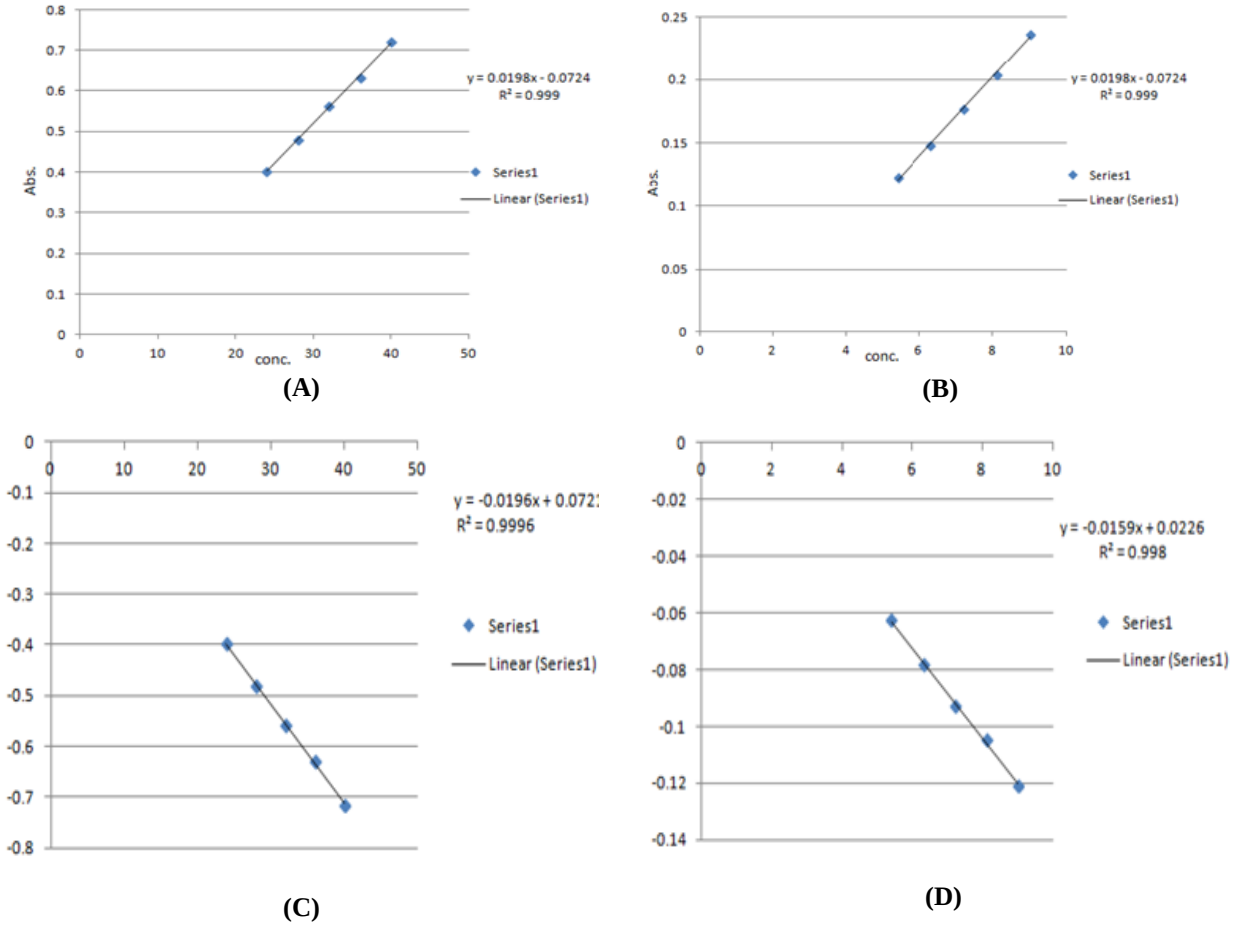


Figure-3: Calibration curve for (A) SOFO and (B) LEDI using ACM (C) SOFO and (D) LEDI using FDZC.

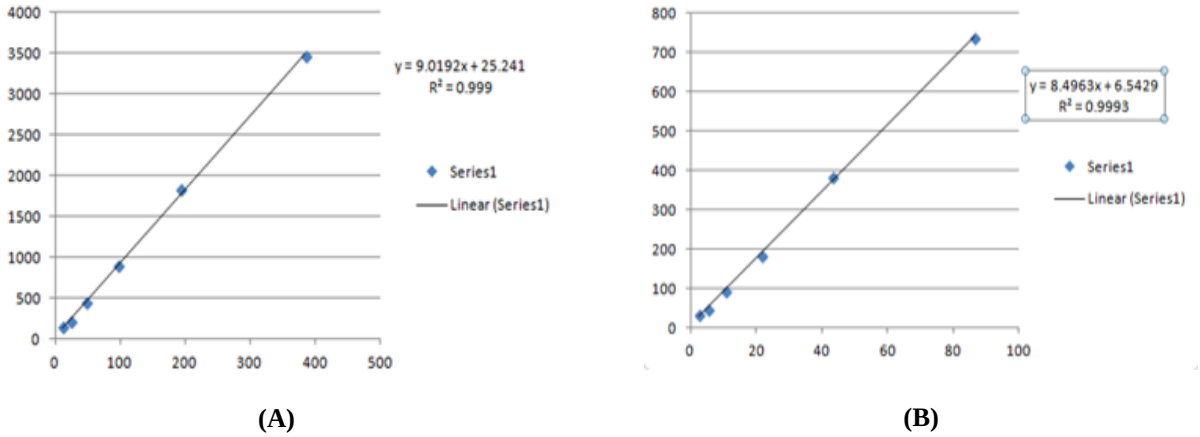
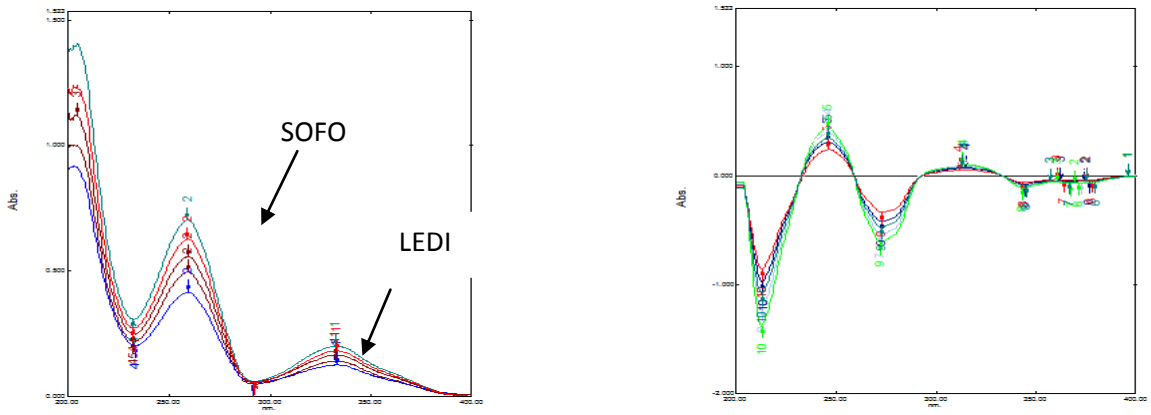


Figure-4. Calibration curve for (A) SOFO and (B) LEDI in RP-HPLC.



(A) **(B)**

Figure-5. Overlain calibration spectra of SOFO andLEDIusing (A) ACM and (B) FDZC.

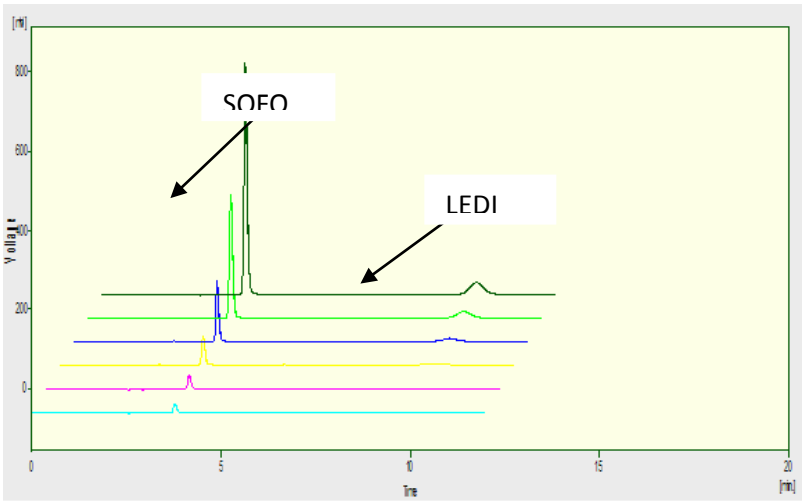


Figure-6. Overlain calibration chromatogram of mixture of SOFO and LEDI in RP-HPLC.

Table 2. Validation results for SOFO and LEDI.

PARAMETERS	UV METHODS				RP-HPLC	
	Absorbance correction method		First derivative zero crossing point method			
	SOFO	LEDI	SOFO	LEDI	SOFO	LEDI
Wavelength(nm)	260	334	274.5	260.6	253nm	253nm
Beer's law limit (µg/mL)	24-40	5.4-9	24-40	5.4-9	12-384	2.7-86.4
Regression equation (y=mx+c)	y= 0.0159x+0.018	y= 0.022x+0.001	y= -0.0196x+0.072	y= -0.0159x+0.022	y= 9.019x+25.24	y= 8.496x+6.5429
Correlation coefficient (R^2)	0.9997	0.9998	0.9996	0.998	0.999	0.9993
Intra-day Precision(%RSD)	0.845	0.106	0.8695	0.9062	0.957	0.882
Inter-day Precision(%RSD)	1.14	0.8721	1.049	0.8721	0.919	1.194
LOD (µg/mL)	0.200	0.184	0.791	0.632	0.1986	0.0160
LOQ(µg/mL)	0.522	0.558	0.90	0.89	0.6020	0.0487
Accuracy (% recovery)	100.21	100.7	100.3	100.7	100.21	100.50

Sensitivity (Limit of detection and Limit of quantitation)

The limit of detection (LOD) and limit of quantitation (LOQ) parameters were calculated using standard deviation of response and slope of calibration curve method implementing the following equations. Six replicates of the drug sample with lowest detectable and quantifiable concentration were analysed and %RSD was determined (Table 2).

$$LOD: \frac{3.3\sigma}{S}$$

$$LOQ: \frac{10\sigma}{S}$$

Where, σ is standard deviation of intercept of calibration curve (n = 6) and S is slope of regression equation

Precision

Precision is a degree of reproducibility. Reproducibility of spectrophotometric and chromatographic methods was checked by performing intra-day precision (on same a day) and inter-day precision (repeated trials on consecutive days). Results were expressed in terms of standard deviation and %relative standard deviation (%RSD). It was observed that the %RSD was less than 2 for all the proposed methods summarized in table 2.

Accuracy

To check the accuracy of different methods, recovery studies were carried out from pre-analyzed sample at three deferent level of standard addition 80%, 100% and 120%. Results of recovery studies are shown in Table 2. For each of the method explained above, %Recovery was the average of three determinations at each standard addition level. %Recovery for different methods was found to be between 97%-103% which prove that all the methods were accurate.

Robustness

Parameters taken to perform robustness study were organic to aqueous phase ratio for UV and RP-HPLC method; variation in pH and flow rate as in case of RP-HPLC method. The result of robustness study of the developed assay method was established in table 3 and table 4 for UV and RP-HPLC methods correspondingly. The result shown that during all conditions, assay value of the test preparation solution were not affected and in accordance with that of actual.

Table 3: Robustness result for SOFO and LEDI using UV spectrophotometric methods.

PARAMETERS	CONDITION	SOFO				LEDI			
		METHOD-I		METHOD-II		METHOD-I		METHOD-II	
		Absorbance Value	%RSD	Absorbance Value	%RSD	Absorbance Value	%RSD	Absorbance Value	%RSD
Mobile Phase composition (Methanol : Water)	52:48	0.0772 0.0761 0.0766	0.718	0.5011 0.5030 0.5100	0.928	0.0856 0.0854 0.0852	0.2341	0.3612 0.3590 0.3621	0.44
	48:52	0.0781 0.0782 0.0789	0.555	0.5034 0.5041 0.5033	0.075	0.0842 0.0849 0.0851	0.5577	0.3452 0.3445 0.3510	1.028
Brand of Methanol (Methanol:Water :: 50:50)	Rankem	0.798 0.0797 0.0789	0.583	0.4961 0.4941 0.4901	0.619	0.0876 0.0872 0.090	1.715	0.3473 0.3474 0.3455	0.308
	Emparta	0.0766 0.0769 0.0782	1.101	0.5041 0.5052 0.520	1.741	0.0850 0.0853 0.087	1.257	0.342 0.3412 0.3510	1.578

%Assay

%Assay was determined by analysing similar concentration of SOFO and LEDI laboratory mixture by UV and HPLC. The value of %assay was calculated by comparing absorbance (for UV methods) and area of chromatographic peak (for HPLC method) of laboratory mixture with drug standard and their results are listed in Table 5.

Table 4: Robustness result for SOFO and LEDI using RP-HPLC chromatographic method.

PARAMETERS	VALUES	SOFO				LEDI			
		Retention time		Peak area		Retention time		Peak area	
		Value (min)	%RSD	Value	%RSD	Value (min)	%RSD	Value	%RSD
pH (unit)	2.7	3.83		2053.3		9.94		424.5	
		3.81	0.261	2053.4	1.24	9.93	0.100	433.7	1.343
		3.82		2098.1		9.95		435.2	
	2.8	3.77		2054.9		9.92		425.7	
		3.78	0.152	2063.7	0.483	9.92	0.058	430.9	0.619
		3.78		2074.8		9.91		427.4	
	2.9	3.81		2055		9.90		426.7	
		3.82	0.399	2067.3	1.132	9.91	0.058	431.9	1.047
		3.84		2100.4		9.90		435.7	
Flow rate (mL/min)	0.9	3.88		2107.2		10.01		435.9	
		3.89	0.257	2160.3	1.720	10.02	0.099	430.8	0.757
		3.87		2178.3		10.00		429.8	
	1	3.77		2054.9		9.92		425.7	
		3.78	0.152	2063.7	0.483	9.92	0.058	430.9	0.619
		3.78		2074.8		9.91		427.4	
	1.1	3.67		2109.2		9.85		430.8	
		3.66	0.157	2119	0.571	9.84	0.155	429.8	0.630
		3.67		2133.3		9.82		425.7	
	43	3.58		2025		9.77		435.2	
		3.57	0.280	2089.2	1.944	9.79	0.102	425.7	1.104
		3.56		2099.2		9.78		430.9	
Volume of buffer in mobile phase (mL)	45	3.77		2054.9		9.92		425.7	
		3.78	0.152	2063.7	0.483	9.92	0.058	430.9	0.619
		3.78		2074.8		9.91		427.4	
	47	3.88		2067.2		10.01		429.8	
		3.89	0.257	2098.7	1.865	10.05	0.207	433.7	0.643
		3.87		2145.2		10.04		435.2	

Table 5: % Assay of SOFO and LEDI using UV and RP-HPLC method.

APIs	Labelled Claim	% Assay by Absorption Correction method	% Assay by First derivative method	% Assay by RP-HPLC method
SOFO	400 mg	100.2±0.280	100.3±1.01	101.25±0.428
LEDI	90 mg	100.7±1.41	100.74±0.14	100.17±0.320

System suitability parameters

For chromatographic method, system suitability testing was carried out on freshly prepared standard solutions (n=6) containing SOFO and LEDI. System suitability parameters obtained with 20µL injection volumes are summarized in Table 6.

Table 6. Result of system suitability parameters of SOFO and LEDI.

Parameters	Data Obtained*	
	SOFO	LEDI
Retention time ± SD	3.78±0.123	9.92±0.06
Theoretical plate ± SD	8987±18.98	3720±14.32
Tailing factor ± SD	1.29±0.001	0.99±0.043
Resolution ± SD	4.084±0.501	3.98±0.451

*Six replicated were taken to perform system suitability parameters.

DISCUSSION

In both the UV methods, the primary requirement for developing a method for analysis is that the entire spectra should follow the Beer's law at all the wavelength, which was fulfilled in case of both these drugs. The two wavelengths were used for the analysis of the drugs were 260nm (λmax of SOFO) and 334nm (λmax of LEDI) at which calibration curves were prepared for both the drugs.The validation parameters were studied at all the wavelengths for the proposed method. Accuracy was determined by calculating the recovery. The method was successfully used to determine the amount of SOFO and LEDI present in tablet dosage form. The results obtained were in good agreement with the corresponding labeled amount. Precision was calculated as intra-day and inter-day variations (%RSD) for both the drugs.

CONCLUSIONS

The proposed methods were simple, rapid, accurate, precise and robust and were successfully validated according to ICH Q2 (R1) guidelines. The sample recovery was in good agreement with the respective label claim, which suggested non-interference of formulation additives in its estimation. Hence, the developed UV method and RP-HPLC method could be successfully applied for estimation of SOFO and LEDI in bulk and laboratory mixture

ABBREVIATIONS

SOFO : SOFO
LEDI : LEDI
LOD : Limit of detection
LOQ : Limit of quantitation
HPLC : High performance liquid chromatography
UV : Ultraviolet
MeOH : Methanol
ACN : Acetonitrile
ICH : International Conference on harmonization
%RSD : % Relative standard deviation

REFERENCES

1. Link JO, Taylor JG, Xu L, Mitchell M, Guo H, Liu H, Kato D, Kirschberg T, Sun J, Squires N, Parrish J. Discovery of ledipasvir (GS-5885): a potent, once-daily oral NS5A inhibitor for the treatment of hepatitis C virus infection. Journal of medicinal chemistry. 2014 Jan 10;57(5):2033-46.
2. Gentile I, Borgia F, R Buonomo A, Castaldo G, Borgia G. A novel promising therapeutic option against hepatitis C virus: an oral nucleotide NS5B polymerase inhibitor sofosbuvir. Current medicinal chemistry. 2013 Oct 1;20(30):3733-42.
3. Traveset J, Such V, Gonzalo R, Gelpi E. Derivative and graphical procedures for correction of irrelevant UV spectrophotometric absorption in changeable matrixes. Journal of pharmaceutical sciences. 1980 Jun 1;69(6):629-33.
4. Maurice-Estepa L, Levillain P, Lacour B, Daudon M. Advantage of zero-crossing-point first-derivative spectrophotometry for the quantification of calcium oxalate crystalline phases by infrared spectrophotometry. Clinica Chimica Acta. 2000 Aug 31;298 (1):1-1.
5. Brown P, Hartwick RA. High performance liquid chromatography.
6. Pan C, Chen Y, Chen W, Zhou G, Jin L, Zheng Y, Lin W, Pan Z. Simultaneous determination of ledipasvir, sofosbuvir and its metabolite in rat plasma by UPLC–MS/MS and its application to a pharmacokinetic study. Journal of Chromatography B. 2016 Jan 1;1008:255-9.
7. German P, Moorehead L, Pang P, Vimal M, Mathias A. Lack of a clinically important pharmacokinetic interaction between sofosbuvir or ledipasvir and hormonal oral contraceptives norgestimate/ethinyl estradiol in HCV-Uninfected female subjects. The Journal of Clinical Pharmacology. 2014 Nov 1;54(11):1290-8.
8. Swain D, Samanthula G, Bhagat S, Bharatam PV, Akula V, Sinha BN. Characterization of forced degradation products and in silico toxicity prediction of Sofosbuvir: A novel HCV NS5B polymerase inhibitor. Journal of pharmaceutical and biomedical analysis. 2016 Feb 20;120:352-63.
9. Zaman B, Siddique F, 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 Dec 1;79(23-24):1605-13.
10. El Hamd MA, Ali R, Marzouk AA, Abdelmageed OH. Validated Ultraviolet-Spectrometric Method for Determination of Sofosbuvir in Tablets Formulation. Journal of Applied Pharmaceutical Science Vol. 2017 Feb;7(02):114-9.

5487847845**1170357**