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Development and Validation of RP-HPLC Method for the Simultaneous Determination of Ledipasvir and Sofosbuvir in Bulk and Formulation

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ABSTRACT

A simple and economic reverse phase liquid chromatographic method was developed for the simultaneous estimation of Sofosbuvir and Ledipasvir. The method was validated as per ICH guidelines for accuracy, precision, specificity, linearity, solution stability, robustness, sensitivity, and system suitability. The chromatographic separation was carried out using RP C18 column and the mobile phase composed of 1 % orthophosphoric acid and acetonitrile (30:70%v/v) at a flow rate of 0.8 ml/min. Linearity was established for both the drugs in the range of 1-10µg/ml. Both the drugs were subjected to stressed conditions like acidic, basic, oxidative, photolytic, and thermal conditions. The degradation results were found satisfactory. The proposed method is, therefore, suitable for the purpose in quality-control laboratories for quantitative analysis of both the drugs individually and combined dosage form, as it is simple and rapid with tremendous precision and accuracy.

INTRODUCTION

Sofosbuvir [1] (SOF) is a direct-acting antiviral medication used as a part of a combination medical aid to treat chronic viral hepatitis, associate degree infectious disease caused by infection with viral hepatitis virus (HCV). It is chemically propane-2-yl (2S)-2-{[(S)-{[(2R, 3R,4R,5R)-5-(2,4-dioxo-1,2,3,4-tetrahydro pyrimidine-1-yl)-4-fluoro-3-hydroxy-4-methyl oxolan-2yl]methoxy}(phenoxy)phosphoryl]amino}propanoate[2]. Sofosbuvir is a nucleotide analog inhibitor, which specifically inhibits HCV NS5B (non-structural protein 5B) RNA-dependent RNA polymerase. Following intracellular metabolism to form the pharmacologically active uridine analog triphosphate (GS-461203), sofosbuvir incorporates into HCV RNA by the NS5B polymerase and acts as a chain terminator. More specifically, Sofosbuvir prevents HCV viral replication by binding to the two Mg2+ ions present in HCV NS5B polymerase's GDD active site motif and preventing further replication of HCV genetic material.

Ledipasvir [3] (LPV) is a drug for the treatment of hepatitis C that was developed by Gilead sciences. It inhibits an important viral phosphoprotein, NS5A which is involved in viral replication assembly and secretion. It is chemically methyl [(2S)-1-{(6S)- 6-[5-(9,9-difluoro-7-{2-[(1R,3S,4S)-2-{(2S)-2 [(methoxycarbonyl) amino] methyl butanoyl}2azabicyclo [2.2.1]hept-3-yl]-1H-benzimidazol-6-yl}-9Hfluoren-2-yl)-1H-imidazol-2-yl]-5 aza spiro [2.4] hept-5-yl}-3-methyl-1-oxobutan-2-yl]carbamate.

Ledipasvir is an inhibitor of the Hepatitis C Virus (HCV) nonstructural protein 5A (NS5A) protein [4] required for viral RNA replication and assembly of HCV virions. Although its exact mechanism of action is unknown, It is postulated to prevent hyperphosphorylation 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.

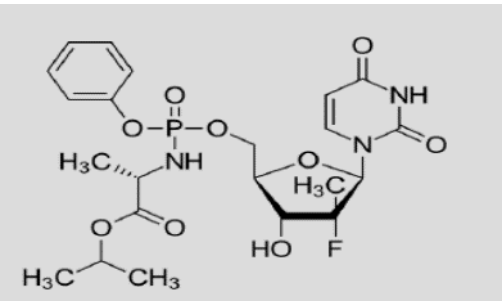

Fig. No. 1: Structure of Sofosbuvir

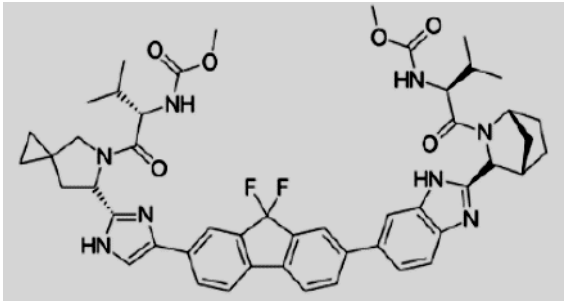

Fig. No. 2: Structure of Ledipasvir

Literature survey revealed that several instrumental methods based on HPLC [5-6], LC-MS/MS [7], LCQTOF-MS/MS, NMR [8] were reported for the determination of LPV and SOF as an individually and HPTLC [9], HPLC [10], Chemometric models [11], LC-MS/MS [12] in plasma and a UV spectroscopy [13] for the analysis of simultaneous estimation of LPV and SOF in the formulation were reported. The ideal stability-indicating method is the one that resolves the drug and its degradation products efficiently. Hence it is necessary to conduct stability studies of SOF and LPV under acidic, alkaline, oxidative, thermal, and photolytic conditions. The present work aims to develop a novel validated stability-indicating HPLC technique for the simultaneous determination of SOF AND LPV in bulk and formulation.

MATERIALS AND METHODS

SOF and LPV were received as a gift sample from SNR Sons Charitable Trust.

HPLC System and Chromatographic Conditions

A High-Performance Liquid Chromatographic system (Shimadzu), equipped with an autosampler and PDA detector (Model-SPD 20A) was used for the analysis. An analytical reversed-phase Hibar C-18 column was used to analyze the standards and samples. The mobile phase was run at a flow rate of 0.8 ml/min for 15 minutes. The injection volume was 20 µL for standard and samples. Before analysis, every standard and sample was filtered through 0.45 µm filter tips. The column eluent was monitored with UV detection at 254nm.

METHODS

Preparation of Standard Mixture:

A blend of 1% orthophosphoric acid: acetonitrile in a ratio of 30:70%v/v was used as a mobile phase. A stock solution was prepared by dissolving 10mg of SOF and LPV using mobile phase in a 100mL standard flask (100 µg/ml). It was further diluted to get SOF and LPV concentrations in the range of 1-10µg/ml respectively. All the solutions were filtered through a syringe filter (0.45µm) into vials.

Preparation of Sample Solution

To determine the amount of ledipasvir and sofosbuvir in tablet dosage form (label claim 90 & 400mg per tablet respectively) 20 tablets were weighed, their average weight was determined, and they were finely powdered. An accurately weighed powder sample equivalent to 9mg of

ledipasvir and 40mg of sofosbuvir was transferred into a 100ml volumetric flask then added 10ml acetonitrile, followed by sonication for 10 min and further dilution up to the mark with acetonitrile. The resulting solution was filtered through Whatman filter paper (No.1) and using a syringe 0.45µm. The above stock solution was further diluted to get a sample solution of 0.9µg/ml of ledipasvir and 4µg/ml of sofosbuvir respectively. A 20µl volume of sample solution was injected into HPLC, six times, under the conditions described above. The peak areas were measured at 254nm.

METHOD VALIDATION

The method validation was made according to guidelines about the validation and verification of analytical methods as described in the United States Pharmacopoeia, Food and Drug Administration (FDA), and International Conference on Harmonization (ICH). The developed HPLC method was validated according to ICH guidelines in terms of specificity, linearity, accuracy, precision, robustness, and LOD and LOQ.

The specificity of the method towards the drug was established through the study of the resolution factor of the drug peak from the nearest resolving peak. The peak purity of SOF and LPV were determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M), and peak-end (E). The effect of excipients of the formulation was studied for whether it interfered with the assay. The linearity of the method was studied by injecting ten concentrations of the drug prepared in the mobile phase in triplicate into the LC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentration to obtain the calibration graphs.

Accuracy of the method was carried out by applying the method to drug sample (SOF and LPV combination tablet) to which known amount of SOF and LPV standard powder corresponding to 50, 100, and 150% of label claim had been added (standard addition method) mixed and the powder was extracted and analyzed by running chromatogram in optimized mobile phase. The precision of the method was verified by intraday, interday (4, 8µg/ml for SOF and LPV) and repeatability study was carried out using 4 µg/ml and the precision was expressed as %RSD amongst responses using the formula [%RSD = (Standard deviation/Mean) x 100 %].

To evaluate the robustness of an HPLC method, a few parameters were deliberately varied. The parameters included a variation of flow rate, percentage of acetonitrile in the mobile

phase. The response factors like retention time, resolution, asymmetric factor for these changed conditions were noted. The system suitability parameters like peak area, tailing factor, theoretical plate count, resolution, and retention time were calculated from the standard chromatograms. The lowest concentration detected (LOD) and lowest concentration quantified (LOQ) were estimated from the set of five calibration curves used to determine method linearity. LOD = 3.3×σ/S and LOQ= 10×σ/S. Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

STRESS STUDIES

In this stage, stressed degradation studies were carried out to degrade the sample (e.g., drug product or Active Pharmaceutical Ingredient) deliberately. These studies were carried out to evaluate the analytical method's ability for the measurement of an active ingredient and its degradation products without interference. The degraded samples were then analyzed using the method to determine if there are interferences between the active and degradation compound(s).

Forced degradation studies for Ledipasvir and sofosbuvir:

For conducting the forced degradation studies, four samples were taken viz., the blank and analyte solution was stored under normal as well as stress conditions. The study was conducted separately for SOF and LPV individually and also for combination.

Acid Hydrolysis:

Weighed accurately 100 mg of SOF and LPV and transferred into 25ml standard flasks separately. To this 10 ml of 0.1M hydrochloric acid was added and made up to 25ml with acetonitrile. These solutions were refluxed for 5 hours at 80˚C. Further 0.25 ml was made up to 100ml (10µg/ml) for SOF and LPV individually and also for the mixture.

Alkaline Hydrolysis:

Weighed accurately 100 mg of SOF and LPV and transferred into 25ml standard flasks separately. To this 10 ml of 0.1M sodium hydroxide was added and made up to 25ml with acetonitrile. This solution was refluxed for 4 hours at 80˚C. Further 0.25 ml was made up to 100ml (10µg/ml) for SOF and LPV individually and also for the mixture.

Neutral hydrolysis:

Weighed accurately 100 mg of SOF and LPV and transferred into 25ml standard flasks separately. To this 10 ml of water was added separately and made up to 25 ml acetonitrile. This solution was refluxed for 4 hours at 80˚C. Further 0.25 ml was made up to 100ml (10µg/ml) for SOF and LPV individually and also for the mixture.

Oxidative Degradation:

Weighed accurately 100 mg of SOF and LPV and transferred into 25ml standard flasks separately. To this 10 ml of 6% hydrogen peroxide was added separately and made up to 25ml with acetonitrile. Further 1 ml was withdrawn 1 hour once for about 5 hours and Further 0.25 ml was made up to 100ml (10µg/ml) for SOF and LPV individually and also for the mixture.

Thermal degradation:

Weighed accurately 100 mg of SOF and LPV and transferred to a Petri dish separately**.** It was then placed in a hot air oven at 80ºC for 5 hours then dissolved and made up to 25 ml with acetonitrile. Further 0.25 ml was made up to 100ml (10µg/ml) for SOF and LPV individually and also for the mixture.

Photolytic degradation:

Weighed accurately 100 mg of SOF and LPV and transferred to a Petri dish separately**.** It was then exposed to sunlight for about 5 hours. The drug solution was prepared using acetonitrile. Further 0.25 ml was made up to 100ml (10µg/ml) for SOF and LPV individually and also for the mixture.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

The Ledipasvir and Sofosbuvir are polar hence RP-HPLC method and C_{18} column were used for the HPLC method development and stability-indicating for the Ledipasvir and Sofosbuvir.

Selection of wavelength:

Good analytical separation can be obtained only by careful selection of wavelength for detection. The smooth obtained from which 254nm was selected for both drugs was recorded on the HPLC system.

Fixed chromatographic conditions

Chromatographic method	:	RP-HPLC
Column (stationary phase)	:	Hibar, C_{18} column(250mmx 4.0mm, 5µm)
Mobile phase	:	1% orthophosphoric acid (pH6.4): Acetonitrile
Ratio of mobile phase	:	30:70 v/v
Detection of wavelength	:	254nm
Flow rate	:	0.8ml/min
Retention time	:	Sofosbuvir 3.7 ± 0.02 Ledipasvir 7.1 ± 0.02
Temperature	:	Room temperature

METHOD VALIDATION

a) Linearity and range:

A calibration graph was plotted with measured peak areas against concentration. From the graph, it was found that ledipasvir and sofosbuvir show good linearity in the concentration range 1-10µg/ml and 1-10µg/ml. The peak area of these solutions was measured at 254nm. The slope, intercept, and correlation coefficient values were calculated respectively (table 1). The linear graph and standard chromatogram obtained are shown in fig.3. The linearity table is shown in Tables 2 and 3.

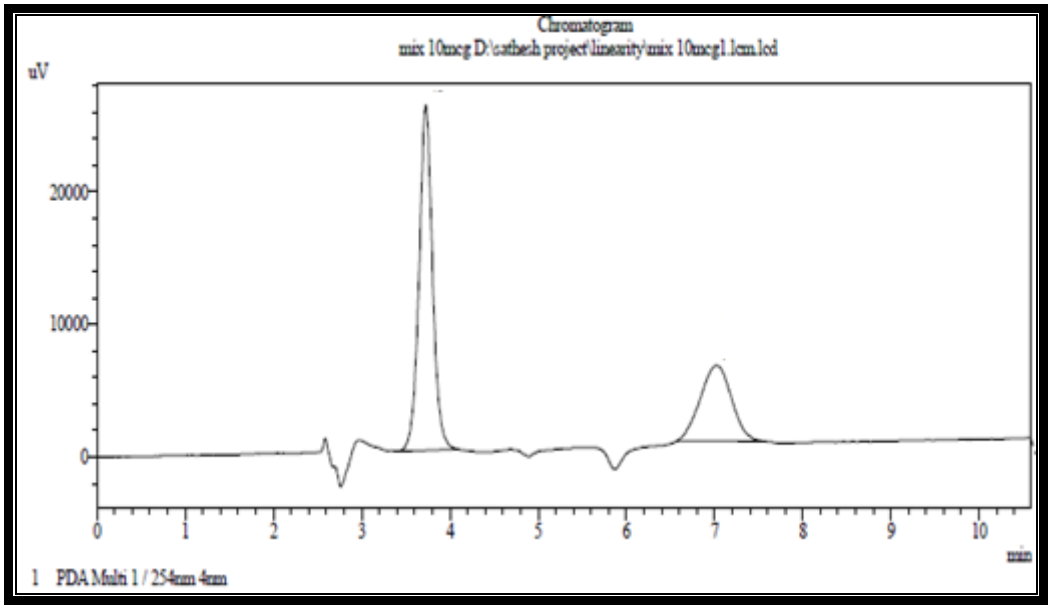

Fig. No. 3: Chromatogram of Ledipasvir (10µg/ml) and Sofosbuvir (10µg/ml)

Table. No. 1: Regression data

Linear regression	Ledipasvir	Sofosbuvir
Slope	5976.13	0.13807.7
Intercept	14699.1	24637.8
Correlation coefficient	0.9987	0.9995

b) Recovery study:

Recovery studies were carried out at 50,100, and 150% levels. The percentage recovery and its %RSD were calculated, shown in table 2a & 2b.

Table. No. 2a: Recovery study for Ledipasvir

Level	% Recovery	%RSD*
50%	98.34%	0.05
100%	98.01%	0.84
150%	99.27%	0.94

*RSD of six determination

Table. No. 2b: Recovery study for Sofosbuvir

Level	% Recovery	%RSD*
50%	99.73%	0.54
100%	100.36%	0.25
150%	101.48%	0.14

*RSD of six determination

c) Precision:

Intra-day and inter-day precision:

Intra-day and inter-day precision was determined by injecting standard solutions in between linearity range (4 and 8µg/ml for ledipasvir and 4and 8µg/ml for sofosbuvir) were injected three times and % RSD was calculated (table3, and 4).

Table. No. 3: Intra-day precision

Concentration (ng/spot)	Peak area		%RSD*	
	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir
Ledipasvir(4µg/ml) & Sofosbuvir (4 µg/ml)	65221	84645	0.0123	0.0270
	65531	84660		
	65537	84690		
Ledipasvir (8 µg/ml) & Sofosbuvir (8 µg/ml)	125378	181499	0.0300	0.0185
	125319	181491		
	125308	181437		

*RSD of three determinations

Table No. 4: inter-day precision

Concentration (ng/spot)	Peak area		%RSD*	
	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir
Ledipasvir (4µg/ml) & Sofosbuvir (4 µg/ml)	65310	84650	0.0177	0.0343
	65319	84666		
	65333	84609		
Ledipasvir (8 µg/ml) & Sofosbuvir (8 µg/ml)	124219	181409	0.0065	0.0128
	124225	181410		
	124009	181450		

*RSD of three determinations

d) Repeatability:

Repeatability of injection was determined by injecting standard solutions (4µg/ml) of ledipasvir and (4µg/ml) of sofosbuvir for six times, noted peak areas and % RSD was calculated table 5a & 5b.

Table. No. 5a: Repeatability injection of Ledipasvir

Concentration (µg/ml)	Repeatability	% RSD*
4	65251	0.0215
	65242	
	65255	
	65235	
	65260	
	65275	

*RSD of six determinations

Table. No. 5b: Repeatability injection of Sofosbuvir

Concentration (µg/ml)	Repeatability	% RSD*
4	84734	0.0102
	84712	
	84722	
	84730	
	84725	
	84735	

*RSD of six determinations

e) Limit of detection and limit of quantification (LOD and LOQ):

Limit of detection and limit of quantification that were calculated from the equation. The result was shown in table 6a & 6b, which proved the sensitivity of the method.

Table. No. 6a: Results of LOD & LOQ of Ledipasvir

Correlation coefficient ± SD*	0.9987 ± 0.0001342
Slope ± SD*	3.123 ± 2.608
Intercept ± SD*	1112.65 ± 2.436
LOD (ng / spot)	0.008µg / ml
LOQ (ng / spot)	0.27µg / ml

*RSD of six determinations.

Table. No. 6b: Results of LOD & LOQ of Sofosbuvir

Correlation coefficient ± SD*	0.9995 ±0.4080
Slope ± SD*	3.123 ±2.611
Intercept ± SD*	1112.65 ±2.822
LOD (ng / spot)	0.0006µg /ml
LOQ (ng / spot)	0.0020 µg / ml

*RSD of six determinations.

f) Specificity:

There were no additional peaks observed while injecting solvents or mobile phase alone. The peak purity index of standard ledipasvir is 0.9998 and sofosbuvir 0.9995.

g) Robustness:

To demonstrate the robustness of the method, the following optimized conditions were slightly changed.

± 0.1ml flow rate

± 2% organic solvent

± 0.5 P^H

The responses for these changed chromatographic parameters were almost the same for the fixed chromatographic parameters and hence the developed method was said to be robust.

h) Stability of solution:

The solution at room temperature was stable for 24 hours. (Table 7)

Table. No. 7: Stability of solution

Hours	Ledipasvir		Sofosbuvir	
	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
0 hrs	5	82453	5	110043
6 hrs		82412		110012
24 hrs		82219		109702

i) System suitability studies:

The system suitability parameters like peak area, tailing factor, theoretical plate count, resolution, and retention time were calculated from the standard chromatogram (table 8).

Table. No. 8: System suitability studies

Drug	Theoretical plate(N)	Retention time(min)	Tailing factor (10%)	Resolution
Ledipasvir	2703	3.7	1.062	-
Sofosbuvir	1762	7.1	0.994	6.90

ANALYSIS OF TABLET FORMULATION

The % label claim was calculated and the estimated amount was close to the labeled value that was shown in figure 4 and table 9.

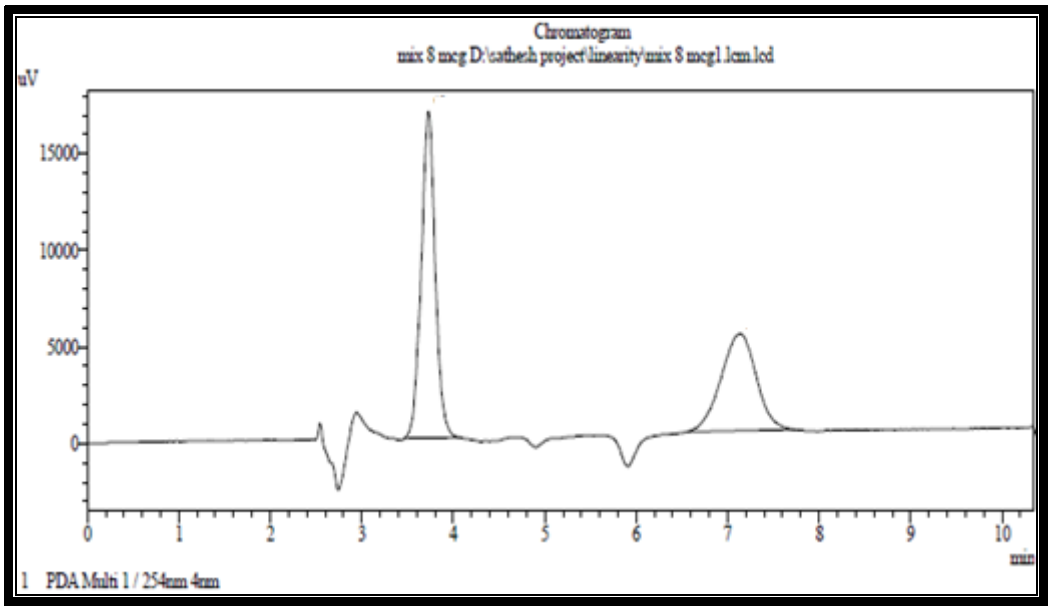

Figure. No. 4: Chromatogram of Ledipasvir and Sofosbuvir

Table. No. 9: Analysis of formulation

Drug Name (cimivir-L)	Amount (mg/tab)		% Label claim	%RSD*
	Labeled	Estimated		
Ledipasvir	90	89.34	99.30	0.41
Sofosbuvir	400	398.17	99.54	0.67

*RSD of six observations

RESULTS OF FORCED DEGRADATION STUDIES:

Acid and Alkaline hydrolysis:

The drug was subjected to acid and alkaline hydrolysis two additional peaks were observed at R_t 3.1 and 4.2 value which was shown in figure 5a and 5b.

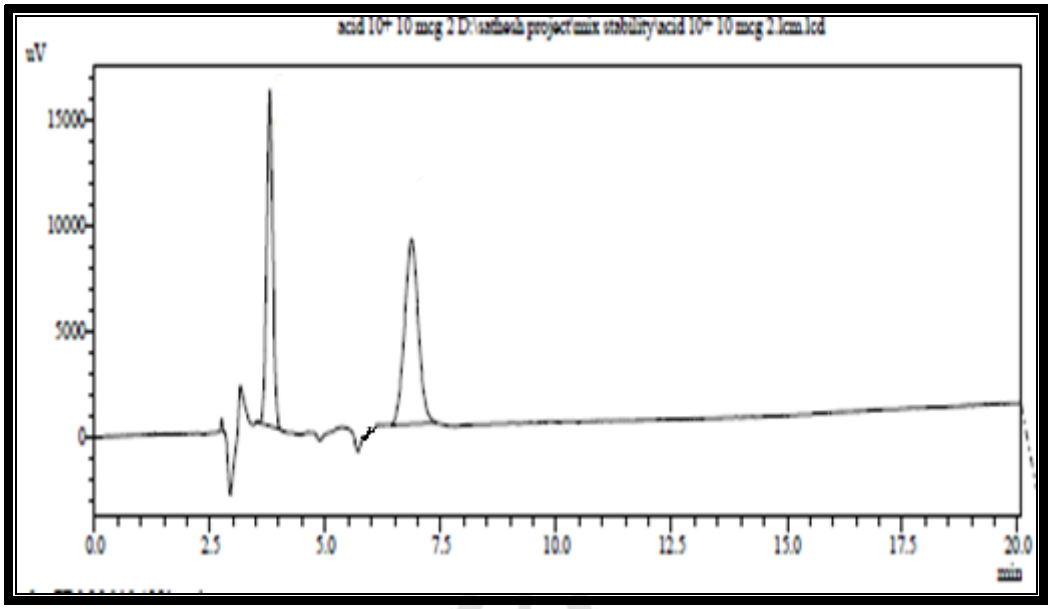

Figure. No. 5a: Chromatogram of acid hydrolysis of Ledipasvir and Sofosbuvir

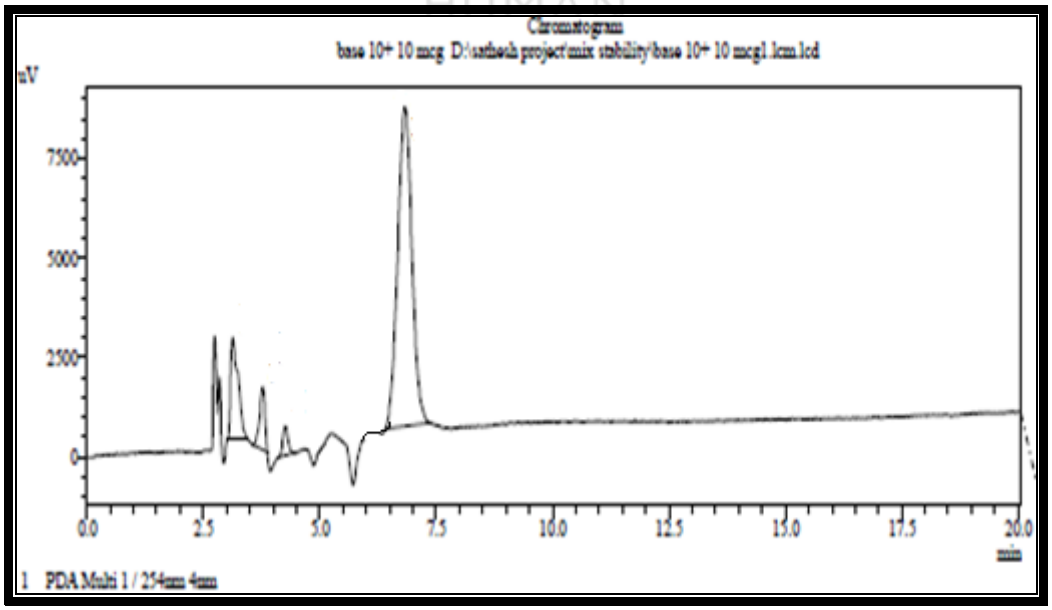

Figure. No. 5b: Chromatogram of alkaline hydrolysis of Ledipasvir and Sofosbuvir

Table. No. 10: Summary of forced degradation studies for Ledipasvir and Sofosbuvir

Type of stress	Stress condition	Retention time(min)			
		Ledipasvir	Sofosbuvir	Ledipasvir Degradant	Sofosbuvir Degradant
Acid hydrolysis	Drugs refluxed with 0.1M HCl for about 5 hours	7.1	3.7	No additional peak	No additional peak
Basic hydrolysis	Drugs refluxed with 0.1M NaOH for about 5 hours	7.1	3.7	No additional peak	2.8±0.02 4.1±0.02
Neutral hydrolysis	Drugs treated with water and refluxed for about 5 hours	7.1	3.7	No additional peak	No additional peak
Oxidative degradation	Drugs treated with 6% hydrogen peroxide at normal room temperature	7.1	3.7	No additional peak	No additional peak
Photodegradation	Drugs exposed to Sunlight for 5 hours	7.1	3.7	No additional peak	No additional peak
Thermal degradation	Drugs introduced in Hot air oven for 5 hours	7.1	3.7	No additional peak	No additional peak

CONCLUSION

A simple, fast, accurate, and precise stability-indicating HPLC analytical method has been developed and validated for the quantitative analysis of Sofosbuvir and Ledipasvir in bulk drugs and combined dosage forms. Stress testing is a significant part of the drug development process and the pharmaceutical industry has a lot of interest in this area. The results of stress testing undertaken according to the ICH guidelines reveal that the method is specific and stability-indicating. The proposed method can separate these drugs from their degradation products in tablet dosage forms and hence can be applied to the analysis of routine quality control samples and samples obtained from stability studies. As the precision, accuracy, and robustness are a concern the % RSD is less than 2 which is within the range of ICH guidelines. So, this HPLC method should be useful for monitoring plasma drug concentrations, and pharmacokinetic studies in patients diagnosed with the Ledipasvir and Sofosbuvir formulations. This method was undergone a careful and extensive validation process maintaining the guidelines outlined in USP, ICH, and FDA, and met all the requirements of a reliable analytical method.

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REFERENCES

1. National Center for Biotechnology Information. PubChem Compound Database;CID=45375808,https://pubch em.ncbi.nlm.nih.gov/compound/45375 808 (accessed Apr. 10, 2018). Sofosbuvir structure.
2. P. Mohan Vikas, T. Satyanarayana, D. Vinod kumar, E. Mounika, M. Sri latha, R. Anusha, Y. Sathish, Method development and validation of new RP- HPLC method for the determination of sofosbuvir in pure form, *World Journal of Pharmacy and Pharmaceutical Sciences*, 2016, 5 (5),775-781.
3. National Center for Biotechnology Information. PubChem Compound Database;CID=67505836,https://pubchem.ncbi.nlm.nih.gov/compound/67505836 (accessed Apr. 10, 2018). Ledipasvir structure.
4. Pol, S., Vallet-Pichard, A., and Corouge, M, Daclatasvir sofosbuvir combination therapy with or without ribavirin for hepatitis C virus infection: from the clinical trials to real life. Hepatic Medicine: Evidence and Research, 2016, 8, 21–26.
5. Mohan Vikas, P., Satyanarayana, T., Vinod Kumar, D., Mounika, E., Sri Latha, M., Anusha, R., and Sathish, Y, *Journal of Global Trends Pharmaceutical Sciences*, 2016, 7: 3013-3015.
6. A. P., j. Alhat, and a. A. Kulkarni. "Development and validation of RP-HPLC method for the simultaneous estimation of Ledipasvir and Sofosbuvir in bulk and pharmaceutical dosage form". *International journal of pharmaceutical sciences and drug research*, 2017, 9, (6), 291-8.
7. Nebsen, Eman S. Elzanfaly, Stability-Indicating Method and LC-MS-MS Characterization of Forced Degradation Products of Sofosbuvir, *Journal of Chromatographic Science*, 2016, 54, (9), 1631–1640.

8. Chakravarthy V, A., S. Bbv, and P. K. A. "Method development and validation of an ultraviolet-visible spectroscopic method for the estimation of hepatitis-c drugs - Daclatasvir and Sofosbuvir in active pharmaceutical ingredient form". *Asian Journal of Pharmaceutical and Clinical Research*, 2016, 9, (9), 61-66.
9. A Suganthi*, S Satheshkumar, T K Ravi, Development of Validated Specific Stability-Indicating HPTLC Method for the Simultaneous Determination of Ledipasvir and Sofosbuvir in Fixed-Dose Tablet Formulation. *Asian Journal of Nanoscience and Materials*, 2019, 2(2), 228-243. DOI: 10.26655/ajnanomat.2019.3.9**.**
10. Zaman, B., Siddique, F. & Hassan, W, RP-HPLC Method for Simultaneous Determination of Sofosbuvir and Ledipasvir in Tablet Dosage Form and Its Application to In Vitro Dissolution Studies, *Chromatographia*,2016, 79, 1605–1613.
11. Fathy, M. S., Khalid, A. A., Ahmed, A. A., Ahmed El-Olemy., and Ebrahim, A, Multivariate Chemometric Models and Application of Genetic Algorithm for Simultaneous Determination of Ledipasvir and Sofosbuvir in Pure Form and in Pharmaceutical Preparation; A Comparative Study**,** *Journal of Advanced Pharmacy Research*, 2017, 1,(4), 185-192.
12. Ehab F Elkady, Ahmed A Aboelwafa, A Rapid and Optimized LC-MS/MS Method for the Simultaneous Extraction and Determination of Sofosbuvir and Ledipasvir in Human Plasma, *Journal of AOAC INTERNATIONAL*, 2016, 99, (5), 1252–1259.
13. Sherif Abdel-Naby Abdel-Gawad. "Simple chromatographic and spectrophotometric determination of Sofosbuvir in pure and tablet forms". *European Journal of Chemistry*, (2016),7(3) 375-379.