IJPSR (2020), Volume 11, Issue 2 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 17 April 2019; received in revised form, 09 August 2019; accepted, 06 November 2019; published 01 February 2020

STABILITY INDICATING UHPLC METHOD FOR SIMULTANEOUS ESTIMATION OF SOFOSBUVIR AND LEDIPASVIR IN TABLET DOSAGE FORM

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Keywords:

Ledipasvir, Sofosbuvir, UHPLC, Stability Indicating, Forced Degradation, ICH

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ABSTRACT: A stability-indicating UHPLC method was developed for the simultaneous estimation of Sofosbuvir (SOF) and Ledipasvir (LPS) in bulk and tablet dosage form. The study was performed using C18 BEH column (210 mm × 50 mm, 1.7 µm), with a mobile phase consisting of acetonitrile (55% v/v) and phosphate buffer of pH 3 (45% v/v) at a flow rate of 0.3 ml/min. The detection was carried at 247 nm using PDA detector and the retention times were found to be 0.7 and 1.2 min for SOF and LPS respectively. The developed method was validated according to ICH guidelines and the results were statistically validated. The method was found to be linear in the concentration range of 80 - 240 µg/ml for SOF with r2 value 0.999 and 18 - 54 µg/ml for LPS with r2 value 0.999. The % RSD value for precision, measured as repeatability and intermediate precision was less than 2 for both the analytes. The recovery percentages were found to be 100.13 and 99.93 for SOF and LPS respectively. The analytes were subjected to stress conditions (forced degradation studies) such as acidic, basic, peroxide, thermal, and photodegradation and the results showed that % degradation was within limits and drugs can be estimated in the presence of degradants. The method was successfully applied to the assay of Sofosbuvir and Ledipasvir in tablet dosage form. Thus, the proposed method is simple, accurate, precise and can be applied confidently for the quantitative determination of Sofosbuvir and Ledipasvir in tablets even in the presence of degradants.

INTRODUCTION: Hepatitis C is an infectious liver disease caused by the Hepatitis C virus (HCV) [1]. The virus (HCV) can cause both acute and chronic hepatitis [2]. HCV infection is a major global health problem affecting approximately 160 - 180 million individuals.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.11(2).784-90

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(2).784-90

Chronic HCV infection may progress to liver cirrhosis, hepatic decompensation, hepatocellular carcinoma, liver failure, and death especially in HIV-positive patients [3]. Treatment of hepatitis C virus (HCV) infection has progressed considerably with the approval of interferon-free, direct-acting antiviral (DAA)-based combination therapies [4].

An interferon-free combination of direct-acting antiviral agents was recommended as the first-line standard-of-care treatment for chronic HCV infection. Interferon-based therapy should be considered as a second-line option after an individual benefit-risk assessment [5].

Direct-acting antivirals (DAA) show high potency, favorable tolerability profile, shorter duration of treatment, all-oral regimen and fewer drug interactions [6]. A fixed-dose combination of Sofosbuvir (SOF) (400 mg) and Ledipasvir (LPS) (90 mg) was approved in October 2014 by USFDA for the treatment of chronic HCV infection [7, 8] Sofosbuvir, also known as GS-7977 is a nucleotide inhibitor of NS5B polymerase and is among recent prodrugs, having high SVR rate and can be used in the treatment of HCV infection in combination with other drugs [9, 10]. SOF is white to off white crystalline powder, with IUPAC name of (S)-isopropyl 2- (((2R, 3R, 4R, 5R)-5-(2, 4-dioxo-3, 4-dihydropyrimdin- 1(2H)- yl)- 4- fluoro- 3-hydroxy- 4-methyltetrahydrofuran-2-yl) methoxy) (phenoxy) phosphoryl) amino) propanoate. The chemical structure of sofosbuvir is shown in **Fig. 1**. LPS, also is known as GS-5885, is a new HCV NS5A inhibitor, potent antiviral agent against HCV (genotype 1a and 1b) and approved as a fixed-dose combination with sofosbuvir for treatment of chronic HCV infection (genotype 1) [11]. LPS is off white to yellow coloured, amorphous powder with IUPAC name of (1- {3- [6- (9, 9-difluoro– 7-{2-[5-(2- methoxy carbonyl amino-3-methyl -butyryl) -5-aza-spiro [2.4] hept-6-yl] -3H-imidazol -4-yl}-9H fluoren- 2- yl)- 1H- benzoimidazol- 2- yl] -2-aza-bicyclol [2.2.1] heptanes-2-carbonyl}-2-methyl-propyl)-carbamic acid methyl ester. **Fig. 2** represents the chemical structure of Ledipasvir.

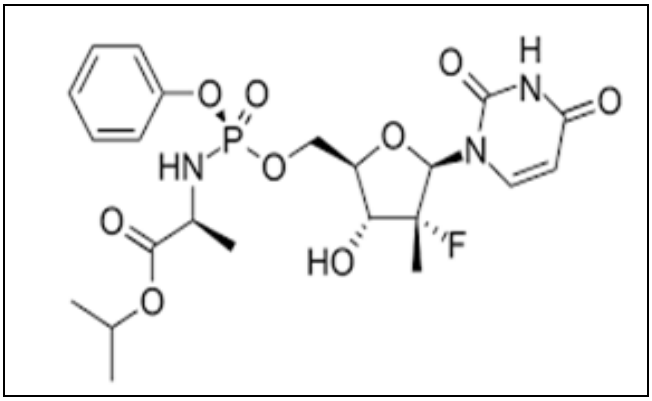

FIG. 1: CHEMICAL STRUCTURE OF SOFOSBUVIR

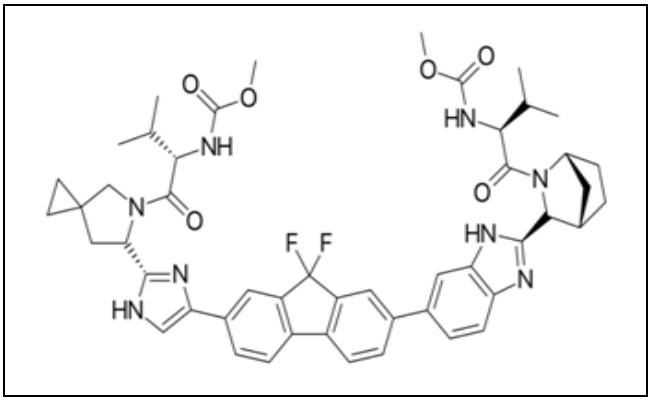

FIG. 2: CHEMICAL STRUCTURE OF LEDIPASVIR

Various analytical methods have been described in the literature for the estimation of SOF and LPS in combination and individually by LC-MS/MS [12-16] and RP-HPLC [17]. To the best of our knowledge, there is no UHPLC method for the simultaneous estimation of SOF and LPS. In this study a simple, isocratic UHPLC method was developed for the simultaneous determination of SOF and LPS in tablet dosage form for assay determination and would help to quantify the content of SOF and LPS in dosage form. The method was validated according to ICH guidelines [18].

MATERIALS AND METHODS: Generic product of a fixed-dose combination of SOF 400 mg LPS 90 mg (LEDIFOS) was purchased from the local market. Reference standards SOF and LPS were obtained as gift sample from Hetero Labs, Nakkapally. Acetonitrile, (UPLC Lichrosolv), Potassium dihydrogen orthophosphate (Merck, Mumbai), Orthophosphoric acid (Qualigens) were of analytical grade.

Instrumentation: The UHPLC system(waters Acquity) comprised of quaternary pump, autosampler, PDA 2996 detector, controlled by EMPOWER 2 Software, BEH C18 analytical column (210 × 50 mm, 1.7 µm) was used for analysis. A double beam UV visible spectrophotometer (Lab India, UV- 3000+) with matched 1 cm quartz cells was used for optimizing the wavelength. An electronic balance (Afcoset ER- 2C0A), pH meter (Adwa- AD1020), hot air oven, ultra sonicator, Millipore vacuum Filtration Assembly were also used in the study.

Preparation of Mobile Phase: Phosphate buffer (pH 3.0) was prepared by dissolving 3.4 g of KH_2PO_4 in 1000 ml Millipore water. The pH was adjusted to 3.0 with orthophosphoric acid. The mobile phase was prepared by mixing phosphate buffer pH 3.0 and acetonitrile in the ratio of 45:55% v/v, sonicated for 10 min and filtered through 0.45 µm membrane filter using vacuum filtration assembly.

Preparation of Standard and Sample Solutions: Standard stock solutions were prepared by dissolving 40 mg of SOF and 9 mg of LPS separately in the mobile phase in a 10 ml volumetric flask. Further dilutions were made to obtain composite and individual standard solutions as per the requirement. The sample solution of tablet dosage form was prepared by weighing suitable quantity of tablet powder equivalent to 40 mg of SOF and 9 mg of LPS in 10 ml volumetric flask containing mobile phase. Further dilution is made to obtain a composite sample stock solution containing SOF 160 µg/ml and LPS 36 µg/ml.

Method Development and Optimization: SOF and LPS having chromophores are UV active and show maximum absorbance at 260 nm and 332 nm respectively. To determine the optimum wave-length for simultaneous determination, the standard solutions of SOF (160 µg/ml) and LPS (36 µg/ml) and a composite sample were scanned from 200 to 400 nm. The suitable wavelength for measurement of both drug analytes from the UV Spectrum was found to be 247 nm **Fig. 3**.

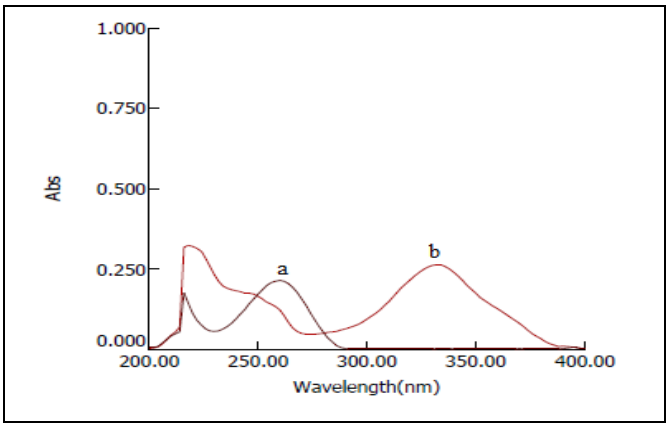

FIG. 3: OVERLAY UV SPECTRA OF SOF (A) AND LPS (B)

Optimization of method was done to achieve better resolution, by considering the solubility of analytes, by applying different set of conditions. Method optimization was performed by considering different mobile phases at different ratios and different flow rates over different stationary phases. Individual and composite solutions of both analytes were analyzed in isocratic mode using methanol, acetonitrile and phosphate buffer on C8 and C18 columns in different ratios. The combination of acetonitrile (55% v/v) and phosphate buffer (45% v/v) pH 3.0 at a flow rate of 0.3 ml/min over BEH C18 column (210 × 50, 1.7 µm) showed better resolution and peak symmetry for both analytes. Each analyte has a different retention time and can be identified and quantified by comparing with individual reference solutions. The optimized set of chromatographic conditions were validated as per ICH guidelines.

Method Validation: The analytical method was validated properly according to the ICH guidelines for accuracy, precision, intermediate, linearity, specificity, robustness, and ruggedness. For linearity assessment five solutions (n = 3) with known concentrations of SOF (80, 120, 160, 200, 240 µg/ml) and LPS (18, 27, 36, 45, 54 µg/ml) were analysed. The calibration curve was plotted between peak response on y-axis and concentration on x-axis. The relation between concentration and response was evaluated by least-square linear regression method y = mx + c, where m is slope, y = peak area, c = intercept and X = concentration.

The accuracy of the method was established by analyzing the solutions of 3 concentrations (n = 3) at 3 different levels (50%, 100%, 150%) of target assay concentration. The % recovery and relative standard deviation (RSD) were evaluated against acceptable limits of ± 2%. Repeatability and intermediate precision were checked by analyzing replicate composite reference solutions (n = 6) of known concentrations. The overall % RSD for peak response on day 1 and day 2 was checked against acceptable limits of ± 2% for precision and intermediate precision. Specificity is important to check the interference of excipients on analytes response.

A placebo solution was prepared from all tablet excipients except the active ingredient in the same diluent. The solution was analyzed under the same chromatographic conditions, and the baseline was evaluated for peak response. Placebo interference was determined by spiking reference solution with appropriate level of excipients and evaluating for additional peaks other than peaks of SOF and LPS. Robustness of method was assured by analyzing replicates (n = 6) of solution used for precision with small changes in chromatographic conditions such as composition of mobile phase, Flow rate, *etc.* The influence of variables is determined by evaluating value of % RSD against acceptable limit of ± 2% for peak response and R_t of each analyte.

Limit of Detection (LOD), Limit of Quantification (LOQ) were established based on the Signal to Noise (S/N) ratio method.

Forced Degradation Studies: These studies of analytes were conducted under acid, alkaline, peroxide (oxidation), thermal and photolytic conditions. The reference solutions of analyte were exposed to these conditions, and the main peak was studied for peak purity to indicate that the method effectively separates the degradation products from the pure active ingredient. Degradation studies can be performed in solid-state or solution for both drug substance and drug product. Acid degradation was carried out by placing 0.4 ml of sample solution in 10 ml volumetric flask, and 3 ml of 1N HCl was added. The flask was kept aside for 2 h at room temperature and then neutralized with 1 N NaOH, diluted to 10 ml with diluent, and analyzed in UHPLC system.

Alkaline degradation was performed by adding 3 ml of 1N NaOH to 0.4 ml of the sample solution in a 10 ml volumetric flask and stands it for 2 h at room temperature. Neutralize the solution with 1N HCl and diluted to 10 ml with diluent. Oxidative degradation was done by using 30% v/v H_2O_2 solution. To 0.4 ml of sample solution 3 ml of 30% v/v H_2O_2 solution was added and allowed to stand for 2 h at room temperature. Then the solution was made up to 10 ml with diluent and is injected into UPLC system. Thermal degradation was simply carried out by placing the flask containing the drug solution in a hot air oven at 110 ℃ for 3 h and diluted with diluent to 10 ml. The solution was then used for analysis. The photodegradation of sample solution was checked by exposing the sample solution to sunlight for 8 h. The solution was then diluted to 10 ml with diluent and injected for analysis.

RESULTS AND DISCUSSION:

System Suitability: The optimized chromatographic conditions *i.e.*, a combination of Acetonitrile and Phosphate buffer pH 3.0 (55:45% v/v) at a flow rate of 0.3 ml/min over BEH column (C18, 210 × 50 mm, 1.7 µm) were checked for the system suitability parameters such as Retention time (Rt), Theoretical plates (N), peak area (A), Symmetry factor (As), Resolution of LPS. The statistical data of parameters were calculated using Empower 2.0 software. The results in **Table 1** showed that the performance parameters of the developed analytical method comply with USP requirements of system suitability.

TABLE 1: SYSTEM SUITABILITY RESULTS

Parameters	SOF	LPS	Acceptable limit
RSD of Peak area (A)	0.1	0.3	<1 for n ≥ 6
RSD of Rt	0.93	0.88	< 1 for n ≥ 6
Theoretical plates (N)	3606.91	3661.89	N > 2000
Symmetry factor (As)	1.19	1.14	As < 2
Resolution(R)	-	3.41	R > 2

The % RSD for peak area(A), retention time (R_t) for both analytes was less than 2.0, resolution of LPS compared to SOF was more than 2.0, tailing factor was less than 2.0, and the number of theoretical plates was more than 2000. The method was found to be suitable for simultaneous determination of SOF and LPS after successful application of their estimation in tablet dosage form.

TABLE 2: STATISTICAL DATA OF LINEARITY OF SOFOSBUVIR AND LEDIPASVIR

Parameters	SOF	LPS
Linearity range (µg/ml)	80-240	18-54
Correlation, r	0.999	0.999
Slope	7763.24	12287.67
Y-Intercept	158054.4	1036.2
LOD (µg/ml)	0.08	0.108
LOQ (µg/ml)	0.28	0.32

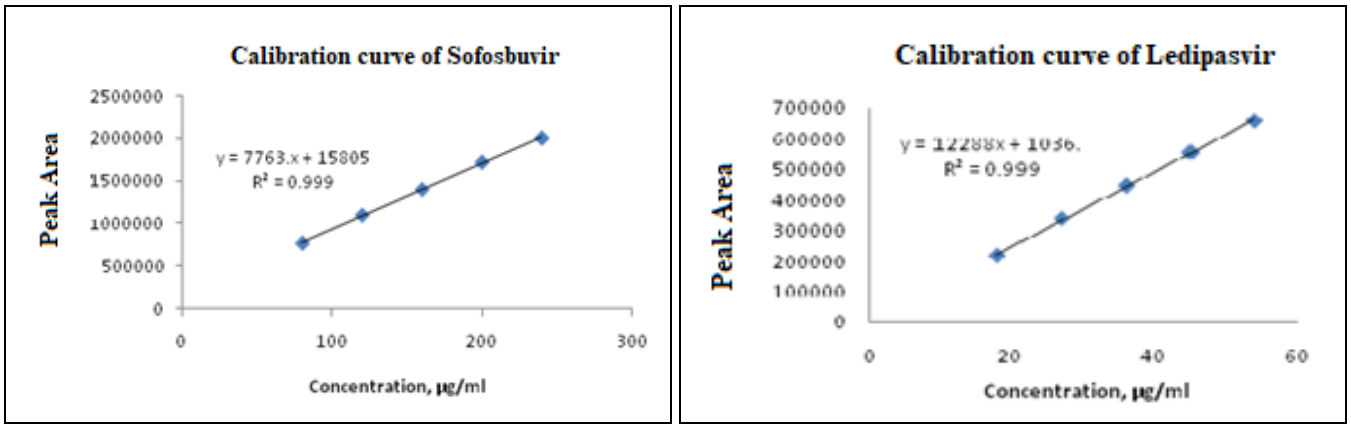

FIG. 4: CALIBRATION CURVE OF SOFOSBUVIR AND LEDIPASVIR

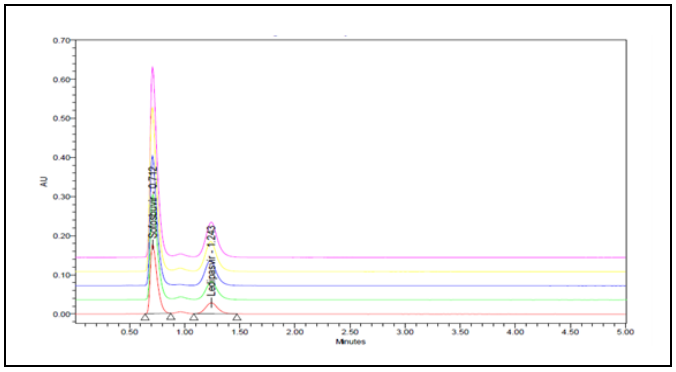

FIG. 5: LINEARITY OVERLAY CHROMATOGRAM

Linearity: The statistical analysis of linearity data in **Table 2** showed a good correlation between concentration and response. The linear regression equations for linearity of SOF and LPS were Y = 7763x + 15805, Y = 12288x + 1036 respectively and the correlation coefficient for both analytes is 0.999.

The calibration curves and overlay chromatogram of SOF and LPS are shown in **Fig. 4** and **5**, respectively.

TABLE 3: RESULTS OF RECOVERY STUDIES OF SOFOSBUVIR AND LEDIPASVIR

Concentration (% level)	SOF		LPS	
	% Recovery	% RSD	% Recovery	% RSD
50	99.75	0.6	99.56	0.6
100	100.49	0.6	99.84	0.1
150	100.16	0.3	100.40	0.7

% RSD = Percentage Relative Standard Deviation

Accuracy: The sample solutions of three concentrations (n = 3) at three different levels of 50%, 100% and 150% of target assay concentration were analyzed, and the results were given in **Table 3**.

The overall recovery of SOF and LPS at each concentration is 100 ± 1%, and % RSD was less than 2. The method is suitable for the assay of SOF and LPS in tablet dosage form as the results indicate that the method is accurate.

Precision: The results of precision and intermediate precision studies **Table 4** shows that the method is precise and repeatable within acceptable limits. The % RSD for peak response of six replicates (n=6) for both analytes is less than 2.

TABLE 4: PRECISION DATA OF SOFOSBUVIR AND LEDIPASVIR

Days	SOF		LPS	
	Peak area	Assay (%)	Peak area	Assay (%)
1	1447821.3	99.5	442304.0	99.8
% RSD	0.1	0.5	0.3	0.3
2	1444900.3	99.48	446048.0	100.86
% RSD	0.2	0.1	0.2	0.1

% RSD = Percentage Relative Standard Deviation

Specificity: The specificity studies revealed the absence of significant peaks at the given retention times of both analytes SOF and LPS in placebo.

Robustness: The chromatographic conditions (flow rate, mobile phase composition) were deliberately variated to obtain the results of robustness, shown in **Table 5**. The change in chromatographic conditions did not influence the results of SOF and LPS.

The ruggedness of method was carried out by different analysts on different days. The results indicate that the method is robust and rugged. The % RSD values were less than 2.0.

LOD and LOQ: The LOD values were 0.08 µg/ml and 0.108 µg/ml for SOF and LPS respectively. The LOQ values were 0.28 µg/ml and 0.32 µg/ml for SOF and LPS respectively **Table 2**.

Forced Degradation Studies: The results of forced degradation studies carried on drug samples indicate that the % degradation was within the limit of 5% to 20%. The degradants formed under stress conditions were resolved from the active ingredients. The results were shown in **Table 6**. The chromatograms of acid, base, peroxide, photolytic and thermal degradation of SOF and LPS were shown in **Fig. 6**.

TABLE 5: ROBUSTNESS RESULTS OF SOFOSBUVIR AND LEDIPASVIR

Parameter	SOF		LPS	
	Rt	USP Tailing	Rt	USP Tailing
Flow Rate (ml/min)				
0.27	0.804	1.14	1.371	1.15
0.30	0.715	1.15	1.24	1.14
0.33	0.650	1.10	1.107	1.08
Change in organic phase composition				
10% less	0.684	1.17	1.266	1.11
Actual	0.715	1.15	1.24	1.14
10% more	0.602	1.48	0.892	1.15

Rt = Retention time

TABLE 6: RESULTS OF FORCED DEGRADATION STUDIES

Stress condition	% Assay of Active Ingredient			
	SOF	% degradation	LPS	% degradation
Acid	92.28	7.72	94.59	5.41
Base	90.45	9.55	94.65	5.35
Peroxide	91.28	8.72	94.36	5.64
Thermal	91.97	8.03	95.55	4.45
Photolytic	91.97	8.03	95.30	4.70

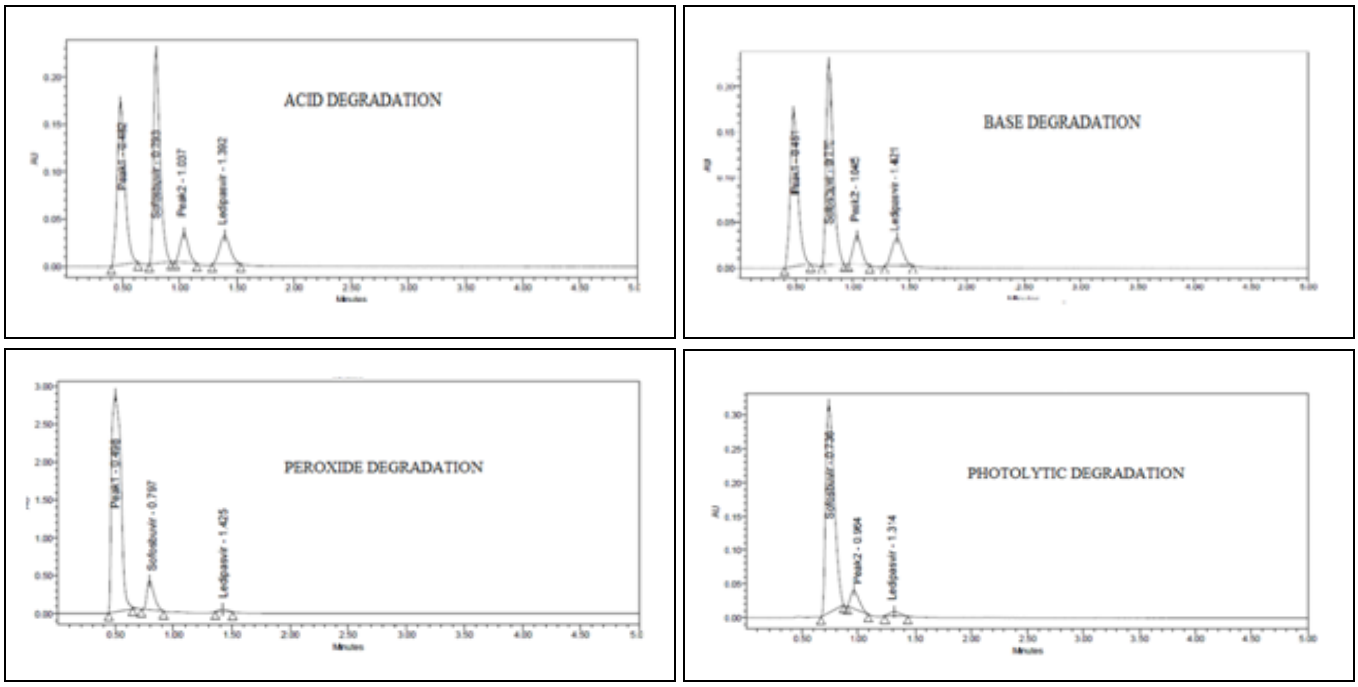

FIG. 6: DEGRADATION CHROMATOGRAMS

Assay: The developed method was successfully applied to analyze Sofosbuvir and Ledipasvir in tablet dosage form. The chromatograms of standard and sample were shown in **Fig. 7** and **8** respectively. The results of the analysis of the tablet dosage form are shown in **Table 7**.

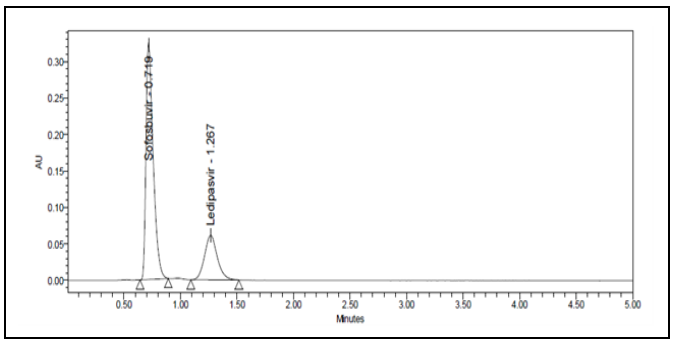

FIG. 7: STANDARD CHROMATOGRAM

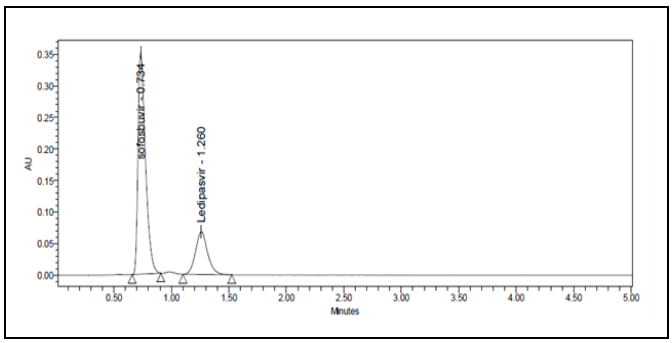

FIG. 8: SAMPLE CHROMATOGRAM

TABLE 7: ASSAY RESULT OF SOFOSBUVIR AND LEDIPASVIR

Compound	Content found (%)	Theoretical content (%)
Sofosbuvir	99.56	100
Ledipasvir	99.48	100

CONCLUSION: The proposed UHPLC analytical method was validated according to ICH guidelines. The test results of method validation comply with the acceptance criteria. The validated method was successfully applied for the simultaneous determination of SOF and LPS in the combined dosage form. It is concluded that the method can be applied for routine control of SOF and LPS analysis in dosage form.

ACKNOWLEDGEMENT: The authors would like to express their gratitude to the principal and management of Aditya College of Pharmacy, surampalem for providing the necessary facilities required to carry out the work.

CONFLICTS OF INTEREST: The authors declare no conflicts of interest.

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How to cite this article:
Narla D and Pappula N: Stability indicating U-HPLC method for simultaneous estimation of sofosbuvir and ledipasvir in tablet dosage form. Int J Pharm Sci & Res 2020; 11(2): 784-90. doi: 10.13040/IJPSR.0975-8232.11(2).784-90.