ISSN- 2230 - 7346

Journal of Global Trends in Pharmaceutical Sciences

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF LEDIPASVIR AND SOFOSBUVIR IN FIXED DOSAGE FORM

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ARTICLE INFO

Key Words

Sofosbuvir,
Ledipasvir,
RP-HPLC,
QC,
Validation

Access this article online
Website:
https://www.jgtps.com/
Quick Response Code:

ABSTRACT

Ledipasvir and Sofosbuvir Combination have been approved for the treatment of Chronic Hepatitis C Viral Infection. Here an accurate, valid, elementary and error free reverse phase liquid chromatography strategy was developed for the quantitation of Ledipasvir and Sofosbuvir in its bulk form as well as in fixed dosage form. Effective chromatographic separation of Ledipasvir and Sofosbuvir was achieved by using Kromasil C-18(250×4.6 mm, 5µm) column using Phosphate buffer (pH 3.5) and Methanol in proportion of 45:55 v/v. The Mobile phase was siphoned at a flow rate of 1.0 mL min^{-1} with a column temperature of 35^0 C and detection wavelength was carried out at 259 nm. Retention time was found to be 3.294 min for Sofosbuvir and 4.630 min for Ledipasvir. The dimensionality of Sofosbuvir and Ledipasvir was in linear range with a parametric static of 0.999 and 0.999. Method Validation was carried out in terms of Specificity, Linearity, Precision, Accuracy, LOD, LOQ as per ICH Guidelines. Results obtained from the validation studies shows that the developed method can be useful in the quality control analysis of bulk and pharmaceutical formulations of Ledipasvir and Sofosbuvir.

INTRODUCTION

Chronic Hepatitis C affects large number of people worldwide[1]. It is a viral infection that attacks the liver and leads to inflammation[2]. Chronic Hepatitis C treatment has continued to evolve and interferon free, oral treatment with combination of Sofosbuvir and Ledipasvir which are two direct acting anti-viral agents[3]. The Oral administration of Sofosbuvir and Ledipasvir combination was well tolerated and suppresses the effect of predictive factors of Chronic Hepatitis[4]. Ledipasvir is anti-viral drug chemically (2S)-1-[(6S)-6-[5-(9,9-difluoro-7-{2-[1R,3S,4S)-2-[(2S)-2 {[hydroxyl (methoxy) methydene] amino}- 3-methylbutanoyl] -2-azabicyclo [2.2.1] heptan -3-yl]-1H-1,3-benzodiazol-6-yl} - 9H-Fluoren-2-yl)-1 H-imidazole-2-yl]-5-azaspirol [2.4] heptan-5-yl]-2-{[hydroxyl(methoxy)methylidene]amino}-3-methylbutan-1-one having formula $C_{49}H_{54}F_2N_80_6$ and relative molecular mass of 889.00 g/mol[5]. It acts by inhibiting NS5A protein which is mainly responsible for viral

RNA Replication[6]. The chemical structure of Ledipasvir is exhibited in figure 1. Sofosbuvir is [1-4] isopropyl(2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxoprrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetrahydrofuran-2-yl] methoxy-phenoxyphosphoryl]amino]propanoate having formula $C_{22}H_{29}FN_3O_9P$ and relative molecular mass of 529.45 g/mol[5]. It acts by inhibiting NS5B polymerase used in the treatment of hepatitis C[7]. The chemical structure of Sofosbuvir is exhibited in figure 2.

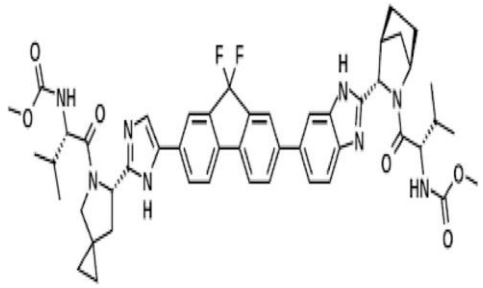

Figure 1: Chemical Structure of Ledipasvir

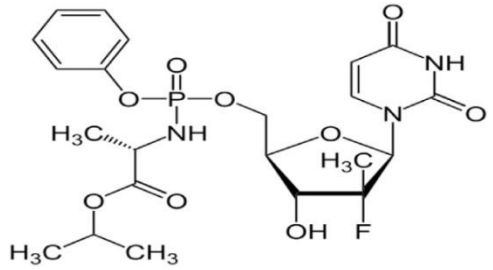

Figure 2: Chemical Structure of Sofosbuvir

The pharmaceutical dosage form having combination of Ledipasvir and Sofosbuvir provides a new method of treatment effectively for several people suffering from chronic hepatitis C Virus Infection[8]. The present strategy focused on isocratic high performance liquid chromatography method for the estimation of Ledipasvir and Sofosbuvir. After performing extensive literature review an attempt was made to develop a smooth plain sailing, unambiguous, valid, speedy and decisive strategy for the estimation of Ledipasvir and Sofosbuvir in fixed dosage form[9-13].

MATERIALS AND METHODS

Chemicals and Reagents: Ledipasvir and Sofosbuvir were obtained as a gift samples from Nutech Biosciences Pvt Ltd, Hyderabad, India certified to contain acceptable purity limit and were used without any refinement. HPLC Grade solvents were used in chromatographic separation of Ledipasvir and Sofosbuvir and 0.45µ membrane filter was obtained from Millipore. Ledifos Tablets (label claim 90 mg of Ledipasvir and 400 mg of Sofosbuvir) of Hetero Health care obtained from local pharmacy were used in analysis

Instrument: Liquid chromatography system used was Waters Alliance having empower software for processing the data with 2695 separation module equipped with PDA detector with universal loop injector of injection capacity 20µl. The analytical column that was selected for ideal separation was Kromasil C-18(250×4.6 mm, packed with particle size of 5µm) column. Several solvents in different proportions were tested in order to determine the suitable conditions for the separation of drugs

Optimized Chromatographic conditions: The mobile phase selected was a mixture of Phosphate buffer (pH 3.5) and Methanol in proportion of 45:55 % v/v at a flow rate of 1.0 mL/min as it resolves the height with retention times of 3.294 min and 4.630 min for Ledipasvir respectively. Standard drug solutions were scanned over a range from 200 to 400 nm and detection was carried out at 259 nm as both the drugs showed reasonably good response with characteristic UV spectrum as exhibited in Figure 3.

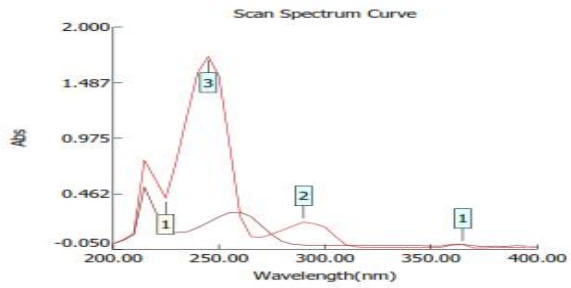

Figure 3: Isobestic point of Ledipasvir and Sofosbuvir

Buffer Preparation: Accurately weighed and transferred a quantity equivalent to 1.732 g of Potassium Dihydrogen Ortho phosphate into a 500 ml clean and dried volumetric flask. Into the above volumetric flask 500 ml of HPLC water was added and subjected to sonication for three minutes to dissolve phosphate buffer completely and the volume was made up to the mark with same solvent and pH was adjusted to

3.5 by adding few drops of Orthophosphoric acid.

Mobile Phase Preparation: Accurately measured 450 milliliter of Phosphate buffer (pH 3.5) (45%) and 550 ml of Methanol (55%) were mixed and subjected to sonication in inaudible water tub for five minutes and after sonication the mobile phase was filtered using 0.45µ membrane filter under vacuum before its use. Diluent: Mobile phase was used as diluent.

Preparation of Standard Solution: Accurately weighed and transferred a quantity which is equivalent to 6 mg of Ledipasvir and 15 mg of Sofosbuvir working standard into a 10 ml clean and dry volumetric flask and add 7 ml of diluent. The above solution was sonicated for few minutes until the drug dissolves and volume was made up to the mark with the same solvent. Further pipette out 1 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent. The chromatogram of standard solution was exhibited in Figure 4.

Preparation of Sample solution: Accurately 10 tablets were taken and crushed in mortar and pestle and transferred an amount equivalent to 6 mg of Ledipasvir and 15 mg of Sofosbuvir sample into a 10 ml clean dry volumetric flask and add 7 ml of diluent. The above solution was sonicated for few minutes until the drug dissolves and volume was made up to the mark with the same solvent. Further pipette out 1 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent. Inject 20µL of the standard, sample into the chromatographic system and measure the peak areas for Sofosbuvir and Ledipasvir and calculate the % Assay by using the formulae. The chromatogram of sample solution was exhibited in Figure 5.

RESULTS AND DISCUSSION

Method Validation: The proposed method was validated for specificity, accuracy, and precision, limit of detection, limit of quantitation as well as robustness of the method as per ICH guidelines. Replicate injections of the standard and sample were used to carry out all the studies.

Specificity: According to ICH Q2(R1) specificity is defined as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present which may be impurities and other products and was verified by injecting blank, standard and sample and was found that no interference from the excipients of Formulation. Chromatograms of blank and placebo are exhibited in Figure 6 and 7.

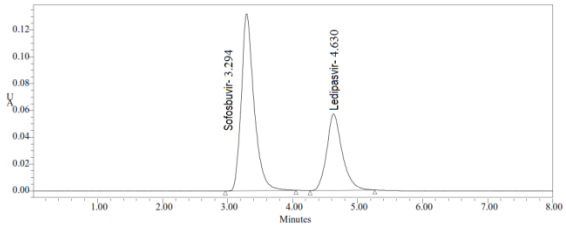

Figure 4: Standard Chromatogram of Ledipasvir and Sofosbuvir

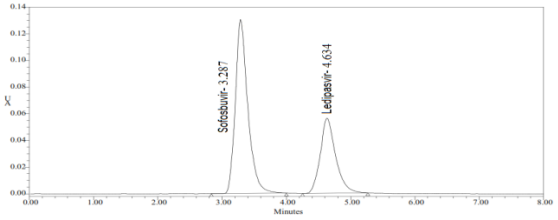

Figure 5: Sample Chromatogram of Ledipasvir and Sofosbuvir

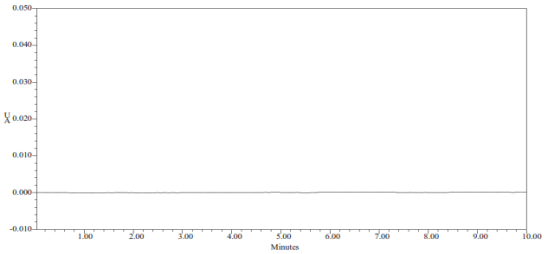

Figure 6: Chromatogram of Blank

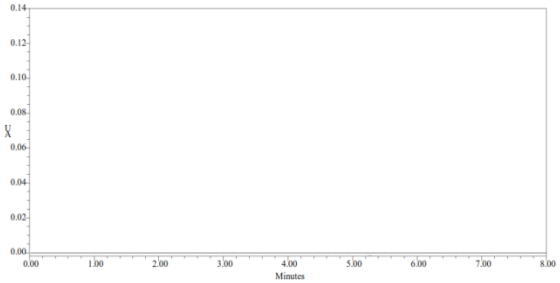

Figure 7: Chromatogram of Placebo

Linearity: Linearity was determined between the concentration ranges of 5-25 µg/ml for Sofosbuvir and 2-10 µg/ml for Ledipasvir. Injection was done twice for each concentration of Sofosbuvir and Ledipasvir. The correlation coefficient value was found to be 0.999 for Sofosbuvir and Ledipasvir. Linearity Results were shown in Table 1 and Calibration graphs were shown in figure 8 and 9.

S. No.	Sofosbuvir		Ledipasvir	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	5	668029	2	293657
2	10	1247781	4	557449
3	15	1944421	6	798552
4	20	2491191	8	1111601
5	25	3230791	10	1395268

Table 1: Linearity Values of Sofosbuvir and Ledipasvir

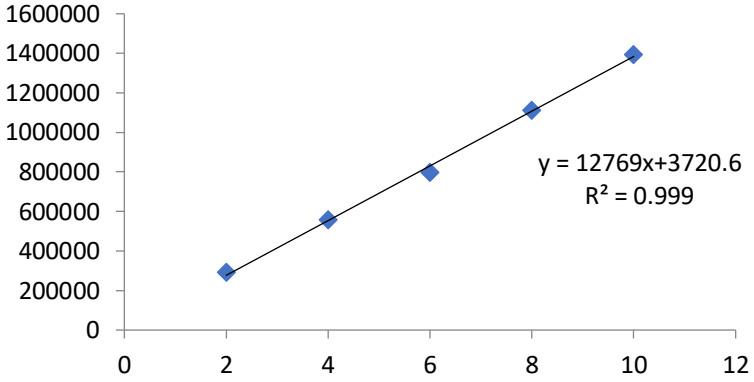

Fig 9: Calibration Graph of Sofosbuvir

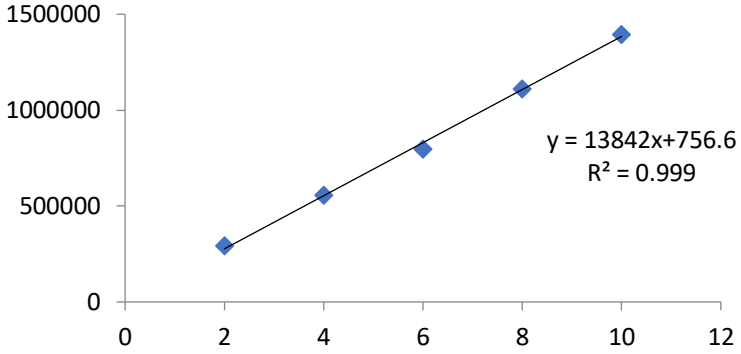

Fig 10: Calibration Graph of Ledipasvir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	% Mean Recovery
50%	7.5	7.39	98.55	99.17%
	7.5	7.40	98.72	
	7.5	7.52	100.33	
100%	15	14.931	99.54	
	15	15.018	100.12	
	15	15.076	100.51	
150%	22.5	22.108	98.26	
	22.5	22.104	98.24	
	22.5	22.101	98.23	

Table 2: Accuracy Report of Sofosbuvir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	% Mean Recovery
50%	3	2.968	98.96	99.83%
	3	3.021	100.72	
	3	3.019	100.65	
100%	6	6.046	100.77	
	6	6.036	100.61	
	6	6.043	100.72	
150%	9	8.923	99.15	
	9	8.842	98.25	
	9	8.875	98.62	

Table 3: Accuracy Report of Ledipasvir

S. No	Area of Sofosbuvir	Area of Ledipasvir
1.	1944421	798552
2.	1943452	798672
3.	1944521	799456
4.	1945397	798662
5.	1944425	798561
6.	1944731	798565
Mean	1944492	798745
S.D	7972.9	5034.7
%RSD	0.3	0.4

Table 4: Precision report of Sofosbuvir and Ledipasvir

Compound	LOD	LOQ
Sofosbuvir	0.24	0.73
Ledipasvir	0.06	0.19

Table 5: LOD & LOQ Values of Sofosbuvir and Ledipasvir

S.no	Condition	%RSD of Sofosbuvir	%RSD of Ledipasvir
1	Flow rate (-) 0.9ml/min	0.4	0.5
2	Flow rate (+) 1.1ml/min	0.6	1.1
3	Mobile phase (-)	0.4	1.8
4	Mobile phase (+)	0.4	0.3
5	Temperature (-)	0.5	0.2
6	Temperature (+)	0.4	0.4

Table 6: Robustness Report of Sofosbuvir and Ledipasvir

Accuracy: The accuracy of an approach is the measurement of intimacy with respect to actuality worth for the sample and was determined by preparing concentration levels of 50%, 100%, 150% and was injected thrice into the chromatographic system and percentage recovery was calculated. The results are tabulated in Table 2 and Table 3.

Precision: Precision of an analytical strategy was expressed by closeness of agreement between a series of measurements obtained when multiple sampling of homogenous sample under the prescribed conditions within the same day and inter day. For precision six repeated injections of standard and sample were made and Percentage Relative Standard Deviation of each study was calculated and was found to be less than 2 showing the strategy was precise and the results were shown in Table 4.

Limit of Detection and Limit of Quantitation

Limit of Detection is the lowest amount of analyte in sample which can be detected but not quantitated and Limit of Quantitation is the lowest amount of analyte in the sample that can be quantitatively determined and were calculated by using the formula LOD = 3.3 *σ / s, LOQ = 10 *σ/S Where, σ = Standard deviation of the response S = Slope of the calibration curve. The results of LOD and LOQ were tabulated in Table 5

Robustness: Robustness study was carried out by performing the flow rate variations from 0.9 mL min^{-1} to 1.1 mL min^{-1} and changes in mobile phase composition ranging from more organic phase to less organic phase ratio. The proposed strategy was found to be robust only in less flow and also by change in composition of mobile phase±5%. Ledipasvir and Sofosbuvir standard and samples were injected by changing the conditions of chromatography and no significant difference in tailing factor and Plate Count was observed and results are tabulated in Table 6.

Recovery studies: Standard addition method is performed at 50,100,150 % levels for Ledipasvir and Sofosbuvir and interference of formulation additives was tested. The Recovery was calculated based on amount of Drug found and was found to be in the range of 98-102%.

CONCLUSION

The Validated Chromatographic Strategy was found to be accurate, simple and decisive for the quantitative estimation of Ledipasvir and Sofosbuvir in bulk and fixed dosage form. Different trials were carried out to determine the optimized chromatographic conditions and initial attempt was performed by utilizing low proportion of organic solvents for the elution of compounds by reducing retention time of the compounds which made the strategy economical. The proposed method is easy, speedy and measurably substantial. During the analysis of drug no interfering peak was found within the chromatogram indicating that there is no excipient interference. Hence this method can be employed for routine quality control analysis of Ledipasvir and Sofosbuvir samples.

Acknowledgement: Authors are very thankful to Nutech Biosciences Pvt Ltd for providing the required facilities to carry out the research work.

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