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Development and Validation of RP-HPLC Method for the estimation of Sofosbuvir and Ledipasvir in combined dosage form

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ABSTRACT

Nowadays scientists aim at developing generic and specific methods for drugs in combined pharmaceutical dosage forms. The main aim was to develop and carry out validation using precision and accuracy for Sofosbuvir and Ledipasvir by applying RP-HPLC method. The dosage form in fixed combination of Sofosbuvir-Ledipasvir helps in effective treatment of genotypes of chronic hepatitis C virus. This combination of Sofosbuvir-Ledipasvir was the first FDA approved direct acting antiviral to treat hepatitis C. Both the drugs were optimized using mobile phase as acetonitrile: 0.1% orthophosphoric acid (55:50v/v) with pH 3.0. The mobile phase was optimized at maximum wavelength of 283nm. The separation was achieved using C_{18} Cosmosil (4.6 x 250mm) column with a particle size of 5μ. The method was specific eluting retention times of 3.7 for Sofosbuvir and 6.0 for Ledipasvir. Intra and interday precision was carried and %RSD was found to be less than 2%. The results were found to be accurate with percentage recovery of 99.76% and 99.10% for Sofosbuvir and Ledipasvir respectively. Linearity was carried out in the concentration ranging from 40-200μg/mL and 9-45μg/mL for Sofosbuvir and Ledipasvir respectively. Both the drugs showed the regression coefficient of 0.999. Deliberate changes in flow rate, pH and wavelength were made and found that method was robust. The developed method was found to be accurate, simple, specific, robust and precise for the simultaneous estimation of Sofosbuvir and Ledipasvir in tablet dosage form.

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INTRODUCTION

Sofosbuvir (SOF)

Sofosbuvir is chemically known to be isopropyl (2S)-2-[[[(2R, 3R, 4R, 5R)-5-(2,4-dioxopyrimidin-1-yl)-4-fluro-3-phosphoryl]amino]propanoate with structural formula of $C_{22}H_{29}FN_3O_9P$ (Vikas *et al.*, 2016; Vejendla *et al.*, 2016). The active antiviral agent 2'-deoxy-2'-α-fluro-β-C-methyluridine-5'-triphosphate (Ramesh *et al.*, 2016; Hassouna *et al.*, 2017a). The defective substrate for the NS5B which is served by the triphosphate acts as an inhibitor for the viral RNA synthesis (Rote *et al.*, 2017; Naazneen

and Sridevi, 2017).

Ledipasvir (LED)

Ledipasvir is chemically known to be Methyl N-[(2S)-1-[(6S)-6-[5-[9,9-Difluoro-7-[2-[1S,2S,4R)-3-[(2S)-2(methoxycarbonylamino)-3methylbutanoyl] 3azaabicyclo[2.2.1]hetan-2-yl]-3H-benzimidazole-5-yl]fluoren-2-yl]-1H-imidazol-2-yl-5-azapiro[2.4] heptan-5-yl]-3H-methyl-1-oxobutan-2-yl] carbamate with structural formula of $C_{49}H_{54}F_2N_8O_6$ (Devilal *et al.*, 2016; Nagaraju *et al.*, 2017). The viral phosphoprotein NS5A is inhibited by Ledipasvir (Rao *et al.*, 2017; Rai *et al.*, 2017). It leads to viral replication, assembly and secretion (Trivedi *et al.*, 2018; Hassouna *et al.*, 2017b).

Literature survey reveals that very less methods or studies are done on combined dosage form for Sofosbuvir and Ledipasvir (Madhavi and Rani, 2017). Thus the objective of the present work was to develop a very simple, economical, precise and accurate method with reproducible assay results for Sofosbuvir and Ledipasvir in simultaneous dosage form to validate as per ICH Guidelines. (Ravichandran *et al.*, 2010; Vejendla *et al.*, 2016).

MATERIALS AND METHODS

Chemicals

The pure standard gift samples of Sofosbuvir and Ledipasvir were obtained from Zydus Cadila Pvt. Ltd. (Goa India). Harvoni, the commercial formulation in tablet form was procured from medical stores.

Instrumentation

The HPLC instrument included Agilent HPLC gradient system equipped with G13104A ISO Pump, G1314A Detector and Chemstation software. Separation and quantitation was done using C_{18} COSMOSIL column with 4.6 x 250 mm as dimensions and 5 mm particle size packing with flow rate 0.7 mL/min and sample size of 20 ml. The filtration assembly with Nylon 66 membrane filter of 13mm diameter having a pore size of 0.45 micron was used to filter both the solutions. Wavelength of detection was obtained from the PDA detector where the two drugs showed the absorption maxima at 283nm. A Digital Weighing Balance (Make: Shimadzu AUX 220) was used for preparations of all standard and sample solutions required.

Chromatographic conditions

The Chromatographic conditions were optimized using isocratic mode. A mixture of Acetonitrile and orthophosphoric acid (0.1%) with a ratio of 55:50 v/v at 3.0 pH expressed a good peak symmetry with efficient separation of drugs. Acetonitrile and 0.1% orthophosphoric acid mixture in the ratio of 55:50v/v pH adjusted to 3.0 provided an efficient separation of the drugs with good peak symmetry. The eluents were monitored at 283nm with a run-time of 10mins. 0.7mL/min expressed optimum retention time of 3.7min and 6.0min for SOF and LED respectively. An ambient column oven temperature was maintained.

Preparation of Mobile phase

Acetonitrile and orthophosphoric acid (0.1%) in the ratio of 55:50 v/v was optimized as the mobile phase with pH adjusted to 3.0. The mobile phase was sonicated for 15 minutes and the same was used as diluent.

Preparation of Standard stock solution

An accurate amount of Sofosbuvir (40mg) and Ledipasvir (90mg) was weighed and dissolved in 10 mL of methanol. The stock solution obtained of 4000 μg/mL for Sofosbuvir and 90 μg/mL of Ledipasvir was diluted further to obtain the required concentration.

Optimization of method

Wavelength of detection of the standard solution of the drugs was obtained from the PDA detector. Both the drugs were scanned between 190 nm to 400nm. The isobestic wavelength after scanning overlain spectra was 283nm.

RESULTS AND DISCUSSION

Method Validation

The method validation parameters were determined experimentally according to International Conference on Harmonization (ICH) guidelines.

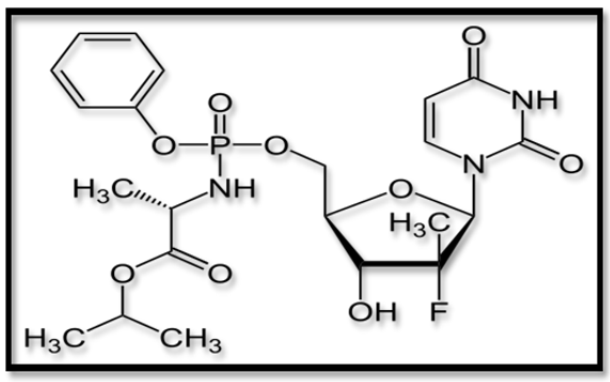

Figure 1: Structure of Sofosbuvir

Figure 1 illustrates structure of Sofosbuvir whereas Figure 2 illustrates structure of Ledipasvir. Fig-

Table 1: Chromatographic conditions

Chromatographic conditions	
HPLC	Agilent (1100) Gradient System VWD Detector
Detector & pump No.	G13104A ISO Pump & G1314A Detector
Software	Chemstation
Column Size	4.6 mm x 250 mm
Particle size	5 μ
Stationary Phase	C18 (COSMOSIL)
Mobile Phase	Acetonitrile: Orthophosphoric acid (0.1%), 55:50 v/v pH adjusted to 3.0
Detection Wavelength	283 nm
Flow Rate	0.7 mL/min
Temperature	Ambient
Sample Size	20 mL

Table 2: System suitability parameters

Parameters	Sofosbuvir	Ledipasvir
Retention Time (Rt)	3.7	6.0
Resolution Factor	-	11.68
Number of Theoretical Plates	5524	17824
Tailing Factor (T)	0.93	0.80
No. of injections: 6 replicates		

Table 3: Intraday precision

Sr. No.	Concentration		Amt Found		% Amt Found	
	Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir
1	80.00	18.00	79.84	18.22	99.80	101.22
2	80.00	18.00	78.54	18.18	98.18	101.00
3	80.00	18.00	78.85	18.32	98.56	101.78
4	80.00	18.00	79.23	18.24	99.03	101.38
5	80.00	18.00	79.41	18.23	99.22	101.28
6	80.00	18.00	79.41	18.21	99.22	101.17
	Mean		79.21	18.23	99.00	101.31
	SD		0.46	0.05	0.57	0.26
	%RSD		0.58	0.26	0.57	0.26

Table 4: Intermediate Precision

	Sr.No.	Mng	Aft	Evg		Mng	Aft	Evg
	Conc	80.00	80.00	80.00		18.00	18.00	18.00
	Area Mean	467.08	467.36	467.52		452.28	451.85	451.64
Sofosbuvir	Amt Found	78.97	79.02	79.05	Ledipasvir	18.34	18.31	18.32
	%Amt Found	98.73	98.78	98.82		102.20	102.08	101.70
	SD	0.301	0.252	0.946		0.65	0.32	0.100
	%RSD	0.305	0.255	0.958		0.63	0.31	0.098

Table 5: Linearity data of Sofosbuvir

Conc	Area	SD	%RSD
40	238.45	0.77	0.32
80	469.87	2.34	0.50
120	714.38	1.81	0.25
160	939.80	3.59	0.38
200	1202.62	3.70	0.31

Table 6: Linearity data of Ledipasvir

Conc	Area	SD	%RSD
9	216.2594	0.44	0.20
18	445.7317	1.35	0.30
27	666.2158	1.57	0.24
36	898.4667	0.87	0.10
45	1134.264	6.92	0.61

Table 7: Accuracy data of Sofosbuvir and Ledipasvir

Analyte	Spiked Amount (μg/mL)	Mean Peak area of Sample	Amount Found (μg/mL)	Recovered Amount (μg/mL)	Recovery (%)
Sofosbuvir	32	422.43	71.54	31.54	98.56
	40	471.32	79.69	20.58	99.23
	48	520.33	87.87	47.87	101.58
Ledipasvir	7.2	394.34	16.07	7.07	98.19
	9	437.43	17.78	20.58	97.54
	10.8	486.93	19.71	10.71	101.58

Table 8: Robustness data of Sofosbuvir and Ledipasvir

		Flow rate			Flow rate	
		0.6mL/min	0.8mL/min		0.6mL/min	0.8mL/min
	Mean Area	877.86	652.07		788.32	589.53
	Std Devn	2.02	0.92		1.73	0.93
	%RSD	0.23	0.14		0.22	0.16
Sofosbuvir		pH		Ledipasvir	pH	
		2.9	3.1		2.9	3.1
	Mean Area	754.8	754.25		674.0	674.87
	Std Devn	0.48	0.82		2.33	2.26
	%RSD	0.06	0.11		0.35	0.33
		Λmax			Λmax	
		282	284		282	284
	Mean Area	729.9	773.35		817.8	520.58
	Std Devn	0.24	3.12		0.72	2.53
	%RSD	0.03	0.40		0.09	0.49

Table 9: Assay results of Sofosbuvir and Ledipasvir

Drug	Area	% Purity
Sofosbuvir	471.32	99.23
Ledipasvir	437.43	97.54

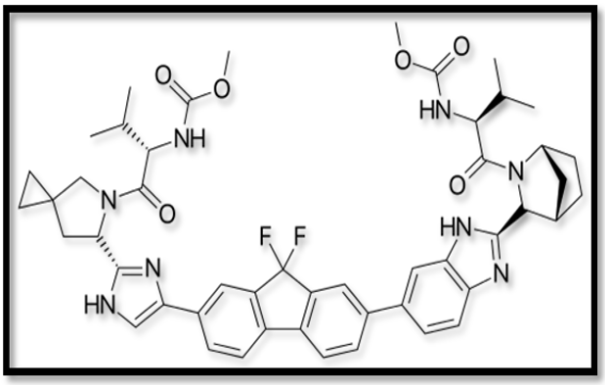

Figure 2: Structure of Ledipasvir

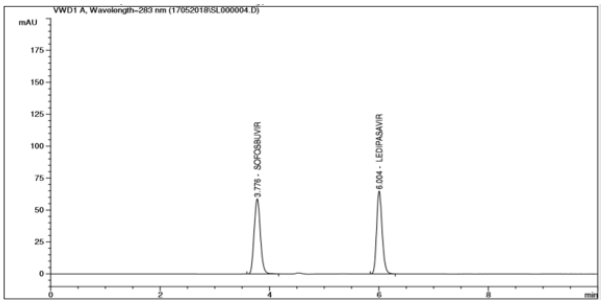

Figure 3: Standard drug chromatogram of Sofosbuvir and Ledipasvir

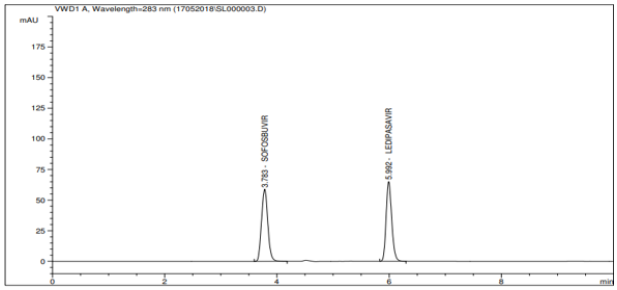

Figure 4: Chromatogram of Sofosbuvir and Ledipasvir Assay sample

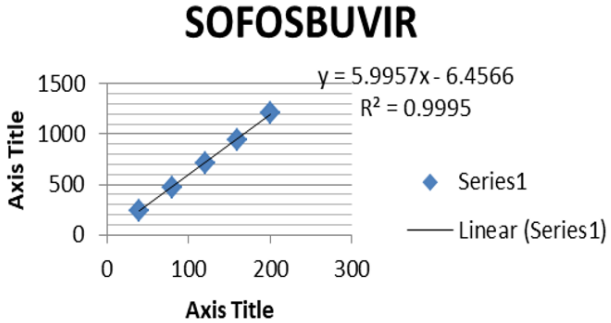

Figure 5: Linearity Graph of Sofosbuvir

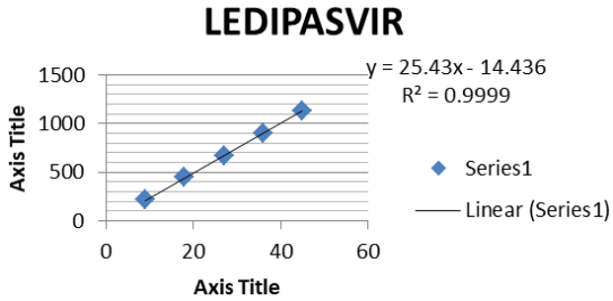

Figure 6: LinearityGraph of Ledipasvir

ure 3 illustrates Standard chromatogram drug chromatogram of Sofosbuvir and Ledipasvir.

Figure 4 illustrates Chromatogram of Sofosbuvir and Ledipasvir in assay sample. Figure 5 and Figure 6 illustrates Linearity graph of Sofosbuvir and Ledipasvir respectively.

Table 1 illustrates Chromatographic conditions of Sofosbuvir and Ledipasvir. Table 2 illustrates System suitability parameters of Sofosbuvir and Ledipasvir. Table 3 illustrates Intraday precision results of Sofosbuvir and Ledipasvir. Table 4 illustrates Intermediate Precision results of Sofosbuvir and Ledipasvir. Table 5 and Table 6 illustrate Linearity data of Sofosbuvir and Ledipasvir respectively. Table 7 illustrates Linearity data of Sofosbuvir and Ledipasvir. Table 8 illustrates Robustness data of Sofosbuvir and Ledipasvir. Table 9 illustrates Assay results of Sofosbuvir and Ledipasvir.

Selectivity/Specificity

Selectivity is demonstrated by the resolution of the two compounds, Sofosbuvir and Ledipasvir. The sample was assessed unequivocally in the presence of other matrix components. Retention times were specific at 3.7 and 6.0 for Sofosbuvir and Ledipasvir respectively.

System Suitability

System suitability was evaluated for analytical operations and samples as whole. Six replicated were recorded of the standard chromatogram and the parameters determined were tailing factor (T), capacity factor (k'), resolution (R) and theoretical plates (N).

Precision

Precision is measured either as degree of reproducibility or of repeatability. The assessment was achieved by evaluating relative standard deviation.

Linearity and range

Linearity was expressed in terms of relationship between concentration and assay method. This relationship was achieved by studying the concentra-

tion levels of Sofosbuvir and Ledipasvir in the range of 40-200 μg/mL and 9-45 μg/mL respectively in HPLC system noting the peak areas.

Accuracy

Accuracy of the method is determined by applying the analytical procedure to an analyte of known purity and evaluating its recovery studies. The standard addition method at concentration levels of 80%, 100%, 120% of both the drugs was performed to evaluate the percentage recovery of spiked samples.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit test of detection and quantitation are characteristic of low level of compounds in sample matrices. The method expresses the concentration of analyte based on signal to noise ratio of 3:1 for LOD and 10:1for LOQ.

Robustness

The robustness expresses deliberate variations in the method developed by changing the conditions of the experiment. Such changes were made in the parameter conditions of flow rate, pH and wavelength.

Assay

An accurate amount of tablet powder equivalent to 40 mg of Sofosbuvir was weighed. The powder was transferred in methanol and mixed for around 20 mins to dissolve the drugs. The volume was made up to 10 mL. Further dilutions were made as per requirement and filtered using Sample Filtration Assembly.

CONCLUSIONS

Fixed dose combination containing Sofosbuvir (400mg) and Ledipasvir (90mg) is introduced lately as a direct-acting antiviral and also for Hepatitis C treatment. Literature survey reveals about the several methods used to assess the drug individually and in combination along with other drugs in various pharmaceutical drug dosage form and biological fluids. Nevertheless, very few methods are reported of these two drugs in fixed dosage form. Thus an effort was made to undertake and develop an accurate, economical and sensitive HPLC method for estimating these drugs in combined dosage form. Both the drugs were resolved using acetonitrile: orthophosphoric acid in a ratio of 55:50 v/v with a pH adjustment of 3.0. The resolution was obtained on C18 Cosmosil (4.6 x 250 mm) column at a maximum wavelength of 283 nm. An outstanding resolution was obtained between both the drugs with an optimum retention time. The developed analytical method accounted to be simple, precise and accurate for Sofosbuvir and Ledipasvir in combined tablet dosage form.

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Conflict of interest

The authors declare that they have no conflict of interest for this study.

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