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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SOFOSBUVIR AND LEDIPASVIR IN RAW MATERIAL AND TABLET FORMULATION BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC (HPTLC) METHOD

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ABSTRACT

A simple, sensitive and rapid high performance thin layer chromatographic (HPTLC) method has been developed and validated for quantitative determination of Sofosbuvir (SFB) and Ledipasvir (LDP) in bulk and formulations. The chromatographic development was carried out on HPTLC aluminum plates precoated with silica gel 60 F254 using a mixture of ethyl acetate, methanol, toluene, acetone and acetic acid (2:1.5:4.5:2.0:0.2) (v/v/v/v) as mobile phase. Detection was carried out densitometrically at 269 nm. The R_f value of SFB and LDP were found to be 0.19 and 0.06 respectively. The method was validated as per ICH guidelines with respect to linearity, accuracy, precision, robustness etc. the calibration curve were found to be linear over a range of 200-1200 ng/spot and 45-270 ng/spot for SFB and LDP respectively, with a regression coefficient of 0.9979 and 0.9986 for SFB and LDP respectively. The accuracy was found to be very high 99.41-100.04 and 99.64-100.64 for SFB and LDP respectively. %RSD values for intra-day and inter-day variation were less than 2. The method has demonstrated high sensitivity and specificity. The method

is new, simple and economic for routine estimation of SFB and LDP in bulk and pharmaceutical formulation to help in industries as well as researchers for their sensitive determination of SFB and LDP rapidly at low cost in routine analysis.

KEYWORDS: HPTLC; Sofosbuvir; Ledipasvir; Validation; Estimation; Raw material.

1. INTRODUCTION

Sofosbuvir (SFB) is nucleotide analog inhibitor, which specifically inhibits HCV NS5B polymerase. SFB prevents HCV viral replication by binding to the two Mg2+ ions present in HCV NS5B polymerase's GDD active site motif. SFB is used in combination therapy to treat chronic hepatitis C virus (HCV) infected patients with HCV genotype 1,2,3, or 4, and to treat HCV and HIV co-infected patients. The combination therapy includes either Ribavirin alone or Ribavirin and Peg-interferon alfa. SFB acts against HCV and is categorized as a direct-acting antiviral agent (DAA). SFB (Fig. 1) has a terminal half-life of 0.4 hours and 27 hours (active metabolite GS-331007). The bioavailability of SFB is high approximately 92%. Ledipasvir (LDP) is an inhibitor of the Hepatitis C Virus (HCV) NS5A protein required for viral RNA replication and assembly of HCV virions. Although its exact mechanism of action is unknown, it is postulated to prevent hyper phosphorylation of NS5A which is required for viral production. It is effective against genotypes 1a, 1b, 4a, and 5a and with a lesser activity against genotypes 2a and 3a of HCV. LDP is used in combination therapy to treat chronic hepatitis C virus (HCV) infected patients with HCV genotype 1,2,3, or 4, and to treat HCV and HIV co-infected patients. The combination therapy includes either Ribavirin alone or Ribavirin and Peg-interferon alfa. LDP acts against HCV and is categorized as a direct-acting antiviral agent (DAA). LDP (Fig. 2) has a terminal half-life of 47 hours. The bioavailability of SFB is high approximately 88 %. FOSTER CITY, Calif.--(BUSINESS WIRE)--Oct. 10, 2014-- Gilead Sciences, Inc. (Nasdaq: GILD 2014) today announced that the U.S. Food and Drug Administration (FDA) has approved Harvoni® (Ledipasvir 90 mg/Sofosbuvir 400 mg), the first once-daily single tablet regimen for the treatment of chronic hepatitis C genotype 1 infection in adults. Harvoni combines the NS5A inhibitor LDP with the nucleotide analog polymerase inhibitor SFB, approved under the tradename Sovaldi® in December 2013. Harvoni's efficacy has been established in patients with chronic hepatitis C virus (HCV) genotype 1 infection, with a treatment duration of eight, 12 or 24 weeks depending on prior treatment history, cirrhosis status and baseline viral load. Eight weeks of treatment with

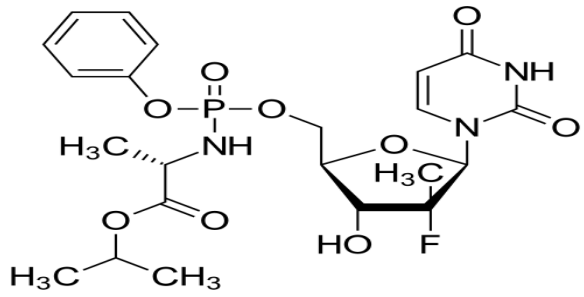
Harvoni can be considered for treatment-naïve patients without cirrhosis who have baseline HCV viral load below 6 million IU/mL.

Extensive literature survey revealed that there is no official high performance thin layer chromatography (HPTLC) method reported in major pharmacopeias like USP, EP, JP, BP and IP. There are few analytical methods available for estimation of SFB alone and in combination with some other drugs. Some liquid chromatographic (LC) methods for determination of SFB alone (Ravikumar Vejendla et al., 2016; P Mohan Vikas et al., 2016; Ramesh Guguloth et al., 2016) in combination with Simeprevir (B Raj Kumar et al., 2016) and with LDP (Bakht Zaman et al., 2016). There are few analytical methods available for estimation of LDP alone and in combination with some other drugs (Bakht Zaman et al., 2016; J Devilal et al., 2016; Mohamed El-Kassem M Hassouna et al., 2017). The few references for LC methods published for determination of SFB and LDP in pharmaceuticals but with sensitivity in microgram levels.

The above literature reveled that all available methods are sensitive upto microgram level only or require large amount of organic solvents or require sophisticated instruments or costly in determination of SFB and LDP in routine analysis.

Nowadays, HPTLC has become a routine analytical technique due to its advantages of reliability in quantitation of analytes at micro and even in nanogram levels and cost effectiveness. The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of mobile phase unlike HPLC. This reduces the time and cost of analysis. In addition, it minimizes exposure risks and significantly reduces disposal problems of toxic organic effluents, thereby reducing possibilities of environment pollution. HPTLC also facilitates repeated detection (scanning) of the chromatogram with same or different parameters. Simultaneous assay of several components in a multi component formulation is possible (Kulkarni et al., 2000; Renger et al., 2011; Shewiyo et al., 2012).

The objective of the present study was to develop and validate a new, simple, accurate, specific and reproducible HPTLC method for determination of SFB and LDP in bulk and pharmaceutical formulation to help the industries as well as researchers for their sensitive determination of SFB and LDP rapidly at low cost in routine analysis.



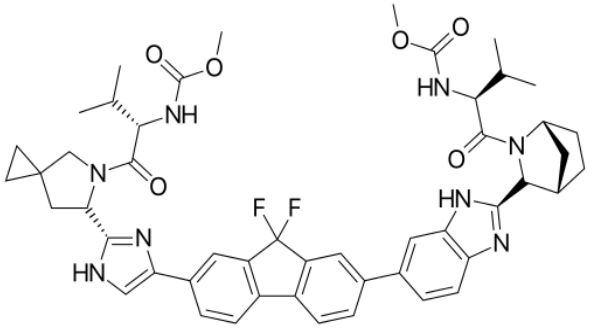


Figure 1: Chemical structure of Sofosbuvir. Figure 2: Chemical structure of Ledipasvir.

2. Experimental

2.1. Reagents and Chemicals

Sofosbuvir and Ledipasvir were obtained as a gift sample from Natco Pharmaceutical Limited (Hyderabad, India). All chemicals used were of analytical grade and solvents were of HPLC grade. Chloroform and Acetone were procured from Qualigens Fine Chemicals Ltd (Mumbai, India), Acetic acid, Toluene and Ethyl acetate were procured from SD Fine Chemicals Ltd (Gujarat, India), n-Hexane procured from CDH (New Delhi, India) and Methanol procured from E - Merck Specialties Pvt. Ltd (Mumbai, India).

2.2.Instrumentation and Chromatographic conditions

Chromatography was performed on 5 cm X 5 cm TLC aluminum plate pre coated with silica gel 60 F_{254} (Make: E. Merck, Germany). Before use, the plates were prewashed with methanol and water mixture then dried in the current of dry air and activated at 120^0C for 5 min. Samples were applied as bands 8 mm wide, 15 mm apart, by use of a CAMAG (Switzerland) Linomat 5 applicator with a CAMAG microlitre syringe. A constant application rate of 2 µl/spot was used. Different mobile phase containing chloroform, ethyl acetate, methanol, n-hexane, toluene, acetone and acetic acid in different proportion were examined. Of these, the mixture of ethyl acetate, methanol, toluene, acetone, acetic acid (2:1.5:4.5:2:0.2) (v/v/v/v) was found to be most suitable for the studies. The Retardation Factor (R_f) values of SFB and LDP were found to be 0.19 ± 0.04 and 0.06 ± 0.02 for SFB and LDP respectively, in optimized mobile phase. The densitogram obtained from a standard solution of SFB and LDP is shown in Fig.3.

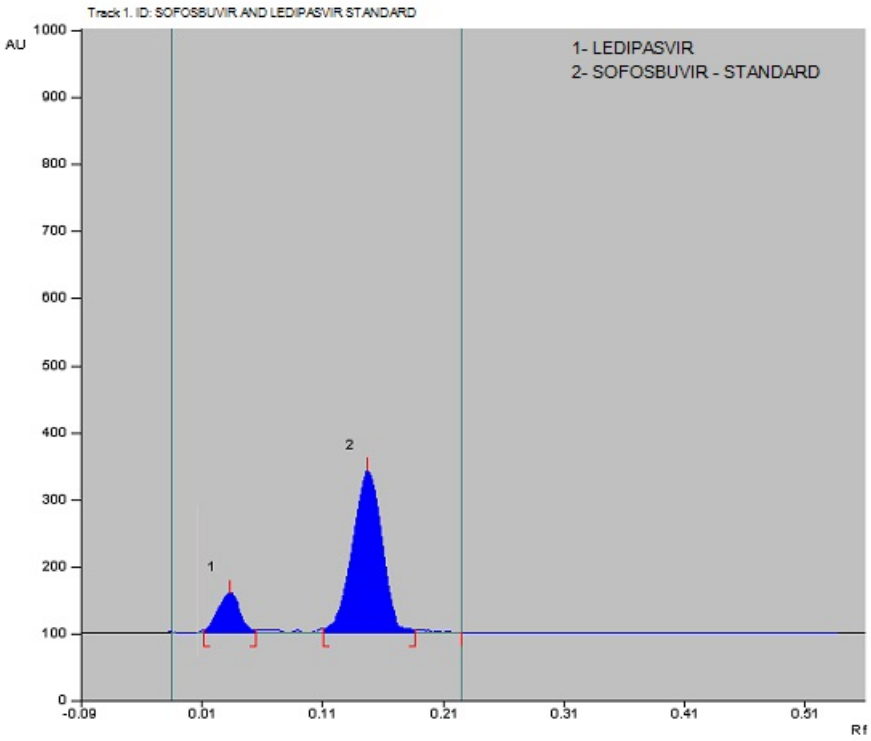


Figure 3: Chromatogram of standard peaks of Sofosbuvir (R_f = 0.19) and Ledipasvir (R_f = 0. 0.06).

2.3. Calibration Curve and Linearity

A stock solution containing 400 µg/mL or 400 ng/µL of SFB and 90 µg/mL or 90 ng/µL of LDP were prepared by dissolving an accurately weighed 400 mg of SFB and 90 mg of LDP portion of the drug in methanol in 100 mL volumetric flask. Further dilution was made with methanol to yield final concentration of SFB (400 µg/mL or 400 ng/µL) and LDP (90 µg/mL or 90 ng/µL). Different volumes of stock solution (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µL) were spotted on an HPTLC plate in triplicate to obtain concentration of 200,400,600,800,1000 and 1200 ng/band for SFB and 45,90,135,180,225 and 270 ng/band for LDP, respectively. The data of peak area versus drug concentration were treated by linear least-squares regression.

3. Method validation

The analytical method was validated for specificity, sensitivity, accuracy, precision, robustness and ruggedness in accordance with ICH guidelines **(**ICH: Q2 (R1)**)**.

3.1. Specificity and Sensitivity

The specificity of the developed method was established analyzing the sample solution containing SFB and LDP standard and marketed tablets in relation to interference from

formulation ingredients. The spot for SFB and LDP in the sample was confirmed by comparing R_f values of the spot with that of the standard.

The sensitivity of measurement was estimated in terms of the limit of quantification (LOQ) and the limit of detection (LOD). The LOQ and LOD were calculated by the use of equation LOD = 3 x *N/B* and LOQ = 10 x *N/B* where *N* is the standard deviation of the peak area of the drug (n = 3), taken as measure of noise and *B* is the slop of the corresponding calibration plot.

3.2. Accuracy

To check the accuracy of the method, recovery measurements were performed by the addition of a standard drug solution at three different levels (80%, 100% and 120%) to a pre-analyzed sample solution (800 ng/band and 180 ng/band for SFB and LDP, respectively. So that after addition of standards, sample would be in the linear range). Three replicate estimations were carried out for each concentration level.

3.3. Precision

Precision of the method, determined from replicate analysis of the sample, was expressed as repeatability (intra-batch) and intermediate precision (inter-batch). The intra-day and inter-day precision studies were carried out by estimating the response of standards in triplicates under same experimental conditions three times on the same day and on three different days. From the results obtained, the precision was expressed as percentage relative standard deviations (%RSD) from mean intra and inter-day assays.

3.4. Robustness and Ruggedness

Robustness of the method was performed by spotting 800 ng/band and 180 ng/band for SFB and LDP, respectively. Making small deliberate changes in various chromatographic conditions. The duration of saturation (30 ± 2 min), migration time (15 ± 2) and nm change (269 ± 2).

Ruggedness of the method was performed by spotting 800 ng/band and 180 ng/band for SFB and LDP drugs, respectively and it was analyzed by two different analysts maintaining same experimental and environmental conditions.

3.5. Analysis of SFB and LDP in tablet dosage form

To determine the SFB and LDP content of marketed tablet dosage form, powder equivalent to 400 mg of SFB and 90 mg of LDP was transferred to a 100 mL volumetric flask, extracted

with methanol, sonicated for 15 min, and diluted to volume with same solvent. The resulting solution was filtered through a 0.45 µm filter (Millifilter; Milford, MA; USA). Further dilution was made by taking 1 mL from the above solution and transfer to a 10 mL volumetric flask and dilute up to the mark with methanol to yield concentration of SFB (400 µg/mL or 400 ng/µL) and LDP (90 µg/ml or 90 ng/µl). The solution (2 µL, 800 ng/spot of SFB and 180 ng/spot of LDP) was applied in triplicate on an HPTLC plate for quantification using the proposed method.

4. RESULTS AND DISCUSSION

4.1. Calibration Curve and Linearity

A calibration curve was constructed by plotting peak area against concentration of Sofosbuvir and Ledipasvir (ng per spot). The results of regression analysis were shown in Table 1. They confirm the linearity of the standard curves over the range studied (200-1200 ng/spot of SFB and 45-270 ng/spot of LDP). Linear regression of concentration versus peak area plots results in an average coefficient of determination (r^2) value 0.9979 for SFB and 0.9986 for LDP. The 3-D chromatograph of all calibration concentrations were shown in Fig.4.

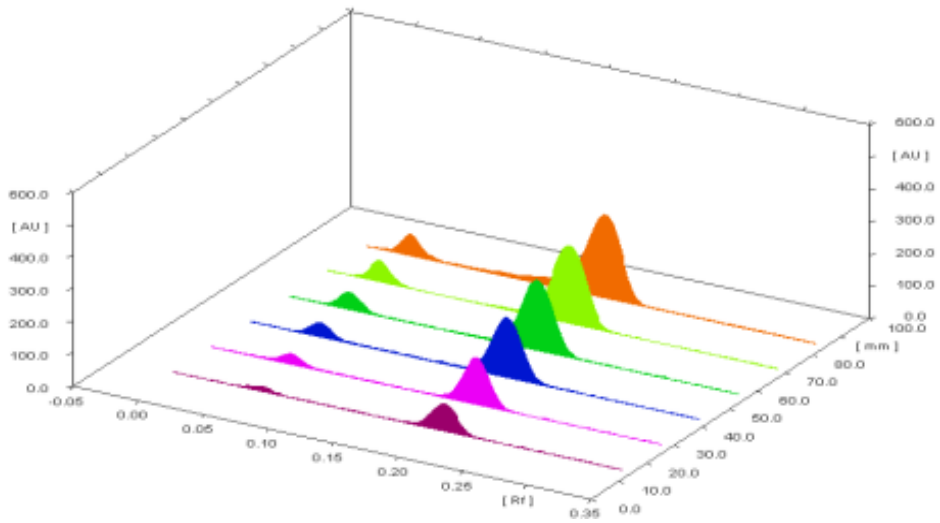


Figure 4: 3D graph of calibration linearity graph: 200-1200 ng/spot of Sofosbuvir with R_f at 0.19 ± 0.04 and 45-270 ng/spot of Ledipasvir with R_f at 0.06 ± 0.02, showing the peak linearity.

4.2. Specificity and Sensitivity

The peak purity of SFB and LDP was assessed by comparing the spectra at three different levels, that is, peak start *(S)*, peak apex *(M)* and peak end *(E)* positions of the spot and the

results obtained as r^2 *(S,M)* = 0.996 and r^2 *(M,E)* = 0.997. Good correlation was obtained between standard and sample spectra of SFB and LDP.

Limit of detection and Limit of quantification were found to be 5.3411 ng/spot and 16.1853 ng/spot for SFB, 0.6598 ng/spot and 1.999 ng/spot for LDP, respectively. Hence the method was found to be highly sensitive for determination of SFB and LDP.

Table 1: Results of regression analysis of linearity data and sensitivity of Sofosbuvir and Ledipasvir.

Parameter	Sofosbuvir	Ledipasvir
Linearity range (ng/spot)	200-1200	45-270
Correlation coefficient (r^2)	0.9979	0.9986
Slop *(m)* ± % RSD		
Intercept *(c)* ± % RSD		
R_f	0.19 ± 0.04	0.06 ± 0.02
LOD (ng/spot)	5.3411	0.6598
LOQ (ng/spot)	16.1853	1.999
Ruggedness (%RSD)	0.6038	0.2598

4.3. Accuracy

The developed method showed high and consistent recoveries at all studies. The results obtained from recovery studies are presented in Table 2. The mean % recovery ranged from 98.94% to 100.81% for SFB and 99.05% to 101.24% for LDP. Additionally, the obtained recoveries were found to be normally distributed with low % RSD (0.7923) at all concentration levels.

Table 2: Results of recovery studies of Sofosbuvir and Ledipasvir.

Parameters	Sofosbuvir			Ledipasvir		
	Amount Present (ng/spot)	Amount Added (ng/spot)	% Amount Recovered	Amount Present (ng/spot)	Amount Added (ng/spot)	% Amount Recovered
80 %	800	640	99.03	180	144	100.19
			98.94			100.56
			99.33			101.24
100%	800	800	100.59	180	180	100.60
			100.81			99.05
			100.70			99.06
120%	800	960	99.55	180	216	98.87
			99.64			99.94
			99.69			99.31
Average			99.80			99.86
SD			0.6755			0.7912

%RSD	0.6768			0.7923
SE	0.2388			0.2798
CI (Confidence Interval 99%)	99.04-100.55			98.97-100.74

4.4. Precision

% RSD values for intra-day variations were not more than 0.499351 and 0.539213 for SFB and LDP, respectively. Inter-day variations were also not more than 0.319968 and 0.428837 for SFB and LDP, respectively. Low % RSD values indicated the good repeatability and intermediate precision of the method as shown in Table 3.

Table 3: Results of precision study of Sofosbuvir and Ledipasvir.

Sofosbuvir						
Intra-day				**Inter-day**		
	Concentration (ng/spot)	% Recovery (± SD)	%RSD		% Recovery (± SD)	%RSD
0 Hours	800	99.25 ± 0.495	0.4993	Day – 1	99.14 ± 0.317	0.3199
3 Hours				Day – 2		
6 Hours				Day – 3		
Ledipasvir						
0 Hours	180	100.44 ± 0.541	0.5392	Day – 1	100.12 ± 0.429	0.4288
3 Hours				Day – 2		
6 Hours				Day – 3		

4.5. Robustness and Ruggedness

The results obtained in the new conditions were in accordance with the original results as shown in Table 4. The % RSD values obtained in robustness and ruggedness was found to be less than 2% indicating the method was highly robust and rugged when estimation was done by two different analysts.

Table 4: Results of robustness, Ruggedness studies of Sofosbuvir and Ledipasvir.

Parameters	**Sofosbuvir**		**Ledipasvir**	
	% Mean recovery (± SD)	**%RSD**	**% Mean recovery (± SD)**	**%RSD**
nm plus (271)	98.89 ± 0.2071	0.2569	100.15 ± 0.2946	0.2843
nm minus (267)	98.72 ± 0.2283	0.2495	101.14 ± 0.3817	0.3694
Chamber saturation (28 min)	98.85 ± 0.2412	0.2658	101.55 ± 0.3742	0.3952
Chamber saturation (32 min)	99.07 ± 0.2594	0.2439	100.68 ± 0.2753	0.2947
Migration time (13 min)	99.47 ± 0.2847	0.2615	100.81 ± 0.3385	0.3214
Migration time (17 min)	98.88 ± 0.2117	0.2573	101.06 ± 0.3394	0.3486
Change of analyst	99.49 ± 0.6355	0.6038	100.25 ± 0.2586	0.2598

4.6. Analysis of SFB and LDP in tablet dosage form

Drug bands at R_f 0.18 and 0.07 corresponding to SFB and LDP were observed in chromatograms obtained from tablet extracts. There was no interference from excipients present in the tablets. The assay of marketed tablet was found to be 99.36% (SD ± 0.3474) and 100.36% (SD ± 0.8809) for SFB and LDP, respectively. The good performance of the method indicated the suitability of this method for routine analysis of SFB and LDP determination in pharmaceutical dosage form.

4.7. Advantages of developed method

Extensive literature survey revealed that there is no official high performance thin layer chromatography (HPTLC) method reported in major pharmacopeias like USP, EP, JP, BP and IP. There are few analytical methods available for estimation of SFB alone and in combination with some other drugs. Some liquid chromatographic (LC) methods for determination of SFB alone in combination with Simeprevir and with LDP. There are few analytical methods available for estimation of LDP alone and in combination with some other drugs. The few references for LC methods published for determination of SFB and LDP in pharmaceuticals but with sensitivity in microgram levels.

The above literature reveled that all available methods are sensitive upto microgram level only or require large amount of organic solvents or require sophisticated instruments or costly in determination of SFB and LDP. There is a need of a new, simple, rapid, highly sensitive and economic HPTLC method for determination of Sofosbuvir and Ledipasvir.

It can be seen that this simple and reproducible HPTLC method has enough sensitive to evaluate preformulation samples and pharmaceutical dosage form of SFB and LDP at low cost. Thus this presented work will meet the industrial and research laboratories requirements.

5. CONCLUSION

A new, simple and sensitive HPTLC method has been successfully developed and validated for determination of SFB and LDP in bulk and pharmaceutical dosage form. The method was found to be accurate, precise and reproducible with good stability under various processing and storage conditions. In addition, the method was successfully employed for compatibility testing of SFB and LDP with various excipients for formulation design. Thus this developed

and validated method will help the industries as well as researchers for their sensitive determination of SFB and LDP rapidly at low cost.

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