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R E S E A R C H A R T I C L E

Stability Indicating RP-HPLC Method Development and Validation for the Simultaneous Estimation of Ledipasvir and Sofosbuvir in Bulk and Pharmaceutical Dosage Form

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A B S T R A C T

A simple, Accurate, precise method was developed for the simultaneous estimation of the Ledipasvir and Sofosbuvir in bulk and its Tablet dosage form. Chromatogram was run through Std Kromosil C18 (150 x 4.6 mm, 5µ) column. Mobile phase containing Buffer 0.01N KH_2PO_4: Acetonitrile taken in the ratio 55:45 was pumped through column at a flow rate of 1.0 ml/min. pH 5.4 adjust with orthophosphoric acid. Temperature was maintained at 30°C. Optimized wavelength selected was 220.0 nm. Retention time of Ledipasvir and Sofosbuvir were found to 2.402 min and 3.526 min. Linearity concentrations forLedipasvir and Sofosbuvir 2.25-13.5µg/ml and 10-60 µg/ml. %Recovery was obtained as 100.28% and 100.25% for Ledipasvir and Sofosbuvir respectively. %RSD of the Ledipasvir and Sofosbuvir were and found to be 0.4 and 0.7 respectively. LOD, LOQ values obtained from regression equations of Ledipasvir and Sofosbuvir were 0.05, 0.16 and 0.41, 1.23 respectively. 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: Ledipasvir, Sofosbuvir, RP-HPLC

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

Ledipasvir chemically known as **:**(2S)-1-[(6S)-6-[5-(9,9-difluoro-7-{2-[(1R,3S,4S)-2-[(2S)-2 {[hydroxyl (methoxy) methylidene]amino}-3-methylbutanoyl]-2-azabicyclo [2.2.1] heptan-3-yl]-1H-1,3-benzodiazol-6-yl}-9H-fluoren-2-yl)-1H-imidazol-2-yl]-5-azaspiro[2.4]heptan-5-yl]-2 {[hydroxyl (methoxy) methyl idene]amino}-3-methylbutan-1-one[1.2]. It 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 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[3].

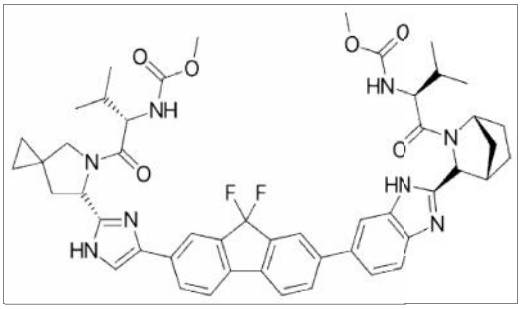

Fig 1: Structure of Ledipasvir

Sofosbuvir chemically known as propan-2-yl (2S)-2-{[(S)-{[(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[4]. It is nucleotide analog inhibitor, which specifically inhibits HCV NS5B polymerase. Sofosbuvir prevents HCV viral replication by binding to the two Mg2+ ions present in HCV NS5B polymerase's GDD active site motif. It is used to treat chronic hepatitis C virus (HCV) infected patients with HCV genoptype 1,2,3, or 4, and to treat HCV and HIV co-infected patients[5,6].

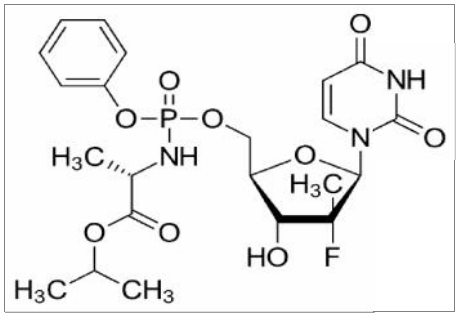

Fig 2: Structure of Sofosbuvir

Literature survey reveals that few analytical methods have been reported for the simultaneous estimation of Ledipasvir and Sofosbuvir Ledipasvir and Sofosbuvir in single analyte and combined dosage form7-13. Therefore an attempt has been made to develop simple, precise, accurate and cost effective RP-HPLC method was developed for the simultaneous estimation of Ledipasvir and Sofosbuvir in bulk and its dosage form.

2. Materials and Methods

Materials:

In this study using chemicals and reagents are Ledipasvir and Sofosbuvir pure drugs (API), Combination tablets of Ledipasvir and Sofosbuvir (Harvoni), Distilled water, Acetonitrile, Phosphate buffer, , Methanol, Potassium dihydrogen ortho phosphate buffer(KH_2PO_4), Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

Instrument:

Liquid chromatographic system was made up of WATERS HPLC 2695 equipped with quaternary pumps, Photo Diode Array detector and Auto sampler integrated with Empower 2 Software. The UV Spectra was monitored at 220.0 nm.

Chromatographic conditions:

Table 1: Chromatographic Conditions

Parameters	Description
Mobile phase	0.01N KH_2PO_4:Acetonitrile (55:45%v/v)
Flow rate	1.0 ml/min
Column	Kromosil C18 (4.6 x 150mm, 5µm)
Detector wavelength	220 nm
Column temperature	30°C
Injection volume	10µL
Run time	6 min
Diluent	Water and Acetonitrile in the ratio 50:50

Preparation of Mobile phase:

The mobile phase was prepared by taking 55% 0.01N KH_2PO_4buffer and 45% Acetonitrile. It was filtered through 0.45µm membrane filter and degassed under ultrasonic bath prior use. The mobile phase was pumped through the column to stabilize the column.

Preparation of Standard solution:

Accurately weighed 4.5 mg of Ledipasvir, 20 mg of Sofosbuvir and transferred to 50ml volumetric flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution (90µg/ml of Ledipasvir and 400µg/ml Sofosbuvir).1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent (9µg/ml of Ledipasvir and 40µg/ml of Sofosbuvir).

Preparation of Sample solution:

5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 100ml 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 (900µg/ml of Ledipasvir and 4000µg/ml of Sofosbuvir). 0.11ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent(9µg/ml of Ledipasvir and 40µg/ml of Sofosbuvir).

3. Results and Discussion

Method Validation

Method validation was done for the according to ICH guidelines (Q_2R_1).Validation parameters like specificity, linearity, accuracy, precision, robustness and system suitability.

System suitability:

The system suitability parameters were determined by preparing standard solutions of Ledipasvir (9ppm) and Sofosbuvir (40ppm) 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%. Results were shown in table 1.

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. Results were shown in table 2 and fig 3,4.

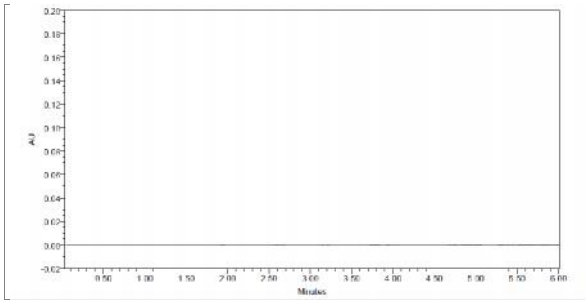

Fig 3: Chromatogram for blank

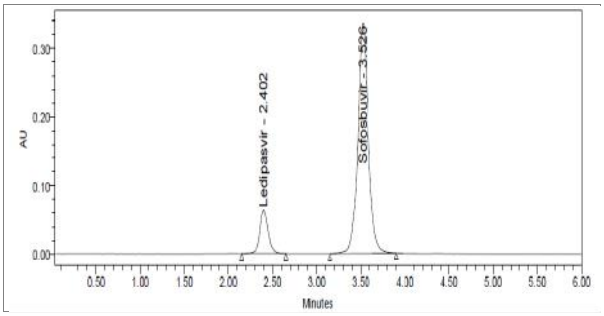

Fig 4: Standard Chromatogram

Linearity:

Solution of the drug at six different concentrations was analyzed and calibration curve was constructed by plotting mean response factor against the respective concentration. The linearity data was shown in table 3 and fig 5,6.

Preparation of 25% Standard solution: 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (2.25µg/ml of Ledipasvir and 10µg/ml of Sofosbuvir).

Preparation of 50% Standard solution: 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (4.5µg/ml of Ledipasvir and20µg/ml of Sofosbuvir)

Preparation of 75% Standard solution: 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (6.75µg/ml of Ledipasvir and 30µg/ml of Sofosbuvir).

Preparation of 100% Standard solution: 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (9µg/ml of Ledipasvir and 40µg/ml of Sofosbuvir)

Preparation of 125% Standard solution: 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (11.25µg/ml of Ledipasvir and 50µg/ml of Sofosbuvir)

Preparation of 150% Standard solution: 1.5ml each from two standard stock solutions was pipettede out and made up to 10ml (13.5µg/ml of Ledipasvir and 60µg/ml of Sofosbuvir).

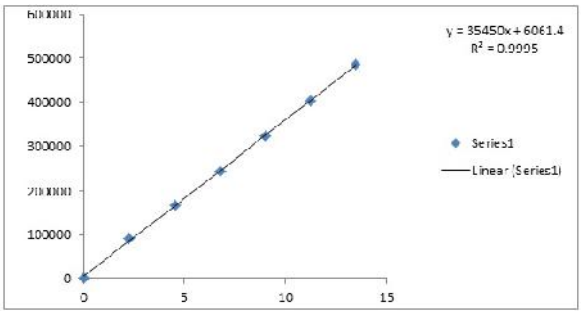

Fig 5: Calibration curve of Ledipasvir

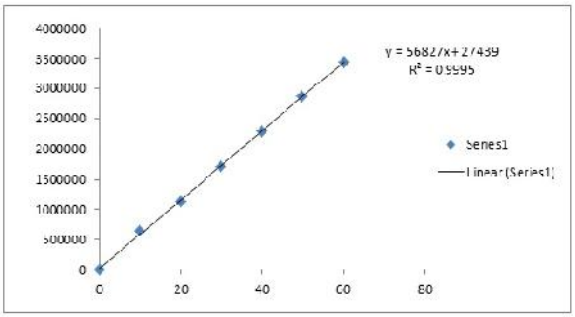

Fig 6: Calibration curve of Sofosbuvir

Precision:

In precision studies Repeatability (intraday) and intermediate precision was carried. The % RSD was found to be lessthan 2%. The results were shown in table 4.

Accuracy: Recovery assessment was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels in 50%, 100% and 150% to the pre analyzed sample formulation. Then calculate the % recovery and results were tabulated in table 5.

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.

LOD & LOQ:

Preparation of sample solution: 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 Ledipasvir and Sofosbuvir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent. Results were shown in table 6.

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. 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 affected and all the parameters were passed. %RSD was within the limit. Results were shown in table 7.

Degradation studies:

To understand the degradation behavior, degradation studies performed. Prepared samples were employed for acid, base, peroxide, water, UV and thermal conditions. Results were shown in table 8.

Table 2: System suitability parameters for Ledipasvir and Sofosbuvir

S no	Ledipasvir			Sofosbuvir			USP
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	Resolution
1	2.402	3332	1.08	3.525	3.525	1.05	5.7
2	2.403	3161	1.05	3.526	3.526	1.05	5.9
3	2.404	3185	1.08	3.526	3.526	1.06	5.8
4	2.404	3220	1.07	3.529	3.529	1.05	5.7
5	2.408	3249	1.06	3.529	3.529	1.05	5.8
6	2.408	3148	1.08	3.533	3.533	1.06	5.8

Table 3: Results for Specificity

S.No	Drug		Observation
1	Blank		Nill
2	Placebo		Nill
3	Standard	Ledipasvir	2.402 min
		Sofosbuvir	3.526 min

Table 4: Linearity Results

Ledipasvir		Sofosbuvir	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
2.25	92597	10	654389
4.5	167714	20	1146923
6.75	244720	30	1719648
9	323447	40	2289104
11.25	404070	50	2868980
13.5	484887	60	3446759

Table 5: Results for Precision

	Ledipasvir		Sofosbuvir	
S.no	**Intra day**	**Inter day**	**Intra day**	**Inter day**
1	322166	313366	2236268	2142436
2	323773	314443	2259241	2167642
3	324070	315593	2229620	2108656
4	325152	315682	2210507	2169510
5	324088	310605	2230541	2138675
6	325757	311176	2237110	2140013
Avg	324168	313478	2233881	2144489
STD	1238.8	2183.0	15715.7	22379.3
%RSD	0.4	0.7	0.7	1.0

Table 6: Results for accuracy

Sample	Spike level	Amount added (µg/ml)	Amount Recovered (µg/ml)	Recovery (%)	%Mean Recovery

Ledipasvir	50%	4.5	4.51	100.25	100.27%
	100%	9	9.04	100.55	
	150%	13.5	13.50	100.03	
Sofosbuvir	50%	20	20.03	100.18	100.24%
	100%	40	40.16	100.42	
	150%	60	60.08	100.14	

Table 7: Results for LOD & LOQ

Molecule	LOD	LOQ
Ledipasvir	0.05	0.16
Sofosbuvir	0.41	1.23

Table 8: Robustness data for Ledipasvir and Sofosbuvir

S.no	Condition	%RSD of Ledipasvir	%RSD of Sofosbuvir
1	Flow rate (-) 0.9ml/min	0.4	1.1
2	Flow rate (+) 1.1ml/min	0.8	0.3
3	Mobile phase (-) 50:50A	1.6	0.2
4	Mobile phase (+) 60B:40A	0.5	0.7
5	Temperature (-) 25°C	0.5	0.2
6	Temperature (+) 35°C	0.9	1.3

Table 9: Results for Degradation Study

Type of degradation	Ledipasvir			Sofosbuvir		
	Area	% Recovered	% Degraded	Area	% Recovered	% Degraded
Acid	309039	95.30	4.70	2137002	95.15	4.85
Base	314890	97.10	2.90	2187782	97.41	2.59
Peroxide	318917	98.35	1.65	2209597	98.39	1.61
Thermal	321331	99.09	0.91	2225389	99.09	0.91
UV	322559	99.47	0.53	2229412	99.27	0.73
Water	322219	99.36	0.64	2233027	99.43	0.57

4. Conclusion

A simple, Accurate, precise method was developed for the simultaneous estimation of the Ledipasvir and Sofosbuvir in Tablet dosage form. Retention time of Ledipasvir and Sofosbuvir were found to be 2.402 min and 3.526 %RSD of the Ledipasvir and Sofosbuvir were and found to be 0.4 and 0.7 respectively. %Recovery was obtained as 100.28% and 100.25% for Ledipasvir and Sofosbuvir respectively. LOD, LOQ values obtained from regression equations of Ledipasvir and Sofosbuvir were 0.05, 0.16 and 0.41, 1.23 respectively. Regression equation of Ledipasvir is y = 35450x + 6061. y = 56827x + 27439 of Sofosbuvir. 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.

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