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

Method Development and Validation of Sofosbuvir and Ledipasvir in API & Its Pharmaceutical dosage forms by RP-HPLC

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A B S T R A C T

Ledipasvir is indicated in patients with hepatitis C virus (HCV) genotype 1 for treatment of chronic hepatitis as a combination therapy, which includes peg interferonalfa and ribavirin. Ledipasvir is a protease inhibitor for HCV NS5A protease, which is required for replication of the virus. SOVALDI is the brand name for sofosbuvir, a nucleotide analog inhibitor of HCV NS5B polymerase. It is a hepatitis C virus (HCV) nucleotide analog NS5B polymerase inhibitor indicated for the treatment of chronic hepatitis C (CHC) infection as a component of a combination antiviral treatment regimen. A novel reversed phase high performance liquid chromatography (RP-HPLC) method has been developed for the quantitative determination of Ledipasvir and Sofosbuvir in active pharmaceutical ingredients and in its pharmaceutical dosage form by using Inertsil-C18 ODS column as stationary phase and a mobile phase containing a mixture of Methanol: Water (60:40% v/v). The flow rate was 1.0ml/min and effluent was monitored at 254nm and a peak eluted at 2.9min, 3.4min and column oven temperature was maintained ambient. Calibration curve was plotted with a range from 20-80ppm. The developed RP-HPLC method was validated according to the current International Council for Harmonisation (ICH) guidelines for specificity, LOD, LOQ, linearity, accuracy, precision, intermediate precision and robustness. The result of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine analysis of Ledipasvir & Sofosbuvir in bulk drug and in its pharmaceutical dosage form.

Key words: Ledipasvir, Sofosbuvir, RP-HPLC and Validation.

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

Sofosbuvir drug profile

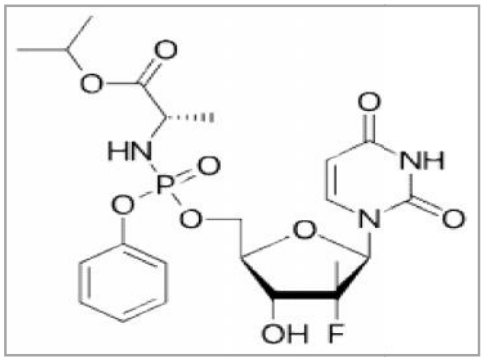

Fig 1: Structure of Sofosbuvir

Generic name: Genotypes
IUPAC: Isopropyl (2S)-2-[[[(2R, 3R, 4R, 5R)-5-(2, 4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetra hydrofuran-2-yl] methoxy-phenoxy-phosphoryl] amino] propanoate
Molecular frame work: Aromatic heteromonocyclic compounds
Molecular formula: $C_{22}H_{29}FN_3O_9P$
Molecular weight: 529.453 g/mol
Monoisotopic: 529.162544258
XLogP3:1
Smiles:CC(C)OC(=O)[C@H](C)N[P@](=O)(OCC1O[C@@H](N2C=CC(=O)NC2=O)[C@](C)(F)[C@@H]1O)OC1=CC=CC=C1
CAS No: 1190307-88-0
Official in: Indian Pharmacopoeia
Appearance: White to off-white crystalline powder.
Solubility: It is soluble in methanol and acetonitrile.
Melting point: 133.189°C
Boiling point: 361.451°C at 760 mmHg
pKa: Strongest Acidic 9.38
pKa: Strongest Basic -3.9
logP :1.63
Indication: Sofosbuvir is used in combination therapy 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. The combination therapy includes either ribavirin alone or ribavirin and peg-interferon alfa.

Pharmacology

Therapeutic Categories: Antiviral Agents
Mechanism of action: Sofosbuvir 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.

Pharmacokinetics

Absorption: When given orally, Sofosbuvir reaches its maximum plasma concentration in about 0.5 to 2 hours.
Metabolism: In vitro studies in human liver microsomes showed that sofosbuvir was an efficient substrate for Cathepsin A (Cat A) and carboxyl esterase 1 (CES1). Sofosbuvir was cleared by CatA and CES1 and subsequent activation steps included amino acid removal by histidine triad nucleotide-binding protein 1 (HINT1) and phosphorylation by uridine monophosphate-cytidine monophosphate (UMP-CMP) kinase and nucleoside diphosphate (NDP) kinase. In vitro data indicated that Cat A preferentially hydrolysedsofosbuvir (the S-diastereomer) while CES1 did not exhibit stereo selectivity.
Route of elimination: Sofosbuvir is eliminated by three routes: urine (80%), feces (14%), and respiration (2.5%); however, elimination through the kidneys is the major route.
Half-life: Sofosbuvir has a terminal half-life of 0.4 hours.
Uses: Sofosbuvir (brand name Sovaldi) is a nucleotide analog used in combination with other drugs for the treatment of hepatitis C virus (HCV) infection. It has been marketed since 2013. Compared to previous treatments, sofosbuvir-based regimens provide a higher cure rate, fewer side effects, and a two- to four-fold reduced duration of therapy. Sofosbuvir allows most patients to be treated successfully without the use of peginterferon, an injectable drug with severe side effects that is a key component of older drug combinations for the treatment of HCV.

Ledipasvir drug profile

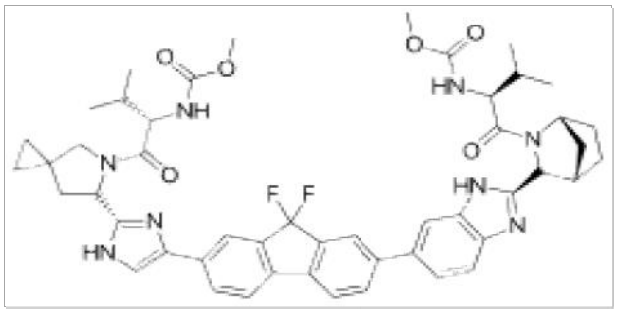

Fig 2: Structure of Ledipasvir

Generic name: Formerly GS-5885
IUPAC: MethylN-[(2S)-1-[(6S)-6-[5-[9,9-Difluoro-7-[2-[(1S,2S,4R)-3-[(2S)-2-(methoxycarbonylamino)-3-methyl butanoyl]-3-azabicyclo[2.2.1]heptan-2-yl]-3H-benzimidazol-5-yl]fluoren-2-yl]-1H-imidazol-2-yl]-5-azaspiro[2.4]heptan-5-yl]-3-methyl-1-oxobutan-2-yl]carbamate
Molecular frame work: Aromatic heteromonocyclic compounds
Molecular formula: $C_{49}H_{54}F_2N_8O_6$
Molecular weight : 889.00 g/mol
Monoisotopic : 888.413452 Da
XLogP3:7.4
Smiles:CC(C)C(C(=O)N1CC2(CC2)CC1C3=NC=C(N3)C4=CC5=C(C=C4)C6=C(C5(F)F)C=C(C=C6)C7=CC8=C(C=C7)N=C(N8)C9C1CCC(C1)N9C(=O)C(C(C)C)NC(=O)OC)NC(=O)OC
CAS No: 1256388-51-8
Official in: Indian Pharmacopoeia
Appearance: Orange to white solid
Solubility : Ledipasvir is practically insoluble (<0.1 mg/mL) across the pH range of 3.0-7.5 and is slightly soluble below pH 2.3(1.1mg/mL).
Melting point : 186 - 190ºC
pKa : pKa1 is 4.0 and pKa2 is 5.0

logP :3.8
Indication: Indicated for adults with chronic hepatitis C virus (HCV) genotypes 1, 4, 5, or 6 infection.1 tablet (90 mg/400 mg) PO qDay.
Pharmacology
Therapeutic Categories : Antiviral Agents
Mechanism of action : Ledipasvir inhibits an important viral phosphoprotein, NS5A, which is involved in viral replication, assembly, and secretion. Sofosbuvir, on the other hand, is metabolized to the active uridine analog triphosphate, which acts as a RNA chain terminator when incorporated into the RNA via the NS5B polymerase.
Pharmacokinetics
Bioavailability : 76%
Protein binding : > 99%
Metabolism : No cytochrome metabolism
Biological half-life : 47 hrs
Absorption : Ledipasvir median peak concentrations were observed 4.0 to 4.5 hours post-dose.
Route of elimination: Feces and urine
Half-life:37-45 h
Uses: Ledipasvir is most commonly used in combination with Sofosbuvir for treatment in chronic hepatitis C genotype 1 patients. This drug has been tested and shown efficacy in treatment-naive and treatment experienced patients.

2. Materials and Methods

Instruments:
HPLC –Waters Model NO.2690/5 series Compact System Consisting of Inertsil-C18 ODS column, Electronic balance (Sartorious) and Sonicator (Fast Clean)
Chemicals: Methanol HPLC Grade and Water HPLC Grade.
Raw Material: Ledipasvir and Sofosbuvir Working Standards.
Method Development for HPLC: The objective of this experiment was to optimize the assay method for simultaneous estimation of Ledipasvir and Sofosbuvir on the literature survey made. So here the trials mentioned describes how the optimization was done.
Trial: 1
Mobile Phase: Degassed Acetonitrile 100%.
Preparation of Standard Solution: Weigh down 10mg's of Ledipasvir and Sofosbuvir drugs and dissolved in 10ml of Mobile phase taken into two 10ml of volumetric flasks separately and sonicated for 20 minutes to get 1000ppm and 1 ml was taken from each solution into a 10ml volumetric flask and diluted to 10 ml with mobile phase.

Table 1: Chromatographic conditions for Trial -1

Flow rate	1.0ml/min
Column	Inertsil - C18, ODS column
Detector wavelength	254nm
Colum température	Ambient
Injection volume	20µl
Run time	10min
Retention time	4.092min for Ledipasvir and 4.560 min for Sofosbuvir.

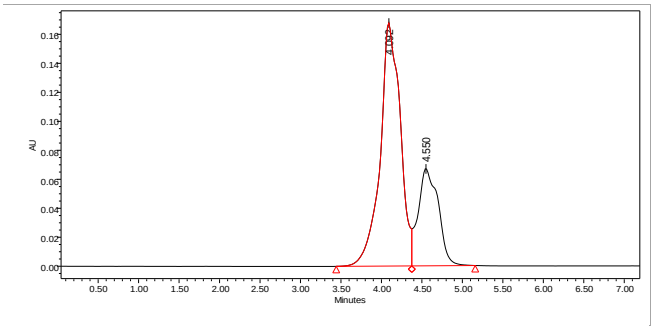

Fig 3: Chromatogram for Trial- 1

Observation:
Two peaks are merged and not separated completely. The trial 1 chromatogram results were showed in Figure 3.
Trail: 2
Mobile Phase: Degassed Acetonitrile and methanol in the ratio of 90:10 V/V.
Preparation of Standard Solution:
Weigh down 10mg's of Ledipasvir and Sofosbuvir drugs and dissolved in 10ml of Mobile phase taken in two 10ml of volumetric flasks separately and sonicated for 20 minutes to get 1000ppms and 1 ml was taken from each solution and diluted to 10 ml with mobile phase.

Table 2: Chromatographic Conditions for Trial – 2

Flow rate	**1.0ml/min**
Column	Inertsil -C18, BDS column
Detector wavelength	254nm
Colum température	Ambient
Injection volume	20µl
Run time	10min
Retention time	3.757 min for Ledipasvir and 4.0 min for Sofosbuvir.

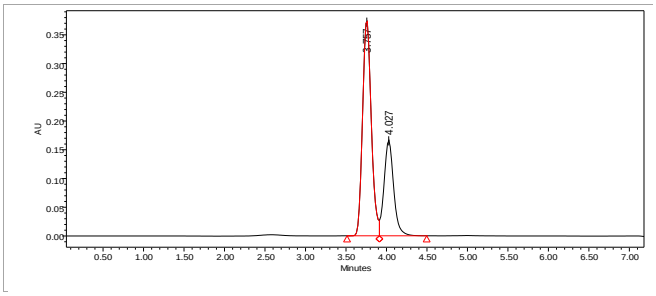

Fig 4: Chromatogram of Trial – 2

Observation:
The two peaks are separated completely but peak shapes are not good. The trial 2 chromatogram result were showed in Figure 4.
Trail: 3
Mobile Phase: Degassed Acetonitrile and Methanol in the ratio of 80:20 V/V.
Preparation of Standard Solution:
Weigh down 10mg's of Ledipasvir and Sofosbuvir drugs and dissolved in 10ml of Mobile phase taken in two 10ml of volumetric flasks separately and sonicated for 20 minutes to get 1000ppms and 1 ml was taken from each solution and diluted to 10 ml with mobile phase.

Table 3: Chromatographic Conditions for Trial – 3

Flow rate	1.0ml/min
Column	Inertsil - C18, BDS column
Detector wavelength	254 nm
Column temperature	Ambient
Injection volume	20µl
Run time	10min
Retention time	1.747 min for Ledipasvir and 2.821 min for Sofosbuvir.

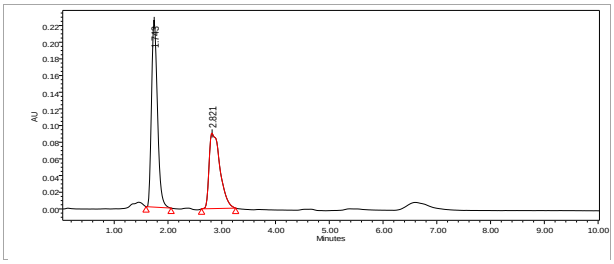

Fig 5: Chromatogram of Trial – 3

Observation:
Sofosbuvir got peak fronting and base line between two peaks is not straight. The trial 3chromatogram result was showed in Fig: 5.

Optimized Method

Mobile Phase: Degassed Methanol and Water in the ratio of 60:40 V/V.

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

Table 4: Optimized Chromatographic conditions

Parameters	**Method**
Stationary phase	Inertsil -ODS C_{18} (250 x 4.6 mm, 5 µ
Mobile phase	Methanol : Water (60:40)
Flow rate (ml/min)	1.0 ml/min
Run time (min)	10 min
Column temperature (^{0}c)	Ambient
Injection volume	20µl
Detection wavelength	254nm
Drug RT (min)	2.9min for Ledipasvir and 3.9 for Sofosbuvir.

3. Results and Discussions

The present study was carried out to develop a sensitive, precise and accurate RP-HPLC method for the simultaneous analysis of Sofosbuvir and Ledipasvir in bulk drug and pharmaceutical dosage forms. The retention time for Sofosbuvir and Ledipasvir was found to be 3.4 min and 2.9 min for a run time of 20 minutes. A good relationship (r=0.999) was observed between the concentrations of Sofosbuvir and Ledipasvir and the respective peak areas. The calibration graph was found to be linear in the range of 0-80ppm, when the Sofosbuvir and Ledipasvir solution was analyzed by the proposed RP-HPLC method.

Precision was determined for the standard drug and tablet sample and the results are represented in the table 7, 8 and 9, 10 which shows that the proposed RP-HPLC method was highly precise. The amount of drug recovered was indicating the high accuracy of the proposed RP-HPLC method. The method was robust as observed from insignificant variation in the results of analysis by changes in the flow rate and column temperature. The drug content in the parenteral was quantified using the proposed analytical method. The proposed RP-HPLC method was found to be simple, precise, highly accurate, specific and less time consuming.

Parameters fixation:
For developing the method, a systematic study for optimization of chromatographic condition was taken up. This was done by varying one parameter at a time keeping all other conditions constant. The fallowing studies were conducted for this purpose. Inertsil-C18 ODS column was chosen as the stationary phase for this study.

The mobile phase characteristics:
In order to get sharp and symmetric peaks, number of experiments were carried out by varying the commonly used solvents, there compositions and flow rate. Mixtures of commonly used solvents like water, methanol and acetonitrile with or without different buffers in different combination were tested as mobile phase on Inertsil-C18 ODS stationary phase. A binary mixture of mobile phase consisting of Methanol and Water (60:40 V/V)was proved to be the most suitable of the all the combination since the chromatographic peaks obtained were better defined and almost free from tailing. In flow rate of 1 ml/min mobile phase was found to be suitable in the range of study (0.8-1.2ml/min).

Detection Characteristics:
To test whether the Sofosbuvir and Ledipasvir has been linearly eluted from the column in this method, different amounts of Sofosbuvir and Ledipasvir were taken and all the solutions were analyzed by this procedure separately. Qualitative determinations were made by comparison of peak area of the sample injection to the corresponding peak area from standard injection in this method.

Analytical method validation

Precision: The precision of the method was ascertained separately from the peak area obtained by actual determination of six replicates for a fixed amount of the drug and formulation.

Specificity:
Got a peak for standard at an RT of 2.866min for Ledipasvir and 3.938min for Sofosbuvir .Got a peak for sample at an RT of 2.871min for Ledipasvir and 3.946min for Sofosbuvir Results were shown in fig 6 and 7.

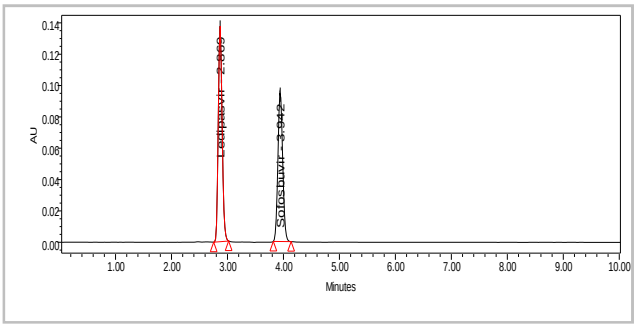

Fig 6: Chromatogram of Standard

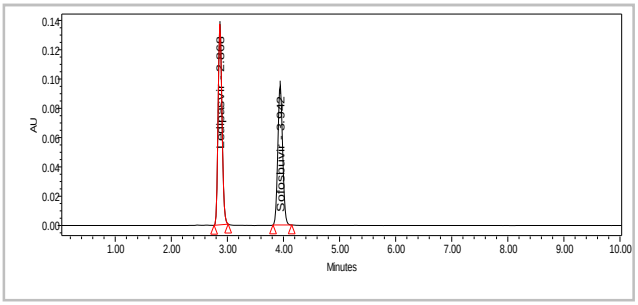

Fig 7: Chromatogram of Sample

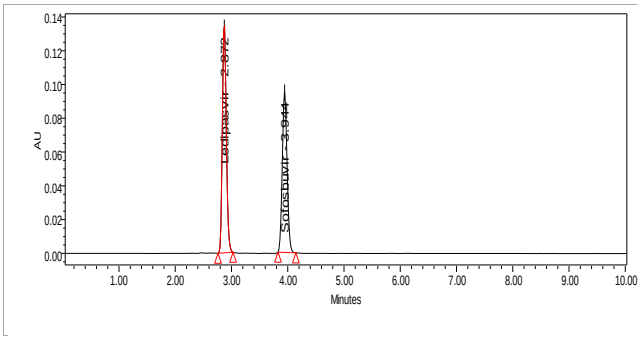

Fig 8: System suitability Chromatogram for standard – 1

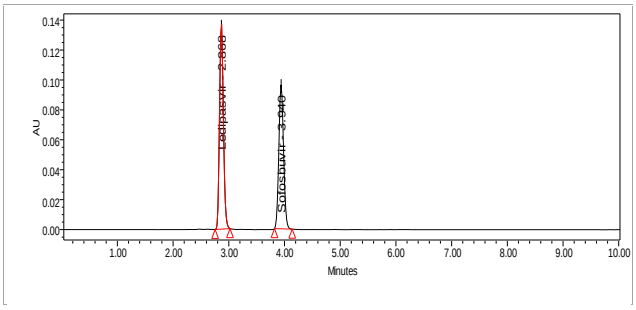

Fig 9: System suitability Chromatogram for standard – 2

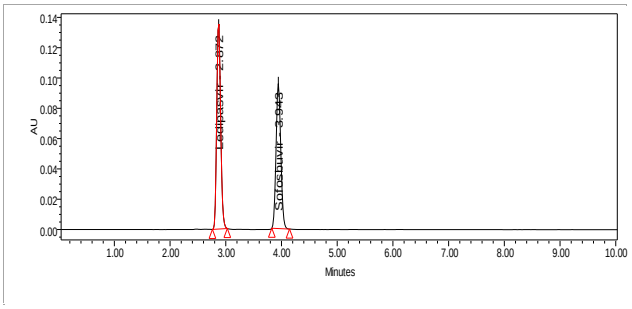

Fig 10: System suitability Chromatogram for standard – 3

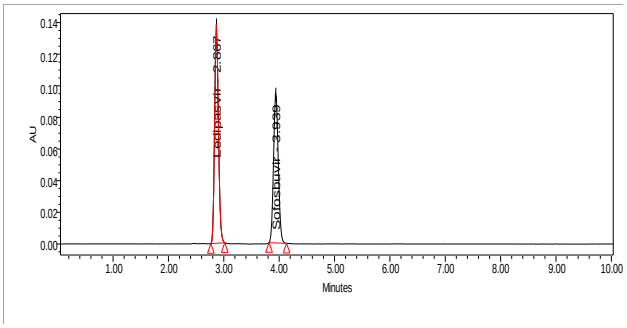

Fig 11: System suitability Chromatogram for standard – 4

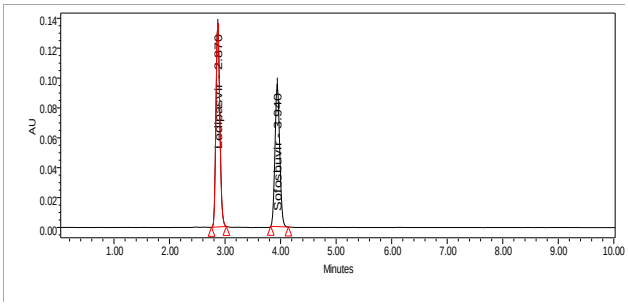

Fig 12: System suit5bility Chromatogram for standard – 4

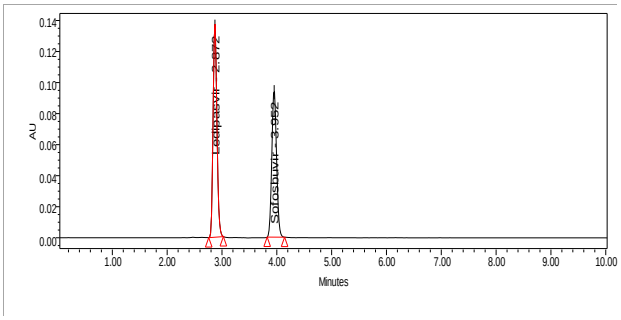

Fig 13: Chromatogram for system precision (standard - 1)

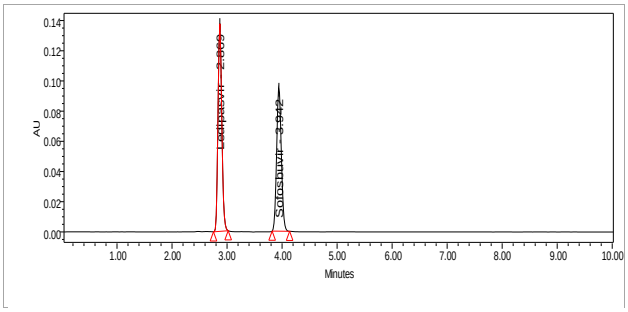

Fig 14: Chromatogram for system precision (standard - 2)

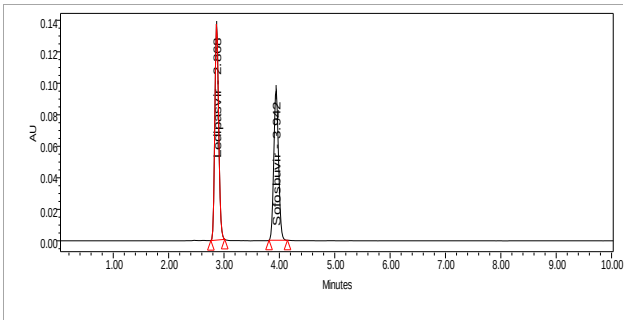

Fig 15: Chromatogram for system precision (standard - 3)

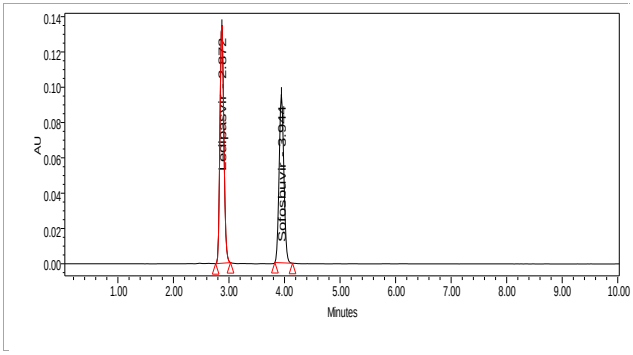

Fig 16: Chromatogram for system precision (standard - 4)

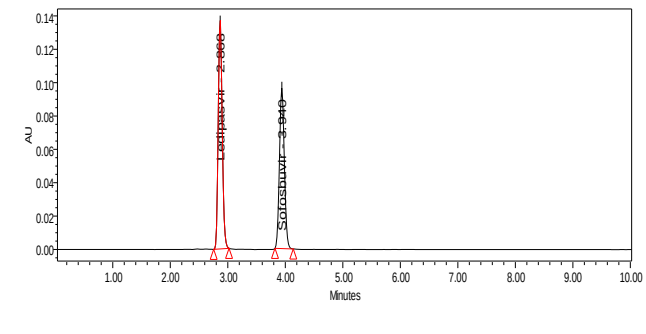

Fig 17: Chromatogram for system precision (standard - 5)

Table 5: Data of System Suitability for Ledipasvir

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	2.864	9438247	1023.845712	1.14721
2	2.867	9436021	1010.547812	1.13384
3	2.863	9431581	1036.874214	1.18742
4	2.868	9432036	1027.254178	1.16547
5	2.864	9433819	1084.658952	1.17485
Mean	2.865	9434340.8	1036.825471	1.1852313
SD	0.002168	2796.53	-------	-------
% RSD	0.08	0.03	-------	-------

Table 6: Data of System Suitability for Sofosbuvir

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	3.948	323209	8325.874512	1.284572
2	3.942	323181	8384.547862	1.254872
3	3.945	323028	8314.875424	1.278451
4	3.946	323915	8372.784518	1.287451
5	3.948	324059	8392.084512	1.298745
Mean	3.946	323478.4	8358.8754210	1.255471
SD	0.00249	472.12	-------	-------
% RSD	0.06	0.15	-------	-------

Table 7: Data of Repeatability (System precision) forLedipasvir

	Injection	Peak Areas of Ledipasvir	%Assay
Concentration 40ppm	1	9437784	99.74
	2	9437412	99.14
	3	9430257	99.62
	4	9438431	99.72
	5	9438754	99.42
Statistical Analysis	**Mean**	9436527.6	99.53
	SD	3544.75	0.25124
	% RSD	0.04	0.25

Table 8: Data of Repeatability (System precision) for Sofosbuvir

	Injection	Peak Areas of Sofosbuvir	%Assay
Concentration 40ppm	1	323112	99.98
	2	323452	99.30
	3	323742	99.60
	4	323047	99.84
	5	323087	99.72
Statistical Analysis	**Mean**	323288	99.69
	SD	301.02741	0.259

	% RSD	0.09	0.26

Table 9: Data of Repeatability (Method precision) for Ledipasvir

	Injection	**Peak Areas of Ledipasvir**	**%Assay**
Concentration 40ppm	1	9432571	99.25
	2	9438475	99.12
	3	9434752	98.12
	4	9430487	99.52
	5	9436547	98.84
	6	9437841	99.54
Statistical Analysis	**Mean**	9435112.2	99.07
	SD	3123.88671	0.53170
	% RSD	0.03	0.54

Table 10: Data of Repeatability (Method precision) for Sofosbuvir

	Injection	**Peak Areas of Sofosbuvir**	**%Assay**
Concentration 40ppm	1	323584	99.54
	2	323054	99.72
	3	323847	99.31
	4	323751	99.84
	5	323814	99.42
	6	323745	99.32
Statistical Analysis	**Mean**	323632.5	99.53
	SD	297.54512	0.21760
	% RSD	0.09	0.22

Table 11: LOD & LOQ Results

Drug	**LOD**	**LOQ**
Ledipasvir	0.33	1.01
Sofosbuvir	0.32	0.98

4. Conclusion

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 232nm for Sofosbuvir and 274nm for Ledipasvir. Common wavelength will be 254nm and the peaks purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was Inertsil C_{18}, ODS chosen good peak shape. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area, satisfactory retention time and good resolution. Different ratios of mobile phase were studied, mobile phase with ratio of 60:40 Methanol: Water was fixed due to good symmetrical peaks and for good resolution. So this mobile phase was used for the proposed study. The present recovery was found to be 98.0-101.50 and was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. Detection limit was found to be 0.0554 Sofosbuvir and 0.17727 for Sofosbuvir. Linearity study was, correlation coefficient and curve fitting was found to be. The analytical method was found linearity over the range of 20-80ppm of the target concentration for both the drugs. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was found well satisfactory.

5. References

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