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DEVELOPMENT OF ASSAY METHOD AND FORCED DEGRADATION STUDY OF LEDIPASVIR AND SOFOSBUVIR BY RP-HPLC IN TABLET FORMULATION

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ABSTRACT

Chronic hepatitis C virus (HCV) infection is one of the most common etiologies of liver-related mortality throughout the world. Sofosbuvir and ledipasvir are inhobits HCV NS5B and HCV NS5A polymerase respectively. No published LC-MS/MS and HPLC based methods for simultaneous estimation of ledipasvir and sofosbuvir. Therefore, A stability indicating high performance liquid Chromatographic (HPLC) method was developed and validated for estimation of both drugs. Chromatographic separation was achieved on a C18 column [Xterra, 250 x 4.6 mm, 5µ] utilizing a mobile phase consisting a mixture of 0.1% trifluro acetic acid and methanol in the ratio of 40:60 v/v at a flow rate of 1ml/min with UV detection at 246nm. The retention time of Ledipasvir and Sofosbuvir was 3.13 min and 4.17 min respectively. Good linearity obtained over the range of 25µg/ml to 150µg/ml for Ledipasvir and sofosbuvir. Correlation coefficient was found to be 0.998&0.998 for Ledipasvir& sofosbuvir respectively. The % RSD of precision for Ledipasvir and sofosbuvir was found to be 0.23and 0.86respectively. The % mean recovery was found to be 98.76-99.26% for Ledipasvir and 99.03-100.73.% for sofosbuvir. Thus the validated economical method was applied for forced degradation study of Ledipasvir and Sofosbuvir tablet.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection is one of the most common etiologies of liver-related mortality throughout the world. Among the six HCV genotypes, genotype 1 was significantly more aggressive Among the available treatments for HCV genotype 1, the combination therapy of ledipasvir/sofosbuvir provides several advantages compared to other regimens, including use of a single-pill regimen, possibility to shorten the duration of treatment to 8 weeks, efficacy in patients exposed to protease inhibitors, safety in decompensated cirrhosis, and potential to avoid ribavirin[1-3]. Sofosbuvir and ledipasvir are inhobits HCV NS5B and HCV NS5A polymerase respectively. A fixed-dose combination of sofosbuvir–ledipasvir was approved in 2014for treatment of patients chronically infected with genotype 1HCV[4]. Literature search reveals that two LC-MS/MS methods were reported for simultaneous estimation of ledipasvir and sofosbuvir from biological matrixes.[5-6] Limited analytical methods were reported including UV[7], HPLC[8-11], and LC-MS[12-14] for the analysis of ledipasvir and sofosbuvir alone or in combination with other drugs. To best of our knowledge, no published LC-MS/MS and HPLC based methods for simultaneous estimation of ledipasvir and sofosbuvir. Therefore, in this study, a quick, simple and sensitive stability indicating HPLC method was developed and validated for simultaneous estimation of ledipasvir and sofosbuvir from bulk and pharmaceutical dosage form.

MATERIALS AND METHODS

Chemicals and reagents:

HPLC grade methanol, acetonitrile and analytical grade trifluro acetic acid were purchased from Merck (Mumbai, India). Sofosbuvir working standard was obtained as a gift sample from Natco Pharma Ltd., Hyderabad., and Ledipasvir working standard from Hetero drugs Ltd, Hyderabad, India.

Instrumentation:

Shimadzu gradient HPLC (JAPAN) ,HPLC column Phenomenex (250 x 4.6mm, 5µm), Mobile phase filtration unit (Pall Life sciences, Mumbai, India), LAB-INDIA U.V with UV Win software, Sonicator, P^H meter (LAB-INDIA), digital balance (Denver).

Preparation of standard solutions:

Stock solutions (1mg/ml) of ledipasvir and sofosbuvir were prepared in methanol. Further dilutions were carried out using 60% methanol as diluent. Ledipasvirand sofosbuvir working standards of different concentrations ranging from 12-68µg/ml were prepared by diluting several aliquots of standard solutions of ledipasvir and sofosbuvir.

RESULTS

Chromatographic Conditions:

Chromatographic Conditions the HPLC system consisted of Shimadzu gradient HPLC (JAPAN) with dual λ Absorbance UV detector. The wavelength of detection as set at 246nm. Separation was carried out in isocratic mode on Xterra C18 column (4.6x250mmx5µm) and the retention time of Ledipasvir and sofosbuvir was found to be 3.13 min and 4.27 min respectively. (Fig 1), using mobile phase consisting a a mixture of 0.1% trifluro acetic acid and methanol in the ratio of 40:60 v/v at a flow rate of 1ml/min. The mobile phase filtered through nylon milli pore (0.2µm) membrane filter, purchased from pall life sciences, Mumbai and degassed with Ultra sonicator prior to use. Chromatography was carried out at room temperature 25°C and maintains the column temperature at 32°C. The developed Method was validated for linearity, precision, accuracy, ruggedness and is applied for forced degradation studies as per the ICH guidelines[15-16].

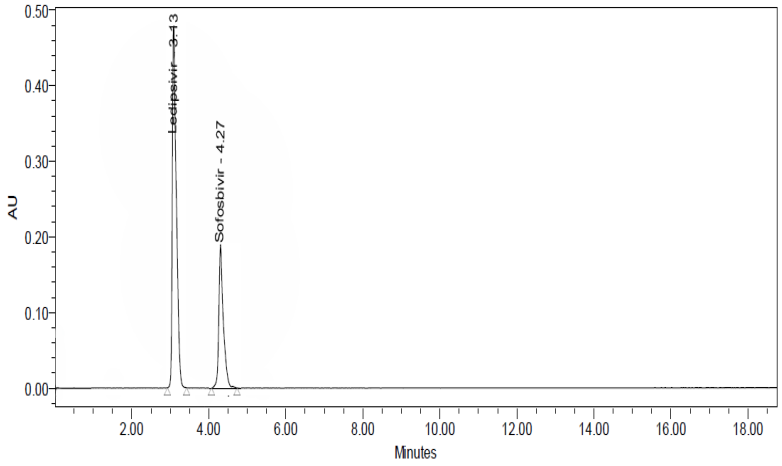

Fig 1: Chromatogram of ledipasvir and sofosbuvir.

METHOD VALIDATION:

Linearity:

Linear concentrations of both drugs were prepared and the best fit line was calculated. Wide range calibration was determined by solutions containing 12µg/ml to 68µg/ml (Table 1) for Ledipasvir and sofosbuvir. Correlation coefficient was found to be 0.998&0.998 for Ledipasvir& sofosbuvir respectively (Fig 2; Fig 3).

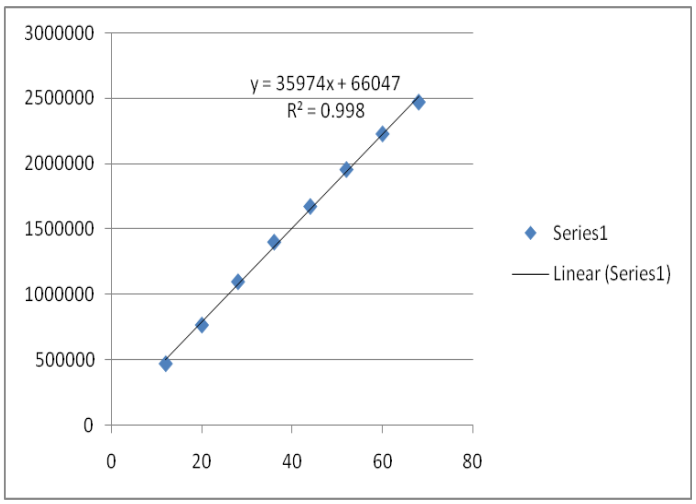

Figure 2: Calibration curve of ledipasvir.

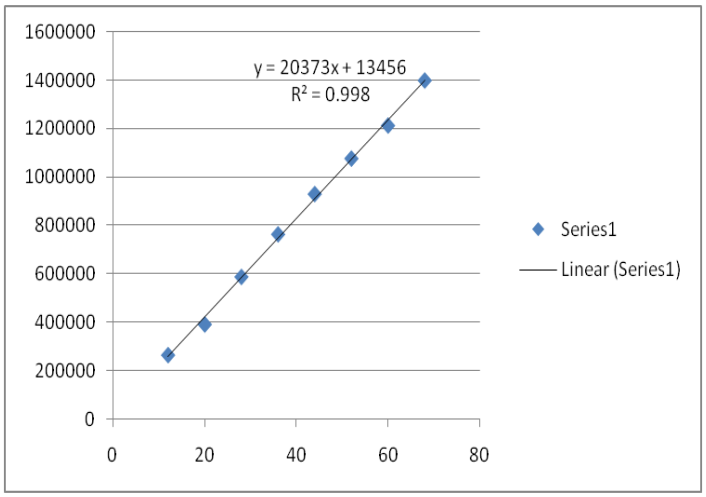

Figure 3: Calibration curve of sofosbuvir.

Table 1: Linearity data for ledipasvir and sofosbuvir.

S.no	Ledipasvir		Sofosbuvir	
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area
1	12	467150	12	264445
2	20	762941	20	391176
3	28	1094510	28	587594
4	36	1397408	36	763718
5	44	1670363	44	930257
6	52	1953156	52	1076996
7	60	2225942	60	1213327
8	68	2468704	68	1399456

Limit of Detection (LOD) and Limit of Quantification (LOQ):

The LOD is calculated using the formula 3.3 times σ/s where "σ" is standard deviation of the intercept obtained for calibration curve and "s" is the slope of the calibration curve. Similarly LOQ is calculated using the formula 10 times σ/s. The calculated LOD and LOQ are shown in Table 2 &3.

Table 2: LOD & LOQ Results of Ledipasvir.

Conc (µg/ml)	Area 1	Area 2	Area 3	Avg Area
12	467146	467238	467066	467150
20	762943	763064	762815	762941
28	1094519	1094632	1094378	1094510
36	1397397	1397538	1397290	1397408
44	1670374	1670454	1670261	1670363
52	1953151	1953258	1953059	1953156
60	2225928	2226056	2225843	2225942
68	2468705	2468799	2468609	2468704
Intercept	66049	66161	65930	66046.66667
slope	35974	35974	35975	35974.33333
Intercept Standard Deviation				115.51
LOD (µg/ml)				0.01
LOQ(µg/ml)				0.03

Table 3: LOD & LOQ Results of Sofosbuvir.

Conc (µg/ml)	Area 1	Area 2	Area 3	Avg Area
12	264443	264547	264344	264445
20	391173	391269	391087	391176
28	587602	587690	587490	587594
36	763731	763808	763617	763718
44	930261	930370	930140	930257
52	1076990	1077106	1076892	1076996
60	1213319	1213440	1213224	1213327
68	1399448	1399572	1399349	1399456
Intercept	13463	13544	13360	13456
Slope	20373	20373	20373	20373
Intercept Standard Deviation				92.21
LOD (µg/ml)				0.014
LOQ(µg/ml)				0.045

Precision:

The intraday precision was demonstrated by injecting standard solutions of Ledipasvir and sofosbuvir with 20 µg/ml and 60µg/ml respectively as per the test procedure (Table 4) & recording the chromatograms of six standard solutioins. The % RSD of Ledipasvir and sofosbuvir was found to be 0.84 and 0.67 respectively.

Table 4: Method Precision data of Ledipasvir and sofosbuvir.

Ledipasvir (20µg/ml)		Sofosbuvir (60µg/ml)
S.No	Area	Area
1	762674	1279313
2	751991	1287315
3	749132	1270927
4	762543	1269331
5	751837	1281849
6	762143	1262025
Mean	756720	1275126.667
SD	6364.76	8506.38
%RSD	0.84	0.67

Intermediate Precision:

Intermediate precision of the analytical method was determined by performing method precision on in three successive days by different analysts under same experimental condition by injecting six replicate standards preparations was determined and the mean % RSD of Ledipasvir(20µg/ml) and Sofosbuvir (60µg/ml) was found to be 0.23 and 0.86 respectively (Table 5).

Table 5: Precision Data for Ledipasvir& Sofosbuvir.

Ledipasvir Area for 20µg/ml					Sofosbuvir Area for 60µg/ml			
S.No	**Day-1**	**Day-2**	**Day-3**	**Avg**	**Day-1**	**Day-2**	**Day-3**	**Avg**
1	751148	740385	759623	750385	1276754	1275475	1274195	1275475
2	750487	749735	748983	749735	1294740	1283453	1282165	1286786
3	747633	746884	746135	746884	1268385	1267114	1295843	1277114
4	741017	750255	749492	746921	1266792	1265523	1264253	1265523
5	750333	749581	748829	749581	1299285	1278003	1276721	1284670
6	730618	749856	759094	746522	1259500	1258238	1256976	1258238
Mean	745206	747783	752026	748338	1277576	1271301	1275026	1274634
SD	8071.31	3820.72	5801.32	1738.08	16087.70	9290.51	13636.25	11018.97
%RSD	1.08	0.51	0.77	0.23	1.25	0.73	1.06	0.86

Accuracy:

Accuracy of the method was established by performing recovery studies according to the ICH guidelines. Spiked samples were prepared by spiking pre-analyzed sample solutions with pure drug at three different concentration levels each in triplicate. Mean percentage recovery values at three different concentrations of the two drugs was calculated. The % mean recovery of Ledipasvir(98.76-99.26%) & Sofosbuvir (99.03-100.73.%) at each level was within the limits of 98% and 102% (Table 6)

Table-6: Accuracy of ledipasvir & Sofosbuvir.

Accuracy of Ledipasvir						
S.N0.	**Conc.**	**Calculated Concn.**	**%Recovery**	**Mean Recovery**	**SD**	**%RSD**
1	34	34	100.01			
2	34	33.75	99.29	99.26%	0.77	0.78
3	34	33.48	98.47			
1	68	67.06	98.62			
2	68	67.06	98.63	98.76%	0.23	0.24
3	68	67.34	99.03			
1	102	102.25	100.25			
2	102	100.55	98.58	99.16%	0.94	0.95
3	102	100.62	98.65			
Accuracy of Sofosbuvir						
S.N0.	**Conc.**	**Calculated concn.**	**%Recovery**	**Mean Recovery**	**SD**	**%RSD**
1	34	33.93	99.79			
2	34	33.54	98.65	99.03%	0.66	0.67
3	34	33.53	98.64			
1	68	67.54	99.32			
2	68	67.15	98.76	99.14%	0.33	0.34
3	68	67.55	99.35			
1	102	102.57	100.56			
2	102	103.08	101.06	100.73%	0.28	0.28
3	102	102.58	100.57			

Ruggedness:

The ruggedness of method for Ledipasvir and sofosbuvir was calculated with six injections of 68µg/ml in two batches using two different columns. The % CV of ruggedness for Ledipasvir was 0.33 with column-1 and 0.09 with column-2 and the % CV of ruggedness for sofosbuvir was 0.11 with column-1 and 0.07 with column-2 (Table-7), which is within acceptance limits.

Table 7: Results of Ruggedness.

	Ledipasvir 68µg/ml		Sofosbuvir 68µg/ml	
S.NO	**Column 1**	**Column 2**	**Column 1**	**Column 2**
1	67.06	67.12	67.18	67.14
2	67.06	67.2	67.21	67.01
3	67.34	67.09	67.14	67.04
4	67.54	67.22	67.02	67.02
5	67.15	67.11	67.15	67.09
6	67.55	67.24	67.04	67.11
Mean	67.28	67.16	67.12	67.06
± SD	0.22	0.06	0.07	0.05
% CV	0.33	0.09	0.11	0.078
% Accuracy	98.94	98.76	98.71	98.62

Results of Stress Degradation Studies:

Stress degradation studies were performed as per the ICH guidelinesQ1A (R2) Stability Testing of New Drug Substances and Products, using the proposed validated analytical method.(Table 8&9)

Table 8: Results of stress degradation studies of sofosbuvir.

Sno	Stress conditions	Time	% Assay	% Degradation	Purity angle	Purity threshold
1	Acid Degradation	30 min	90.6	9.4	0.14	0.18
2	Base Degradation	30 min	91.3	8.7	0.21	0.24
3	Peroxide Degradation	30 min	89.5	10.5	0.21	0.26
4	UV Degradation	7 days	99.6	0.4	0.20	0.22
5	Thermal Degradation	24hrs	94.3	5.7	0.18	0.21

Table 9: Results of stress degradation studies of ledipasvir.

Sno	Stress conditions	Time	% Assay	% Degradation	Purity angle	Purity threshold
1	Acid Degradation	30 min	91.8	8.2	0.15	0.18
2	Base Degradation	30 min	90.6	9.4	0.17	0.23
3	Peroxide Degradation	30 min	93.1	6.9	0.21	0.24
4	UV Degradation	7 days	99.2	0.8	0.15	0.21
5	Thermal degradation	24hrs	93.7	6.3	0.17	0.23

Acid Degradation studies:

To 1ml of stock solution Ledipasvir and sofosbuvir, 1ml of 2N HCl was added and refluxed for 30min at 60^0c. From the above solution10 µl was injected into the system and the chromatograms were recorded to detect the stability of sample. (Figure 4).

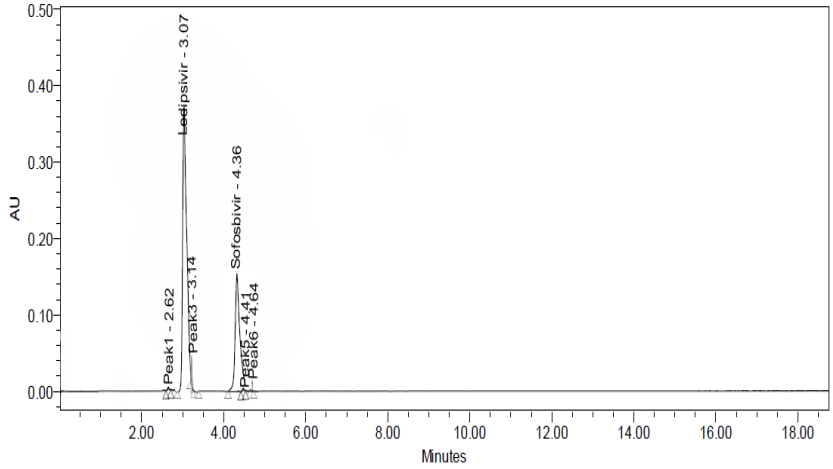

Figure 4: Chromatogram of Acid Degradation.

Alkali Degradation Studies:

To 1ml of stock solution of of standard drug and sample Ledipasvir and sofosbuvir, 1ml of 2N NaOH was added and refluxed for 30min at 60^0c. From the above solution10 µl was injected into the system and the chromatograms were recorded to detect the stability of sample. (Figure 5).

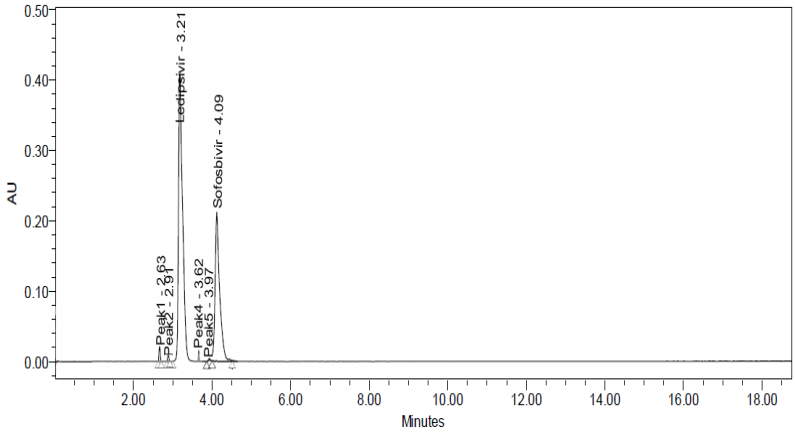

Figure 5: Chromatogram of Base Degradation.

Oxidative Degradation:

To 1ml of stock solution of standard drug and sample of Ledipasvirand sofosbuvir, 1ml of 20% $H_2O_2$was added and refluxed for 30min at 60^0c. From the above solution10 µl was injected into the system and the chromatograms were recorded to detect the stability of sample. (Figure 6).

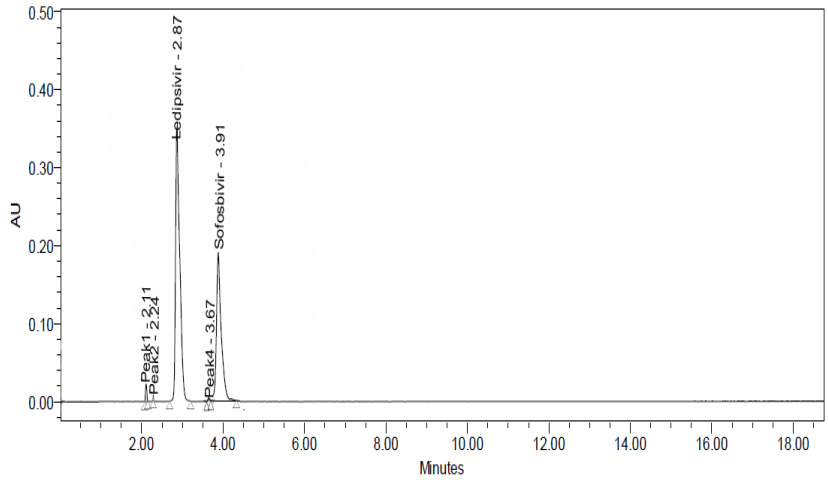

Figure 6: Chromatogram of Oxidative Degradation.

Photo Stability Studies:

The photochemical stability of the drug was also studied by exposing the 36 µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber . For HPLC study, from the above solution10 µl was injected into the system and the chromatograms were recorded to detect the stability of sample. (Figure 7).

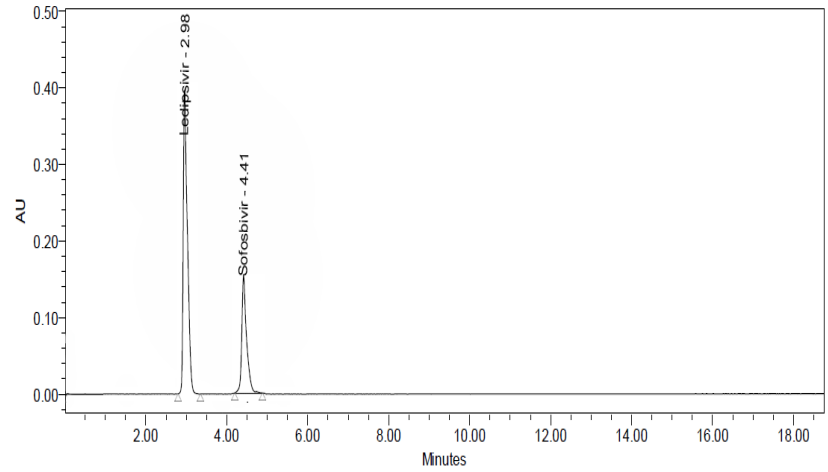

Figure 7: Chromatogram of UV Degradation.

Thermal degradation studies:

The 1ml of stock solution of standard drug and sample of Ledipasvir and sofosbuvir was exposed to temperature 105^{o}C for 24hrs for HPLC study, from the above solution10 µl was injected into the system and the chromatograms were recorded to detect the stability of sample.(Figure: 8).

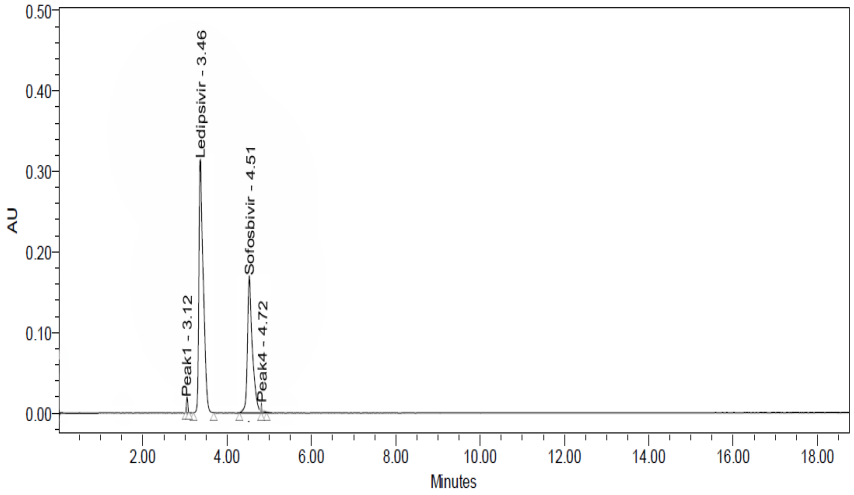

Figure 8: Chromatogram of Thermal Degradation Study.

DISCUSSION

Chromatographic Separation was carried out in isocratic mode on Xterra C18 column (4.6x250mmx5µm) and the retention time of Ledipasvir and sofosbuvir was found to be 3.13 min and 4.27 min respectively. using mobile phase consisting a a mixture of 0.1% trifluro acetic acid and methanol in the ratio of 40:60 v/v at a flow rate of 1ml/min. Good linearity obtained over the range of 12µg/ml to 68µg/ml for Ledipasvir and sofosbuvir. Correlation coefficient was found to be 0.998&0.998 for Ledipasvir& sofosbuvir respectively. The % RSD for intraday precision of Ledipasvir and sofosbuvir was found to be 0.84 and 0.67 respectively. The mean % RSD of Intermediate precision for Ledipasvir(20µg/ml) and Sofosbuvir (60µg/ml) was found to be 0.23 and 0.86 respectively. The % mean recovery of Ledipasvir(98.76-99.26%) & Sofosbuvir (99.03-100.73.%) at each level was within the limits of 98% and 102%. Ledipasvir and Sofosbuvir undergoes significant degradation in acidic, oxidation, alkaline, and UV. Comparatively More degradation was found with base for sofosbuvir and with peroxide for atazanavir. As per ICH guidelines peak purity angle should be less than peak purity threshold. Hence, method of the analysis of Ledipasvir and sofosbuvir in tablet dosage form shows that the degradation product doesn't interfere with the analytical determination. hence the proposed analytical method is also useful for the determination of Ledipasvirand sofosbuvir stability in sample of pharmaceutical dosage form.

CONCLUSION

A simple, precise, accurate, robust & cost-effective method was developed for the routine analysis. The method was successfully validated in terms of linearity, precision, accuracy as per ICH guidelines. Hence it can be concluded that the proposed method was a good approach for obtaining reliable results & found to be suitable for the routine analysis and quality control and percentage degradation of pharmaceutical preparations containing these drugs either individually or in combination.

REFERENCES

1. Veldt BJ, Heathcote EJ, Wedemeyer H, Reichen J, Hofmann WP, Zeuzem S, et al. M.P. Sustained virologicresponse and clinical outcomes in patients with chronic hepatitis C andadvanced fibrosis. Ann. Intern. Med. 2007; 147:677.
2. Bruno S, Crosignani A, Facciotto C, Rossi S, Roffi L, Redaelli A, et al. Sustained virologic response prevents thedevelopment of esophageal varices in compensated, Child-Pugh class Ahepatitis C virus-induced cirrhosis. A 12-year prospective follow-up study, Hepatology 2010;51:2069.
3. Ng V, Saab V. Effects of a sustained virologic response on outcomes of patients with chronic hepatitis C. Clin. Gastroenterol. Hepatol. 2011;9:923.
4. Afdhal N, Reddy KK, Nelson DR, Lawitz E, Gordon SC, Schiff E, et al. Investigators, Ledipasvir and sofosbuvir for previously treated HCV genotype1 infection, N. Engl. J. Med. 2014;370:1483.
5. Pan C, Chen Y, Chen W, Zhou G, Jin L, Zheng Y, et al. Simultaneous determination of ledipasvir, sofosbuvir and its metabolite in rat plasma by UPLC-MS/MS and its application to a pharmacokinetic study. J Chromatogr B Analyt Technol Biomed Life Sci. 2016;1008:255-259.
6. Elkady, Ehab F, Aboelwafa, Ahmed A. 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.
7. Suryawanshi R, Shinde N, Todkar G. Development and Validation of Simple UV Spectrophotometric Method for the Determination of Ledipasvir in Bulk Form and Stress Degradation Studies. Inventi Rapid: Pharm Analysis & Quality Assurance, 2016;3:1-5.
8. Devilal J, Durgaprasad B, Narottam P, Srinivasa Rao A. New method development and validation for the determination of ledipasvir in bulk drug form by using reverse phase HPLC technique. World Journal of Pharmacy and Pharmaceutical Sciences. 2016;5(8), 1312-1321.

9. Raj Kumar B, Subrahmanyam KV. A new validated RP-HPLC method for the simultaneous determination of simeprevir and sofosbuvir in pharmaceutical dosage form. Indo American Journal of Pharmaceutical Research.2016; 6(02):4508-4520.
10. Ravikumar V, Subramanyam CVS, Veerabhadram G. Estimation and validation of sofosbuvir in bulk and tablet dosage form by RP-HPLC. Int J Pharm 2016; 6(2): 121-127.
11. Mohan Vikas P, Satyanarayana T, Vinod Kumar D, Mounika E, Sri Latha M, Anusha R, et al. Development and validation of new RP-HPLC method for determination of sofosbuvir in pure form. J Global Trends Pharm Sci 2016; 7(1): 3013-3015.
12. Bahrami M, Mohammadi T, Miraghaei B, Babaei S, Ghaheri A, Bahrami G. Quantification of sofosbuvir in human serum by liquid chromatography with negative ionization mass spectrometry using the parent peak and its source-induced fragment: Application to a bioequivalence study. J. Sep. Science, 2016;39: 2702–2709.
13. Rezk MR, Basalious EB, Amin ME. Novel and sensitive UPLC–MS/MS method for quantification of sofosbuvir in human plasma: application to a bioequivalence study. Biomed. Chromatogr. 2016;30:1354-1362.
14. Nebsen M, Eman S. Elzanfaly Stability-Indicating Method and LC–MS-MS Characterization of Forced Degradation Products of SofosbuvirJ Chromatogr Sci first published online July 19, 2016. (E pub ahead of print)
15. ICH guidelines for the stability of new drug substances and products. Q1A(R2) ICH, Geneva; 2005. p. 1-13.
16. ICH guidelines for validation of analytical procedures: text and methodology. Q2(R1) ICH, Geneva; 2005. p. 1-14.