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UPLC Method for Simultaneous Estimation of Ledipasvir and Sofosbuvir in Bulk and Dosage Forms and Their Stress Degradation Studies

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Abstract

Ultra-performance liquid chromatographic (UPLC) method, for ledipasvir and sofosbuvir determination in bulk mixtures, and in tablets was developed and validated. The determination of the drugs, ledipasvir and sofosbuvir was carried out applying BEH C18 column (50 mm × 2.1 mm i.d, 1.6 µm particle size), with UV detector at λ max of 220 nm. The mobile phase applied for the current study, composed of two solvents, A (0.01 N w/v potassium di-hydrogen orthophosphate buffer of pH 3.5 modified with dilute orthophosphoric acid) and B (acetonitrile) in the ratio of 50:50 v/v. The isocratic mobile phase was moved at a flow rate of 0.3 ml/min. The validation study with proportionate to selectivity, linearity, robustness, precision, accuracy, stability, (LOD) limit of detection and (LOQ) limit of quantification, was performed utilizing the ICH Guidelines. Ledipasvir and Sofosbuvir displaying a linear response between 22.5-135 µg/ml for Ledipasvir 100-600 µg/ml for Sofosbuvir, with correlation coefficient (R2) 0.9997 and 0.998 for Ledipasvir and Sofosbuvir respectively. The % recovery for Ledipasvir and Sofosbuvir was 99.72 ± 0.61% and 100.21 ± 0.68 respectively. The LOD and LOQ values for Ledipasvir and Sofosbuvir were obtained to be 0.31 and 0.95 µg/ml and 1.29 and 3.91 µg/ml, respectively. The method also exhibits good robustness for different chromatographic conditions like mobile phase, temperature and flow rate. So, the approach can be successfully implemented for the quantitative analysis of ledipasvir and sofosbuvir in the (Q.C) Quality Control of in-house developed tablets, and the same can be exercised for the industrial use.

Keywords: Ledipasvir; Sofosbuvir; UPLC; ICH guidelines

Introduction

Sofosbuvir along with ledipasvir are directly acting antiviral drugs used in combination to treat long lasting hepatitis C infection [1]. Sofosbuvir [2] acts as an inadequate substrate for NS5B, A Ribonucleic Acid-dependent RNA polymerase which is essential for the transcription of Hepatitis C viral RNA and prevents further replication of virus. The structural formula and molecular mass of sofosbuvir are $C_{22}H_{29}FN_3O_9P$ and 529.458 g/mol respectively [3]. Chemical structure is shown in Figure 1. Ledipasvir [4] is orally available inhibitor of hepatitis C virus, it acts by binding to virus non-structural protein 5 A and blocks viral RNA production and viral replication. The structural formula and molecular mass of ledipasvir are $C_{49}H_{54}F_2N_8O_6$ and 888.99 g/mol respectively. Chemical structure is shown in Figure 2.

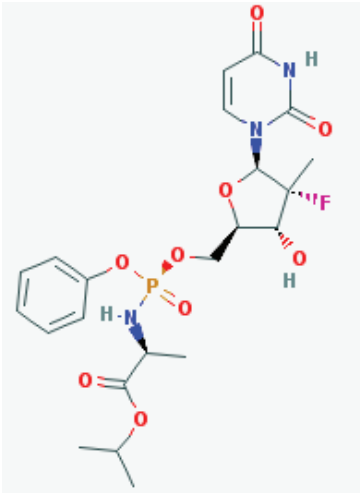

Figure 1: Chemical structure of Sofosbuvir.

Few HPLC and LC-MS/MS methods for concurrent analysis of sofosbuvir and ledipasvir are reported in literature [5-10]. The developed method was less time taking and more precise, accurate when compared to existing methods. So, it is applicable for the regular qualitative analysis of both bulk and formulation.

Experimental

Chemicals

The drugs sofosbuvir and ledipasvir samples are gifted by Spectrum Labs, Hyderabad. The reagents and chemicals used in the method are Potassium di-hydrogen ortho phosphate (Molychem) is of analytical grade, Acetonitrile (Merck) HPLC grade, Orthophosphoric acid analytical grade (RFCL,limited Molychem) and milli-Q water. Hepcinat LP tablets were obtained from Natco pharma, Hyderabad.

Equipment

Ultra performance chromatographic system (Acquity waters) euipped with UV detector processed with Empower software, weighing balance (Saritorius), pH meter (Mestar), sonciator (Labman) and vaccum pump (Crompton) are used for the present study.

Chromatographic conditions

Chromatographic separations were performed on an Acquity

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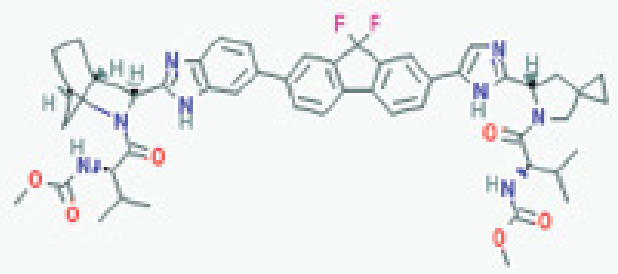

Figure 2: Chemical structure of Ledipasvir.

BEH C18 50 × 2.1 mm, 1.6 µ column using an Acquity Waters UPLC system. Acetonitrile and phosphate buffer in the ratio of 50:50 v/v was employed as mobile phase. Mobile phase composition was same during the entire elution process i.e., Isocratic mode of elution. The column was maintained at temperature of 30°C. The overall run time was 3 min at a flow rate of 0.3 ml/min. The peaks were detected at wavelength of 220 nm.

Preparation of buffer solution

Weighed 1.36 g of potassium di-hydrogen phosphate was dissolved in 900 ml of milli-Q water, sonicate and make the solution to 1000 ml. finally pH adjusted to 3.5 using dil. Orthophosphoric acid.

Preparation of standard stock solution

Sofosbuvir (100 mg), ledipasvir (22.5 mg) standards were weighed and transferred into 25 ml volumetric flask, 10 ml of diluent (water: Acetonitrile 50:50 v/v) was added and subjected to ultra-sonication for 10 min and final volume was adjusted using diluent. The resulting standard stock solution contains 4000 µg of sofosbuvir and 900 µg of ledipasvir.

Preparation of working standard solution

The working standard solution was prepared by diluting 1 ml of standard stock solution containing 4000 µg of sofosbuvir and 900 µg of ledipasvir to 10 ml of diluent to obtain the required concentration of 400 µg Sofosbuvir and 90 µg of ledipasvir.

Preparation of pharmaceutical samples

5 tablets Hepcinat LP weight was measured and turned in to powder form and calculated the average weight of each tablet. Then the equivalent weight of 1 tablet in the form of powder form was transmitted into a volumetric flask of 100 mL and 50 mL of diluent was added and ultra- sonicated for 25 min, further the volume made up to 100 ml using the diluent and filtered. 1 ml of filtered prepared sample stock solution was transferred to the 10 ml volumetric flask and made up to the volume of 10 ml with the diluents.

Method development and optimisation

The method was developed by choosing different columns and mobile phase to obtain good resolution of the peaks and to satisfy the system suitability parameters drafted by ICH. The columns used for development were HSS C18, SB C18, hibra C18, BEH C18. Mobile phase consists of different solvents such as methanol, orthophosphoric acid; water and Acetonitrile of different composition were used for this study.

Method validation

Method validation was done according to ICH guidelines [11]. The parameters tested are system suitability, accuracy, specificity, robustness, precision, linearity, limit of detection (LOD), limit of quantitation (LOQ), forced degradation and stability.

System suitability

This is to check the ability of system with respect to parameters such as theoretical plates, asymmetry and reproducibility by injecting six replicas of working standard solution containing sofosbuvir (400 µg/ml) and ledipasvir (90 µg/ml).

Specificity

This is to check whether any other compounds were eluting at the same retention of sofosbuvir and ledipasvir by injecting blank and working standard solution.

Accuracy

Additional spiking method was used to determine accuracy. Accuracy was determined by spiking 50%, 100%, and 150% of working standard solution to pharmaceutical sample solution in three replica injections at each level. The % recovery was calculated.

Precision

Six injections of pharmaceutical sample solution were analysed, the % RSD of assay values was calculated to determine method precision. Intermediate precision was evaluated by injecting six sample solutions by different analyst on other day and % RSD of assay value was calculated. The % RSD should be not more than 2.0%.

Linearity

Six different concentrations (25 %, 50 %, 75 %, 100 %, 125% and 150 % of working standard concentration) were injected and peak responses were recorded. Concentration of standard solution on X-axis versus peak response on Y-axis was plotted from the recorded data; correlation coefficient, slope and intercept were measured from the plot. If the correlation coefficient was more than 0.99 the method was found to be linear over the given concentration range.

LOD and LOQ

The formulas for calculating LOD and LOQ are 3.3 s/n and 10 s/n respectively, where s/n indicates signal-to-noise ratio.

Robustness

Method robustness was established by considering the variations in mobile phase ratio (± 10% organic phase), flow rate (± 10%), Column oven temperature (± 5°C). From the analyzed data % Relative Standard Deviation (RSD) was calculated.

Forced Degradation studies

The standard solution was degraded by using acid (2N HCl, 30 min, 60°C), base (2N NaOH, 30 min, 60°C), oxidation (20 % peroxide, 30 min, 60°C), Photolytic (UV, 7 days), neutral (water,6 h, 60°C) and thermal (6 h,105°C) degradation. The resulting solutions were neutralized and analysed to check the interference of degradants with the sample peaks.

Stability of solution

The prepared standard and sample solutions were kept on bench top for 24 h. The peak area and retention time of these solutions were compared with freshly prepared solution.

Results and Discussion

Method development

The method was developed by choosing different columns and mobile

phase composition. The columns used for the study were HSS C18, Hibra C18, and BEH C18. In trial 1, HSS C18 column with 50:50 ratio of methanol and orthophosphoric acid was used, the peak shapes were not good and system suitability test was failed because of less theoretical plates. In trial 2, Hibra C18 column with 50:50 ratio of acetonitrile and orthophosphoric acid was used, poor resolution of peaks was observed. In third trial using the same column mobile phase composition was changed to 60:40 ratio of acetonitrile and orthophosphoric acid, two drugs were eluted and failure of theoretical plates was found. In next trial BEH C18 column, with 50:50 ratios of acetonitrile and orthophosphoric acid was used, two peaks were eluted well with theoretical plates less than 2000. To improve the separation, the pH of the mobile phase becomes an important factor. Finally, BEH 50 mm × 2.1 mm, 1.6 µ with an isocratic mobile phase composed of 0.01 N KH_2PO_4 buffer (pH 3.5) and Acetonitrile (50:50) at a flow rate of 0.3 ml/min was used. The drugs were eluted well and satisfying all the system suitability parameters. The retention times were found to about 0.867 min and 1.549 min for ledipasvir and sofosbuvir respectively. This is the optimized method; Figures 3-5 shows for chromatograms.

Method validation

System suitability: The system suitability factors such as theoretical plates (not less than 2000), tailing factor (not more than 2.0) and % RSD (not more than 2.0%) were measured and results were summarised in Table 1.

Specificity: The method was found to be specific because there were no interferent peaks found at the retention time of the sofosbuvir and ledipasvir. Comparing Figures 3-6 gives a clear picture of specificity.

Linearity: The peak area was directly proportional to concentration over a range of 100-600 µg/ml for sofosbuvir and 22.5-135µg/ml for ledipasvir. Least squares method was used to obtain regression line. The regression equation for sofosbuvir was y=7447x+13787 (r^2=0.9998) and y=6279.6x+1538.2 (r^2=0.9997) for ledipasvir. The linear graphs of sofosbuvir and ledipasvir are given in Figures 7 and 8 respectively.

Accuracy: The % Mean recovery for sofosbuvir and ledipasvir are 100.21% and 99.72% respectively. In Table 2, the amount of drug added and amount of drug found and % recovered results are given. 98%-102% is the acceptable limit.

Precision: The % RSD of the six replica injections was calculated to determine the precision of the method. It should be less than or equal to 2.0. For Reproducibility testing, the % RSD found to be 0.8 and 0.5 for sofosbuvir and ledipasvir respectively. While evaluating Intermediate precision, the % RSD for Sofosbuvir and ledipasvir is found to be 0.6 and 0.6 respectively. Method Precision: The pharmaceutical sample was

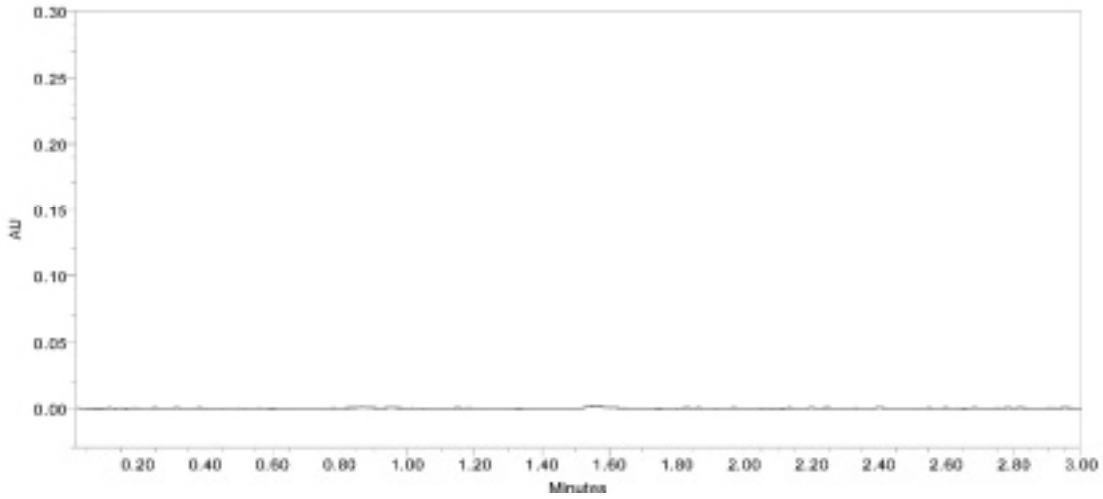

Figure 3: Typical chromatogram of blank solution.

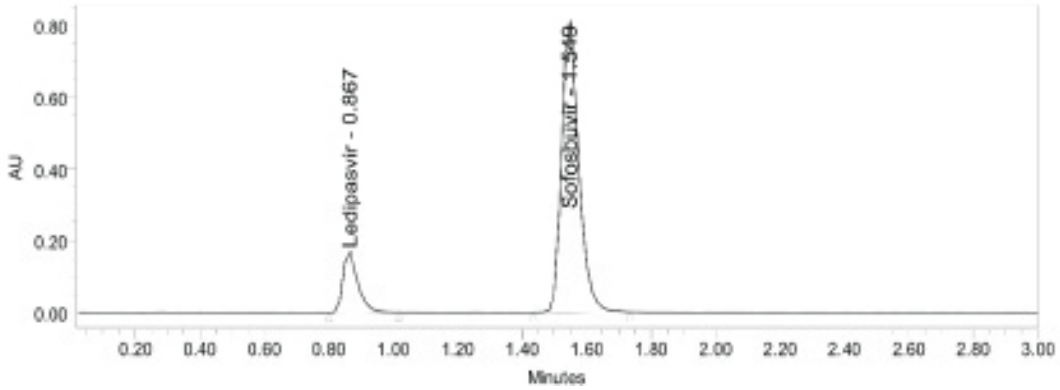

Figure 4: Typical chromatogram for sofosbuvir and ledipasvir standard solution.

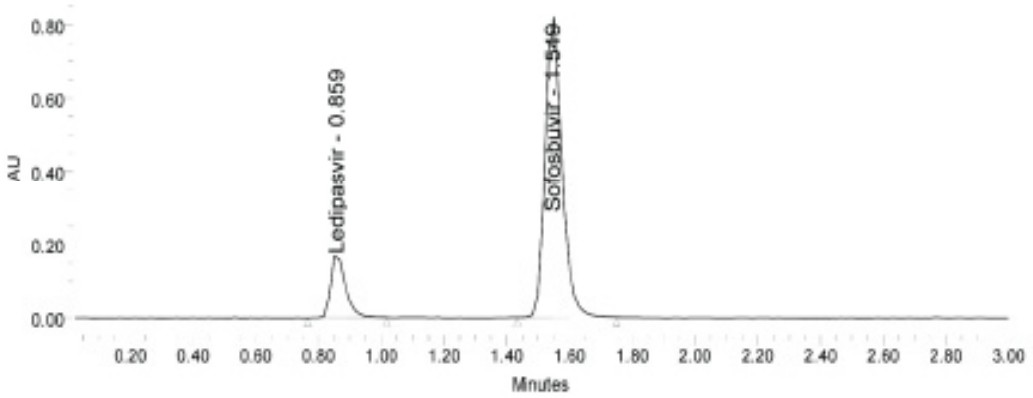

Figure 5: A representative chromatogram of marketed sample.

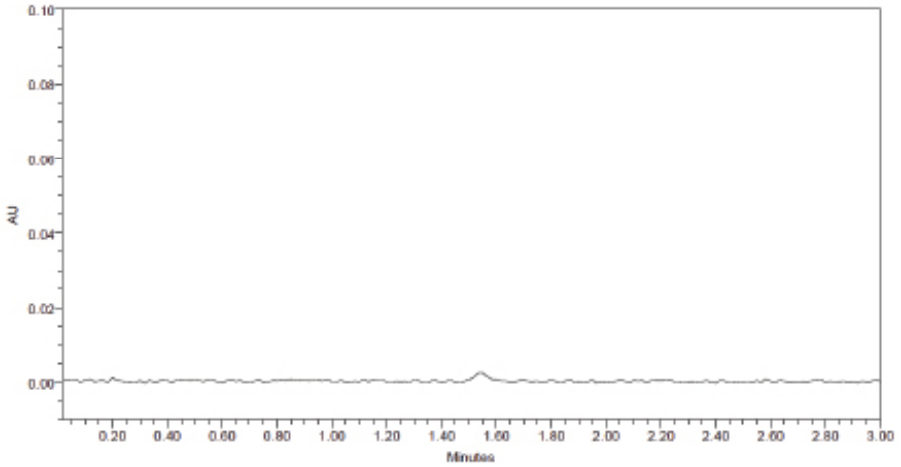

Figure 6: A representative chromatogram of placebo.

S. No.	Retention time		Tailing factor		Number of theoretical plates		Ledipasvir	
Drug Name	Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir
1	1.542	0.858	1.25	1.44	4036	3702	3052155	565404
2	1.548	0.859	1.18	1.43	4077	3668	3087026	563462
3	1.549	0.859	1.2	1.45	4189	3617	3035316	562443
4	1.55	0.865	1.23	1.3	4225	3618	3093643	569742
5	1.551	0.867	1.25	1.25	4275	3567	3093448	569844
6	1.556	0.872	1.24	1.45	4290	3694	3053519	566744
Mean							3069185	566447
Standard Deviation(SD)							25249.7	3109.1
%RSD								

Table 1: System suitability parameters for sofosbuvir and ledipasvir.

prepared, injected in six replicates and analyzed as per the optimized method for the percentage of the drug (% assay) in the marketed formulation.100.54 ± 0.59 of sofosbuvir and 99.48 ± 0.4 of ledipasvir was found in pharmaceutical samples (Table 3).

LOD and LOQ: The method was found to be sensitive because of the lowest values of LOD and LOQ. The LOD and LOQ for sofosbuvir and ledipasvir were 1.29 and 0.31 µg/ml and 3.91 and 0.95 µg/ml respectively.

Robustness: With little variations in flow rate, column temperature, and composition of the mobile phase no major difference was observed in the retention of the sofosbuvir and ledipasvir. Table 4 shows for % RSD values when the conditions are varied within allowable limits.

Forced degradation studies

The sample was stressed and analyzed as per the optimized method. In Table 5, the percentage of degradation under all the stress conditions was given. The sample was found to be stable in thermal, photolytic and hydrolytic degradation. In acid and alkali degradation two degradant peaks were found. When treated with peroxide one degradant peak was found.

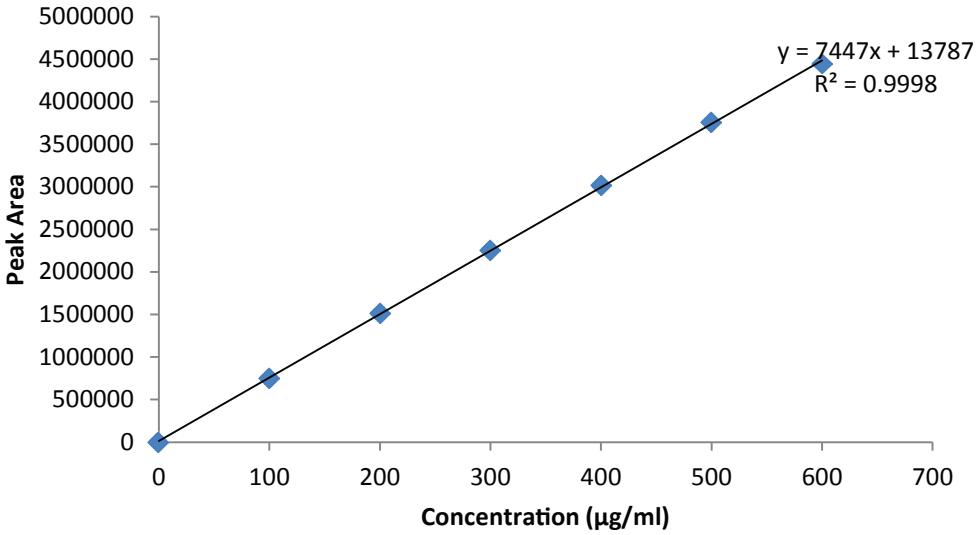

Figure 7: Linearity graph of sofosbuvir.

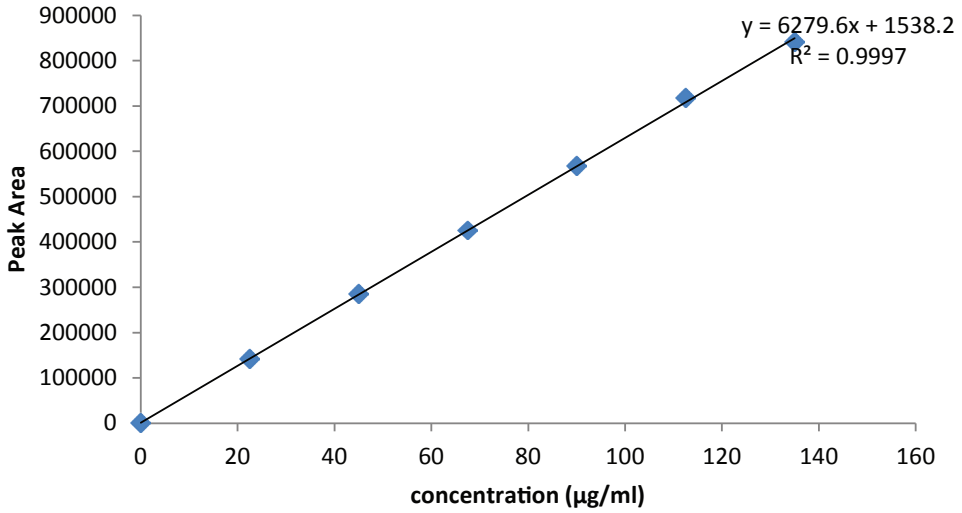

Figure 8: Linearity graph of Ledipasvir.

Accuracy level	Sofosbuvir				Ledipasvir			
	Amount added	Amount Found	% Recovery	Stastical analysis	Amount added	Amount Found	% Recovery	Stastical analysis
50%	200	198.05	99.02	Mean=100.21	45	45.24	100.54	Mean=100.21
	200	201.64	100.82	SD = 0.556	45	45.23	100.51	SD=0.556
	200	201.57	100.79	%RSD=0.555	45	44.8	99.56	%RSD=0.555
100%	400	398.7	99.68	Mean=100.19	90	89.35	99.28	Mean=99.17
	400	402.09	100.52	SD= 0.451	90	88.96	98.84	SD= 0.291
	400	401.49	100.37	%RSD=0.450	90	89.45	99.39	%RSD=0.291
150%	600	599.78	99.96	Mean=100.22	135	134.42	99.57	Mean=99.78
	600	606.46	101.08	SD=0.762	135	134.15	99.37	SD=0.554
	600	597.7	99.62	% RSD=0.761	135	135.56	100.42	% RSD=0.553

Table 2: Accuracy data for sofosbuvir and ledipasvir.

S.NO	Peak Areas			
	Repeatability		Intermediate Precision	
	Sofosbuvir	ledipasvir	Sofosbuvir	ledipasvir
1	3052155	565404	2933110	562042
2	3087026	563462	2926534	562042
3	3035316	562443	2933893	563807
4	3093643	569742	2951739	569837
5	3093448	569844	2966492	560528
6	3053519	566744	2963616	560709
Mean	3069185	566447	2945897	563161
SD	25249.7	3109.1	17057.4	3476.7
%RSD	0.8	0.5	0.6	0.6

Table 3: Reproducibility & Intermediate precision data for Sofosbuvir and ledipasvir.

S.NO	Condition	%RSD (Retention time)	
		Sofosbuvir	Ledipasvir
1	Flow rate (-10%)	0.38	0.68
2	Flow rate (+10%)	0.13	0.24
3	Mobile phase (-10% organic phase)	0.17	0.12
4	Mobile phase (+10% organic phase)	0.49	0.88
5	Temperature (-10%)	0.73	0.82
6	Temperature (+10%)	0.11	0.07

Table 4: Results for robustness.

S.NO	Degradation Study	% Drug Degraded	
		Sofosbuvir	ledipasvir
1	Acid	6.09	6.21
2	Base	5.49	4.84
3	Peroxide	3.83	3.14
4	Thermal	2.69	2.35
5	UV	1.99	1.52
6	Water	0.72	0.83

Table 5: Results of forced degradation studies of sofosbuvir and ledipasvir.

No degradant peaks are found during thermal, photolytic and hydrolytic degradation.

Solution stability

The solution is stable for 24 h on bench top. From freshly prepared standard solution, the RT and peak response of sofosbuvir and ledipasvir was calculated and compared with that of the standard solution kept on bench top for 24 h. Found that there is no major difference between the observed values.

Conclusion

Simultaneous estimation of sofosbuvir and ledipasvir and its stability studies by UPLC was developed and validated according to International Conference on Harmonisation (ICH) guidelines. Short run time (3 min) with better resolution of compounds ensures fast analysis. So, it can be applicable for regular analysis of sofosbuvir and ledipasvir in analytical laboratories. With 0.7%-8% degradation the method was found to be stable under forced degradation conditions with no interference of degradants.

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Conflict of Interest

No Conflict of Interest.

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