Available online at www.ijpsdr.com

International Journal of Pharmaceutical Sciences and Drug Research
2017; 9(6): 291-298

Research Article

ISSN: 0975-248X
CODEN (USA): IJPSPP

Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Ledipasvir and Sofosbuvir in Bulk and Pharmaceutical Dosage Form

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ABSTRACT
The day by day new combinations drugs are being introduced in market. Then the multiple therapeutic agents which acts at different sites are used in the management of various diseases and disorders are done. Thus it is necessary to develop methods for analysis with the help of number of analytical techniques which are available for the estimation of the drugs in combination. The analyst were determine the Specific, accurate, simple, selective and stability-indicating RP-HPLC method is developed and validated for simultaneous determination of sofosbuvir and ledipasvir in tablet dosage form. RP-HPLC method was performed on the systronics isocratic HPLC System equipped with SP930 D HPLC pump and dual wavelength UV-VIS detector and C_{18} column (250 mm × 4.6 mm, 5µm), using the mobile phase (Methanol: Water 83:17 v/v) pH 3.0 with 0.05% acidic acid at a flow rate of 1.0 ml/min, injection volume 20µl and UV detection at 245 nm. This method is validated according to BP, USP and ICH requirements for new methods, which include accuracy, precision, robustness, ruggedness, lod, loq, linearity and range. Linear relationships were obtained in the ranges of 10-50µg/ml and 40-200µg/ml with correlation coefficients of 0.9991 and 0.9994 at Rt value of 7.45 min and 3.50 min for sofosbuvir and ledipasvir respectively. The forced degradation studies as acidity, alkalinity, oxidation and hydrolytic degradation were performed according to ICH guidelines.

Keywords: Sofosbuvir and Ledipasvir, HPLC, Development, Forced degradation, Validation.

DOI: 10.25004/IJPSDR.2017.090602 **Int. J. Pharm. Sci. Drug Res. 2017; 9(6): 291-298**

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Received: 28 September, 2017; **Revised:** 18 October, 2017; **Accepted**: 25 October, 2017; **Published:** 20 November, 2017

INTRODUCTION
Pharmaceutical Analysis plays a vital role in quality assurance and quality control of bulk drugs and their formulations. Pharmaceutical analysis is a particular branch of analytical chemistry, which includes isolating, identifying and determining the relative

amounts of compounds in a sample matter. It is concerned with chemical characterization of matter both quantitative and qualitative. In recent years many analytical techniques have been developed. Analytical method is a particular utilization of a procedure to solve a problem. Analytical instrumentation assumes an imperative part in the production and evaluation of new products and protection of Consumers and the environment. This instrumentation provides the lower detection limits required to assure safe foods, medications, water and air.

Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications. There are two important reasons for validating assays in the pharmaceutical industry. The first, and by for the most important, is that assay validation is an integral part of the quality control system. The second is that current good manufacturing practice regulation requires assay validation.

Globally, 130-150 millions of people have chronic hepatitis C infection. A significant number of those who are chronically infected will develop liver cirrhosis or liver cancer. Gilead Sciences overcome most common related liver diseases by its Great invention (Harvoni). Harvoni (90 mg ledipasvir/400 mg sofosbuvir) approved by United States FDA. It is indicated for the treatment of chronic HCV genotypes 1, 4, 5, and 6 in adults and also indicated for the treatment of chronic HCV in patients co-infected with HIV. [1-2]

Sofosbuvir is chemically known as (S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)-(phenoxy) phosphorylamino) propanoate. It has a molecular formula of $C_{22}H_{29}FN_3O_9P$ and a molecular weight of 529.45. [2]

Ledipasvir is chemically known as Methyl [(2S)-1-{(6S)-6-[5-(9,9-difluoro-7-{2-[(1R,3S,4S)-2-{(2S)-2-[(methoxycarbonyl)amino]-3-methylbutanoyl} 2azabicyclo[2.2.1]hept-3-yl]-1H-benzimidazol-6-yl}-9H-fluoren-2-yl)-1H-imidazol-2-yl]-5-azaspiro[2.4]hept-5-yl}-3-methyl-1-oxobutan-2-yl]carbamate. It has a molecular formula of $C_{49}H_{54}F_2N_8O_6$ and a molecular weight of 889.00. [2]

The combination of these two drugs is not official in any pharmacopoeia. Very recently, a limited number of methods have been developed for the individual and simultaneous determination of both drugs. The degradation products of sofosbuvir and ledipasvir under several stress conditions have been determined by HPLC.

MATERIALS AND METHODS

Chemicals and Reagents

I. Pure samples: pure samples of sofosbuvir and ledipasvir were obtained as a generous gift samples from the Mylan Pharmaceutical Pvt. Ltd. (India), Mylan Pharmaceutical Company which is india's third largest exporter of antiretroviral for HIV/Aids care, hepato care, critical care, onco care and women's care.

II. Pharmaceutical dosage form: MyHep LVIR^{tm} fixed dose combination tablets Containing ledipasvir (90mg) and sofosbuvir (400 mg) were manufactured by Mylan Pharmaceutical Pvt. Ltd. (India).

III. Chemicals: all chemicals and reagents of analytical grade and hplc grade were purchased from Merck Chemicals, Mumbai, India.

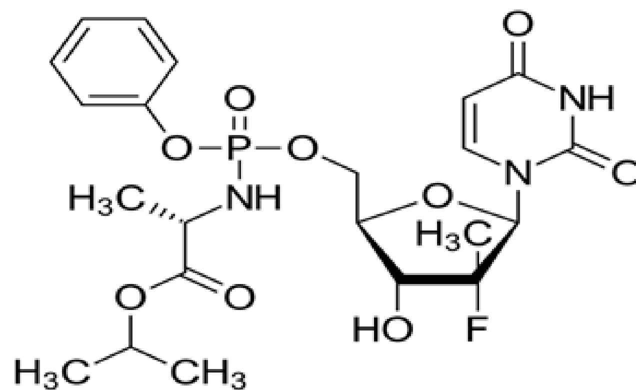

Fig. 1: Chemical structure for sofosbuvir

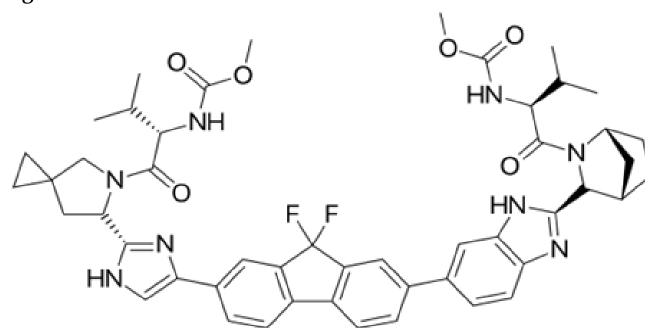

Fig. 2: Chemical structure for ledipasvir

Instruments

Chromatographic experimentations were performed using Systronics isocratic HPLC System equipped with SP930 D HPLC pump and dual wavelength UV-VIS detector, Data acquisition and processing was performed using Chemitochrom automation Chromatograph data system software and methods were conducted using an isocratic Reverse phase HPLC techniques. The mobile phase was prepared freshly filtered through 0.45µm membrane filters (Millipore, USA) and sonicated for 30 min, before use in order to degas the mobile phase. A C_{18} RP-Purosphere column (5µm, 4.6 mm × 250 mm), Germany was used for analysis. Column was prewashed before the analysis of samples with boiling water and methanol alternately.

Selection of Chromatographic Mode

Proper selection of the method depends upon the nature of the sample (ionic/ ionizable/ neutral molecule), its molecular weight and solubility. The drugs selected in present study are neutral in nature. Both the drugs are freely soluble in organic solvents. Hence, reversed phase HPLC was selected for the initial separations because of its simplicity and suitability. [3-4]

Selection of Stationary Phase

On the basis of reversed phase HPLC mode and number of carbon present in the molecule (analyte) RP-Purosnosphere C_{18} (Grace) column (5µm, 4.6 mm × 250 mm), of following configuration was selected for further study. [4]

Selection of Mobile Phase

Ledipasvir and Sofosbuvir are soluble in HPLC grade water and methanol on ultrasonication, insoluble in hexane and are freely soluble in methanol and water, DMSO. Both the drugs on ultrasonication are soluble in methanol, acetonitrile and water mixture. Hence, mixture of methanol: water was used for initial separation. [4]

Selection of Detector and Detection Wavelength

UV-Visible detector was selected, as it is reliable and easy to set at the correct wavelength. An overlay spectrum of drugs in methanol water (83:17) was recorded. From the overlay spectrum 245 nm was selected as a wavelength of measurement.

Standard Sample Preparation Ledipasvir and Sofosbuvir [A]

1) An accurately weighed quantity of Ledipasvir 10 mg and 40 mg Sofosbuvir was taken in 25 ml volumetric flask and dissolved in 10 ml Methanol, with the help of ultrasonication for about 10 min. Then the volume was made up to the mark using methanol to get standard stock solution. 1000µgm/ml Ledipasvir and 4000µgm/ml Sofosbuvir----- STOCK.

2) Ledipasvir and Sofosbuvir Standard Solution [A1]: Standard stock Solution [A] 0.1 ml and make vol. With mobile phase 10 ml, = 10µgm/ml Ledipasvir and 40µgm/ml Sofosbuvir

3) Ledipasvir and Sofosbuvir Standard Solution [A2]: Standard stock Solution [A] 0.2 ml and make vol. With mobile phase 10 ml, = 20µgm/ml Ledipasvir and 80µgm/ml Sofosbuvir

4) Ledipasvir and Sofosbuvir Standard Solution [A3]: Standard stock Solution [A] 0.3 ml and make vol. With mobile phase 10 ml, = 30µgm/ml Ledipasvir and 120µgm/ml Sofosbuvir

5) Ledipasvir and Sofosbuvir Standard Solution [A4]: Standard stock Solution [A] 0.4 ml and make vol. With mobile phase 10 ml, = 40µgm/ml Ledipasvir and 160µgm/ml Sofosbuvir

6) Ledipasvir and Sofosbuvir Standard Solution [A5]: Standard stock Solution [A] 0.5 ml and make vol. With mobile phase 10 ml, = 50µgm/ml Ledipasvir and 200µgm/ml Sofosbuvir.

Optimization of HPLC Parameters

Optimizations of HPLC process is to find a set of conditions that adequately separate and enable the quantification of the analytes from the endogenous material with acceptable accuracy, precision, cost, ease and speed. [5]

Optimization of Mobile Phase Strength

Initially methanol and water in different ratios were tried, and then methanol and water at different pH were tried. It was found that in methanol: water 83:17 (v/v), pH 3.0 adjusted using 0.05% acidic acid, this mobile phase at flow rate 1.0 mL/min gave good resolution of peaks with minimum tailing as compared to other mobile phases. [5]

The effect of change in composition on retention time is shown in Table 1.

Optimization of Detection Wavelength

On the basis of overlay spectra different wavelengths were selected. A fixed concentration of analyte mixture was analyzed at selected wavelengths. As per the response of analyte 245 nm wavelength was chosen. At wavelength 245 nm, analyte peak area was greater than that of at other wavelength observed, which is shown in Table 2.

Table 1: Optimization of Mobile Phase Strength

S. No.	Mobile Phase Strength (methanol: water v/v)	t_R of Ledipasvir	t_R of Sofosbuvir
1	81: 19	7.36	3.35
2	85:15	7.33	3.30
3	83:17	7.45	3.50
4	80:20	7.35	3.43
5	87:23	6.47	4.12

Table 2: Optimization of Detection Wavelength

Mobile Phase Strength (methanol: water v/v)	Wavelength (nm)	Area of Analytes	
		Ledipasvir	Sofosbuvir
83:17	244	143.97	212.53
83:17	245	145.17	214.62
83:17	265	146.27	216.41

Table 3: Final Chromatographic Conditions

Chromatographic Mode	Chromatographic Condition
Standard Solutions	1000µg/mL Ledipasvir and 4000µg/mL Sofosbuvir, in mobile phase
HPLC System	Younglin (S.K) Gradient System UV Detector
Pump	SP930 D HPLC Pump
Detector	UV 730 D UV-Visible detector
Stationary Phase	C_{18} (Grace) column (250 × 4.6mm, 5µm)
Mobile Phase	Methanol :water in ratio of 83:17 v/v pH 3.0 with 0.05% acidic acid
Detection Wavelength	245 nm
Flow Rate	1 mL/min
Sample Size	20µl
Column Temperature	Ambient

Table 4: Results of Analysis of Mixed Laboratory Standards.

Drug	Amount Present (µg/mL)	Mean Area	Amount Found ± S.D.	% Drug Estimation
Ledipasvir	10	146.32	9.98 ± 1.08	99.8
Sofosbuvir	40	216.29	39.95 ±1.56	99.87

Preparation of Mobile Phase

In volumetric flask Methanol: Water (83:17 v/v), pH 3.0, adjusted using 0.05% acidic acid. Then the mobile phase was filtered through 0.45 micron membrane filter paper using suction pump. The content was ultrasonicated for 20 min for degassing.

Preparation of Standard Mixture

Accurately weighed quantity of Ledipasvir (1 mg) and Sofosbuvir (4 mg) was transferred to 10 ml volumetric flask and dissolved in 10 ml methanol by means of ultrasonication so as to get Ledipasvir (100µg/ml) and

Sofosbuvir (400µg/ml). The above solution was filtered through 0.45µ Millipore Membrane filter. This solution (1 ml) was diluted to 10 ml using mobile phase to get the Ledipasvir (10µg/ml) and Sofosbuvir (40µg/ml) solution. The content was ultrasonicated for 20 min.

Preparation of Sample Mixture

Twenty tablets were weighed accurately and finely powdered. The tablet powder equivalent to Ledipasvir (1 mg) and Sofosbuvir (4 mg) was weighed accurately. Then it was transferred to a 100 ml volumetric flask containing in methanol: water 83:17 v/v. Then the content was ultrasonicated for 30 min. And volume was made up to the mark using methanol. The above solution was filtered through Whatman filter paper No. 1. This solution was again filtered through 0.45µm Millipore Membrane filter. This solution (1ml) was diluted to 10 ml using mobile phase to get Ledipasvir (10µg/ml) and Sofosbuvir (40µg/ml) solution. The content was ultrasonicated for 20 min.

Procedure [6-7]

Standard (20µL) and sample (20µL) solutions were injected separately after the equilibrium of stationary phase. The chromatograms were recorded and the response i.e. AUC of major peaks were measured. The content of Ledipasvir and Sofosbuvir were calculated by comparing a sample peak with that of standard.

The replicate analysis of Ledipasvir and Sofosbuvir by proposed method showed the content of Ledipasvir and Sofosbuvir as 99.8 and 99.87% respectively (Table 4). The retention time of Ledipasvir and Sofosbuvir were found to be 7.6333 and 3.5167 min respectively (Fig. 1) and the results of the analysis of tablet were given in Table 5. Chromatogram of blank i.e. only mobile phase was also recorded (Fig. 3). The amount of each drug estimated in laboratory mixture was calculated using following formula:

$$\% \text{ Label claim} = \frac{At}{As} \times \frac{Ds}{Dt} \times \frac{Ws}{Wt} \times \frac{A}{Lc} \times 100$$

Where;

At = AUC for sample solution

A_s= AUC for standard solution

Ds = Dilution of standard

Dt = Dilution of sample

Ws = Weight of standard (mg)

Wt = Weight of sample (mg)

Lc = Label claim

A = Average weight

Validation of the Method

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

Linearity

The linearity range of Ledipasvir and Sofosbuvir was evaluated by varying concentrations of standard solutions were injected into HPLC system. The linearity graph was plotted from Fig. 9-10. A calibration curve was constructed for each sample by plotting the peak area obtained the concentration. The correlation coefficient for the data was calculated as 0.9991 for Ledipasvir and 0.9994 for Sofosbuvir. Linearity results were shown in Table 6-7.

Accuracy

Accuracy of the method was expressed in terms of recovery of added compound at 80%, 100% and 120% level of sample. Mean % recovery and % RSD were calculated and were summarized in Table 8. The Results within 99% to 102% of true concentration of each drug were obtained at each added concentration, indicating that the method was accurate.

Precision

Precision studies were carried out using parameters like different days (intraday and inter-day). Results were shown in Table 9. i.e. % RSD is in the range of 0.30-1.78 i.e. less than 2 for different days.

Repeatability

The % RSD of Ledipasvir and Sofosbuvir for Repeatability were calculated as 0.83 and 0.51. Hence low values of % RSD (less than 2) indicate high precision of the method as shown in Table 10.

Robustness

The changes in flow rate, composition of mobile phase and wavelength performed to evaluate the robustness of the method; each parameter selected was varied at three levels. The results indicate that the insignificant differences in peak areas and less variability in retention time and theoretical plates were observed. Results were shown in Table 11-12.

Ruggedness

Ruggedness studies were carried out using different analyst parameter. (Analyst I) and (Analyst II) Results showed that the % RSD is less than 2 for different analyst studies at different days. This study signifies the ruggedness of the method under varying conditions of its performance (Table 13).

Limit of Detection and Limit of Quantitation

The LOD and LOQ were determined by RP HPLC for Ledipasvir (1.09µg/ml), (3.30µg/ml), and Sofosbuvir (3.36µg/ml), (10.19µg/ml) respectively (Table 14).

Forced Degradation Studies [7-9]

The Forced degradation of API was carried out as per ICH guidelines (ICH, Q2B) in acid, base, oxidation, hydrolytic. The results were displayed in Table 16.

Acid degradation

Weigh accurately 1 mg of Ledipasvir and 4 mg of Sofosbuvir into 10 ml volumetric flask, dissolve in 10 ml of diluent and 1.0 N aqueous HCL solution and close the volumetric flask by stopper. Keep the solution at room temperature up to 24 hours. Neutralize with 1.0 N aqueous NaOH solution and make up to the mark with the diluent, inject into the chromatographic system and calculate the percent of degradation (Table 16).

Base degradation

Weigh accurately 1 mg of Ledipasvir and 4 mg of Sofosbuvir into 10 ml volumetric flask, dissolve in 10 ml of the diluent 1.0 N aqueous NaOH solution and

close the volumetric flask by stopper. Keep the solution at room temperature up to 24 hours. Neutralize with 1.0 N aqueous HCl solution and make up to the mark with the diluent, inject into the chromatographic system and calculate the percent of degradation (Table 16).

Peroxide degradation

Weigh accurately 1 mg of Ledipasvir and 4 mg of Sofosbuvir into 10 ml volumetric flask, dissolve in 10 ml of the diluent, add 0.3 ml of 3.0% aqueous H_2O_2 solution and close the volumetric flask by stopper. Keep the solution at room temperature up to 24 hours. Inject into the chromatographic system and calculate the percent of degradation. No interference was found, although there was some degradation products appeared after H_2O_2 treatment due to the peak of hydrogen peroxide. And the degradation percent was calculated as shown in Table 16.

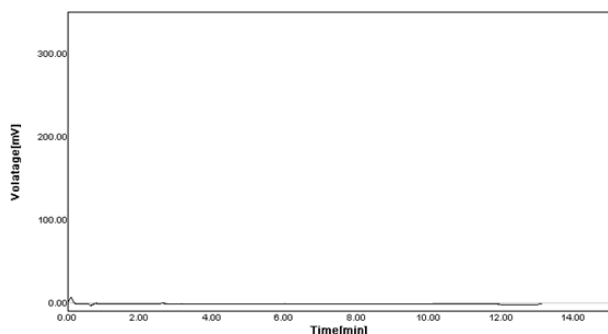

Fig. 3: Chromatogram of Blank

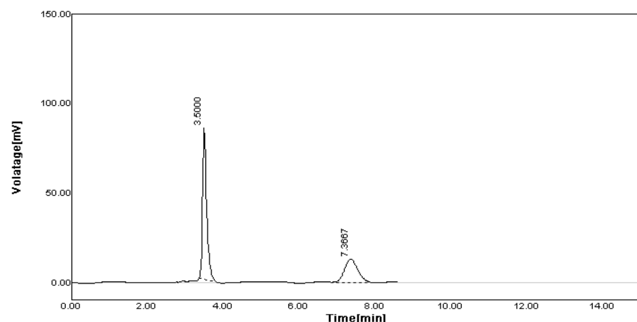

Fig. 4: Typical Chromatogram of Pure Mixed Standards

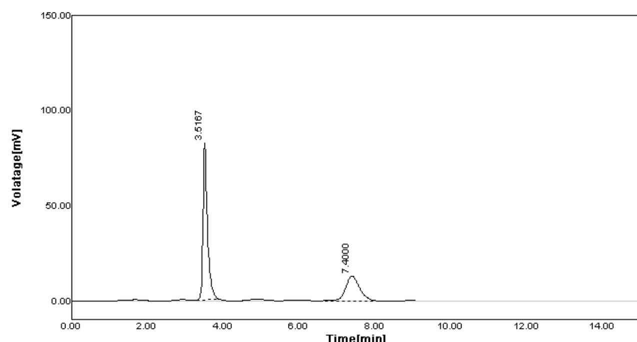

Fig. 5: Typical Chromatogram of Ledipasvir and Sofosbuvir in Tablet Formulation

Hydrolytic degradation

Weigh accurately 1 mg of Ledipasvir and 4 mg of Sofosbuvir into 10 ml volumetric flask, dissolved in 10 ml of diluent and up to the mark. Add H_2O_2 and close the volumetric flask by stopper. Keep the solution at room temperature up to 24 hours. Inject into the chromatographic system and calculate the percent of degradation. No interference was found, although there was some degradation products appeared after H_2O_2 treatment due to the peak of hydrogen peroxide. And the degradation percent was calculated as shown in Table 16.

Table 5: Analysis of Tablet Formulation (MyHep LVIR - Tablet*- Mylan Pharmaceutical)

S. No.	Amount Present (µg/ml)		Area		Amount Found (µg/ml)		% Drug Estimation	
	LEDI	SOFO	LEDI	SOFO	LEDI	SOFO	LEDI	SOFO
1.	10	40	144.1	215.62	9.97	39.83	99.7	99.57
2.	10	40	144.98	217.758	9.98	39.99	99.8	99.97
3.	10	40	145.2	216.259	9.99	39.95	99.9	99.87
4.	10	40	143.53	216.485	9.93	39.98	99.3	99.95
5.	10	40	145.23	217.354	9.99	39.99	99.9	99.97
MEAN					144.608	216.6952	9.972	39.948
SD					0.76	0.86	0.02	0.07
%RSD					0.52	0.40	0.25	0.17

Table 6: Standard Calibration Data of Ledipasvir by RP-HPLC Method

S. No.	Concentrations (µg/mL)	Area Mean (n=3) ± S.D.		%RSD
1	10	146.535	±1.93	1.32
2	20	255.030	±2.97	1.16
3	30	380.505	±3.15	0.83
4	40	483.445	±8.59	1.78
5	50	592.555	±1.84	0.31

Table 7: Standard Calibration Data of Sofosbuvir by RP-HPLC Method

S. No.	Concentrations (µg/mL)	Area Mean (n=3) ± S.D.		%RSD
1	40	216.689	± 2.93	1.35
2	80	469.387	± 4.95	1.05
3	120	736.566	± 6.58	0.89
4	160	963.558	± 9.36	0.97
5	200	1210.895	7±.83	0.65

RESULTS AND DISCUSSION

Careful evaluation of various parameters influencing analysis is an important aspect for the development of analytical method. The mobile phase was found to be most suitable methanol: water (83:17, v/v) at pH 3.0, adjusted using 0.05 % acidic acid at flow rate 1.0 ml/min give good resolution of peaks with minimum tailing as compared to other mobile phases. The wavelength 245 nm was selected. This system gave good resolution (>1.5 min) and optimum retention time with appropriate tailing factor (<2). The retention time of Ledipasvir and Sofosbuvir was found to be 7.45 and 3.50 min respectively.

The above results clearly indicate that HPLC technique can be successfully applied for the estimation of above

mentioned drugs in their combined dosage formulation without prior separation. [10-15]

Linearity Study

In linearity study combined dosage formulation was found to be linear in range of (10-50µg/ml) and (40-200µg/ml) with correlation coefficient values 0.9991 and 0.9994 respectively. At selected wavelength 245 nm. [16]

Accuracy (Recovery Studies)

Three replicate injections, each of three different test concentrations in the range of 80%, 100% and 120% of labeled claim of tablet under study yielded the results within 99% to 102% of true concentration of each drug. The results indicated that the method is accurate. [17]

Precision

Precision studies were carried out using parameters like different days and repeatability. Results showed that the % RSD in the range of 0.30-1.78 i.e. less than 2 for different days. [18]

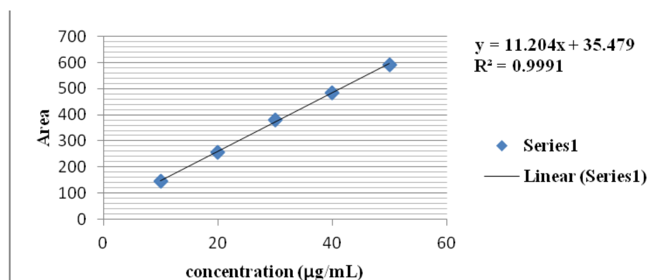

Fig. 6: Calibration Curve for Ledipasvir

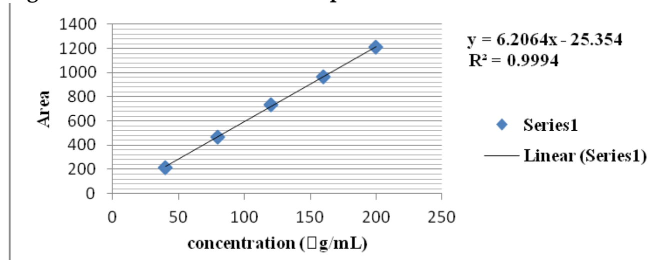

Fig. 7: Calibration Curve for Sofosbuvir

Table 8: Standard Addition Techniques for Determination of Ledipasvir and Sofosbuvir

Drug	Label Claim (µg/ml)	%	Amount Added (µg/ml)	Total Amount (µg/ml)	Amount Recovered (µg/ml)	Recovery (%)	SD	%RSD
				17.98	7.98	99.74		
		80	8	17.87	7.87	98.41	0.88	0.88
				18.08	8.08	100.06		
				20.00	10.0	101.64		
LEDI	10	100	10	20.16	10.16	100.0	089	0.88
				20.20	10.20	100.20		
				22.01	12.01	101.58		
		120	12	21.91	11.91	99.26	0.89	0.88
				21.11	11.11	99.58		
				71.12	31.12	97.25		
		80	32	71.99	30.99	96.84	0.25	0.25
				70.98	30.98	96.81		
				79.77	39.77	99.43		
SOFO	40	100	40	80.16	40.16	100.40	0.49	0.49
				79.82	39.99	99.97		
				87.81	47.81	99.62		
		120	48	88.30	48.30	100.63	0.53	0.53
				88.20	48.20	100.40		

Table 9: Results of Precision Study

Drug	Conc. [µg/ml]	Intra-day				Inter-day			
		Mean Area	Amount Found	S.D.	% R.S.D.	Mean Area	Amount Found	S.D.	% R.S.D.
	10	150.89	10.30	2.68	1.78	142.80	10.02	2.21	1.55
LEDI	30	377.38	30.52	1.14	0.30	380.18	30.77	1.50	0.39
	50	592.33	49.73	1.97	0.33	589.57	49.47	3.65	0.62
	40	218.34	38.85	3.65	1.67	223.80	40.14	2.22	0.99
SOFO	120	740.74	123.44	3.38	0.46	725.68	121.01	6.28	0.87
	200	1145.07	194.40	17.28	1.51	1179.89	194.21	12.21	1.04

Repeatability Study of Ledipasvir and Sofosbuvir

Table 10: Results of Repeatability Study of Ledipasvir and Sofosbuvir

Drugs	Conc. (µg/mL)	Area	Mean area	Amount found	SD	%RSD
		462.5529				
Ledipasvir	20	470.1528	465.9851	19.98	3.85	0.83
		456.2500				
		256.7151				
Sofosbuvir	40	257.3245	257.7544	39.99	1.31	0.51
		259.2235				

Robustness

To evaluate the robustness of the method, each parameter selected was varied at three levels. The results indicate that the insignificant differences in peak areas and less variability in retention time and theoretical plates were observed. [18-19]

Ruggedness

Ruggedness studies were carried out using different analyst parameter. (Analyst I) and (Analyst II) Results showed that the % RSD is less than 2 for different analyst studies at different days. This study signifies the ruggedness of the method under varying conditions of its performance. [19]

Table 11: Results of Robustness Study of variation (Ledipasvir)

Parameters	Variation Level	Retention Time (min)	Area	SD	%RSD
Flow Rate mL/min	0.9 mL	8.4000	393.35	0.88	0.22
	1.0 mL	7.4500	146.32	1.08	0.73
	1.1 mL	7.2333	387.11	2.01	0.52
Wavelength	244 nm	7.9000	377.4	2.87	0.76
	245 nm	7.4500	146.32	1.08	0.73
	246 nm	7.7333	384.01	4.77	1.24
Mobile Phase	84:16	5.1333	373.0	4.80	1.29
	83:17	7.4500	146.32	1.08	0.73
	82:18	8.1333	364.76	4.95	1.36

Table 12: Results of Robustness Study of variation (Sofosbuvir)

Parameters	Variation Level	Retention Time (min)	Area	SD	%RSD
Flow Rate mL/min	0.9 mL	3.8000	740.58	2.88	0.38
	1.0 mL	3.5000	216.29	1.56	0.72
	1.1 mL	4.8887	710.30	5.70	0.80
Wavelength	244 nm	3.5833	793.0	4.70	0.59
	245 nm	3.5000	216.29	1.56	0.72
	246 nm	3.5333	717.83	9.17	1.28
Mobile Phase	84:16	3.8167	724.2	6.38	0.88
	83:17	3.5000	216.29	1.56	0.72
	82:18	5.3333	728.01	10.77	1.48

Table 13: Ruggedness Studies

Drug	Label Claim	Amount Found ± S.D.		% Label Claim	
		Analyst I	Analyst II	Analyst I	Analyst II
Ledipasvir	30	29.45 ± 0.66	30.62 ± 0.27	98.16	102.06
Sofosbuvir	120	119.81 ± 0.12	121.73 ± 0.24	99.84	101.44

LOD and LOQ

The LOD and LOQ were determined by RP HPLC for Ledipasvir (1.09µg/ml), (3.30µg/ml), and Sofosbuvir (3.36µg/ml), (10.19µg/ml) respectively. [19]

Table 14: Results of LOD and LOQ by RP-HPLC Method

Sample	LOD (µg/mL)	LOQ (µg/mL)
Ledipasvir	1.09	3.30
Sofosbuvir	3.36	10.19

System Suitability Parameters

Table 15: System Suitability Test Parameters.

System Suitability Parameters	Proposed Method	
	Ledipasvir	Sofosbuvir
Retention Time (t_R)	7.45	3.5
Number of Theoretical Plate	2512.4	3857.2
Tailing Factor	2.0682	1.6667
Resolution Factor (R)	8.4643	0.000
Area [mV*s]	145.1706	214.6290
Area (%)	40.35	59.65

Forced Degradation Studies [18-19]

The forced degradation studies of Sofosbuvir and Ledipasvir tablet formulation was done on stress degradation by hydrolysis under alkaline conditions by using 0.1N NaOH was found to be 99.01% for 24 hours, stress degradation by using 0.1N HCL and product degradation was found to be 99.2% for 24 hours. Oxidative degradation was done by using hydrogen peroxide 99.78% for 24 hours. Hydrolytic degradation was found to be 99.78% for 24 hours. The Sofosbuvir and Ledipasvir were found to be stable of the condition.

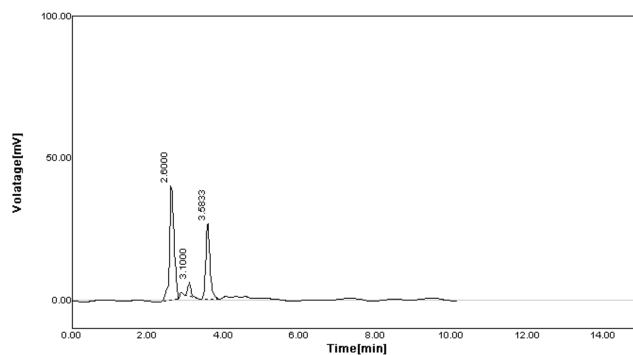

Fig. 8: Chromatogram of Acid degradation

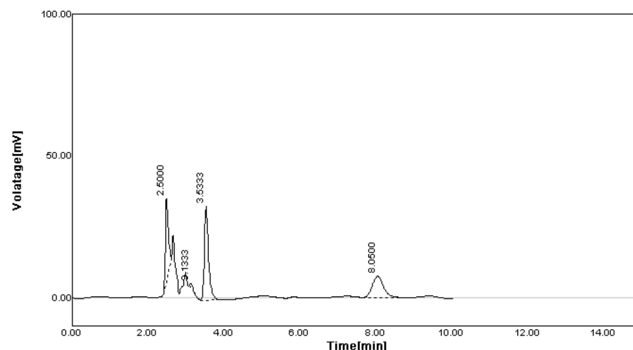

Fig. 9: Chromatogram of Base degradation

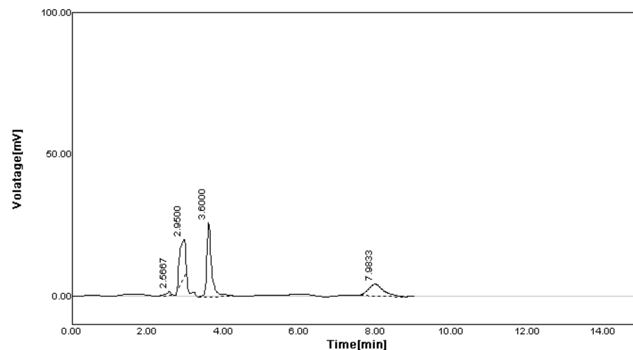

Fig. 10: Chromatogram of Peroxide degradation

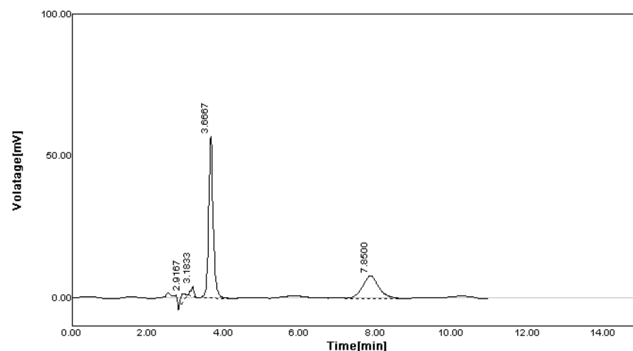

Fig. 11: Chromatogram of Hydrolytic degradation

ACKNOWLEDGEMENTS

The authors are thankful to the Department of Quality Assurance CAYMET's Siddhant College of Pharmacy, Sudumbare, Pune for providing all facilities to carry out the work.

Table 16: Summary of Results of Forced Degradation Study.

Stress condition	Sofosbuvir			Ledipasvir		
	tR	Peak Area	Degradation %	tR	Peak Area	Degradation %
Without Effect	3.5000	216.295	99.97	7.4500	146.32	99.95
Acid hydrolysis (HCl 0.1N)	2.6000	339.524	95.97 % No degradation	3.5833	201.219	99.92 % No degradation
Alkaline hydrolysis (NaOH 0.1N)	2.5000	137.984	98.65 % No degradation	3.5333	250.193	99.01 % No degradation
Oxidative hydrolysis (H_2O_2 30%).	3.6000	226.524	99.65 % No degradation	7.9833	117.169	99.2 % No degradation
Hydrolytic degradation	3.6667	463.038	97.85 % No degradation	7.8500	235.464	99.78 % No degradation

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HOW TO CITE THIS ARTICLE: Rote AP, Alhat J, Kulkarni AA. Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Ledipasvir and Sofosbuvir in Bulk and Pharmaceutical Dosage Form. Int. J. Pharm. Sci. Drug Res. 2017; 9(6): 291-298. **DOI: 10.25004/IJPSDR.2017.090602**