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SIMULTANEOUS ANALYSIS OF LEDIPASVIR AND SOFOSBUVIR IN BULK AND TABLET DOSAGE FORM BY STABILITY INDICATING HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

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ABSTRACT A simple, sensitive, precise, selective and specific stability indicating reverse phase high performance liquid chromatography assay for sofosbuvir and ledipasvir has been developed. The chromatography was conducted using YMC C18 analytical column (5 µm; 4.6 mm × 250 mm). The mobile phase containing disodium hydrogen phosphate solution (0.1 M) pH 6.5 and acetonitrile (60:40 v/v); and the isocratic flow rate of 0.8 ml/min were used. Detection was made with photodiode array detector set at 282 nm. The retention time of sofosbuvir and ledipasvir was found to be 2.731 min and 5.850 min, respectively. Linearity was established for sofosbuvir and ledipasvir in the range of 80-240 µg/ml and 18-54 µg/ml, respectively. The good recovery (sofosbuvir – 100.92%-101.06%; ledipasvir – 99.52%-99.63%) and low relative standard deviation (sofosbuvir – 0.0788%; ledipasvir – 0.0085%) confirm the accuracy and precision of the proposed method, respectively. The applicability of the method was evaluated by the assay of sofosbuvir and ledipasvir in combined tablet dosage form. It is recommended that the proposed method can be used for the routine quality control analysis of sofosbuvir and ledipasvir in pure drug and its tablet dosage forms. Forced degradation study was also done and indicated that the proposed method can also be used for degradation evaluation of sofosbuvir and ledipasvir.

KEYWORDS : Antiviral agents, Tablet dosage, Stability indicating, HPLC, Method development, Validation

INTRODUCTION

Sofosbuvir is a direct acting antiviral agent for hepatitis C treatment [1]. As a nucleotide analog inhibitor, sofosbuvir particularly inhibits hepatitis C virus NS5B polymerase. NS5B polymerase is a RNA-dependent RNA polymerase necessary for viral RNA replication. Sofosbuvir inhibits hepatitis C virus replication by binding to the magnesium ions present in the NS5B polymerase active site [2,3].

Ledipasvir is also a direct acting antiviral agent that acts against hepatitis C virus by inhibiting hepatitis C virus NS5B polymerase [4,5]. The exact mechanism of ledipasvir action is not known. But it is assumed to prevent hyperphosphorylation of NS5A polymerase which is required for the activity of enzyme. The chemical structures of both the drugs are shown in Figure 1.

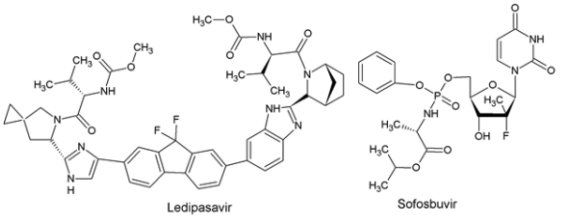

Figure 1: Structure of selected drugs

Hepatitis C is an infectious liver disease caused through infection with Hepatitis C Virus. Sofosbuvir has become available as a fixed dose drug combination product with levipasvir for the treatment of chronic Hepatitis C [6,7]. In October 2014 FDA approved this two direct acting antiviral agents, ledipasvir and sofosbuvir, combination for the treatment of Hepatitis C genotype 1 with cirrhosis or devoid of cirrhosis [8].

The assay of sofosbuvir and ledipasvir in combined dosage form is not official in any pharmacopoeia. Simultaneous determination of sofosbuvir and ledipasvir has been accomplished by UV-spectrophotometry [9,10], ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) [11,12], liquid chromatography with tandem mass spectrometry (LC-MS/MS) [13] and reversed-phase high-performance liquid chromatography (RP-HPLC) [14,15]. Though the UV-spectrophotometric methods [9,10] are simple, they are less selective as it involves measurements in the ultraviolet region where the interference from the exicpients is more. Although UPLC-MS/MS and LC-MS/MS methods are sensitive, they are time consuming, complicated and involve expensive instrumentation. The UPLC-MS/MS and LC-MS/MS methods are not applied to combined tablet dosage forms. The UPLC-MS/MS methods were applied to a pharmacokinetic study of ledipasvir & sofosbuvir in rats [11] and to bioequivalence studies in human volunteers [12]. The LC-MS/MS method was applied to the analysis of the two drugs in the plasma of four healthy volunteers [13]. The reported RP-HPLC methods suffer from few disadvantages such as increased retention time of the drugs (> 8 min) [14], more runtime (> 8 min) [14], increased flow rate of mobile phase (> 1ml/min) [15], less sensitive [14,15], narrow range of linearity [14], and less precise [14,15]. The increased retention time of the drugs, runtime and flow rate of mobile phase will increases the utilization of solvents, time and cost of single analysis.

Therefore, it is apparent that simple, inexpensive, selective, sensitive and precise method is required for the simultaneous determination of sofosbuvir and ledipasvir in bulk and tablet dosage forms. The present study is aimed at developing a stability indicating RP-HPLC method with photodiode array detection for the simultaneous estimation of sofosbuvir and ledipasvir. The method was successfully applied to bulk sample and commercial tablet dosage forms.

MATERIALS AND METHODS

INSTRUMENTATION

- Waters 2695 alliance HPLC system with Waters 2998 photodiode array detector and Waters empower2 software
- YMC C18 analytical column (250 mm × 4.6 mm, 5 µm)
- Electronic balance ELB 300 was used for weighing the materials
- Digisun pH meter was used for all pH measurements

MATERIALS

All the chemicals used were of analytical reagent grade and solvents

were of HPLC grade

- Reference standards of sofosbuvir and ledipasvir (Lara Drugs Private Limited, Telangana, India)
- Ledofos tablets, each tablet contains 90 mg ledipasvir and 400 mg sofosbuvir (Hetero labs limited, Himachal Pradesh, India)
- Milli-Q water (Millipore, USA)
- Acetonitrile (Merck India Ltd., Mumbai, India)
- Hydrogen peroxide (Sd. Fine Chemicals Ltd., Mumbai, India)
- Hydrochloric acid (Sd. Fine Chemicals Ltd., Mumbai, India)
- Sodium hydroxide (Sd. Fine Chemicals Ltd., Mumbai, India)
- Disodium hydrogen phosphosphate (Sd. Fine Chemicals Ltd., Mumbai, India)

CHROMATOGRAPHIC CONDITIONS

- Mobile phase: 0.1 M Na_2HPO_4: acetonitrile (60:40 *v/v*) pH 6.8
- Flow rate: 0.8 ml/min
- Temperature: 30 oC
- Injection volume: 10 µl
- Analytical wavelength: 282 nm
- Runtime: 8 min

STANDARD SOLUTIONS

Stock standard solution (4 mg/ml sofosbuvir and 0.9 mg/ml ledipasvir) was prepared dissolving 400 mg and 90 mg of sofosbuvir and ledipasvir reference substance, respectively in a mixture of 0.1 M Na_2HPO4: acetonitrile (60:40 *v/v*) in a 100 ml volumetric flask. Working standard solutions equivalent to 80 µg/ml, 120 µg/ml, 160 µg/ml, 200 µg/ml and 240 µg/ml of sofosbuvir and 18 µg/ml, 27 µg/ml, 36 µg/ml, 45 µg/ml and 54 µg/ml of ledipasvir was prepared by appropriate dilution of the stock standard solution with the same solvent system.

TABLET SAMPLE SOLUTION

Twenty Ledofos tablets were weighed and the average weight was calculated. Tablets were crushed to a fine powder. Amount equivalent to 400 mg and 90 mg of sofosbuvir and ledipasvir, respectively was transferred to 100 ml volumetric flasks and 30 ml of mobile phase was added. The flasks were shaken ultrasonically for 20 min. The solution was diluted to volume with mobile phase. The solution was filtered through 0.45 µm pore size membrane filter. The prepared solution was diluted with mobile phase to obtain final concentration of 200 µg/ml of sofosbuvir and 45 µg/ml of ledipasvir.

CALIBRATION CURVE

The calibration curve was constructed by analyzing 5 different concentrations of standard solutions using above described chromatographic conditions. The range of solutions varied from 80-240 µg/ml for sofosbuvir and 18-54 µg/ml for ledipasvir. The Y-intercept, slope and regression coefficient were calculated.

ASSAY OF SOFOSBUVIR AND LEDIPASVIR IN COMMERCIAL TABLET DOSAGE FORMS

Tablet sample solution prepared as described earlier was subjected to analysis by applying the proposed method. The measured peak area response was used to calculate the percent of the label claim.

STRESS DEGRADATION STUDIES

Stress degradation study was carried out by subjecting the tablet powder to degradations such as acid, alkaline, oxidative, thermal and photolytic conditions to assess the specificity and stability indicating nature of the proposed method [16].

For acidic hydrolysis, powder equivalent of 400 mg and 90 mg of sofosbuvir and ledipasvir, respectively was mixed with 10 ml of 0.1N HCl in 100 ml volumetric flask. The resultant solution was sonicated for 30 min. Following hydrolysis, the solution was neutralized with sufficient volume of 0.1 N NaOH and diluted with mobile phase up to the mark.

For alkaline hydrolysis, powder equivalent of 400 mg of sofosbuvir and 90 mg of ledipasvir was mixed with 10 ml of 0.1N NaOH in 100 ml volumetric flask and sonicated for 30 min. After hydrolysis, the solution was neutralized with sufficient volume of 0.1 N HCl and diluted with mobile phase up to the mark.

Oxidative degradation was carried out by mixing powder equivalent of 400 mg of sofosbuvir and 90 mg of ledipasvir with 10 ml of H_2O_2 (3% *v/v*) in a 100 ml volumetric flask and the resultant solution was sonicated for 30 min. After oxidation, the solution was diluted with mobile phase up to the mark.

Photo degradation studies were carried out by the exposure of sample powder containing sofosbuvir 400 mg and ledipasvir 90 mg to direct sunlight for 24 hrs. The photo degraded sample was cooled and transferred to a 100 ml volumetric flask containing 30 ml of mobile phase and mixed well. The volume of the flask was completed up to mark with mobile phase.

Dry heating was performed by keeping sample powder containing sofosbuvir 400 mg and ledipasvir 90 mg in oven maintained at a temperature of 105 °C for 30 min. The treated sample was cooled and dissolved in 30 ml of mobile phase in a 100 ml volumetric flask. The contents of the flask were mixed well and diluted up to the mark with mobile phase.

All the degraded samples were diluted with mobile phase to get a final concentration of 200 µg/ml of sofosbuvir and 45 µg/ml of ledipasvir. The degraded samples were analyzed using the chromatographic conditions described.

RESULTS AND DISCUSSION

OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

The major objective of the current investigation was to develop a stability indicating RP-HPLC method for the concurrent estimation of sofosbuvir and ledipasvir in bulk & tablet dosage form and to get good resolved peaks of sofosbuvir, ledipasvir and their stress degradation products. During method development, chromatographic parameters such as mobile phase composition, flow rate of mobile phase, ph of the mobile phase, detection wavelength, analytical column and column temperature were optimized to get improved efficiency of the chromatographic system. After several preliminary experiments, the following chromatographic conditions were finalized:

- Mobile phase: 0.1 M Na2HPO4: acetonitrile (60:40 v/v) pH 6.8
- Flow rate: 0.8 ml/min
- Temperature: 30 oC
- Injection volume: 10 µl
- Analytical wavelength: 282 nm

Under the optimized chromatographic conditions, the retention time for sofosbuvir and ledipasvir was found to be 2.731 min and 5.850 min, respectively.

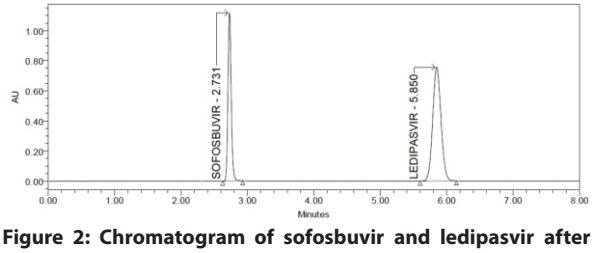

Figure 2: Chromatogram of sofosbuvir and ledipasvir after method optimization

METHOD VALIDATION

The developed RP-HPLC method was validated according to International Conference on Harmonization [17].

SYSTEM SUITABILITY

System suitability was tested with five replicate injections of the working standard solutions (160µg/ml sofosbuvir and 36 µg/ml ledipasvir). The system suitability parameters like relative standard deviation of retention time of drugs, relative standard deviation of peak area of drugs, tailing factor, plate count and resolution were calculated. The system suitability results are shown in Table 1. All the calculated parameters are within the acceptance criteria.

Table 1: System suitability parameters for sofosbuvir and ledipasvir

Parameters	Sofosbuvir		Ledipasvir		Recommended limit
	Value*	RSD (%)	Value*	RSD (%)	
Retention time	2.734	0.066	5.840	0.054	RSD ≤2
Peak area	3780471	0.278	6528785	0.235	RSD ≤2
Resolution	-	-	19.366	0.221	> 1.5
Plate count	15746	0.552	10524	0.926	> 2000
Tailing factor	1.126	0.486	1.076	0.831	≤ 2

*mean of five values

LINEARITY

The calibration curve of peak area of drug against concentration of drug was linear in the range investigated, 80-240 µg/ml for sofosbuvir (Figure 3a) and 18-54 µg/ml for ledipasvir (Figure 3b). The regression equations and regression coefficient are shown in Table 2.

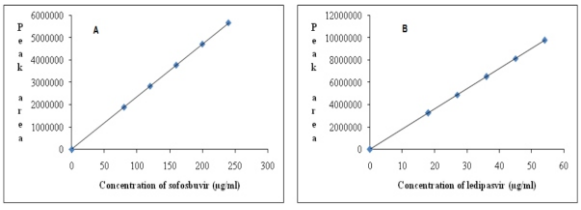

Figure 3: Calibration curve of [A] Sofosbuvir [B] Ledipasvir

Table 2: Linearity studies for sofosbuvir and ledipasvir

Parameter	Sofosbuvir	Ledipasvir
Linearity (µg/ml)	80-240	18-54
Regression equation (y = mx + c)	y = 23631x + 674	y = 18144x - 2795
Slope (m)	23631	18144
Intercept (c)	674	-2795
Regression coefficient (R2)	0.9999	0.9999

LIMIT OF DETECTION (LOD) AND QUANTIFICATION (LOQ)

The LOD and LOQ for sofosbuvir were 0.225 µg/ml and 0.751µg/ml, while for ledipasvir 0.074 µg/ml and 0.249 µg/ml, respectively. 3

SELECTIVITY

The chromatograms of mobile phase blank, placebo blank and tablet sample were obtained by the developed method and compared with those obtained by standard drugs solution. A placebo blank (composition: 20mg talc, 30mg starch, 20mg sucrose, 20mg lactose, 10mg gelatin, 20mg sodium alginate, 30mg magnesium stearate, and 20mg methyl cellulose) was prepared by homogeneous mixing in a mortar. 100 mg of placebo was placed in a 50 ml calibration flask and its extract was prepared as described under Section "Tablet sample solution". Chromatograms of mobile phase blank and placebo blank obtained shows no interference from the components of mobile phase or from the common excipients used in the preparation of tablets. The tablet sample chromatogram obtained is similar to that of drug standard solution, devoid of any interference from excipients. The results proved the selectivity of the proposed method for the simultaneous analysis of sofosbuvir and ledipasvir.

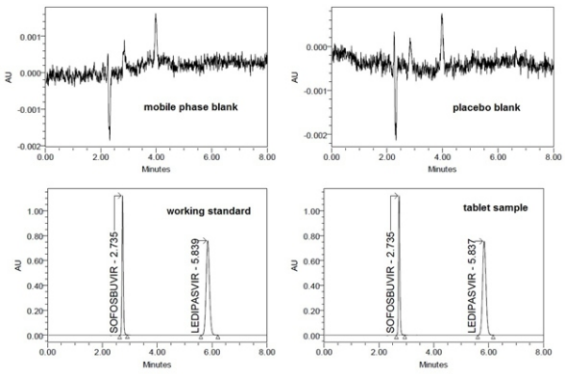

Figure 4: Chromatograms of selectivity studies

ACCURACY AND PRECISION

The precision study was carried at a concentration of sofosbuvir 160 µg/ml and ledipasvir 36 µg/ml and is expressed as relative standard deviation of sofosbuvir and ledipasvir peak area response. The low relative standard deviation values (<0.5%) indicate high precision of the proposed method (Table 3).

The accuracy was also studied at a concentration of 160 µg/ml and 36 µg/ml of sofosbuvir and ledipasvir, respectively and is expressed as percent assay of sofosbuvir and ledipasvir. The excellent percent assay values indicate good accuracy of the proposed method (Table 3).

Table 3: Results of precision and studies of the proposed method

Sample No.	Sofosbuvir		Ledipasvir	
	Peak area response	Assay (%)	Peak area response	Assay (%)
1	3785457	99.33	6521885	99.49
2	3786093	99.35	6527321	99.58
3	3789726	99.44	6527994	99.59
4	3784267	99.3	6527483	99.58
5	3789531	99.44	6528087	99.59
6	3783194	99.27	6528718	99.6
Average	3786562	99.36	6527920	99.58
RSD	0.0788	0.0789	0.0085	0.0084

RECOVERY TEST

Preanalyzed tablet sample was spiked with pure sofosbuvir and ledipasvir at three concentration levels and the percent recovery of the pure drug added was calculated. The recoveries demonstrated that the inactive ingredients in the tablet dosage form have negligible effect on the quantification of sofosbuvir and ledipasvir and the method is found to be accurate (Table 4).

Table 4: Results of recovery test by standard addition method

Spiked Level (%)	Concentration of drug (µg/ml)		Recovery (%)	Mean (%)
	Added	Found		
Sofosbuvir				
50	79.200	80.21	101.27	101.06
	79.200	79.85	100.82	
	79.200	80.06	101.09	
100	158.400	159.78	100.87	100.98
	158.400	160.05	101.04	
	158.400	160.01	101.02	
150	237.600	239.89	100.97	100.92
	237.600	239.84	100.94	
	237.600	239.60	100.84	
Ledipasvir				

50	18.000	17.93	99.61	99.63
	18.000	17.91	99.51	
	18.000	17.96	99.77	
100	36.000	35.82	99.49	99.52
	36.000	35.85	99.58	
	36.000	35.82	99.49	
150	54.000	53.77	99.58	99.59
	54.000	53.79	99.61	
	54.000	53.77	99.58	

DEGRADATION STUDIES

In the forced degradation studies, sofosbuvir and ledipasvir was found to degrade under acidic, alkaline, oxidative, photo and thermal stress conditions employed. Table 5 summarizes the sofosbuvir and ledipasvir recovery after stress degradation and the retention times of the degradation products. Typical chromatograms obtained for sofosbuvir and ledipasvir under different stress conditions are shown in Figures 5-9. The developed RP-HPLC method could effectively resolve the sofosbuvir and ledipasvir from their degradation products. Well separation of degradants from sofosbuvir and ledipasvir peaks shows that the proposed method is specific and stability indicating.

Table 5: Results from degradation studies

Stress condition	Sofosbuvir			Ledipasvir			Retention time of degradants
	Peak area	Recovery (%)	Degra-dation (%)	Peak area	Recovery (%)	Degra-dation (%)	
Untreated	3780471	100	-	6528785	100	-	-
Acid	3702195	97.73	2.27	6426135	98.03	1.97	2.337, 3.147, 3.448 & 3.612
Alkaline	3707127	97.86	2.14	6443131	98.29	1.71	2.346, 3.178, 3.476 % 3.654
Oxidative	3724684	98.33	1.67	6429439	98.08	1.92	2.347, 3.182, 3.489, 3.667 & 3.959
Thermal	3722016	98.26	1.74	6485557	98.94	1.06	2.348, 3.188, 3.501, 3.682 & 3.944
Photo	3735250	98.61	1.39	6468801	98.68	1.32	2.352, 3.193, 3.511, 3.691 & 3.959

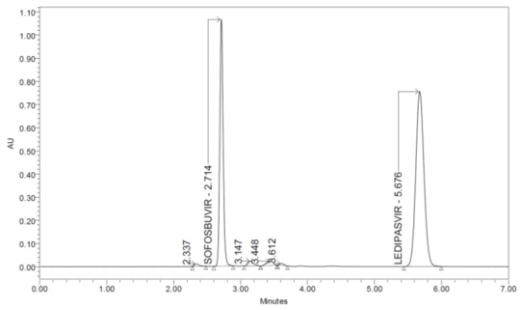

Figure 5: Chromatogram of sofosbuvir and ledipasvir after acid stress

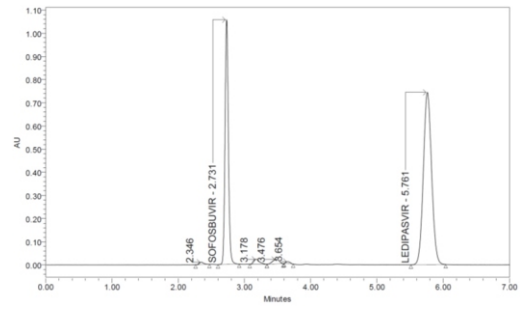

Figure 6: Chromatogram of sofosbuvir and ledipasvir after alkaline stress

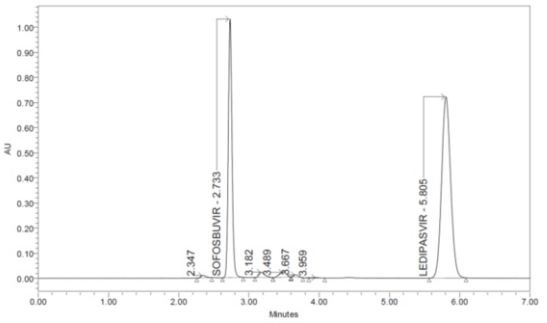

Figure 7: Chromatogram of sofosbuvir and ledipasvir after oxidative stress

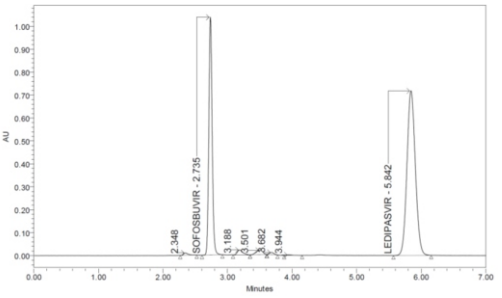

Figure 8: Chromatogram of sofosbuvir and ledipasvir after thermal stress

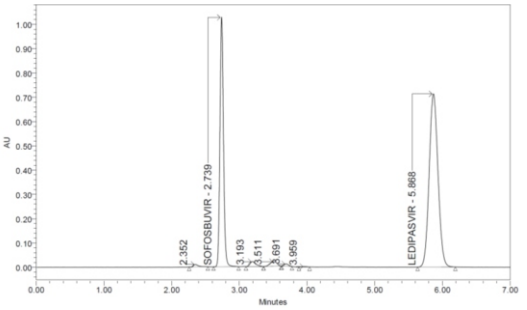

Figure 9: Chromatogram of sofosbuvir and ledipasvir after photo stress

ROBUSTNESS

Robustness of the proposed RP-HPLC method was studied by small but deliberate variations in the method parameters. The effect of changes in the mobile phase flow rate (± 0.1 ml) and column temperature (±5 oC) on system suitability parameters for both drugs was examined. The result of robustness study is abridged in Table 6. The system suitability parameters were not significantly affected. Hence, the proposed method was robust for the simultaneous determination of sofosbuvir and ledipasvir.

Table 6: Robustness study of sofosbuvir and ledipasvir

Parameter varied	Retention time	Peak area	Plate count	Tailing factor	Resolution
Sofosbuvir					
Flow rate – 0.7 ml/min	3.311	4589046	16041	1.09	-
Flow rate – 0.9 ml/min	2.361	3264042	12684	1.12	-
Column temperature-29oC	2.746	3790902	14511	1.11	-
Column temperature-31oC	2.726	3795749	14157	1.09	-
Ledipasvir					
Flow rate – 0.7 ml/min	7.128	7963226	11452	1.08	20.14
Flow rate – 0.9 ml/min	5.121	5659420	8641	1.08	17.88
Column temperature-29oC	5.916	6578974	9877	1.07	18.88
Column temperature-31oC	5.676	6555880	10253	1.06	18.36

CONCLUSION:

It can be concluded that a simple, inexpensive, selective, sensitive and precise stability indicating high-performance liquid chromatographic simultaneous assay for sofosbuvir and ledipasvir has been developed. The method was practically validated showing satisfactory data for all the method validation parameters investigated. The method is free from interference of the components of mobile phase used, inactive ingredients & additives used in the tablet dosage forms and stress degradation products. Therefore the developed and validated method is suitable for use of the routine quality control analysis of sofosbuvir and ledipasvir in bulk and in tablet dosage forms.

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