International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 11, Issue 2, 2019

Original Article

AN INNOVATIVE METHOD DEVELOPMENT AND FORCED DEGRADATION STUDIES FOR SIMULTANEOUS ESTIMATION OF SOFOSBUVIR AND LEDIPASVIR BY RP HPLC

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Received: 27 Aug 2018 Revised and Accepted: 20 Dec 2018

ABSTRACT

Objective: To develop an innovative, rapid, simple, cost-effective, stability indicating reverse phase-high performance liquid chromatography (RP-HPLC) method for simultaneous estimation of ledipasvir (LP) and sofosbuvir (SB) in combination pill dosage form.

Methods: The method was developed using C8 column, 250 mm x 4.6 mm, 5 mm using mobile section comprising of 0.1% (v/v) orthophosphoric acid buffer at pH 2.2 and acetonitrile in the ratio of 45:55 that was pumped through the column at a flow rate of 0.8 ml/min. Temperature was maintained at 30 °C, the effluents were monitored at 260 nm with the help of usage of PDA detector.

Results: The retention time of LP and SB were found to be 2.246 min and 3.502 min. The approach was found to be linear with the variety of 9-36 µg/ml and 40-240 µg/ml for LP and SB respectively, the assay of estimated compounds were found to be 99.65% and 99.73% w/v for LP and SB respectively.

Conclusion: The pressured samples changed into analyzed and this proposed a technique turned into determined to be particular and stability indicating as no interfering peaks of decay compound and excipients were observed. Hence, the approach was easy and economical that may be efficiently applied for simultaneous estimation of both LP and SB in bulk and combination tablet system.

Keywords: Ledipasvir, Sofosbuvir, Assay, ICH guidelines, Force degradation

DOI: http://dx.doi.org/10.22159/ijpps.2019v11i2.29347

INTRODUCTION

Various drugs are used for the treatment of ailments, but sometimes multiple drug therapy is being followed for cure of chronic disease, as well as it has better patient acceptability due to reduced number of dosage forms to be taken at a time [1-3]. Several optimization techniques are also employed using various latest experimental designs for the latest innovations in developmental methods [4]. In the present work we are trying to develop a method for combination of ledipasvir (LP) and sofosbuvir (SB) by RP-HPLC technique [5].

Chemically, LP is methyl [(2S)-1-{(6S)-6-[4-(9,9-difluoro-7-{2-[(1R, 3S,4S)-2-{(2S)-2[(methoxycarbonyl)amino]-3-methylbutanoyl} -2-aza-bicyclo[2.2.1]hept-3-yl]-1H-benzimidazol-5-yl}-9H-fluoren-2-yl)-1H-imidazol-2-yl]-5-azaspiro[2.4]hept-5-yl}-3-methyl-1-oxobutan-2-yl]carbamate. LP is a direct-acting antiviral medication used as part of combination therapy to treat chronic hepatitis c, an infectious liver disease caused by infection with (HCV). LP inhibits an important viral phosphoprotein, NS5A, which is involved in viral replication, assembly, and secretion.

SB chemically comprises of propan-2-yl (2S)-2-{[(S)-{[(2R,3R,4R,5R)-5-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl]methoxy} (phenoxy) phosphoryl] amino} propanoate. Treatment options for chronic Hepatitis C have advanced significantly since 2011, with the development of direct-acting antivirals such as sofosbuvir. SB is a nucleotide analog inhibitor, which specifically inhibits HCV NS5B (non-structural protein 5B) rna-dependent rna polymerase. The broad writing study uncovered that RP-HPLC [6-14] and UV spectrophotometric techniques [15-17] and UPLC strategies were accessible for the assurance of LP and SB separately or in mix with different medications. The investigation performed with a plan to build up a straightforward, financial, touchy, quick, exact, and steadiness demonstrating RP-HPLC technique for the assurance of ledipasvir and sofosbuvir in joined tablet measurements shape.

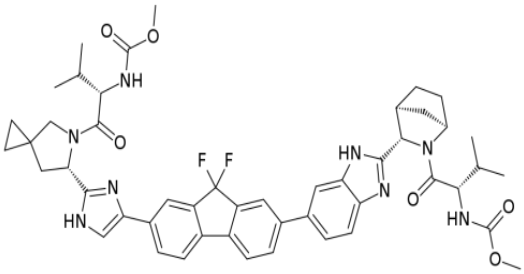

Fig. 1: Structure of ledipasvir

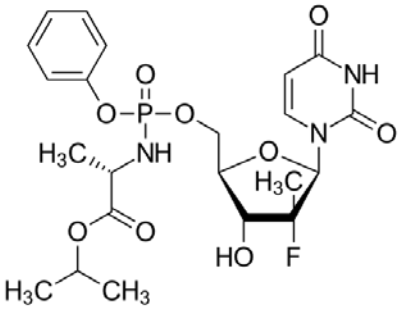

Fig. 2: Structure of sofosbuvir

MATERIALS AND METHODS

Instruments: Waters (2695) PDA detector HPLC using the software Empower 2.

Chemicals and reagent

Material	Source
Reference sample	Spectrum pharma labs, Hyderabad, Telangana
Test sample (LediHep Formulation)	Local pharmacy
HPLC grade: acetonitrile, methanol, and water	Merck chemical division, Mumbai
Potassium dihydrogen ortho phosphate, ortho-phosphoric acid	Rankem

Preparation of mobile phase

(0.1% v/v, opa buffer) 1 ml of orthophosphoric acid solution in a 1000 ml of volumetric flask add about 100 ml of milli-Q water and final volume make up to mark with milli-Q water at pH 2.2 and acetonitrile (45:55 v/v).

Preparation of standard solutions

Accurately weighed and transferred 9 mg of LP and 40 mg of SB working standards into a 25 ml and 25 ml clean dry volumetric flasks, add 10 ml of diluent, sonicated for 10 min and makeup to the final volume with diluents.

Preparation of sample solution

5 tablets (LediHep) were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 50 ml volumetric flask, 20 ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (360µg/ml of LP and 1600µg/ml of SB).

Chromatographic conditions

The chromatographic column used was discovery C8 250 mm x 4.6 mm, 5 µm. The flow rate was maintained at 0.8 ml/min. The detection wavelength was 260 nm. The temperature was at 30 °C. The injection volume was 10 µl. The run time of standard and sample was 7 min.

Optimization of RP-HPLC method

The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of LP and SB. For optimization of method, different mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with 0.1% opa buffer at pH 2.2 and acetonitrile (45:55 v/v) using column discovery C8 (250 mm x 4.6 mm, 5 µm),The results were shown in fig. 3.

Validation of the RP-HPLC method

Validation of the optimized method was performed according to the ICH guidelines [18-19].

System suitability

The system suitability parameters were determined by preparing standard solutions of LP (36µg/ml) and SB (160 µg/ml). The solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%. The results were shown in table 1.

Specificity

Specificity of a method was determined by testing standard substances against potential interferences. There should not be an occurrence of any interfering peaks in the blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Linearity

By appropriate aliquots of the standard LP and SB prepared six working solutions ranging between 9µg/ml to 54µg/ml and 4µg/ml to 240µg/ml. Each experiment linearity point was performed in triplicate according to optimized chromatographic conditions. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficient on curves for LP and SB. The results were shown in table 2.

Accuracy

Accuracy was carried out by % recovery studies of LP and SB at three different concentration levels (50%, 100%, and 150%). Percentage recovery was calculated from the amount added and the amount recovered. The percentage recovery was within the acceptance criteria, this indicates the accuracy of the method. (Acceptance criteria: % recovery between 98 to102). The results were shown in table 3.

Precision

The repeatability of the method was verified by calculating the % RSD of six replicate injections of 100% concentration (36 µg/ml of LP and 160 µg/ml of SB) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. The results were shown in table 4.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were calculated by the use of equations LOD= $3.3\sigma/S$ and LOQ=$10\sigma/S$ where σ is the standard deviation of intercepts of calibration plots and S is the average of the slope of corresponding calibration plots. The results were shown in table 5.

Robustness

Robustness of the method was verified by altering the chromatographic conditions like flow rate, mobile phase ratio and temperature are made, but there was no recognized change in the result and all are within range as per ICH guidelines. Robustness conditions like flow minus (0.7 ml/min), flow plus (0.9 ml/min), Mobile phase ratio (Organic: Aqueous) minus 50:50, Mobile phase ratio (Organic: Aqueous) plus 60:40, temperature minus (25 °C) and temperature plus (35 °C) was maintained and samples were injected in a duplicate manner. System suitability parameter was passed. % RSD was within the limit. The result was shown in table 6.

Degradation studies

Acid degradation: To 1 ml of stock solution LP and SB, 1 ml of 2N hydrochloric acid was added and refluxed for 30 min at 60 °C. The resultant solution was diluted to obtain 36 µg/ml and 160 µg/ml solutions and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of the sample. The results were shown in fig. 8.

Oxidative degradation

To 1 ml of stock solution of LP and SB, 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60 °C. For HPLC study, the resultant solution was diluted to obtain 36 µg/ml and 160 µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample. The results were shown in fig. 9.

Alkali degradation

To 1 ml of stock solution Ledipasvir and sofosbuvir, 1 ml of 2N sodium hydroxide was added and refluxed for 30 min at 60 °C. The resultant solution was diluted to obtain 36µg/ml and 160µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample. The results were shown in fig. 10.

Thermal degradation

The standard drug solution was placed in an oven at 105 °C for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 36µg/ml and 160µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample. The results are shown in fig. 11.

Photodegradation

The photochemical stability of the drug was also studied by exposing the 360µg/ml and 1600µg/ml solution to uv-light by

keeping the beaker in uv-chamber for 7 h or 200 Watt h/m² in photostability chamber. For HPLC study, the resultant solution was diluted to obtain 36µg/ml and 160µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample. The results are shown in fig. 12.

RESULTS

After a number of trials with mobile phases of different composition, and a mobile phase containing 0.1% orthophosphoric acid buffer at pH 2.2 and acetonitrile taken in the ratio of 45:55 v/v was selected as a mobile phase because of better resolution more number of theoretical plates and symmetric peaks.

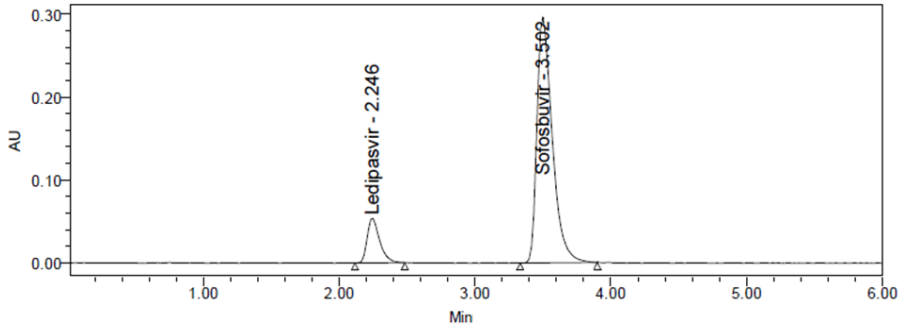

Fig. 3: Optimized chromatogram of ledipasvir and sofosbuvir

System suitability

According to ICH guidelines, plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Linearity

A concentration range of 9-54 µg/ml for ledipasvir and 40-240 µg/ml of sofosbuvir was found to be linear with correlation coefficients 0.999 were within limits. The result was shown in fig. 4 and 5.

Table 1: System suitability parameters

Parameter	Ledipasvir	Sofosbuvir
Retention time (min)	2.246	3.502
Theoretical plates (N)	3039	4771
Tailing factor (T)	1.43	1.56

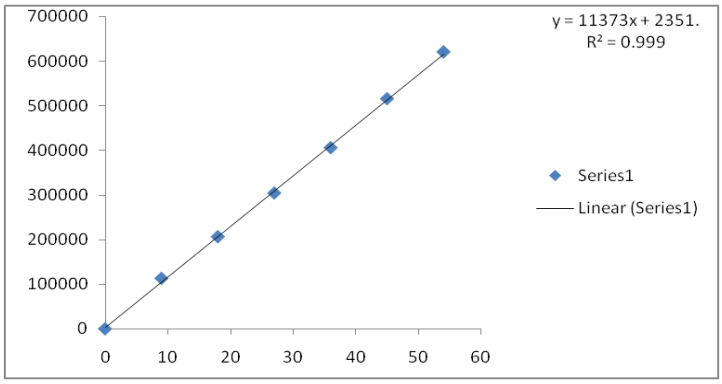

Fig. 4: Calibration curve of sofosbuvir

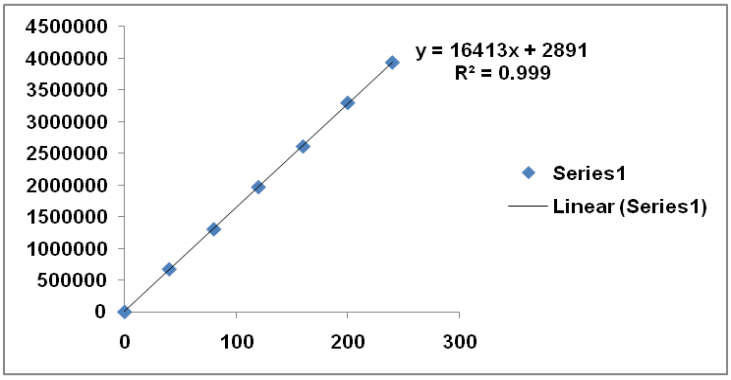

Fig. 5: Calibration curve of ledipasvir

Table 2: Results for linearity

Parameter	Ledipasvir	Sofosbuvir
Y intercept	11373	16413
Correlation coefficient r2	0.999	0.999
Regression Equation	11373x+2351	16413x+2891
Linearity range	9-54 µg/ml	40-240 µg/ml
LOD	0.37	0.25
LOQ	1.13	0.76

n=6 i.e. number of samples for estimation

Accuracy

The percentage accuracy was a relative standard deviation (SD) for accuracy at each level is well within the limit. Overall the percentage recovery of the relative standard deviation was found to be 99.54%-99.50 % for all the levels were within the limit.

Precision

Percentage relative standard deviation of six results was within the limit. Results shown good degree of precision was found to be 0.3% and 0.2%.

Limit of detection

Limit of detection of target assay concentration of ledipasvir and sofosbuvir by using formula method 0.37µg/ml and 0.25µg/ml within the limits. The results were shown in fig. 6.

Limit of quantification

Limit of quantification of the target assay concentration of Ledipasvir and sofosbuvir by using formula method 1.13µg/ml and 0.76 µg/ml were within the limits. The results were shown in fig. 7.

Table 3: Results for accuracy

Ledipasvir				Sofosbuvir		
Recovery level	**Amount added (µg/ml)**	**Amount found**	**% recovery (µg/ml)**	**Amount added (µg/ml)**	**Amount found (µg/ml)**	**% recovery**
50%	18	17.94	99.64	80	79.62	99.52
100%	36	35.83	99.52	160	159.28	99.55
150%	54	53.71	99.47	240	238.59	99.41
Mean recovery			99.54%	Mean recovery		99.49%
±SD			0.09	±SD		0.07
% RSD			0.09	% RSD		0.07

#±SD: Standard deviation, # % RSD: Percentage relative standard deviation

Table 4: Results of precision

Drug	Inter-day precision (%RSD)	Method precision (% RSD)
Ledipasvir	0.7	0.3
Sofosbuvir	0.3	0.2

n=6 i.e. number of injections, # % RSD: Percentage relative standard deviation

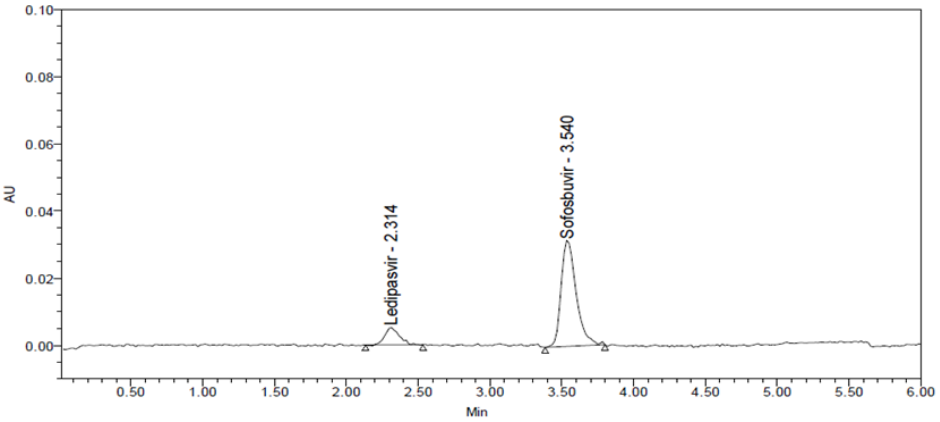

Fig. 6: LOD of ledipasvir and sofosbuvir

Table 5: Results for LOD and LOQ

S. No.	Drug	LOD (µg/ml)	LOQ (µg/ml)
1	Ledipasvir	0.37	1.13
2	Sofosbuvir	0.25	0.76

LOD: Limit of detection, # LOQ: Limit of quantitation

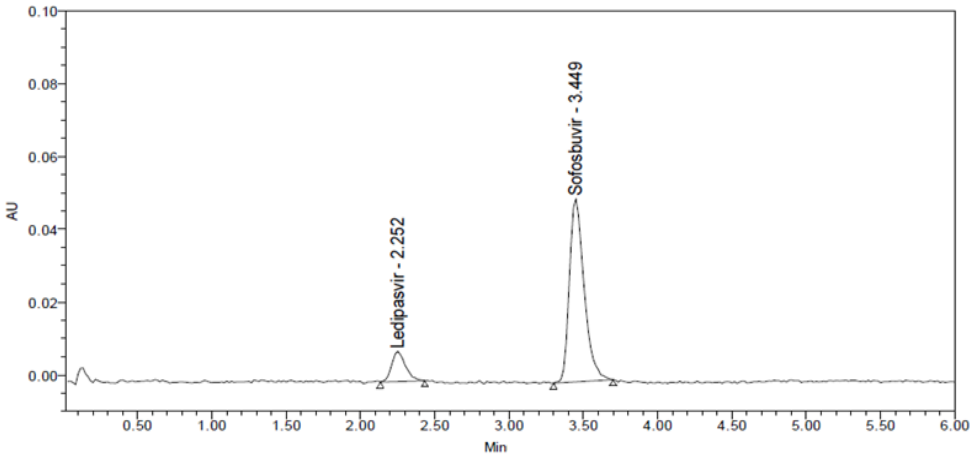

Fig. 7: LOQ of ledipasvir and sofosbuvir

Robustness

Table 6: Result of robustness

S. No.	Condition	Plus	Minus
1	Flow rate	0.9 ml/min	0.7 ml/min
2	Mobile phase ratio (Organic: Aqueous)	60:40	50:50
3	Column temperature	35 °C	25 °C

In the above conditions the parameters like % RSD of peak area, tailing factor and theoretical plates showed were within the limit.

Forced degradation study

Degradation studies demonstrated the specificity of the developed method in the presence of degradation products. Degradation was carried out in combination of two drugs and purity of drug peaks was confirmed by purity angles. Their combination drug products were exposed to acid, alkali, oxidative and thermal stress conditions. Then found to be no degradable substances presence and proved that the proposed method was stable towards acid, alkali, peroxide and thermal conditions within the limits.

Table 7: Results for stability studies of ledipasvir and sofosbuvir

Degradation parameters	Stress condition	% of degradation	
		ledipasvir	sofosbuvir
Acid degradation	30 min refluxed with 2 N HCl at 60 °C	5.05	5.67
Alkaline degradation	30 min refluxed with 2 N NaOH at 60 °C	4.43	3.93
Peroxide degradation	30 min refluxed with 20% H_2O_2 at 60 °C	3.66	3.35
Thermal degradation	6 h kept in dry oven at 105 ° C	2.99	2.67
Photo degradation	UV light-254 nm for 7 h	1.83	1.70

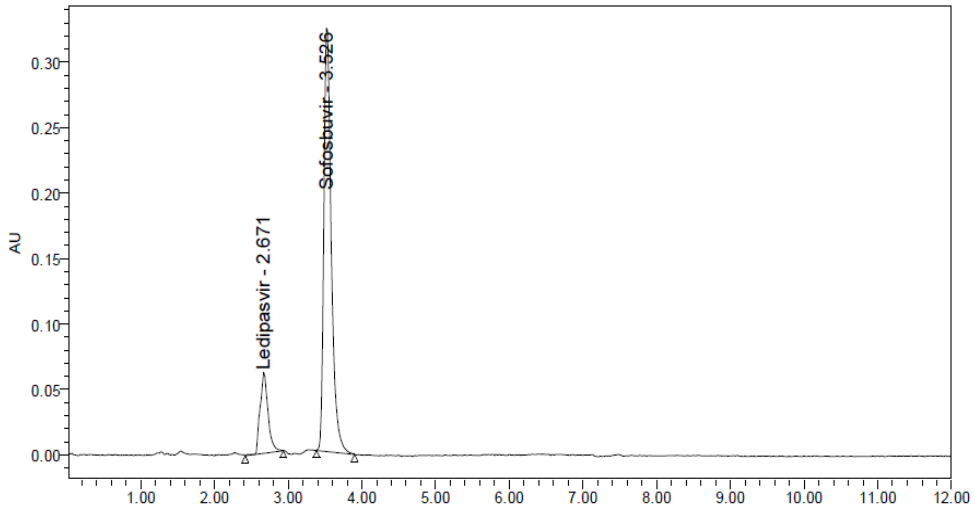

Fig. 8: Acid degradation

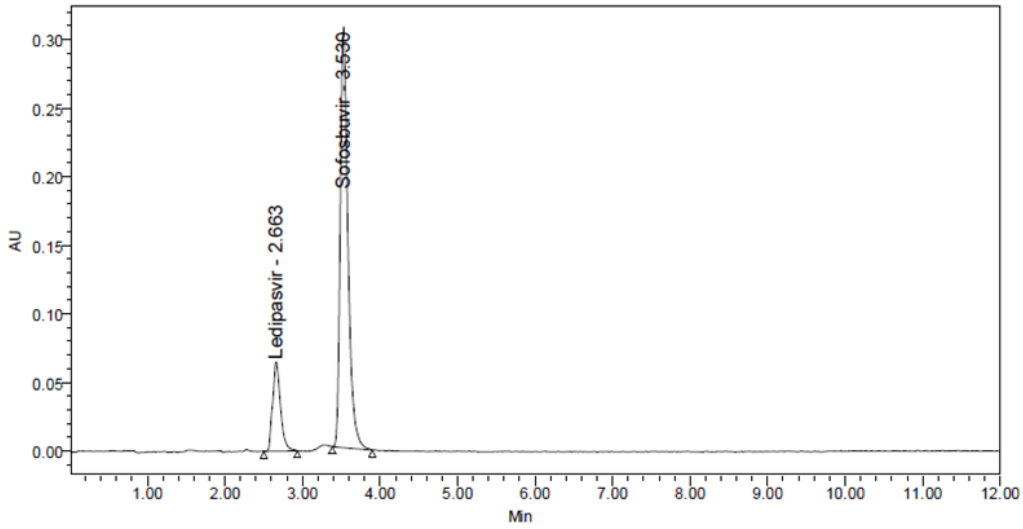

Fig. 9: Alkaline degradation

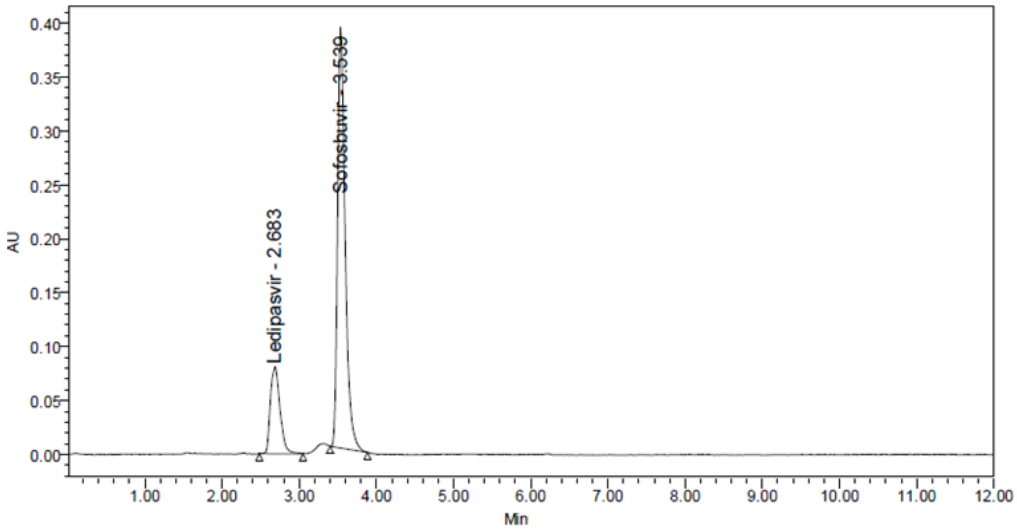

Fig. 10: Peroxide degradation

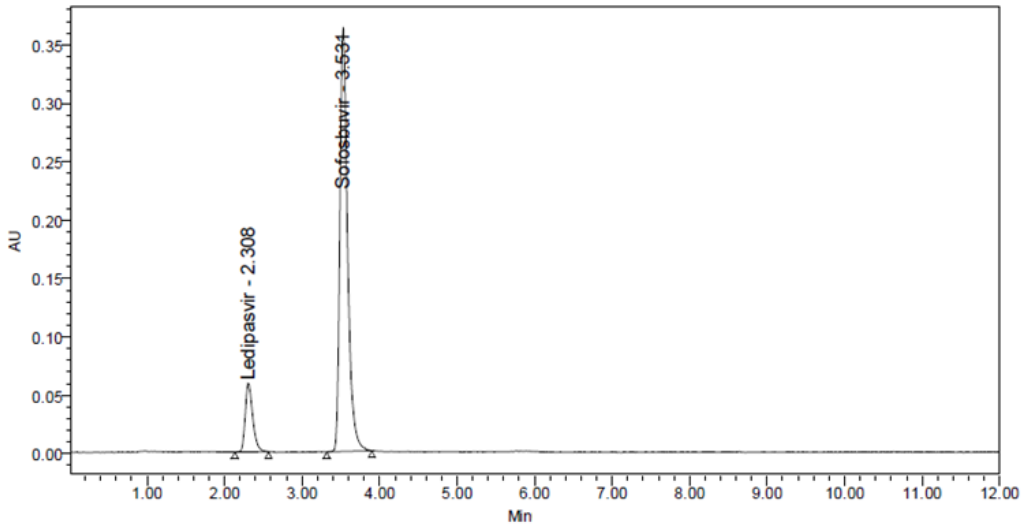

Fig. 11: Thermal degradation

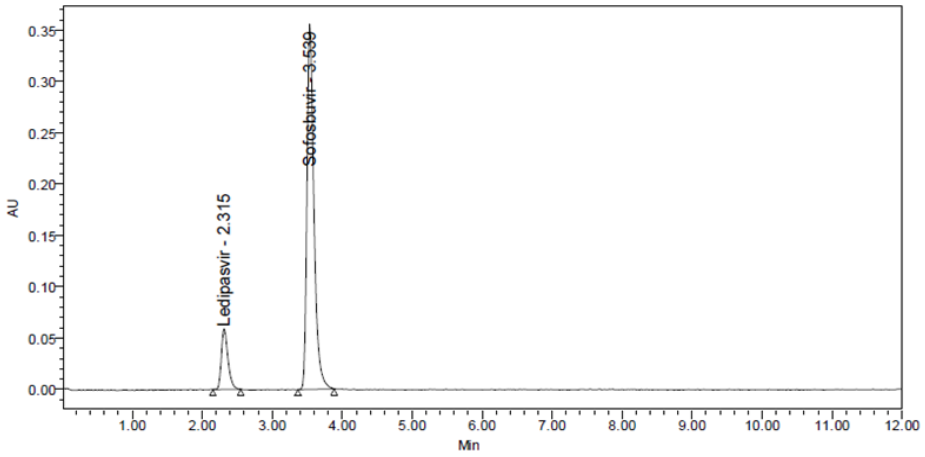

Fig. 12: Photodegradation

DISCUSSION

For novel method development for simultaneous estimation of sofosbuvir and ledipasvir initially, various mobile phases and columns were tried to elute drug peaks with minimum tailing factor and more plate count were tried. For the developed method, Waters (2695) PDA detector HPLC using the software Empower 2 was selected, appreciable absorbance at 260 nm was determined spectro-photo metrically and hence it was selected as the detection wavelength. Then by using column discovery C8 250 mm x 4.6 mm, 5 µm with mobile phase 0.1% opa buffer at pH 2.2 and acetonitrile in the ratio of 45:55 (v/v) by maintaining flow rate at 0.8 ml/min. Initially, prepared standard solution, sample solution and blank solution were injected and system suitability parameters were determined and listed in table 1 for both the drugs and the respective chromatograms were shown in fig. 3. The linearity graph for sofosbuvir and ledipasvir was plotted and shown in fig. 4 and fig. 5 respectively, with the correlation coefficient of 0.999. The developed method was found to be accurate; the percentage recovery for sofosbuvir and ledipasvir was 99.49% and 99.54% respectively, data shown in table 3. The percentage RSD was found to be 0.3% and 0.7%, respectively, hence indicates that the method was precise. As there was no interference of retention time of placebo peak with drug peak, the method was found to be precise. Forced degradation studies for both the drugs revealed that both the drugs were stable during various applied stress conditions and the degradation for both drugs was found to be within limits as shown in table 7. The present developed method for simultaneous estimation of sofosbuvir and ledipasvir by rp hplc has been found to be the most innovative and novel as compared with other available literature. In most of the methods usually, a mixture of acetonitrile and water has been used as mobile phase with eclipse C18 column [20], whereas in present research 0.1% opa buffer is used with discovery C8 column and ultraviolet-visible detector (universal detector). In present work forced degradation study is also performed as compared with other work [15].

CONCLUSION

The present RP-HPLC developed and validated method allows a simple and rapid quantitative determination of ledipasvir and sofosbuvir in bulk and tablet dosage forms. All the validation parameters were found to be within the limits according to ICH guidelines. The proposed method was found to be specific for the drugs of interest irrespective of the excipients present and the short retention time allows the analyst to analyze various numbers of samples in a short period and method was found to be simple, accurate, precise, rugged, robust and stable under forced degradation stress conditions. So the established method can be successfully applied for the routine analysis of the marketed formulations.

ACKNOWLEDGMENT

The authors are thankful to the Spectrum labs, Hyderabad for providing the gift samples of Ledipasvir and sofosbuvir.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally in this work

CONFLICT OF INTERESTS

Authors declare that there is no conflict of interest

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