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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF SOFOSBUVIR AND LEDISPAVIR IN BULK AND TABLET DOSAGE FORM

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ABSTRACT: The day-by-day new combinations drugs are being launched in market. Then the multiple therapeutic agents which acts at diverse sites are used in the management of various diseases and disorders are done. Thus, it is necessary to develop methods for analysis with the help of number of analytical methods which are available for the estimation of the drugs in combination. The analyst was estimating the rapid, selective, specific, simple, RP- HPLC method is developed and validated for simultaneous estimation of Sofosbuvir and Ledipasvir in pharmaceutical tablet dosage form. RP-HPLC method was performed on the Intersil-ODS HPLC System equipped with SP930 D HPLC pump and dual wavelength UV-VIS detector and C18 column (250mm × 4.6mm, 5µm), using the mobile phase (Methanol: Water 60:40 v/v) pH 3.0 with 0.05% acidic acid at a flow rate of 1.0ml/min, injection volume 20µl and UV detection at 254nm. This method is validated according to BP, USP and ICH requirements for new methods, which include accuracy, precision, robustness, ruggedness, lod, loq, linearity and range. Linear relationships were obtained in the ranges of 20-80µg/ml and 20-80µg/ml with correlation coefficients of 0.9991 and 0.9994 at Rt value of 3.94min and 2.86min for Sofosbuvir and Ledipasvir respectively. According to ICH guidelines the developed method was validated. The proposed method can be used for estimation of these drugs in combined pharmaceutical dosage forms.

KEYWORDS

Sofosbuvir and Ledipasvir, RP-HPLC, Development and Validation.

INTRODUCTION

Sofosbuvir

The chemical name is Isopropyl (2S)-2- [[[(2R, 3R, 4R, 5R) -5-(2, 4-dioxopyrimidin-1-yl) -4-fluoro-3- hydroxy-4-methylfuran-2-l] methoxy phenoxy phosphoryl] amino] propionate. It has a molecular formula of C22H29FN3O9P and a molecular weight of 529.453gm/mol. Generously soluble in methanol, dimethyl form amide (DMF) and a little soluble in water. It has the structural formula shown in [Figure No.1]. Ledipasvir The chemical name is (2S)-1-[(6S)-6-[5-(9, 9-difluoro-7-{2-[(1R, 3S, 4S)-2-[(2S)-2- {[hydroxyl (methoxy) methylidene] amino}-3- methylbenzoyl]-2-azabicyclo [2.2.1] heptan-3-yl]- 1H-1, 3-benzodiazol-6-yl}-9H-fluoren-2-yl)-1H- imidazole-2-yl]-5-azaspiro[2.4] heptan -5-yl] -2-{[hydroxyl methoxy) methylidene] amino} -3- methylbutan-1-one. It has a molecular formula of C49H54F2N8O6 and molecular weight of 889.00gm/mol. Liberally soluble in methanol, dimethylformamide (DMF) and to some extent soluble in water. It has the structural formula shown in [Figure No.2]. The literature survey reveals that there are a very few HPLC3-7 methods available for the determination individual Sofosbuvir and Ledipasvir in bulk and dosage forms. There were few analytical methods reported for simultaneous estimation Sofosbuvir and Ledipasvir in bulk and their

combined dosage forms8-11. Hence an author made an attempt to develop specific, sensitive, accurate and precise RP-HPLC method for simultaneous estimation of these drugs with isocratic elution mode.

MATERIAL AND METHODS

Instrumentation

HPLC experiment is carry out on a Waters Alliance 2690 separation module, with waters 2996 photodiode array detector using Auto sampler. Data gathering and processing was prepared using EMPOWER PDA 2 software. The analytical column used for the separation was Inertsil- C18 ODS, 250mm 4.6mm, 5mm Column, Other equipment's used were sonicator (FAST CLEAN), Electronic Balance (SARTORIOUS).

Preparation of solutions

Mobile phase

Mix Degassed Methanol and Water in the ratio of 60:40v/v. Filter through 0.45m. Membrane filter paper.

Diluents

Mobile phase is used as diluents.

Preparation of stock solution

Reference solution

The solution was prepared by dissolving 20.0mg of accurately weighed Ledipasvir and 25.0mg Sofosbuvir in Mobile phase, in two 100.0mL volumetric flasks separately and sonicate for 20min. From the above solutions take 10.0mL from each solution into a 50.0mL volumetric flask and then makeup with mobile phase and sonicate for 10min.

Preparation of working standard solution:

The stock solutions equivalent to 20ppm to 80ppm with respect to both drugs were prepared in combination of Ledipasvir and Sofosbuvir above, sonicated and filtered through 0.45µ membrane.

Chromatographic conditions

HPLC experiment is carry out on a Waters Alliance 2690 separation module, with waters 2996 photodiode array detector using Auto sampler. Data gathering and processing was prepared using EMPOWER PDA 2 software. The analytical column used for the separation was Inertsil-C18, 250mm, 4.6mm, 5mm Column, BDS Column. Other equipment's used were sonicator (FAST CLEAN), Electronic balance (SARTORIOUS).

Selection of wavelength

Absorbance maxima of Sofosbuvir and Ledipasvir were detected at 227nm (λ1) and 264nm (λ2), respectively. Both the spectra's were overlapped at 254nm. Both the drugs showed linearity with absorbance in the range 20-80µg/ml and 20-80µg/ml respectively, when measured at 227nm and 264nm. Calibration curves were plotted from the absorbance values at these wavelengths.

METHOD DEVELOPMENT

To saturate the column, mobile phase was pump for about 20min thus to get the base line corrected. Standard calibration lines were constructed for each drug. A series of aliquots were prepared from the above stock solutions using diluents to get the concentrations 20- 80µg/ml for Ledipasvir, 20- 80µg/ml for Sofosbuvir. Inject each concentration 6 times in to the chromato-graphic system. Every time peak area and retention time were recorded separately for both the drugs. Calibration curves are constructing as by taking average peak area on Y-axis and concentration on X-axis individually for both drugs. Regression equations were calculated from the calibration curves; these regression equations are used to calculate drug substance in formulation.

Estimation of Sofosbuvir and Ledipasvir in tablet dosage forms

20 tablets were taken and their average weight was determined; they were crushed to fine powder. The powder equivalent to 20mg Sofosbuvir and 5mg Ledipasvir was weighed and dissolved in 20ml of mobile phase with the aid of ultra-sonication for 20min. The content was diluted to 50 with mobile phase to furnish a stock test solution. The stock solution is filtered through a 0.5µm nylon syringe filter and 10ml of filtrate was diluted in to a 50ml volumetric flask to give a test solution containing 80µg/ml Sofosbuvir and 20µg/ml Ledipasvir. This solution was estimated by above developed method. The assay procedure was repeated 6 times (n=6) the drug content was estimated using above calculated regression equation.

MEHOD VALIDATION

The described method has been validated for linearity, accuracy, limit of detection, precision, and robustness, as per the ICH guidelines.

Linearity

The linearity of the method was determined by preparing six different concentrations of both Ledipasvir and Sofosbuvir in the concentration range of 20- 80µg/ml and 20-80 µg/ml. Each solution was prepared in triplicate. The calibration curves were obtained by plotting peak area versus concentration. Linearity was checking over the same concentration range on three successive days and the results obtained.

Accuracy, as recovery

The accuracy of the method was determined at three different concentration levels 50%, 100%, and 150% by spike known quantities of the drug analyte and % of recovery were calculated.

Precision

Method precision (repeatability)

The method precision is determined by inject six working standard solutions and six sample injections. The areas of all the injections were taken and standard deviation, % relative standard deviation (RSD), % assay were calculated.

Intermediate precision

The intermediate precision was determined by inject six working standard solutions and six sample injections on different days by different operator or by different instruments. The areas of all the injections were taken and standard deviation, % relative standard deviation (RSD), % assay was calculated.

LOD and LOQ

LOD

It is lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value under the stated experimental conclusions. The detection limit is usually expressed as the concentration of analyte. The standard deviation and response of the slope and the results obtained.

LOQ

The Quantitation limit of an analytical procedure is the lowest amount of an analyte of a sample which can be quantitatively determined with suitable precision and accuracy. The standard deviation and response of the slope and the results obtained.

System suitability parameters

For assessing system suitability, six replicates of working standards samples of Ledipasvir and Sofosbuvir were injected and studied the parameters like plate number(N), tailing factor (K), re-solution, relative retention time and peak asymmetry of samples.

Robustness

The robustness of the assay method was established by introducing small changes in the chromatographic condition which included wavelength (225nm - 229nm) flow rate (1.0 and 1.2mL/min) and organic phase (+5% to -5%).

Specificity and selectivity

Specificity is the degree to which the procedure applies to a single analyte and is checked in each analysis by examining blank matrix samples for any interfering peaks. The specificity of the method was evaluated with regard to interference due to presence of any other placebos. Two different samples were injected and studied with respective placebos. The HPLC chromatograms recorded for the drug matrix (mixture of the drug and placebos) showed almost no interfering peaks with in retention time ranges. The obtained figures shows that the selected drugs were cleanly separated. Thus, the HPLC method proposed in this study was selective.

RESULTS AND DISCUSSION

Optimized chromatographic conditions

Most of all reported HPLC methods till date use C- 8 or C-18 columns. Most of these uses Complex mobile phase compositions. Hence, attempts were directed towards development of a Simple and better method on commonly used Inertsil- C18, BDS column with good resolution. Different logical Modifications were tried to get good separation among the drugs and the degraded products. These changes included change in mobile phase composition in isocratic elution as well as gradient modes on different HPLC columns. The optimized chromatographic conditions Figure No.3. The best peak shape and maximum separation was achieved with mobile phase composition of Methanol and Water (60:40), peak symmetry and reproducibility were obtained on inertsil-C18, 250mm 4.6mm, 5mm Column. The optimum wave-length for detecting the analyte was found to be 254nm, a flow rate of 1.0ml/min yielded optimum separation and peak symmetry. Chromatogram of Sofosbuvir and Ledipasvir (Figure No.3) and optimized chromatographic condition is shown in (Table No.1).

Accuracy and precision

Accuracy as recovery was evaluated by spiking previously analyzed test solution with additional Placebo at three different concentration levels (Table No.4a, b). Recovery of previously analyzed test solution drug concentration added was found to be 99.9% for Sofosbuvir and 100.06% for Ledipasvir with the value of RSD less than 1% indicating that the proposed method is accurate for the simultaneous estimation of both drugs from their combination drug products in presence of their degradation products. The low RSD values indicate the repeatability and reproducibility of the Method (Table No.5a, b).

Linearity, LOD and LOQ

The calibration plot was linear over the concentration range investigated (20-80µg/ml) for Ledipasvir, (20-80µg/ml) for Sofosbuvir respectively. Average correlation coefficient R2¼ 0.999 for both the drugs with %RSD values 2.0 across the concentration ranges studied, was obtained from regression analysis. The LOD that produced the requisite precision and accuracy was found to be 0.32mg/ml Sofosbuvir and 0.33mg/ml Ledipasvir drug. The resultant %RSD values were 1.00% (Table No.3a, b, c). The LOQ for Sofosbuvir and Ledipasvir were found to be 0.98mg/ml and 1.01mg/ml respectively. The Regression results indicate that method was linear in the concentration range studied and can be used for detection and quantification of Sofosbuvir and Ledipasvir in very wide concentration range for Sofosbuvir and Ledipasvir respectively. (Figure No.4a, b).

Specificity and selectivity

Specificity is checked in each analysis by examining blank and placebo samples for any interfering peaks. The specificity of the method was evaluated with regard to interference due to presence of any other excipient.

Robustness

The elution order and resolution for all components were not significantly affected. RSD of peak areas were found to be well within the limit of 2.0%.

System suitability

Parameters calculated for system suitability was a number of theoretical plates, tailing factor, resolution, retention time, and area. Results are shown in (Table No.6).

Assay of the tablet dosage form

(Sofosbuvir 400mg/tab and Ledipasvir 90mg/tab) The proposed validated method was successfully applied to determine Sofosbuvir and Ledipasvir in tablet dosage form. The result obtained for Sofosbuvir and Ledipasvir were comparable with corresponding labeled amounts (Table No.2).

Table No.1: Optimized chromatographic conditions

S.No	Parameters	Method
1	Stationary phase (column)	Inertsil -ODS C_{18}(250 x 4.6 mm, 5 µ)
2	Mobile Phase	Methanol : Water (60:40)
3	Flow rate (ml/min)	1.0 ml/min
4	Run time (minutes)	10 min
5	Column temperature (°C)	Ambient
6	Volume of injection loop (µl)	20
7	Detection wavelength (nm)	254nm

Table No.2: Results for marketed Formulation analysis

S.No	Compound name	Brand name	Label claim (mg)	Test concentration (µg/ml)	Mean amount estimated (µg/ml) (n= 6)	% assay	% RSD
1	SOFOSBUVIR	Resof L	400mg	5.5	5.53	100.5	0.52
2	LEDISPAVIR		90mg	1.25	1.26	100.8	0.271

Table No.3a: Linearity of Ledipasvir and Sofosbuvir

S.No	Concentration (ppm)	Average area for Sofosbuvir	Average area for Ledispavir
1	0	0	0
2	20	219695	572087
3	30	398090	887800
4	40	547437	1239364
5	50	715694	1570861
6	60	885479	1869524
7	70	1022457	2234112
8	80	1199855	2546863

Table No.3b: Statical Analysis

S.No		Sofosbuvir	Ledispavir
1	Slope	16169	33025
2	y-intercept	95766	91183
3	Correlation coefficient	0.999	0.999

Table No.3c: LOQ AND LOD

S.No		SOFOSBUVIR	LEDISPAVIR
1	LOQ	0.98	1.01
2	LOD	0.32	0.33

Table No.4a: Accuracy for Sofosbuvir

S.No	Concentration % of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
1	50%	20	19.98	99.9	MEAN	99.9
2	50%	20	19.94	99.7		
3	50%	20	20.02	100.1	%RSD	0.20
1	100 %	40	39.86	99.65	MEAN	99.9
2	100 %	40	40.05	100.125		
3	100%	40	39.98	99.95	%RSD	0.23
1	150%	60	59.90	99.83	MEAN	99.93
2	150%	60	59.97	99.95		
3	150%	60	60.02	100.03	%RSD	0.10

Table No.4b: Accuracy for Ledipasvir

S.No	Concentration % of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
1	50%	20	19.95	99.75	MEAN	99.81
2	50%	20	19.86	99.3		
3	50%	20	20.08	100.4	%RSD	0.55
1	100 %	40	40.14	100.35	MEAN	99.91
2	100 %	40	39.96	99.9		
3	100%	40	39.80	99.5	%RSD	0.42
1	150%	60	59.89	99.81	MEAN	100.067
2	150%	60	60.04	100.06		
3	150%	60	60.09	100.15	%RSD	0.17

Table No.5a: Method Precision of Sofosbuvir

S.No	Retention Time (min)	Area
1	3.942	547437
2	3.942	549117
3	3.944	546517
4	3.940	550490
5	3.943	547427
Average	3.9422	548197.6
S.D	0.001483	1588.8
%R.S.D	3.942	547437

Table No.5b: Method Precision of Ledispavir

S.No	Retention Time(min)	Area
1	2.869	1239364
2	2.867	1243411
3	2.872	1237979
4	2.868	1246482
5	2.872	1241537
Average	2.8696	1241755
S.D	0.002302	3358.178
%R.S.D	0.080226	0.270438

Table No.6: System Suitability Parameters

S.No	Parameter	Acceptance criteria	Observed value
		Theoritical plates	
1	Ledispavir	(Not less than 3000)	7983.59
2	Sofosbuvir		9827.03
		Tailing factor	
3	Ledispavir	(Not more than 2)	1.13
4	Sofosbuvir		1.07
		Repeatability	
5	Ledispavir	(RSD <1% for N>5)	0.14
6	Sofosbuvir		0.24
7	Resolution (Rs)	(Rs>2)	14.45

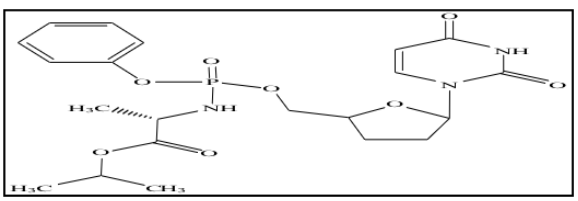

Figure No.1: Chemical structure of Sofosbuvir

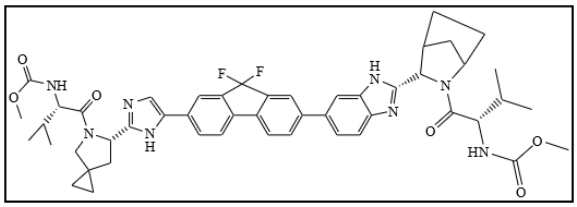

Figure No.2: Chemical structure of Ledipasvir

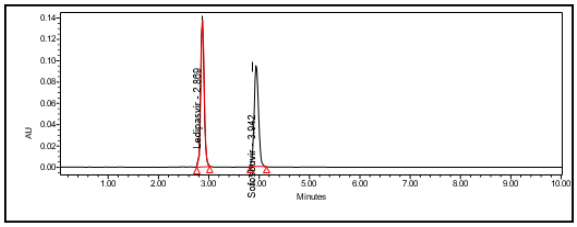

Figure No.3: A typical chromatogram of Sofosbuvir and Ledipasvir

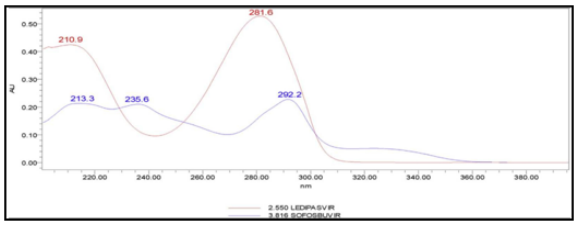

Figure No.4: UV Spectrum of Sofosbivir and Ledipasvir

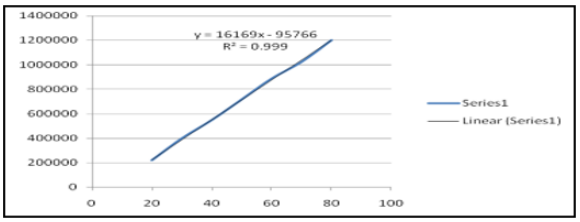

Figure No.4a: Linearity plot for Ledispavir

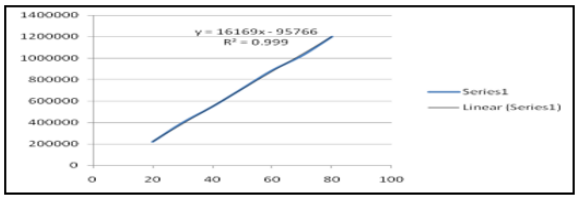

Figure No.4b: Linearity plot for Sofosbuvir

CONCLUSION

The proposed method has benefit of simplicity and convenience for the quantitation and separation of Sofosbuvir and Ledipasvir in the combination and can be used for the assay of their dosage form. Also, the very short analytical run

time and low quantity of solvent utilization lead to environmentally simple chromatographic method. The method is accurate, precise, rapid and selective for simultaneous estimation of Sofosbuvir and Ledipasvir in tablet dosage form. Hence the method can be suitably adopted for routine analysis.

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