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A NEW VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR SIMUTANEOUS ESTIMATION OF SOFOSBUVIR AND LEDIPASVIR IN TABLET DOSAGE FORMS

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ABSTRACT

A combination of Sofosbuvir and Ledipasvir is used to treat Hepatitis C virus (HCV). A selective, accurate and precise RP-HPLC method was developed and validated for simultaneous estimation of these drugs in combined tablet dosage forms. The drugs were resolved on a Kromosil C_{18} column using Acetonitrile: 0.1% orthophosphoric acid (35:65 v/v) as the mobile phase. The detection wavelength was 272 nm. The retention times obtained for sofosbuvir and ledipasvir were 2.516 & 3.743 min respectively. The linearity ranges were 100-600 & 22.5 - 135 µg/ml respectively with Regression coefficients of 0.999. The % R.S.D. of precision studies was found to be 0.92 & 0.64 respectively. The Accuracy of the proposed method was determined by recovery studies and the mean recovery was 100.16 & 99.78% respectively. The method was also applicable for quantitative analyses of the marketed tablet formulations and in studying stability of the drugs under acidic, alkaline, oxidation, thermal and UV conditions.

KEYWORDS: Sofosbuvir, Ledipasvir, Tablets, Degradation studies, RP-HPLC.

INTRODUCTION

Hepatitis C virus (HCV) infection is a significant public health concern. Globally, between 130-150 millions of people have chronic hepatitis C infection. Approximately 3.99 lakh people die each year due to Hepatitis C, mostly from cirrhosis and hepatocellular carcinoma.[1,2] Hepatitis C, is a liver disease caused by the HCV. It is a blood borne virus and most common modes of infection are through exposure to small quantities of blood.[3]

Sofosbuvir is a direct acting antiviral agent against the HCV, it is a nucleotide analog NS5B polymerase inhibitor indicated for the treatment of chronic hepatitis C (CHC) infection as a component of a combination antiviral treatment regimen.[4] Sofosbuvir is chemically known as Isopropyl(2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetrahydrofuran-2-2yl]methoxy-phenoxy-phosphoryl]amino]propanoate.[5]

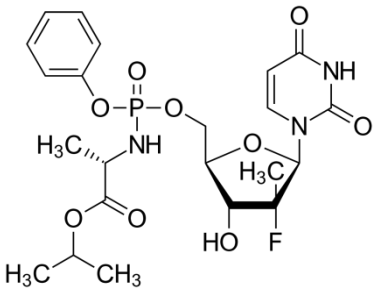

Fig. 1: Structure of Sofosbuvir.

Ledipasvir is HCV NS5A inhibitor, it stop the virus that causes hepatitis C from spreading inside the body. [6] it is chemically known as Methyl N-[(2S)-1-[(6S)-6-[5-[9,9-difluoro-7-[2-[(1S,2S,4R)-3-[(2S)-2-(methoxycarbonylamino)-3-methylbutanoyl]-3-azadicyclo [2.2.1] heptan-2-yl]-3H-benzimidazol-5-yl]- flouoren-2-yl]-1H-imidazole-2-yl]-5-azaspiro[2.4] heptan-5-yl]-3-methyl-1-oxobutan-2-yl]carbamate.[7,8]

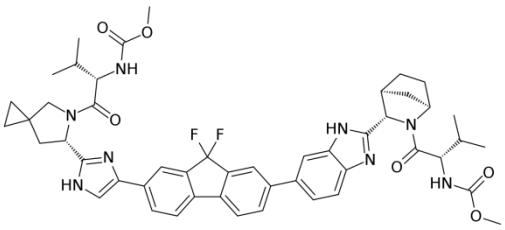

Fig. 2: Structure of Ledipasvir

The literature survey shows that there are few methods for determination of sofosbuvir and ledipasvir individually in tablet dosage form by using various analytical instruments like UV-Vis spectrophotometer[9,12], TLC[13], Capillary zone electrophoresis[14], HPLC [15-21], LC-MS/MS[22,23] and UPLC-MS/MS.[24,26] So, the attempt has been made to develop a new validated stability indicating RP-HPLC method for simultaneous estimation of sofosbuvir and ledipasvir in tablet dosage form as per International Conference on Harmonization (ICH) guidelines.

MATERIALS AND METHODS

The combination of sofosbuvir & ledipasvir tablets were provided by Natco Pharma and API gift samples from Spectrum Pharma Research, Hyderabad. HPLC grade Acetonitrile, water and other chemicals obtained from the Rankem, Hyderabad. WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector and Auto sampler integrated with Empower 2 Software. UV-VIS spectrophotometer T60 with special bandwidth of 2 mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Sofosbuvir and Ledipasvir solutions.

Preparation of Buffer

Accurately pipette 1.0mL of OPA into clean & dried 1000mL volumetric flask, add 900mL of milli-Q water, stir well, Degas to sonicate and make up the volume with milli-Q water. Finally, the pH of the solution was adjusted to 2.5 with dilute NaoH.

Selection of Mobile Phase

The mixture of buffer (0.1% OPA) and Acetonitrile at ratio 65:35 v/v was used as a mobile phase for analysis in which optimum suitability parameters were obtained and this mobile phase was filtered through 0.45µm membrane filters and sonicated for 10min.

Preparation of Diluent

It is a mixture of Acetonitrile and milli-Q water at ratio 50:50 v/v.

Preparation of standard solution

Accurately weighed 40mg of Sofosbuvir and 9mg of Ledipasvir working standards and transferred into clean and dried 10ml volumetric flask. To this add 3/4th volume of diluent, sonicate to degas for 30min and make up to the final volume with diluent. The resultant concentrations are 4000 µg/ml of Sofosbuvir and 900 µg/ml of Ledipasvir.

Preparation of Standard working solutions (100% solution)

Pipette 1ml from each stock solution and transferred into clean and dried 10ml volumetric flask and finally make up to the mark with diluent. The resultant concentrations are 400µg/ml of Sofosbuvir and 90µg/ml of Ledipasvir.

Preparation of Sample stock solutions

10 tablets are randomly selected, weighed and the average weight of each tablet is calculated, all tablets were grounded into fine powder. The weight equivalent to 1 tablet was transferred

into 100ml volumetric flask, add 3/4th volume of diluent, sonicate to degas for 25 minutes and finally make up to the mark with diluent. All the content was passed through 0.45µ filter paper. The resultant concentration 4000µg/ml of Sofosbuvir and 900µg/ml of Ledipasvir.

Preparation of sample working solution (100% solution)

Pipette 1 ml of filtered sample stock solution, transfer it into 10 ml volumetric flask and make up to the mark with diluent. The resultant concentrations were 400µg/ml of Sofosbuvir and 90µg/ml of Ledipasvir.

Optimized chromatographic method

The separation of Sofosbuvir and Ledipasvir was achieved on a Kromosil C_{18} column (250x4.6 mm; 5.6µ) and eluting with a mobile phase consisting of a 35:65 v/v mixture of Acetonitrile and Buffer (pH 2.5) at a flow rate of 1.0mL/min. The analytes were monitored at wavelength of 272 nm. The injection volume was 10 µl. The total run time for elution of compound was 6 min.

Column	**:**	Kromasil C18; 50 x 4.6 mm; 5µ.
Column temperature	**:**	30°C
Flow rate	**:**	1 mL/min
Injection volume	**:**	10 µL
Detector wave length	**:**	272 nm
Run time	**:**	6 min

Method Validation

The US Food and Drug Administration (FDA) and US Pharmacopeia (USP) both refer to ICH guidelines. The most widely applied validation characteristics are accuracy, precision, specificity, linearity, range, robustness, limit of detection, limit of quantification, limit of detection and limit of quantification.

System suitability

It is the checking of a system to ensure system performance before or during the analysis of unknown**.** It tests are an integral part of chromatographic method and are used to verify that the resolution & reproducibility of the system are adequate for the analysis to be performed. In this, plate count (N), tailing factor (T), resolution (Rs) and reproducibility (%RSD) are determined from replicate injection of standard. The data was shown in Table 1.

Table 1: System suitability parameters.

Drug	Retention time (min)	Area	USP Plate Count	USP Tailing
Sofosbuvir	2.516	829577	6477	1.20
Ledipasvir	3.743	383273	10745	1.16

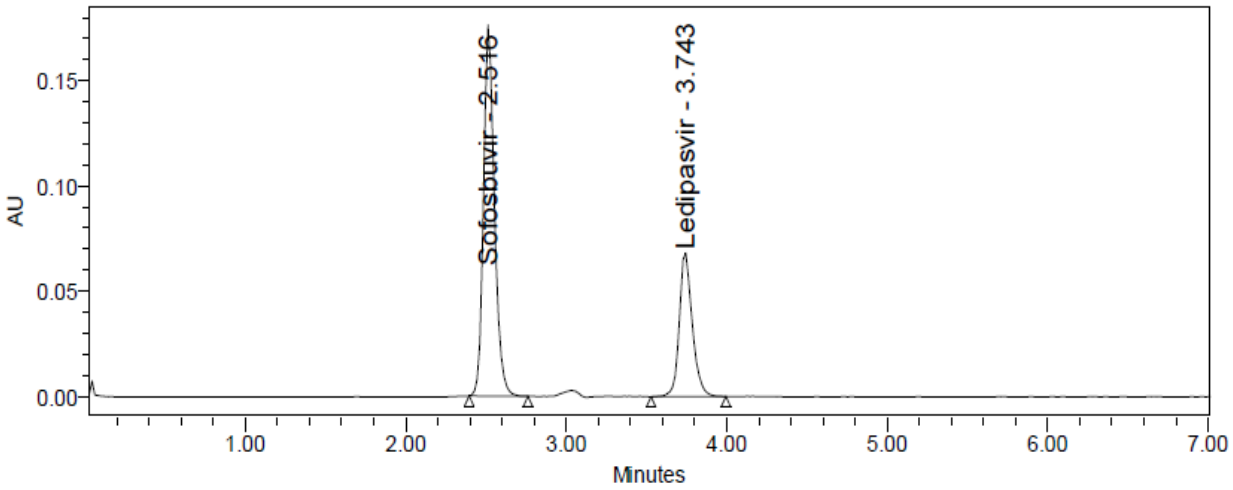

Fig. 3: Optimized Chromatogram of Sofosbuvir and Ledipasvir.

Specificity

The ability of the method is to accurately measure the analyte response in the presence of all potential sample components. In this study, the method was evaluated by injecting 10µl of blank sample, placebo, standard and sample into HPLC. Fig No.4, 5, 6 & 7.

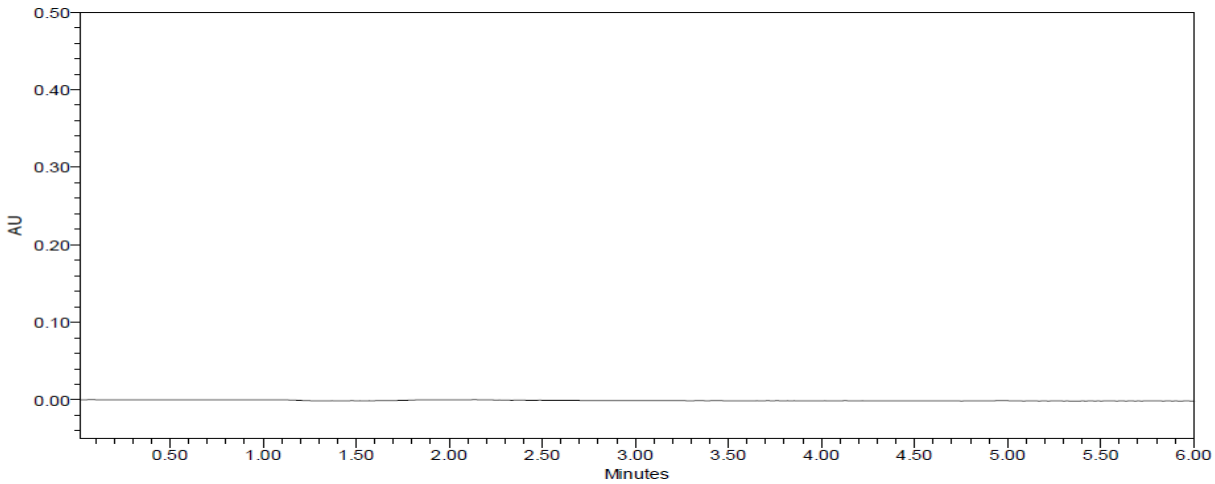

Fig. 4: Chromatogram of Blank solution.

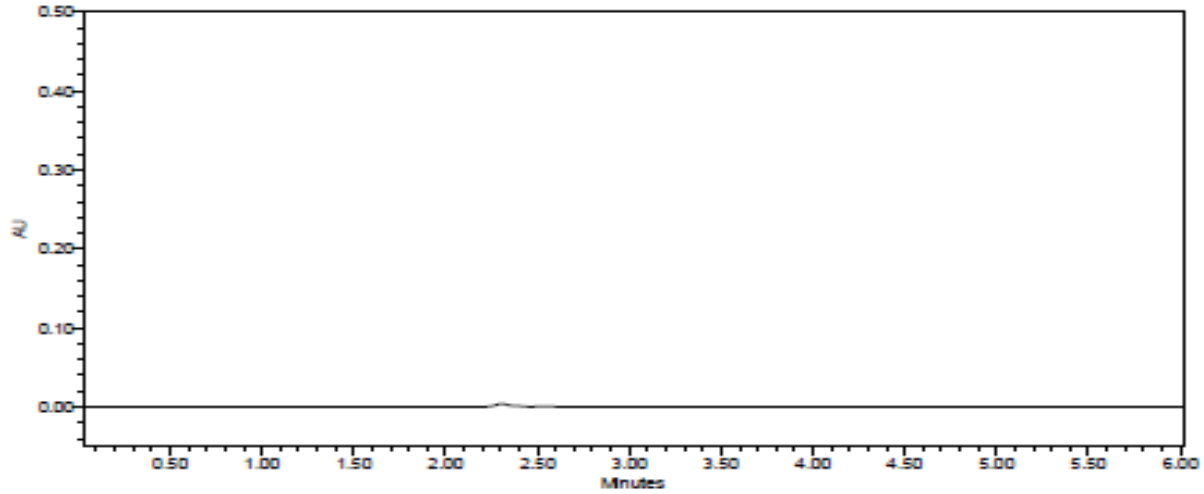

Fig. 5: Chromatogram of Placebo.

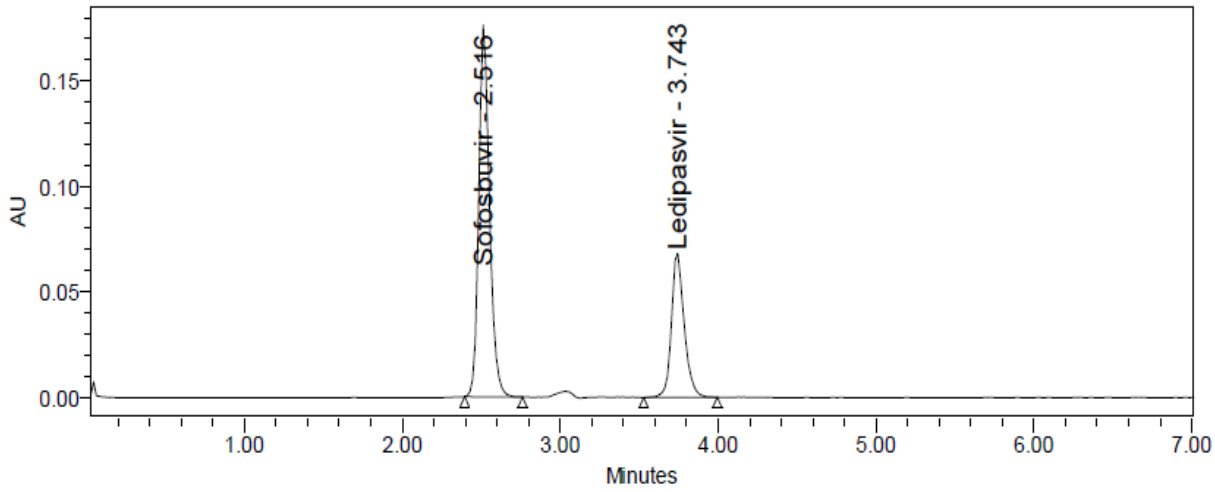

Fig. 6: Standard Chromatogram of Sofosbuvir and Ledipasvir.

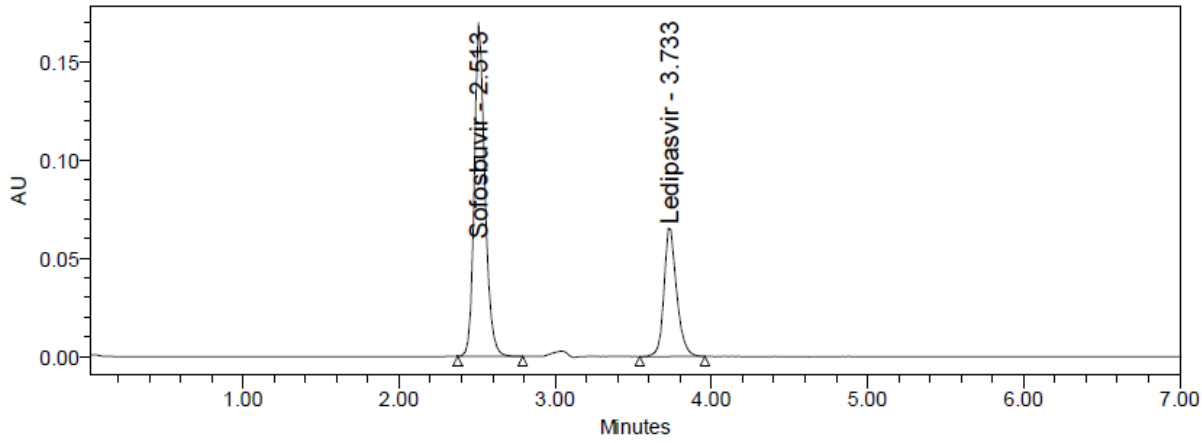

Fig. 7: Typical Chromatogram of Sample (Tablet dosage form).

Accuracy

The accuracy of the method was evaluated by standard addition method. The known amount of the reference standard was added to the known amount of standard solution at three different levels. The solutions were analyzed for mean recovery and %RSD. The studies were performed for both Sofosbuvir & Ledipasvir at three different levels 50%, 100% and 150% solution. The 10 µL was injected into HPLC and % recovery and % RSD were noted as shown in Table 2.

Table 2: Recovery studies of Sofosbuvir and Ledipasvir.

Drug	Level of spike solution	Amount present (mg/mL)	Amount added	Amount recovered	% Recovery	% RSD
Sofosbuvir	50%	400	200	200.128	100.06	0.86
	50%	400	200	203.949	101.97	
	50%	400	200	199.93	99.97	
	100%	400	400	404.191	101.05	
	100%	400	400	398.552	99.64	
	100%	400	400	396.977	99.24	
	150%	400	600	598.115	99.69	
	150%	400	600	601.668	100.28	
	150%	400	600	597.152	99.53	
Ledipasvir	50%	90	45	45.324	100.72	0.88
	50%	90	45	44.585	99.08	
	50%	90	45	44.941	99.87	
	100%	90	90	88.449	98.28	
	100%	90	90	89.194	99.11	
	100%	90	90	90.132	100.15	
	150%	90	135	135.578	100.43	
	150%	90	135	134.215	99.42	
	150%	90	135	136.316	100.97	

Precision

Precision is the degree of agreement among individual test results when an analytical method is used repeatedly to multiple sampling of a homogenous sample. The precision was determined as reproducibility precision and studied for method precision and inter-day precision by injecting 10µL for six times and peak areas of replicated injections as shown in Table 3.

Table 3: Method Precision and Interday precision studies of Sofosbuvir and Ledipasvir.

S.No.	Injection	Method Precision		Interday Precision	
		Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir
1	Injection -1	823083	381990	816602	372686
2	Injection-2	839084	386511	816404	389623
3	Injection-3	834067	380408	827853	381542
4	Injection-4	831221	384823	819428	383333
5	Injection-5	81954	385461	836546	381326
6	Injection-6	839482	380987	837690	371632
Average		**831482**	**383363**	**825754**	**380024**
SD		**7614.0**	**2557.6**	**9742.1**	**6802.4**
% RSD		**0.9**	**0.7**	**1.2**	**1.8**

Linearity and Range

Linearity is the ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation to analyte concentration within a given range. Range is the interval between the upper and lower levels of analyte. The linearity determined were within concentration range of 100-600 µg/ml for Sofosbuvir and 22.5-135µg/ml for Ledipasvir respectively as shown in Table 4 and Fig. 8 & 9. The linearity of the method was evaluated by linear regression analysis.

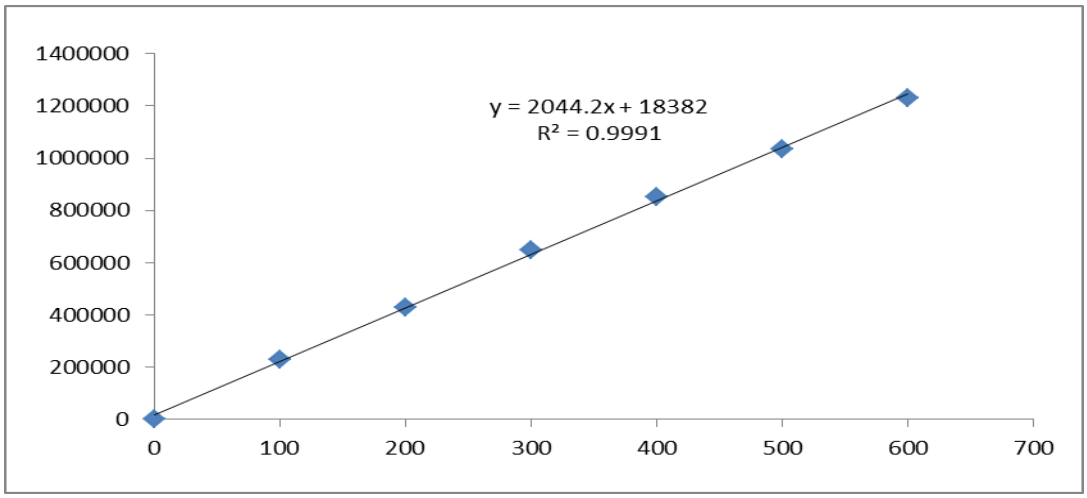

Fig. 8: Linearity curve of Sofosbuvir.

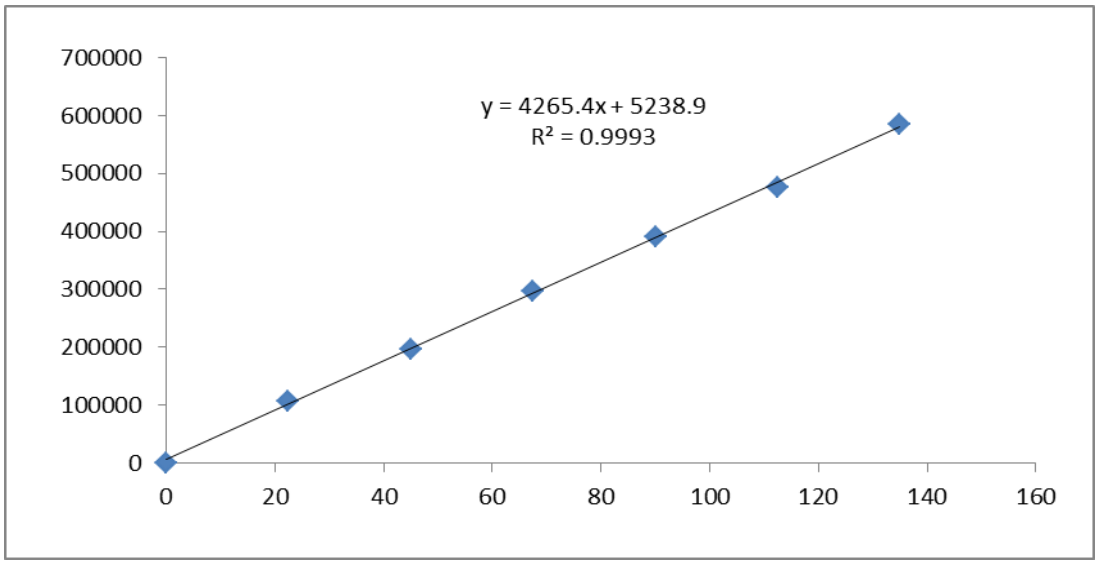

Fig. 9: Linearity curve of Ledipasvir.

Table 4: Linearity data of Sofosbuvir and Ledipasvir.

S.No.	Sofosbuvir		Ledipasvir	
	Concentration (µg/mL)	Peak Area	Concentration (µg/mL)	Peak Area
01.	100	230964	22.5	107209
02.	200	427672	45	195740
03.	300	64534	67.5	297029
04.	400	850638	90	391353
05.	500	1034178	112.5	476182
06.	600	1231415	135	584532
Correlation coefficient (R^2)		**0.9991**	**0.9993**	

Robustness

It is the capacity of a method to remain unaffected by small, deliberate variations in method parameters. It was indicated by changing the flow rate, mobile phase composition and temperature and the samples were injected into HPLC system and data obtained shown in Table 5.

Table 5: Robustness studies of Sofosbuvir and Ledipasvir.

Parameter	Change in parameter	Peak Area		SD		% RSD	
		Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir
Flow rate	0.8mL/min	927461	413995	2201.6	1539.1	0.2	0.4
	1.2 mL/min	743126	329336	815.2	766.4	0.1	0.2
Mobile phase composition	2.4	778454	344813	1144.5	1400.2	0.1	0.4
	2.8	767265	338811	3007.0	695.5	0.4	0.2
Temperature	25^0C	773648	345107	519.8	1067.7	0.1	0.3
	35^0C	843776	374326	2283.3	1743.7	0.3	0.5

Limit of Detection (LOD) & Limit of Quantification (LOQ)

LOD is the lowest concentrations of an analyte in a sample that can be detected. LOQ is the lowest concentration of an analyte in a sample that can be quantized. The LOD and LOQ of Sofosbuvir & Ledipasvir were determined from standard deviation of the response and the slope. Table 6.

Table 6: LOD and LOQ of Sofosbuvir and Ledipasvir.

Parameter	Sofosbuvir	Ledipasvir
LOD	1.22	0.184
LOQ	3.70	0.557

Assay Procedure

The assay performed by the marked product (400mg of Sofosbuvir & 90mg of Ledipasvir). The prepared sample and standard solution were injected into HPLC and peak areas were recorded. Finally percentage amount of drug was calculated. Table 7.

Table 7: Assay of Sample (Table dosage form).

Drug	Label Claim	Amount present (mg)	% Drug Content
Sofosbuvir	400	398.55	99.64
Ledipasvir	90	89.19	99.11

Forced Degradation Studies

Forced degradation is a degradation of new drug substances and drug products at conditions more than accelerated conditions. This studies show the chemical behavior of the molecules which in turn helps the development of formulation and packaging. Hence, in the present study forced degradation studies were established by subjecting the samples of sofosbuvir and ledipasvir standard solution to degradation in Oxidation, Acid, Alkaline, Dry heat, Photo stability and Neutral degradation. As shown in Table 8.

Oxidation

Pipette 1ml of standard stock solution of Sofosbuvir and Ledipasvir into volumetric flask separately, add 1ml of 20% hydrogen peroxide (H_2O_2), and these solutions were kept for 30min at 60^0C. The resultant solutions were diluted to obtain 400µg/ml and 90µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies

Pipette 1ml of stock solution of Sofosbuvir and Ledipasvir into volumetric flask separately, add 1ml of 2N Hydrochloric Acid and reflex for 30 minutes at 60^0C. The resultant solutions were diluted to obtain 400µg/ml and 90µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies

Pipette 1ml of stock solution of Sofosbuvir and Ledipasvir into volumetric flask separately, add 1ml of 2N sodium hydroxide and reflex for 30 minutes at 60^0C. The resultant solutions were diluted to obtain 400µg/ml and 90µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies

The standard Drug solutions were placed into oven at 105^0C for 6hours. The resultant solutions were diluted to obtain 400µg/ml and 90µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Photo Stability Studies

The photo chemical stability of the drug was also studied by exposing the stock solutions to UV light by keeping the beaker in UV chamber for 7 days or 200 watt hours/m^2 in photo

stability chamber. The resultant solutions were diluted to obtain 400µg/ml and 90µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hours at 60^0C. The resultant solutions were diluted to obtain 400µg/ml and 90µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Table 8: Forced Degradation studies of Sofosbuvir and Ledipasvir.

Forced Degradation				
Parameters		**% amount retained**	**Purity Angle**	**Purity threshold**
Sofosbuvir	Acid	97.03	0.128	0.314
	Alkaline	97.83	0.179	0.324
	Oxidation	98.35	0.823	1.386
	Photo Stability	99.62	0.187	0.325
	Thermal	99.15	0.195	0.317
	Neutral	99.84	0.199	0.333
Ledipasvir	Acid	95.28	0.242	0.418
	Alkaline	97.71	0.199	0.392
	Oxidation	98.76	0.102	0.297
	Photo Stability	99.39	0.229	0.413
	Thermal	99.15	0.174	0.384
	Neutral	99.81	0.219	0.401

RESULTS AND DISCUSSION

The proposed method was simple, precise and accurate for the simultaneous determination of sofosbuvir and ledipasvir in combined tablet dosage form. The drugs were resolved on a Kromosil C_{18} column using Acetonitrile: 0.1% orthophosphoric acid (35:65 v/v) as the mobile phase, flow rate of 1ml/min and the detection wavelength was 272 nm. The retention times obtained for sofosbuvir and ledipasvir were 2.516 & 3.743 min respectively.

The developed method was validated for accuracy, precision, linearity, robustness, LOD and LOQ. The linearity of the method was determined by Regression analysis. A linear relationship was evaluated in the concentration range of 100-600 µg/mL of sofosbuvir and 22.5-135 µg/mL of ledipasvir with correlation coefficient of 0.999 respectively. The system suitability studies and method precision were carried and %RSD were found to be less than 2%. The accuracy of the method was determined by recovery studies and mean recovery was

observed to be 100.16% for sofosbuvir and 99.78% for ledipasvir. The LOD and LOQ were found to be 1.22µg/mL & 3.70µg/mL for sofosbuvir and 0.184µg/mL & 0.557µg/mL for ledipasvir. It indicates that the method was very sensitive. The robustness of the method was studies by deliberate changes in the flow rate, mobile phase composition and temperature. The %RSD were found to be not more than 2% and results indicates that the slight variations on the chromatographic conditions have negligible effect and conformed that the method was highly robust. The proposed method was successfully applied to the assay of commercial formulation and showed 99.64% and 99.11% of sofosbuvir and ledipasvir respectively.

Forced degradation of the drug product was carried out as per the ICH guidelines with a view to establishing the stability-indicating property of this method and providing information of degradation/ quality of a drug substance and drug product changes with time under the influence of various stressing conditions like acid, base, oxidation, thermal, photolytic and neutral to sofosbuvir and ledipasvir in combined dosage form. The result obtained indicates that the lower the purity angle when compared to purity threshold revealed that sample was free from interference from its impurities and its degradents.

CONCLUSION

The developed method was simple, precise, accurate and reliable for the simultaneous estimation of sofosbuvir and ledipasvir in combined dosage form and envisages the stability behavior of both the drugs as per ICH guidelines. The %RSD of all results is less than 2% that shows high degree. Hence, the proposed method was simple, easy, cost-effective and can be used for routine analysis of sofosbuvir and ledipasvir in combined dosage form.

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