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A new analytical method for determination of ledipasvir and sofosbuvir in pharmaceutical formulations by HPLC method

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ABSTRACT

A simple, Accurate, precise method was developed for the simultaneous estimation of the Sofosbuvir and Ledipasvir in Tablet dosage form. Chromatogram was run through Std Discovery C8 150 x 4.6 mm, 5μ. Mobile phase containing Buffer 0.1% OPA: Acetonitrile taken in the ratio 60:40 was pumped through column at a flow rate of 1 ml/min. Buffer used in this method was 0.1% OPA buffer. Temperature was maintained at 30°C. Optimized wavelength selected was 260 nm. Retention time of Sofosbuvir and Ledipasvir were found to be 2.367 min and 3.436 min. %RSD of the Sofosbuvir and Ledipasvir were and found to be 0.6 and 0.5 respectively. %Recovery was obtained as 99.61% and 99.80% for Sofosbuvir and Ledipasvir respectively. LOD, LOQ values obtained from regression equations of Sofosbuvir and Ledipasvir were 0.67, 2.02 and 0.70, 2.12 respectively. Regression equation of Sofosbuvir is y = 4266.x + 7700, and y = 4861.x + 2656.of Ledipasvir. Retention times were decreased and run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

Keywords: Sofosbuvir; Ledipasvir; RP-HPLC.

INTRODUCTION

Development of the simple and reproducible analytical methods for estimation of multi component drugs is very important part of quality control and for social awareness which was established in present work.
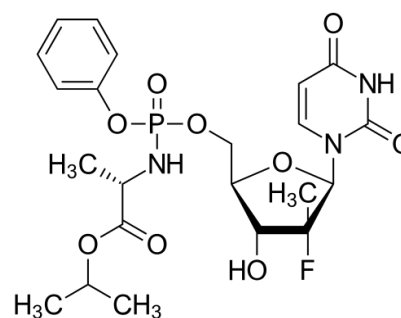

Figure 1: chemical structure of sofosbuvir

Sofosbuvir[1-3] is a prodrug nucleotide analog used as part of combination therapy to treat hepatitis C virus (HCV) infection or to treat co-infection of HIV and HCV. After metabolism to the active antiviral agent 2'-deoxy-2'-α-fluoro-β-C-methyluridine-5'-triphosphate (also known as GS-461203), the triphosphate serves as a defective substrate for the NS5B protein, an RNA-dependent RNA polymerase required for replication of viral RNA. More recently, sofosbuvir has become available as a fixed dose drug combination product with ledipasvir[1-3] (trade name Harvoni

) used for the treatment of chronic Hepatitis C, an infectious liver disease caused by infection with Hepatitis C Virus (HCV). Approved in October 2014 by the FDA, ledipasvir and sofosbuvir are direct-acting antiviral agents indicated for the treatment of HCV genotype 1 with or without cirrhosis.

Ledipasvir, previously known as GS-5885, is an inhibitor of the Hepatitis C Virus (HCV) NS5A protein required for viral RNA replication and assembly of HCV virions. Although its exact mechanism of action is unknown, it is postulated to prevent hyper phosphorylation 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. Ledipasvir is available as a fixed dose drug combination product with sofosbuvir (tradename Harvoni) used for the treatment of chronic Hepatitis C, an infectious liver disease caused by infection with Hepatitis C Virus (HCV). Approved in October 2014 by the FDA,
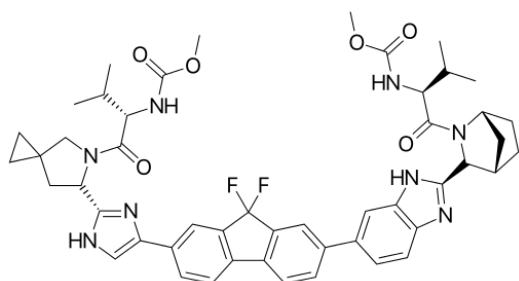

Figure 2: Chemical structure of ledipasvir

MATERIALS AND METHODS

Materials: Sofosbuvir and Ledipasvir pure drugs (API), Combination Sofosbuvir and Ledipasvir tablets (*Harvoni*), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.[3-4]

HPLC method

Instrument: UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Sofosbuvir and Ledipasvir solutions having universal loop injector of injection capacity 20μL[4-6]. The column used was Discovery C18 (4.6 x 250mm, 5μm) at ambient temperature. Different mobile phases were tested in order to find the best conditions, for separating both the drugs simultaneously. Optimised Chromatographic conditions The mobile phase having 60% OPA (0.1%): 40% Acetonitrile was selected because it was found that it ideally resolve the peaks with retention time (RT) 2.380 min and 3.449 min for Sofosbuvir and Ledipasvir respectively[7-8]. Wavelength was selected by scanning all standard drugs over a wide range of wavelength 200nm to 350nm. Both the

components showed reasonably good response at 260 nm.

Methods:

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50

Preparation of Standard stock solutions: Accurately weighed 40mg of Sofosbuvir, 9mg of Ledipasvir and transferred to 25ml & 25ml volumetric flasks and 3/4th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labelled as Standard stock solution. (1600μg/ml of Sofosbuvir and 360μg/ml Ledipasvir)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (160μg/ml of Sofosbuvir and 36μg/ml of Ledipasvir).

Preparation of Sample stock solutions: 5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 50ml volumetric flask, 50ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (800μg/ml of Sofosbuvir and 1800μg/ml of Ledipasvir)

Preparation of Sample working solutions (100% solution): 0.2ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent.(160μg/ml of Sofosbuvir and 36μg/ml of Ledipasvir).

Preparation of buffer:

0.1%OPA Buffer: 1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water.

Typical Chromatogram

Retention times of Sofosbuvir and Ledipasvir were 2.369 min and 3.436 min respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.
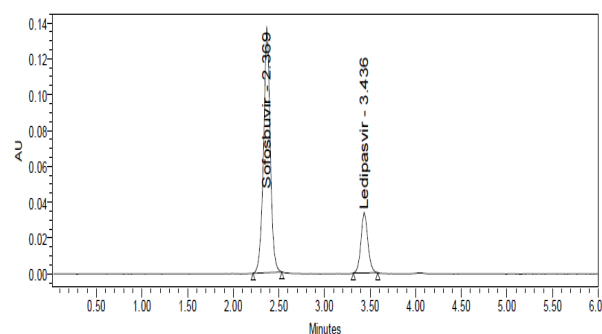

Figure 3: Typical Chromatogram of Sofosbuvir and Ledipasvir

Table 1: System suitability parameters for Sofosbuvir and Ledipasvir

S.no	Sofosbuvir			Ledipasvir			
Inj	RT (min)	USP Plate Count	Tailing	RT (min)	USP Plate Count	Tailing	Resolution
1	2.366	5341	1.08	3.434	9522	1.09	7.5
2	2.367	5497	1.09	3.436	9659	1.09	7.6
3	2.367	5685	1.08	3.436	9776	1.08	7.5
4	2.369	5082	1.04	3.436	9731	1.10	7.5
5	2.369	5104	1.03	3.438	10083	1.09	7.6
6	2.372	5095	1.03	3.447	9852	1.05	7.7

Table 2: Accuracy table of Sofosbuvir and Ledipasvir

% Level	Sofosbuvir			Ledipasvir		
	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery
50%	80	79.779	99.72	18	17.986	99.92
	80	79.543	99.43	18	17.979	99.88
	80	79.306	99.13	18	17.949	99.72
100%	160	159.392	99.62	36	35.902	99.73
	160	159.779	99.86	36	35.915	99.76
	160	159.761	99.85	36	35.889	99.69
150%	240	239.079	99.62	54	53.976	99.95
	240	239.099	99.62	54	53.796	99.62
	240	239.0872	99.62	54	53.959	99.92
Mean %Recovery	99.61%			99.80%		

Linearity: Six linear concentrations of Sofosbuvir (40-240µg/ml) and Ledipasvir (9-54µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Sofosbuvir was y = 4266.x + 7700and of Ledipasvir was y =4861.x + 2656Correlation coefficient obtained was 0.999 for the two drug.

Table 3: Linearity of Sofosbuvir and Ledipasvir

S. NO	Sofosbuvir		Ledipasvir	
	Conc (µg/ml)	Area	Conc (µg/ml)	Area
1	0	0	0	0
2	40	193238	9	48575
3	80	346154	18	92565
4	120	523238	27	135642
5	160	680185	36	174059
6	200	853929	45	219015

Figure 5: calibration curve of ledipasvir

System suitability parameters:

The system suitability parameters were determined by preparing standard solutions of Sofosbuvir (160ppm) and Ledipasvir (36ppm) and 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%.

Specificity: Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision:

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask

Figure 4: Calibration curve of sofosbuvir

and made up with diluent. (160µg/ml of Sofosbuvir and 36µg/ml of Ledipasvir).

Repeatability: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.5% and 0.6% respectively for Sofosbuvir and Ledipasvir. As the limit of Precision was less than "2" the system precision was passed in this method.

Accuracy:

Preparation of 50% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Accuracy: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.61% and 99.80% for Sofosbuvir and Ledipasvir respectively[9-10].

Table 4: Sensitivity table of Sofosbuvir and Ledipasvir

Molecule	LOD	LOQ
Sofosbuvir	0.67	2.02
Ledipasvir	0.70	2.12

Precision: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.5% and 0.6% respectively for Sofosbuvir and Ledipasvir. As the limit of Precision[11] was less than "2", the system precision was passed in this method.

Table 5: System precision table of Sofosbuvir and Ledipasvir

S. No	Area of Sofosbuvir	Area of Ledipasvir
1.	698943	177127
2.	695463	176673
3.	693621	177445
4.	704923	179081
5.	698452	176591
6.	693668	177154
Mean	697512	177345
S.D	4288.8	908.6
%RSD	0.6	0.5

Table 6: Intermediate precision table of Sofosbuvir and Ledipasvir

S. No	Area of Sofosbuvir	Area of Ledipasvir
1.	695241	176978
2.	695200	176075
3.	694259	176873
4.	694723	176902
5.	697754	176596
6.	696181	177083
Mean	695560	176751
S.D	1250.9	368.9
%RSD	0.2	0.2

Robustness: Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

Table 7: Robustness data for Sofosbuvir and Ledipasvir

S.no	Condition	%RSD of Sofosbuvir	%RSD of Ledipasvir
1	Flow rate (-) 0.9ml/min	0.5	0.4
2	Flow rate (+) 1.1ml/min	0.5	0.7
3	Mobile phase (-) 65B:35A	0.5	0.4
4	Mobile phase (+) 55B:45A	0.5	0.5
5	Temperature (-) 25°C	0.3	0.3
6	Temperature (+) 35°C	0.1	0.1

Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus(35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much effected and all the parameters were passed. %RSD was within the limit.

Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus (65B:35A), mobile phase plus (55B:45A), temperature minus (25°C) and temperature plus(35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

LOD sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks

and made up with diluents. From the above solutions 0.1ml each of Sofosbuvir, Ledipasvir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

LOQ sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each of Sofosbuvir, Ledipasvir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

Assay: Radha Kishna Pharmaceuticals, (Hepcvir L)bearing the label claim Sofosbuvir 400mg, Ledipasvir 90mg.Assay was performed with the above formulation. Average % Assay for Sofosbuvir and Ledipasvir obtained was 99.32 and 98.47% respectively.

Table 8: Assay Data of Sofosbuvir

S.no	Standard Area	Sample area	% Assay
1	698943	695241	99.28
2	695463	695200	99.27
3	693621	694259	99.14
4	704923	694723	99.20
5	698452	697754	99.63
6	693668	696181	99.41
Avg	697512	695560	99.32
Stdev	4288.8	1250.9	0.18
%RSD	0.6	0.2	0.18

Table 9: Assay Data of Ledipasvir

S.no	Standard Area	Sample area	% Assay
1	177127	176978	99.59
2	176673	176075	99.09
3	177445	176873	99.53
4	179081	176902	99.55
5	176591	176596	99.38
6	177154	177083	99.65
Avg	177345	176751	99.47
Stdev	908.6	368.9	0.2
%RSD	0.5	0.2	0.2

Degradation studies: Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation.

Oxidation: To 1 ml of stock solution of Sofosbuvir and Ledipasvir, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions we are kept for 30 min at 60⁰c. For HPLC study, there solution was diluted to obtain 160µg/ml&36µg/ml solution and10µlwere injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies: To 1ml of stock solution Sofosbuvir and Ledipasvir, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60⁰c.The

resultant solution was diluted to obtain 160µg/ml & 36µg/ml solution and 10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies: To 1 ml of stock solution Sofosbuvir and Ledipasvir, 1ml of 2N sodium hydroxide was added and refluxed for 30mins at 60⁰c. There sultant solution was diluted to obtain 160µg/ml & 36µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies: The standard drug solution was placed inovenat105°C for1hr to study dry heat degradation. For HPLC study, the resultant solution was diluted to 160µg/ml & 36µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo Stability studies: The photochemical stability of the drug was also studied by exposing the 1600µg/ml Sofosbuvir & 360µg/ml Ledipasvir µg/ml solution to UV Light by keeping the beaker in UV Chamber for 1days or 200 Watt hours/m² in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 160µg/ml & 36µg/ml solutions and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies: Stress testing under neutral conditions was studied by refluxing the drug in water for 1hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to 160µg/ chromatograms were recorded ml&36µg/ml solution and 10µl were injected into the system and the to assess the stability of the sample.

Table 10: Degradation Data of Sofosbuvir

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	4.21	0.051	0.296
2	Alkali	3.96	0.124	0.252
3	Oxidation	3.89	0.159	0.304
4	Thermal	2.61	0.197	0.294
5	UV	1.48	0.133	0.280
6	Water	1.48	0.044	0.287

Table 11: Degradation Data of Bromhexine

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	4.62	0.187	0.320
2	Alkali	4.22	0.162	0.587
3	Oxidation	3.78	0.171	0.328
4	Thermal	2.78	0.197	0.297
5	UV	1.22	0.130	0.296
6	Water	0.90	0.123	0.299

Regarding the pH adjustment in mobile phase for the acid and base degradation studies have movement in retention time of drugs. But due to neutralized acid sample with 2N Base solution and base sample with 2N Acid solution there will be no change in retention time.

CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Sofosbuvir and Ledipasvir in Tablet dosage form. Retention time of Sofosbuvir and Ledipasvir were found to be 2.367 min and 3.436 min. %RSD of the Sofosbuvir and Ledipasvir were and found to be 0.6 and 0.5 respectively. %Recovery was obtained as 99.61% and 99.80% for Sofosbuvir and Ledipasvir respectively. LOD, LOQ values obtained from regression equations of Sofosbuvir and Ledipasvir were 0.67, 2.02 and 0.70, 2.12 respectively. Regression equation of Sofosbuvir is $y = 4266.x + 7700$, and $y = 4861.x + 2656$.of Ledipasvir. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

ACKNOWLEDGEMENT

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