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Simultaneous estimation of ledipasvir and sofosbuvir in bulk and its dosage forms by stability indicating RP-HPLC method

Ismail Y*, Vara Prasad M

Department of Pharmaceutical Analysis, Crescent School of Pharmacy, B.S. Abdur Rahman Crescent Institute of Science and Technology, Chennai-600048, Tamil Nadu, India

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ABSTRACT

The fixed-dose ledipasvir-sofosbuvir combination offers an effective and well-tolerated pill for the treatment of chronic hepatitis C infection. Only a few analytical works were carried out to estimate the Ledipasvir and Sofosbuvir drug combination in the various dosage forms. This work aimed to simplify the estimation process using RP-HPLC methodology. The method was developed on a reversed phase Agilent C18 (4.6 x 150 mm, 5 μm) column. The isocratic elution process was performed using a mobile phase ratio of Methanol (70% v/v): Water (30 % v/v) with 0.6 ml/min flow rate. Elute was scanned using the PDA detector at the wavelength of 235 nm. The results of the elution process showed that the Ledipasvir and Sofosbuvir elute the peak at a concentration of 9 μg/ml and 40 μg/ml with retention times of 7.745 min and 2.345 min respectively. The percentage purity of Ledipasvir and Sofosbuvir was found to be 99.40 % w/v and 98.20 % w/v. The proposed method was found to be a high degree of precision and reproducibility. The percentage recovery was found to be 99.92 % for Ledipasvir and 99.82 % for Sofosbuvir. The LOD and LOQ were measured, and the results were within limits. The developed validation method can be applied for degradation evaluation of Ledipasvir and Sofosbuvir for the various dosage forms.

*Corresponding Author

Name: Ismail Y
Phone: +91-9700550331
Email: ismailpharmacy786@gmail.com

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INTRODUCTION

Ledipasvir is the first approved NS5A inhibitor agent for HCV genotype 1 treatment (Yasir, 2015), (Figure 1). The Ledipasvir possessed multiple hetero rings such as benzimidazole, imidazole, pyrrole, etc. It also contains the carbonyl amino, fluoro and carbamate groups. The pharmacokinetic parameter of the drug showed >99.8% protein binding, the plasma half-life of 47 hrs and the renal clearance of 1% (Devilal *et al.*, 2016). Sofosbuvir, second-hand agent for the combination therapy to treat chronic Hepatitis C with Ledipasvir (Keating, 2014), (Figure 2). It contains a furan ring in the center with the surrounding of various fragments of pyrimidine, phenoxy, phosphoryl amino and methyl group (Bandla and Ganapathy, 2017). Sofosbuvir found 61- 65 % of protein binding, 0.4 hr of plasma half-life and 80% renal clearance (Guguloth *et al.*, 2016). FDA approved the fixed dose combination of ledipasvir-sofosbuvir for the sustained virological response (Akshay *et al.*, 2017; Zaman *et al.*, 2016). The primary data revealed that the combination offers effective treatment of hepatitis C infection, and it is well tolerated in oral dosage forms. It is imperative to have safety and efficacy in drug therapy; pharmacists must take into consideration the

stability of drugs and their therapeutic values. The stability of the drug formulations can be assessed by using stability indicating methods (Sreenivasa *et al.*, 2017). A well-designed stress study is important to help develop and demonstrate the specificity of stability indicating methods (Nebsen and Elzanfaly, 2016). They are also useful in checking rapid and accurate drug quality during stability testing (Ewing, 1997).

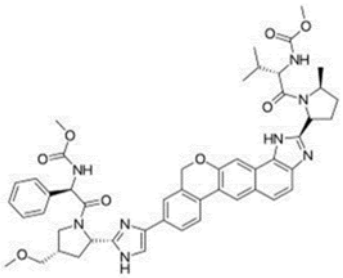

Figure 1: Chemical structure of Ledipasvir

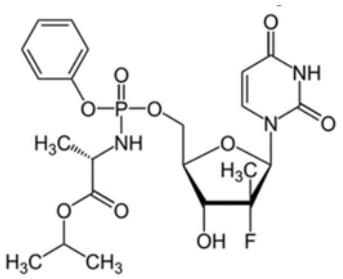

Figure 2: Chemical structure of Sofosbuvir

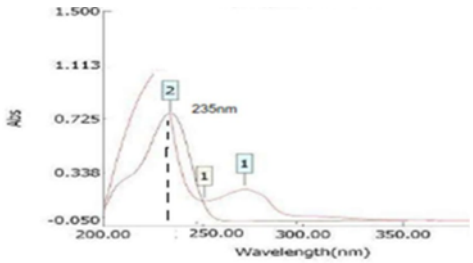

Figure 3: Spectrum showing the overlapping spectrum of Ledipasvir and Sofosbuvir

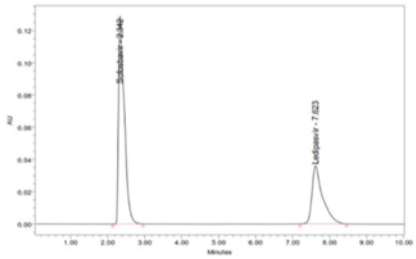

Figure 4: Chromatogram showing specificity of Ledipasvir & Sofosbuvir

There is no cost-effective analytical method reported (consuming less amount of mobile

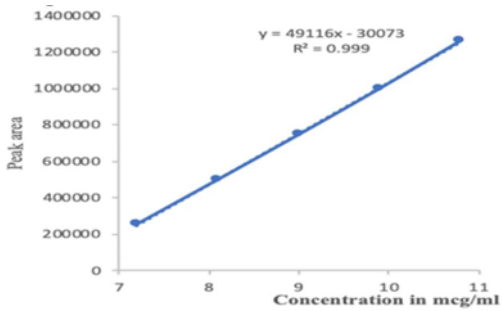

Figure 5: Linearity of Ledipasvir

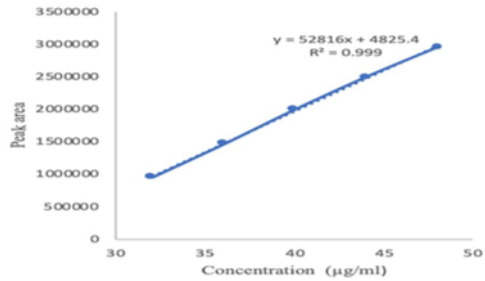

Figure 6: Linearity of Sofosbuvir

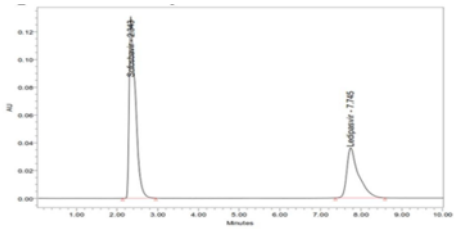

Figure 7: Chromatogram showing system precision of Ledipasvir and Sofosbuvir

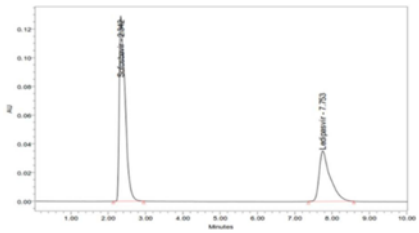

Figure 8: Chromatogram showing method precision of Ledipasvir and Sofosbuvir

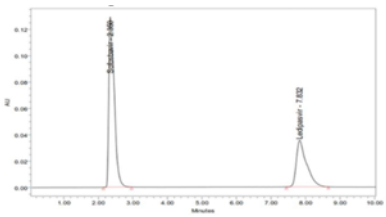

Figure 9: Chromatogram showing system suitability of Ledipasvir and Sofosbuvir

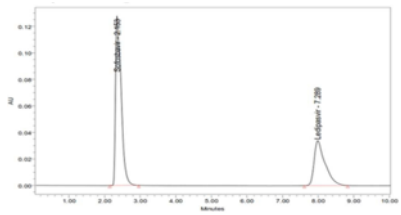

Figure 10: Chromatogram showing more flow rate of 0.8 ml/min of Ledipasvir and Sofosbuvir

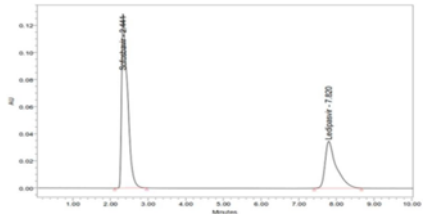

Figure 11: Chromatogram showing less flow rate of 0.4 ml/min of Ledipasvir and Sofosbuvir

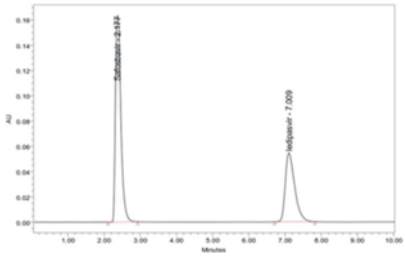

Figure 12: Chromatogram showing more organic phase ratio of Ledipasvir and Sofosbuvir

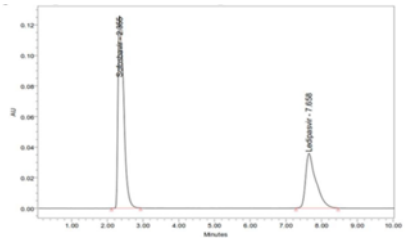

Figure 13: Chromatogram showing less organic phase ratio of Ledipasvir and Sofosbuvir

phase) for simultaneous analysis of Ledipasvir and Sofosbuvir, previous to our work (Hassouna *et al.*, 2017; Kiran *et al.*, 2017; Nagaraju *et al.*, 2017). The present work is aimed at to develop a novel, rapid, affordable, reliable, cost-effective, selective, sensitive, precise, accurate, reproducible, and specific analysis of combination (Ledipasvir and Sofosbuvir) in various dosage forms using stability indicating RP-HPLC method (Uppalapati *et al.*, 2017; Yogendrachari *et al.*, 2016; Madhavi and Rani, 2017).

MATERIALS AND METHODS

Authentic drug samples of Ledipasvir and Sofosbuvir were obtained from Pharma Train Lab, Hyderabad. The commercial samples of the tablets Hepcinat-LP containing Ledipasvir - 90 mg and Sofosbuvir–400 mg were provided by Natco Pharmaceuticals Pvt.Ltd, Hyderabad. All the solvents were HPLC grade and purchased from the Sigma Aldrich.

Chromatographic process and conditions

Waters e2695 Separation Module HPLC autosampler system and a UV-visible detector were used for the chromatographic separation.The chromatographic column utilized in the study was Agilent C18 (4.6×150 mm, 5μm). Different mobile phases were tried, and the one containing Methanol: Water in the ratio of 70: 30 % v/v was appropriate. The mobile phase was used as a diluent. The flow rate selected was 0.6 ml/min. The overlay spectrum of Ledipasvir and Sofosbuvir was obtained, and the isobestic point of Ledipasvir and Sofosbuvir showed absorbance's maxima at 235 nm (Rai *et al.*, 2017), (Figure 3).

Preparation of Mixed Standard and sample Solutions of Ledipasvir Sofosbuvir

Accurately weighed pure drugs of Ledipasvir 90 mg and 400 mg of Sofosbuvir for working standard preparation and 20 ml of diluent (methanol: water) was added in 100 mL standard flask. Further, it was sonicated and made up to the volume. The final concentration of 9.0 μg/ml Ledipasvir and 40.0 μg/ml Sofosbuvir was prepared from the stock solution. Similarly, the marketed sample Hepcinat-LP tablets with the label claim of 90 mg of Ledipasvir and 400mg Sofosbuvir was powdered and placed into a 100 ml volumetric flask. The sample solution is prepared as a similar standard concentration of 9.0 μg/ml Ledipasvir and 40.0 μg/ml Sofosbuvir, respectively.

Assay

Initially, 10μl of standard solution and simultaneously sample solution was injected and the peak areas of the drugs in standard and sample compared. Ledipasvir and Sofosbuvir show the percentage purity values of 99.40 % w/v and 98.20 % w/v respectively (Tables 1 and 2).

RESULTS AND DISCUSSION

Specificity

There are no elutions of interfering peak takes place, which shows that the peak of the analyte was pure, and excipients in the combination do not interfere

Table 1: Quantitative determination for Ledipasvir and Sofosbuvir

		Ledipasvir			
S.No	Standard Area	Sample Area	Amt. Present (mg)	Amt. Found (mg)	% Assay
1	708756	720705	90	89.9	99.89 % w/v
2	710729	715103	90	89.8	99.7 % w/v
3	713024	714243	90	88.9	98.7 % w/v
	Average Assay				99.4 % w/v

Table 2: Quantitative determination for Ledipasvir and Sofosbuvir

		Sofosbuvir			
S.No	Standard Area	Sample Area	Amt. Present (mg)	Amt. Found (mg)	% Assay
1	1394581	1408375	400	399.89	99.9 % w/v
2	1394685	1394807	400	389.9	97.4 % w/v
3	1395686	1391570	400	388.98	97.25 % w/v
	Average Assay				98.20 % w/v

Table 3: Linearity Results for Ledipasvir and Sofosbuvir

Parameters	Ledipasvir	Sofosbuvir
Linear Dynamic Range	7.2 – 10.8 μg/ml	32 – 48 μg/ml
Correlation Coefficient	0.999	0.999
Slope (m)	49116	52816

with the analyte (Figure 4). Therefore, the method is specific and selective for the estimation of Ledipasvir and Sofosbuvir in the combination.

Linearity and Range

Linearity and range measured for the working standard by the various diluted concentration of 7.2, 8.1, 9.0, 9.9 and 10.8 μg/ml for Ledipasvir and 32,36,40,44 and 48μg/ml for Sofosbuvir respectively. From the results of linearity studies, the specified range was determined for Ledipasvir & Sofosbuvir given as 7.2 – 10.8 μg/ml and 32 μg/ml – 48 μg/ml (Table 3). The calibration curves are shown in (Figures 5 and 6). The Correlation Coefficient for Ledipasvir & Sofosbuvir was found to be within the acceptance criteria of 0.999.

System precision

Six replicate injections of a mixed standard solution of Ledipasvir (9.0 μg/ml), and Sofosbuvir (40.0 μg/ml) was used to measure the system precision. The R.S.D [%] of peak area was 0.432, retention time was 0.554, U.S.P Plate count was 1.281 and U.S.P tailing of 0.813 for Ledipasvir. The R.S.D (%) of peak area was 0.391; retention time was 0.266, U.S.P Plate count 1.08 and USP tailing of 1.186 for Sofosbuvir. The reports revealed that all the system parameters were found within the Acceptance criteria of 2 %. The results are reported in Tables 4 and 5. The chromatograms are shown in (Figure 7).

Method Precision

In the same way, method precision was determined by six repeat injections of a mixed combination of the sample solution of 9.0 μg/ml Ledipasvir and 40.0 μg/ml Sofosbuvir. The Tables 5 and 6 elucidated the percentage RSD of peak area was 0.120, Rt of 0.726 and assay of 0.120 for Ledipasvir. The % R.S.D of peak area was 0.354; retention time was 0.318 and assay was 0.354 for Sofosbuvir. The method precision results showed that the obtained values are in the acceptance criteria of 2 % (Tables 6 and 7). The chromatograms are shown in (Figure 8). Thus, the proposed method was found to be a high degree of precision and reproducibility.

Table 9: System suitability parameters of Ledipasvir and Sofosbuvir

Parameters	Ledipasvir	Sofosbuvir
Tailing factor	1.74	1.80
Resolution	6.42	
Retention time	7.745	2.343
Theoretical plates per unit	2741	2529.7

Inter-day Precision

To check the inter-day variations of the method, the same concentration of Ledipasvir and Sofosbuvir were subjected to the proposed HPLC method of

Table 4: System precision data for Ledipasvir

Injection	R_t	Peak Area	USP Plate count	USP Tailing
1	7.839	718391	2760	1.74
2	7.832	724000	2750	1.73
3	7.774	715765	2735	1.74
4	7.753	716230	2673	1.72
5	7.745	717222	2763	1.75
6	7.746	716181	2765	1.76
MEAN	7.781	717964.83	2741	1.74
SD	0.0432	3104.44	35.105	0.0141
% RSD	0.554	0.432	1.281	0.813

Table 5: System precision data for Sofosbuvir

Injection	R_t	Peak Area	USP Plate count	USP Tailing
1	2.358	1394821	2550	1.81
2	2.355	1392238	2554	1.79
3	2.344	1394221	2544	1.79
4	2.343	1403835	2498	1.80
5	2.345	1394286	2492	1.78
6	2.350	1404803	2540	1.84
MEAN	2.349	1397367	2529.667	1.80
SD	0.006	5464.572	27.347	0.021
% RSD	0.266	0.391	1.08	1.186

Table 6: Method precision data for Ledipasvir

S.No	Sample name	R_t	Peak Area	% Assay-Ledipasvir
1	Ledipasvir	7.804	702828	99.85
2	Ledipasvir	7.832	703012	99.87
3	Ledipasvir	7.765	703168	99.89
4	Ledipasvir	7.753	702986	99.87
5	Ledipasvir	7.745	704265	100.05
6	Ledipasvir	7.668	704878	100.14
Mean		7.761	703522.8	99.945
SD		0.056	842.037	0.120
%RSD		0.726	0.120	0.120

Table 7: Method precision data for Sofosbuvir

S.No	Sample name	R_t	Peak Area	% Assay- Sofosbuvir
1	Sofosbuvir	2.345	1399343	99.75
2	Sofosbuvir	2.360	1408881	100.43
3	Sofosbuvir	2.343	1410142	100.52
4	Sofosbuvir	2.342	1403270	100.03
5	Sofosbuvir	2.341	1398220	99.67
6.	Sofosbuvir	2.34	1401224	99.88
Mean		2.345	1403513	100.05
SD		0.007	4969.153	0.355
%RSD		0.318	0.354	0.354

Table 8: Inter-day precision data of the proposed method for Ledipasvir and Sofosbuvir

Days	Ledipasvir		Sofosbuvir	
	R_t	Peak Area	R_t	Peak Area
Day 1*	7.886	721335	2.351	1409240
Day 2*	7.879	724937	2.350	1408068
Day 3*	7.723	717532	2.346	1397300
Day 4*	7.690	719255	2.345	1393047
Day 5*	7.683	718995	2.345	1401990
Day 6*	7.648	716548	2.345	1400851
MEAN	7.751	719767	2.347	1401749.333
SD	0.1042	3013.39	0.0027	6203.67
% RSD	1.344	0.418	0.117	0.442

*Average of six injections

analysis on different days, and the results obtained were noted. The R.S.D % of peak area was 0.4186 and retention time was 1.344 for Ledipasvir. The R.S.D % of peak area was 0.442 and retention time was 0.117 for Sofosbuvir. Statistical evaluation revealed that parameters found were within the acceptance range of R.S. D<2% (Table 8). Thus, the proposed method was found to have a high degree of precision and reproducibility.

System Suitability

To elucidate the system suitability, six repeated injections of a mixed standard solution of Ledipasvir & Sofosbuvir was injected. The parameters including resolution, tailing factor, no. of theoretical plates were mainly used to assess system suitability. Table 9 and Figure 9 showed tailing factor for Ledipasvir & Sofosbuvir was found to be 1.74 and 1.80, respectively. The Theoretical plates per unit for Ledipasvir & Sofosbuvir was found to be 2741 and 2529.7 respectively. The resolution value of Ledipasvir & Sofosbuvir was found to be 6.42. The chromatograms are shown in (Figure 9).

Accuracy

The targeted concentration of 7.2 μg/ml, 9.0 μg/ml and 10.8 μg/ml of Ledipasvir & 32 μg/ml, 40 μg/ml and 48 μg/ml of Sofosbuvir working standards were spiked with Placebo and each injected thrice. The Mean recovery for Ledipasvir & Sofosbuvir was found to be 99.92 % and 99.82 % v respectively. The obtained mean recovery values were present within the acceptance limits of 98 - 102 %. The results are reported in Tables 10 and 11.

Robustness

It was performed changing the HPLC pump flow rate variations $\pm$ 0.2 ml/min and organic composition of the mobile phase $\pm$5%. U.S.P Plate count and U.S.P tailing were found to be 2890.0 and 1.72 for 0.4 ml/min flow rate for Ledipasvir. U.S.P Plate count and U.S.P tailing were found to be 2563.1 and 1.71 for 0.8 ml/min flow rate for Ledipasvir. U.S.P Plate count and U.S.P tailing were found to be 2313.2 and 1.82 for 0.4 ml/min flow rate for Sofosbuvir. U.S.P Plate count and U.S.P tailing were found to be 2684 and 1.74 for 0.8 ml/min flow rate for Sofosbuvir. U.S.P Plate count and U.S.P tailing were found to be 2867 and 1.70 for 5% less organic composition in mobile phase for Ledipasvir. U.S.P Plate count and U.S.P tailing were found to be 2654.4 and 1.61 for 5% more organic composition in mobile phase for Ledipasvir. U.S.P Plate count and U.S.P tailing were found to be 2356 and 1.81 for 5% less organic composition in mobile phase for Sofosbuvir. U.S.P Plate count and U.S.P tailing were found to be 2231.4 and 1.82 for 5% more organic composition in mobile phase for Sofosbuvir. No changes were observed in chromatograms when changing the flow rate and the concentration of the mobile phase. The chromatograms are shown in (Figures 10, 11, 12 and 13) and results are reported in Table 12.

Ruggedness

Mixed standard solution of Ledipasvir & Sofos- buvir was prepared by adding diluent as per test method separately by Analyst – I and Analyst – II. 10 μl of these solutions were injected for six times and the % RSD for the Peak area of six replicate injections were calculated for Analyst – I and Analyst - II. The % R.S.D of Peak area for Analyst I and Analyst II was found to be 0.43 and 0.27 for Ledipasvir. The % R.S.D of Peak area for Analyst I and Analyst II was found to be 0.39 and 0.54 for Sofosbuvir. The % R.S.D of peak area was present within the acceptance criteria of 2%. The low values of the % RSD, indicate the ruggedness of the proposed methods (Table 13).

Estimation of LOD and LOQ

Table 10: Accuracy data for Ledipasvir

%Concentration (at specification level)	Sample Area	Amount added (μg)	Amount found (μg)	% Recovery	Mean recovery
7.2 μg/ml	573624	7.2	7.19	99.86	
	572348	7.2	7.10	98.61	99.11 %
	572564	7.2	7.12	98.88	
9.0 μg/ml	717859	9.0	9.10	101.11	
	717033	9.0	8.98	99.77	100.40 %
	720136	9.0	9.03	100.33	
10.8 μg/ml	860784	10.8	10.81	100.09	
	864542	10.8	10.84	100.37	100.24 %
	863652	10.8	10.83	100.27	
Average recovery	99.92 %				

Table 11: Accuracy data for Sofosbuvir

%Concentration (at specification level)	Sample Area	Amount added (μg)	Amount found (μg)	% Recovery	Mean recovery
32.0 μg/ml	1119764	32	31.76	99.25	
	1127984	32	32.10	100.31	99.39 %
	1112438	32	31.56	98.62	
40.0 μg/ml	1402055	40	40.06	100.15	
	1399863	40	40.02	100.05	100.04 %
	1399038	40	39.97	99.92	
48.0 μg/ml	1679761	48	47.97	99.93	
	1680213	48	48.04	100.08	100.03 %
	1682110	48	48.05	100.10	
Average recovery					99.82 %

Table 12: Results of the Robustness study for Ledipasvir and Sofosbuvir

S.No	Parameters	Ledipasvir		Sofosbuvir	
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing
1	Actual method	2741.0	1.74	2529.6	1.80
2	Changes in the flow rate (0.4 ml/min)	2890.0	1.72	2313.2	1.82
3	Changes in the flow rate (0.8 ml/min)	2563.1	1.71	2684.0	1.74
4	Changes in the Organic phase composition in the mobile phase (5 % less)	2867.0	1.70	2356.0	1.81
5	Changes in the Organic phase composition in the mobile phase (5 % more)	2654.4	1.61	2231.4	1.82

Table 13: Ruggedness data for Ledipasvir and Sofosbuvir

Parameters	% RSD Area- Ledipasvir	% RSD Area- Sofosbuvir
Analyst 1	0.43	0.39
Analyst 2	0.27	0.54

Table 14: Limit of detection and Limit of quantitation data for Ledipasvir and Sofosbuvir

Drug name	Standard deviation(σ)	Slope(s)	LOD(μg)	LOQ(μg)
Ledipasvir	3104.0	49116	0.208	0.631
Sofosbuvir	5464.5	52816	0.341	1.034

Table 15: Results of Degradation Studies for Ledipasvir and Sofosbuvir

S.No	Name	Sample weight	% Assay- Ledipasvir	% Assay- Sofosbuvir	% DEG- Ledipasvir	% DEG- Sofosbuvir
1	Acid	590	88	90	- 12	-10
2	Base	590	89	88	-11	-12
3	Peroxide	590	97	94	- 3	- 6
4	Water	590	85	94	- 15	-6
5	Light	590	96	95	-4	-5

LOD= 3.3xσ/S; LOQ=10Xσ/S

The LOD of Ledipasvir and Sofosbuvir were 0.208 μg/ml and 0.341 μg/ml. The obtained LOD values were very less when equated to the reported method. As per the analytical point of view, the method detects the very least quantity. So when compared to the reported methods, the developed method is most suitable to detected less quantity. The LOQ of Ledipasvir and Sofosbuvir were 0.631 μg/ml and 1.034 μg/ml, respectively. The obtained LOQ values were very less when compared to reported methods. As per the analytical point of view, the method detected the very least quantity. So when compared to the reported methods, the developed method is most suitable to detect less quantity. The results are reported in Table 14.

Degradation studies

Acidic Degradation

The sample solution was prepared with 10 ml of 0.01 M Hydrochloric acid. Reflux under heat at 60°C for one hour. The sample solution was neutralized using 0.01 M Sodium hydroxide and diluted with diluent to make the final concentration as per test method.

Basic Degradation

The sample solution was prepared with 10 ml of 0.1 M Sodium hydroxide. Reflux under heat at 60°C for one hour. The sample solution was neutralized using 0.1 M Hydrochloric acid and diluted with diluent to make the final concentration as per test method.

Neutral Degradation

The sample solution was prepared with10 ml of water. Reflux under heat at 60°C for one hour. The sample solution was diluted with diluent to make the final concentration as per test method.

Oxidative Degradation

The sample solution was prepared with 10 ml of 3 % Hydrogen perox- ide. Reflux under heat at 60°C for one hour. The sample solution was diluted with diluent to make the final concentration as per test method.

Photolytic Degradation

The sample solution for photolytic stability studies was prepared with diluent as per test method. The test solution was exposed to natural sunlight for 8 hours. Results of the degradation studies indicated that the Ledipasvir is slightly not stable in neutral, acidic and basic conditions and stable in oxidative and photolytic. Sofosbuvir is slightly not stable in basic and acidic degradation and stable in neutral, oxidative and photolytic degradation. The results are reported in Table 15.

CONCLUSION

An analytical method was developed and validated by RP-HPLC technique. It consumes less amount of mobile phase, and the required time for analysis is very short. The method was applied for the determination of the potency of the commercial product of Ledipasvir and Sofosbuvir and potency were found within the limit. The results of assay analysis of two drugs from a combined dosage form using this developed method were found to be close to 100 %. The projected method was specific as no interference of excipients was found. The information given in the study will be very useful in quality control, content uniformity test, in-vitro dissolution of the combination of Ledipasvir and Sofosbuvir drug products. The developed method can be efficiently applied for the separation of the drugs from its excipients and its degradation components

in the pharmaceu- tical formulation. It can be used to check rapid and accurate drug quality during stability testing.

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