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A Stability Indicating RP-HPLC Method for Simultaneous Estimation of Ledipasvir and Sofosbuvir in Bulk and its Dosage Form

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ABSTRACT

A new, simple stability-indicating RP-HPLC and UV method for the simultaneous estimation of ledipasvir and sofosbuvir in bulk and dosage form was developed. Chromatography was carried out using Dona-Zorbax C8, 250 × 4.6, 5 µm column with a flow rate of 1.0 mL/min. The mobile phase consisted of 0.1% orthophsophoric acid (OPA) and methanol in the ratio 45:55 and eluate was monitored at 238 nm. The retention times of ledipasvir and sofosbuvir were found to be 3.296 and 7.257 min, respectively. The method obeys Beer's law in the concentration range of 45-135 µg/mL for ledipasvir and 200-600 µg/mL for sofosbuvir. The LOD and LOQ were found to be 0.090 and 0.695, respectively for ledipasvir. The LOD and LOQ were found to be 0.6619 and 2.2063, respectively for sofosbuvir. The accuracy of the method was assessed by recovery study in the dosage form at three concentration levels. The method developed has been statistically validated according to ICH guidelines. The method showed good reproducibility and recovery with % RSD less than 2. The stability indicating capability of the method was established by forced degradation studies under stress conditions like acid, base, peroxide, UV, thermal, humidity. Hence, the chromatographic method developed for the estimation is found to be rapid, simple, specific, sensitive, precise, accurate, robust and reliable that can be effectively applied for routine analysis.

KEYWORDS

Ledipasvir, Sofosbuvir, RP-HPLC, UV spectroscopy.

INTRODUCTION

Hepatitis C is treated with a fixed-dose combination of ledipasvir and sofosbuvir [1]. In patients infected with hepatitis C virus (HCV) genotype 1, cure rates range from 94% to 99% [2]. Muscle pain, nausea, a rash, cough and headache are all common side effects. In 2014, ledipasvir/sofosbuvir was approved for use as medicine in Canada, the European Union and the United States [3,4]. It is on the list of essential medicines by the World Health Organization. Gilead Sciences developed the treatment for hepatitis C known as ledipasvir. While treating chronic hepatitis C genotype 1 patients, ledipasvir is most commonly administered in conjunction with sofosbuvir. Both treatment-naive and treatment-experienced patients were subjected to this medication's effectiveness testing [5,6]. Ledipasvir inhibits NS5A, a crucial viral phosphoprotein involved in viral assembly, secretion and replication [7].

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Ledipasvir is an antiviral medication with a chemical formula of methyl N-[(2*S*)-1-[(6*S*)-6-[5-[9,9-difluoro-7-[2-[(1*R*,3*S*,4*S*)-2-[(2*S*)-2-(methoxycarbonylamino)-3-methyl-butanoyl]-2-azabicyclo[2.2.1]heptan-3-yl]-3*H*-benzimidazol-5-yl]fluoren-2-yl]-1*H*-imidazol-2-yl]-5-azaspiro[2.4]heptan-5-yl]-3-methyl-1-oxobutan-2-yl]carbamate (Fig. 1a), it does this by inhibiting the NS5A protein, which is primarily responsible for the replication of viral RNA. Nucleotide polymerase inhibitors are a class of antiviral medications that include sofosbuvir. It works by lowering the body's levels of the hepatitis C virus (HCV) [1-4]. Sofosbuvir is a isopropyl (2*S*)-2-[[(2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxoprrimidin-yl)- 4-fluoro-3-hydroxy-4-methyl-tetrahydrofuran-2-yl]methoxy-phenoxyphosphoryl]-amino]propanoate with a formula of $C_{22}H_{29}FN_3O_9P$ (Fig. 1b) and a it does this by inhibiting the NS5B polymerase, which is used to treat hepatitis C. Sofosbuvir may not stop the virus from spreading to other people. Sofosbuvir is metabolized into a uridine triphosphate mimic that when incorporated into RNA by NS5B polymerase [7], functions as a RNA chain terminator. Depending on the specific circumstances, genotype and cost-effectiveness based perspective, sofosbuvir may also be used in conjunction with other medications and for longer treatment durations. Sofosbuvir and the viral NS5A inhibitor ledipasvir, for instance, can be used to treat genotypes 1, 4, 5 and 6 hepatitis C infections [8].

An extensive literature review was carried out in an effort to come up with a clear and reliable method for estimating ledipasvir and sofosbuvir simultaneously. A few spectroscopic, chromatographic and other analytical techniques have been used to estimate ledipasvir and sofosbuvir in pharmaceutical preparations either alone or in combination with other drugs [9-21]. In order to separate and quantify a mixture of ledipasvir and sofosbuvir in a single run, this study aims to develop and validate a new RP-HPLC method that is quick, accurate and economic stability-indicating. The developed method can be successfully used for quality control purposes and was validated in accordance with ICH guidelines [22,23].

EXPERIMENTAL

Using a Waters Alliance-HPLC system with a waters 1525 binary HPLC pump, a 2695 separation module connected to a 2996 photo diode array detector and Waters 2707 auto sampler, the method was developed and validated. Empower® version 2 acquired the information. The heating mantle, an ADWA pH meter and an Ascoset electronic balance were also utilized. For sonicating the samples, an ultrasonic bath was used.

Spectrum Pharma Ltd. in Hyderabad, India graciously provided gift samples of the working standards for ledipasvir and sofosbuvir. Working standards were kindly given as gift samples by Spectrum Pharma Ltd., Hyderabad, India. HPLC grade solvents include acetonitrile, water and methanol. Other analytical grade chemicals include sodium hydroxide, hydrochloric acid, 20% hydrogen peroxide, orthophosphoric acid, triethyl amine and potassium dihydrogen phosphate were purchased from Merck Ltd., India.

Chromatographic conditions: Zorbax C8 250 × 4.6, 5.0 m column with a mobile phase of 45:55 methanol and 0.1% OPA were used for separation. During the analysis, the samples were injected with a volume of 10 mL, the flow rate was maintained at 1.0 mL/min, the runtime was 8 min and the temperature was kept at 30 ºC. Using a PDA detector with a wavelength of 260 nm, the drugs were detected and their purity established.

Preparation of working standard solution: A 100 mL clean, dry volumetric flask containing 90 mg of ledipasvir and 400 mg of sofosbuvir working standards was accurately weighed and 10 mg of diluent was added and sonicated for 10 min to dissolve. Ledipasvir was diluted to 900 g/mL using the diluent and sofosbuvir was diluted to 4000 g/mL using a nylon filter with a diameter of 0.45 µ. To achieve the final concentration of 90 g/mL ledipasvir and 400 g/mL sofosbuvir, 2.5 mL of the aforementioned stock solution was pipetted into a 25 mL volumetric flask using diluents. Weighed and transferred 90 mg of ledipasvir and 400 mg of sofosbuvir working standards into a 100 mL clean dry volumetric flask and 10 mL of diluent was added and sonicated for 10 min to dissolve. The final volume was made up with the diluent and filtered through 0.45 µ nylon filter to obtain a concentration of 900 µg/mL ledipasvir and 4000 µg/mL sofosbuvir. From the above stock solution, 2.5 mL was pipetted out in to 25 mL volumetric flask and made up to the final volume with diluents to obtain the final concentration of 90 µg/mL ledipasvir and 400 µg/mL sofosbuvir.

Preparation of sample solution: Ten tablets (Hepcinat-LP, each tablet weighed 1 g, containing 90 mg of ledipasvir and 400 mg of sofosbuvir) were weighed. The average weight of ten tablets was determined. A mortar and pestle were used to break up the tablets. A 100 mL volumetric flask containing a portion of powder that weighed the same as one tablet was

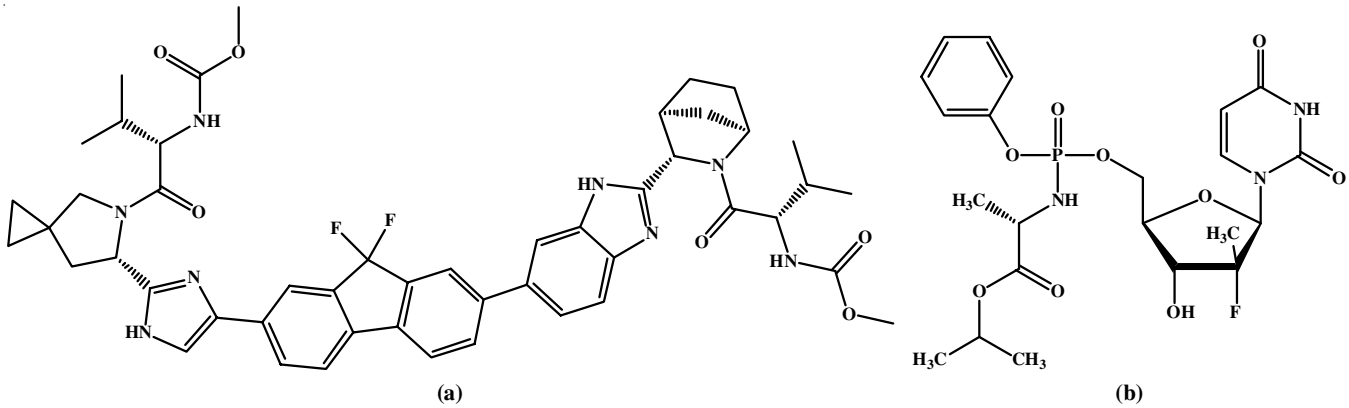

Fig. 1. Chemical structure of (a) ledipasvir and (b) sofosbuvir

accurately weighed. Around 50 mL of diluent was added and the combination was sonicated for 15 min with discontinuous shaking, then, at that point, 20 mL of diluent was added and sonicated for 25 min. The items were reestablished to room temperature and weakened to definite volume with diluent to outfit stock answer for get a convergence of 900 µg/mL ledipasvir and 4000 µg/mL sofosbuvir. In order to arrive at the final concentration of 90 g/mL ledipasvir and 400 g/mL sofosbuvir, 2.5 mL of stock solution was pipetted out of the stock solution into a 25 mL volumetric flask and diluted with diluents.

Method validation: To determine the system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, ruggedness and robustness of the developed and optimized RP-HPLC method, it was validated in accordance with ICH guidelines Q2 (R1).

System suitability: System performance was confirmed by evaluating system suitability parameters. The chromatograms were recorded after five injections of 10 µL of standard solution into the chromatograph. The number of theoretical plates and peak tailing were determined as parameters.

Specificity: In order to investigate interference from the representative peaks, we individually injected the solutions of diluent (blank), placebo, working standards and sample solution to determine the analytical method's specificity.

Precision: Six injections of the same concentrations of ledipasvir and sofosbuvir were used to measure repeatability and method precision and the percentages of assay and RSD for each compound were calculated. In the same laboratory, different analysts and a different instrument were used to perform reproducibility, robustness and intermediate precision.

Accuracy: Accuracy of the proposed method was determined using recovery studies by spiking method. The recovery studies were carried out by adding known amounts (50%, 100% and 150%) of the working standard solutions of ledipasvir and sofosbuvir to the pre-analyzed sample. The solutions were prepared in triplicates to determine the accuracy.

Linearity: Linearity was evaluated by analyzing different concentrations of the standard solutions of ledipasvir and sofosbuvir. Six working standard solutions ranging between 45-135 µg/mL for ledipasvir, 200-600 µg/mL for sofosbuvir were prepared and injected.

Limit of detection and limit of quantification: Limit of detection (LOD) and limit of quantification (LOQ) of ledipasvir and sofosbuvir were determined by calibration curve method. Solutions of ledipasvir and sofosbuvir were prepared in linearity range and injected (n = 3).

Robustness: To examine the robustness of the developed method, the experimental conditions were deliberately changed, resolution, tailing factor and theoretical plates of ledipasvir and sofosbuvir peaks were evaluated.

RESULTS AND DISCUSSION

System suitability: The results of system suitability are depicted in Table-1. The resolution of the peaks of ledipasvir and sofosbuvir were also found to be within the limits.

Specificity: From the obtained chromatograms in Figs. 2-4, it can be inferred that there were no co-eluting peaks at the retention time of ledipasvir and sofosbuvir, which shows that peak of analyte was pure and the excipients in the formulation did not interfere with the analyte of interest.

TABLE-1
SYSTEM SUITABILITY DATA

Parameter	LDP	SFB	Acceptance criteria
USP plate count*	17053	10841	NLT 3000
%RSD	0.7	0.8	NMT 2.0
Peak tailing*	1.10	1.07	NMT 2.0

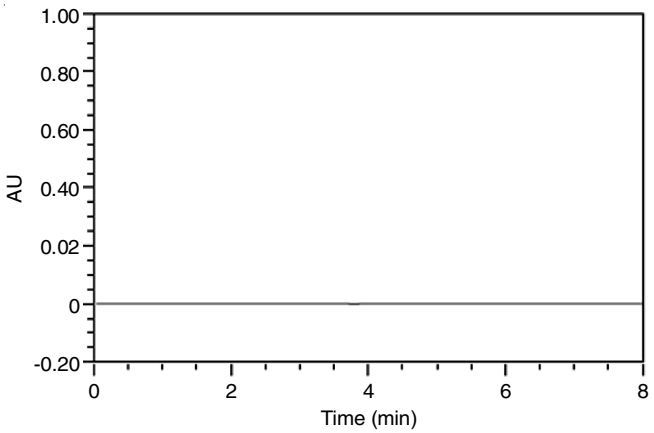

Fig. 2. Chromatogram of blank

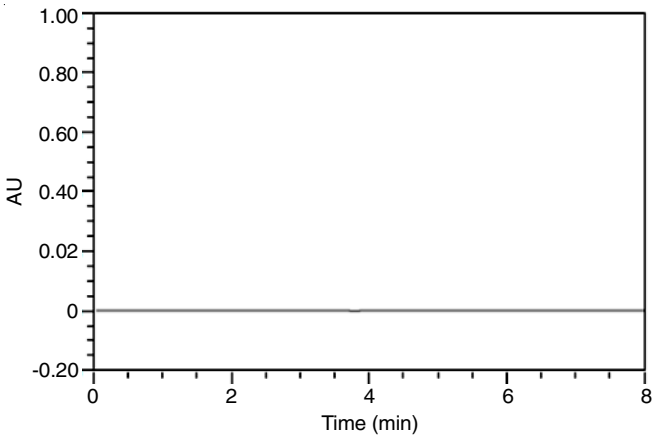

Fig. 3. Chromatogram of placebo

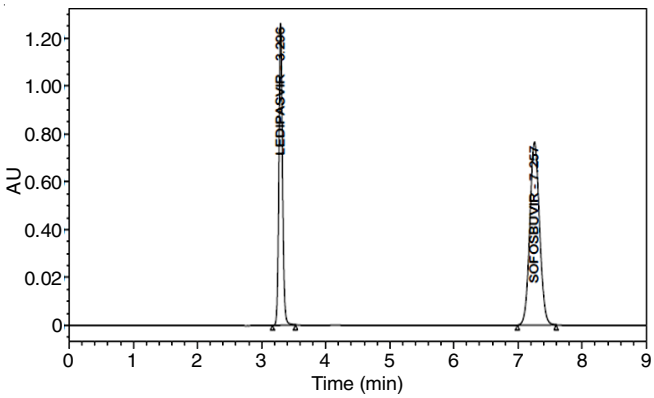

Fig. 4. Chromatogram of ledipasvir and sofosbuvir

Precision: From the results in Table-2, the % assay for ledipasvir and sofosbuvir was found to be in the range of 98-102% and the % RSD for ledipasvir and sofosbuvir to be within 2%. Hence the method is precise, reproducible and rugged for 48 h study.

TABLE-2
PRECISION DATA

S. No.	LDP		SFB	
	Peak areas	Assay (%)	Peak areas	Assay (%)
1	4465231	99.56	7360011	100.78
2	4462350	99.24	7368755	98.41
3	4464645	100.12	7364800	101.07
4	4462083	99.89	7365230	98.80
5	4468154	100.54	7361573	101.75
6	4466897	99.45	7361600	99.47
Mean	4464893	99.81	7363661	100.05
SD	3374.3	0.18	5074.6	1.345
% RSD	1.1	0.18	1.3	1.3

Linearity: Linearity was evaluated by analyzing different concentrations. From the results (Table-3), it is inferred that the correlation coefficient was greater than 0.999. The slope and *y*-intercept values were also provided, which confirmed good linearity between peak areas and concentration.

TABLE-3
LINEARITY DATA

LDP		SFB	
Concentration (µg/mL)	Peak area*	Concentration (µg/mL)	Peak area*
50	2230184	50	3680821
75	3347897	75	5524283
100	4462463	100	7363685
125	5577829	125	9204665
150	6694287	150	11026551
Regression equation y = 43363x + 44843; R^2 = 0.9992		Regression equation y = 73499x + 6964.2; R^2 = 0.9992	

*Average peak area of 3 replicate injections for each concentration

Accuracy: Table-4 shows the % recovery for ledipasvir and sofosbuvir found to be in the range of 98-102% and the % RSD for ledipasvir and sofosbuvir is less than 2%. Hence the proposed method was accurate.

TABLE-4
ACCURACY DATA

Drug name	Conc. (%)	Amount spiked (µg/mL)	Amount recovered (µg/mL)	Recovery (%)	Statistical parameters
LDP	50	44.55	44.72	99.61	Mean %: 99.77
	100	89.10	89.24	99.84	SD: 0.82
	150	133.65	133.81	99.88	%RSD: 0.8
SFB	50	200	199.58	100.21	Mean %: 100.27
	100	400	398.74	100.31	SD: 1.09
	150	600	598.03	100.32	%RSD: 1.09

LOD and LOQ: The limit of detection (LOD) and limit of quantification (LOQ) of ledipasvir and sofosbuvir were calculated by using following equations and the values are reported in Table-5.

$$LOD = 3.3 \times \frac{\sigma}{S}$$

$$LOQ = 10 \times \frac{\sigma}{S}$$

where σ = the standard deviation of the response and S = slope of the calibration curve.

TABLE-5
LOD AND LOQ DATA

Drug name	LOD (µg/mL)	LOQ (µg/mL)
LDP	0.090	0.301
SFB	0.6619	2.2063

Robustness: From the results in Table-6, it is evident that the system suitability parameters such as resolution, RSD, tailing factor and the theoretical plate count of ledipasvir and sofosbuvir remained unaffected by deliberate changes. The results were presented along with the system suitability parameters of optimized conditions. Thus, the method was found to be robust with respect to variability in applied conditions.

TABLE-6
ROBUSTNESS DATA

Parameters		System suitability parameters		
		RT	Theoretical plates	Peak tailing
Optimized method	LDP	3.296	17053	1.10
	SFB	7.257	10841	1.07
Flow rate (1.2 mL/min)	LDP	2.741	15685	1.10
	SFB	6.003	9851	1.07
Flow rate (0.8 mL/min)	LDP	4.127	18129	1.11
	SFB	9.065	12683	1.07
Temperature (30 °C)	LDP	3.266	17367	1.06
	SFB	6.845	12028	1.04
Temperature (20 °C)	LDP	3.288	16823	1.09
	SFB	7.212	10970	1.07

Conclusion

A simple and rugged RP-HPLC method has been developed for the simultaneous determination of ledipasvir and sofosbuvir in active pharmaceutical ingredients and sample. The proposed method was validated in accordance with ICH guidelines by testing its parameters which include system suitability, specificity, precision, linearity, LOD, LOQ, accuracy and robustness. Thus, stress induced studies prove the effectiveness of the proposed stability-indicating RP-HPLC method, which can be adopted in routine analysis in pharmaceutical industries.

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