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Development and Validation of Stability indicating RP- HPLC method for Simultaneous Estimation of Sofosbuvir and Ledipasvir in Bulk Tablet Dosage Form

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ABSTRACT

The present research work describes a simple, accurate, precise, effective, Stability indicating RP-HPLC method for simultaneous estimation of Sofosbuvir and Ledipasvir in their tablet dosage form. A reverse phase high performance chromatographic method was developed for simultaneous estimation of Sofosbuvir and Ledipasvir their combined dosage. The separation was achieved by Inertsil ODS C18 column (150X4.6mm, 5µm) column, and ACN: 0.1% TFA in the proportion of 30:70 %v/v as mobile phase, at a flow rate of 1 ml/min. Detection was carried out at 245 nm. For RP-HPLC method results of the validation indicate that the method was linear in the range of 100-600µg/ml for Sofosbuvir and 22.5-135µg/ml for Ledipasvir. The % recoveries for Sofosbuvir and Ledipasvir obtained in the accuracy study were 99.92-100.31% and 99.84-100.55% respectively. The LOD for Sofosbuvir and Ledipasvir were found to be 0.395µg/ml and 0.132µg/ml respectively. LOQ for Sofosbuvir and Ledipasvir were found to be 1.197µg/ml and 0.401µg/ml respectively. Force degradation study also done and method is stability indicating. Developed methods were found to be accurate, precise, rapid and stability indicating for simultaneous estimation of Sofosbuvir and Ledipasvir.

Keywords: RP-HPLC, Sofosbuvir, Ledipasvir, ACN, TFA.

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INTRODUCTION

1.1 SOFOSBUVIR

propan-2-yl (2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl]methoxy-phenoxyphosphoryl]amino]propanoate.[1] It is indicated for the treatment of chronic HCV genotypes 1, 4, 5, and 6 in adults and also indicated for the treatment of chronic HCV in patients co-infected with HIV. [2] Slightly soluble in water pH 1.2-7.7, freely soluble in ethanol and acetone, soluble in 2-propanol and insoluble in heptanes.[3]

Mol. formula: $C_{22}H_{29}FN_3O_9P$ **Mol. weight**: 529.4525242 gm/mol .

Structure:

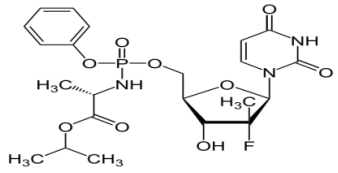

Figure 1: Chemical structure of Sofosbuvir

Mechanism of action: Sofosbuvir is a direct-acting antiviral agent against the hepatitis C virus. The HCV polymerase NS5B protein is an RNA-dependent RNA polymerase (RdRp). It is the essential initiating and catalytic subunit of this replication complex and is critical for the viral replication cycle. There is no human homolog for HCV NS5B RdRp. Sofosbuvir is a monophosphorylated pyrimidine nucleotide prodrug that undergoes intracellular metabolism to form the pharmacologically active uridine analog triphosphate (GS-461203). GS-461203 competes with natural nucleotides for incorporation (by HCV NS5B) into the nascent RNA strand during replication of the viral genome.GS-461203 differs from endogenous pyrimidine nucleotides in that it has been modified at the 2' position with the addition of a methyl and a fluoro functional group. Incorporation of GS-461203 into nascent RNA strongly reduces the efficiency of further RNA elongation by RdRp, resulting in premature termination of RNA synthesis. The stopping of viral replication leads to a rapid decline of HCV viral load and clearing of HCV levels in the body.[4,5]

1.2. LEDIPASVIR

The chemical name is methyl [(2S)-1-{(6S)-6-[4-(9,9-

difluoro-7-{2-[(1R,3S,4S)-2-{(2S)-2-[(methoxycarbonyl)amino]-3-methylbutanoyl}-2-azabicyclo[2.2.1]hept-3-yl]-1H-benzimidazol-5-yl}-9H-fluoren-2-yl)-1H-imidazol-2-yl]-5-azaspiro[2.4]hept-5-yl}-3-methyl-1-oxobutan-2-yl]carbamate [6]. 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 1.1 mg/ml. The partition coefficient for Ledipasvir is 3.8 and the pKa1 is 4.0 and pKa2 is 5.0.[7]

Molecular formula: $C_{49}H_{54}F_2N_8O_6$

Molecular weight: 888.999866 gm/mole.

Structure:

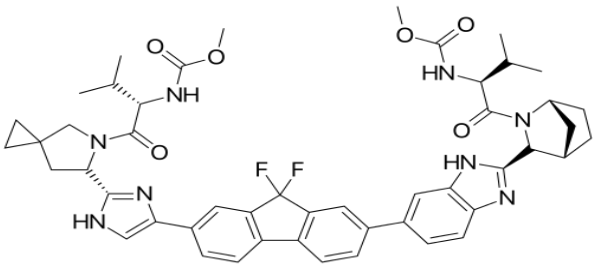

Figure 2: Chemical structure of Ledipasvir

Mechanism of action:

Ledipasvir inhibits an important viral phosphoprotein, NS5A, which is involved in viral replication, assembly, and secretion. Ledipasvir 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 [8,9]

According to the best of our knowledge, only four HPLC methods[10-13] have been published, during the preparation of the present work for publishing. The present study aimed to develop a simple, sensitive, short retention time and accurate RP-HPLC method for the simultaneous determination of both sofosbuvir and ledipasvir together in pure and tablet dosage forms with high sensitivity, selectivity that can be used for the routine analysis of production samples.

2. MATERIAL AND METHOD

2.1. Instruments and Apparatus :

- Ultrasonic Cleaner-5510
- Glass wares – volumetric flask (10, 50 and 100 ml), pipettes beaker (500 ml), measuring cylinder(25, 50, 100 ml) (Borosil)
- What man filter paper no.41
- Thermo separation HPLC (Assemble), UV-200 detector (single wavelength), Rheodyne injector (20 µl).
- 0.45 µm Nylon 66 (Milipore, India)
- Analytical Balance (Swisser)

All instruments and glass wares were calibrated.

2.2. Preparation of Solutions

- **Preparation of the Buffer solution**

Diluted 1.0 ml of Trifluoro acetic acid in 1000ml of purified water and mixed.

Filter the resultant solution through 0.45µm Nylon membrane filter.

- **Preparation of the Mobile phase**

Prepared a filtered and degassed mixture of buffer solution and acetonitrile in the ratio of (70:30 %v/v).

2.3. Preparation of Standard Solutions

- **Preparation of Standard Solution of Sofosbuvir**

Accurately weighed quantity of Sofosbuvir 200.00 mg was transferred into 100 ml volumetric flask, sonicated to dissolve and diluted up to mark with diluent to give a stock solution 2000µg/ml. An aliquot 5.0 ml of the solution was transferred to 50 ml volumetric flask and diluted to the mark with diluent to obtain a working standard solution 200µg/ml of Sofosbuvir.

- **Preparation of Standard Solution of Ledipasvir**

Accurately weighed quantity of Ledipasvir 45.00 mg was transferred into 100 ml volumetric flask, sonicated to dissolve and diluted up to mark with diluent to give a stock solution 450µg/ml. An aliquot 5.0 ml of the solution was transferred to a 50 ml volumetric flask and diluted to the mark with diluent to obtain a working standard solution 45µg/ml of Ledipasvir.

- **Preparation of Combined Standard Solution of Sofosbuvir and Ledipasvir:**

Accurately weighed Sofosbuvir 200.00 mg and Ledipasvir 45.00 mg were transferred into 100 ml volumetric flask, sonicated to dissolve and diluted up to mark with diluent to give a stock solution 2000µg/ml of Sofosbuvir and 450µg/ml Ledipasvir. Designate it as a combine stock solution

Stock solution 5.0 ml was transferred to 50 ml volumetric flask and diluted up to mark with diluent to obtain working standard solution 200µg/ml of Sofosbuvir and 45µg/ml Ledipasvir.

- **Preparation of Sample Solution of Sofosbuvir and Ledipasvir**

Twenty tablets were weighed and finely powdered. Powdered equivalent to 400 mg Sofosbuvir and 90 mg Ledipasvir was accurately weighed and transferred to 200 ml volumetric flask, and 140 ml of diluent was added and sonicated for 20 min finally volume was made up to the mark with diluent. The solution was filtered through whatmann filter paper (0.45µ). From this solution 5.0 ml was transferred to 50 ml volumetric flask and volume was made up to the mark with diluent to give a solution containing 200µg/ml Sofosbuvir and 45µg/ml Ledipasvir.

2.4. Selection of Wavelength for Estimation

Standard solution of Sofosbuvir 40µg/ml and Ledipasvir 9.0µg/ml were prepared using their working standard solution using diluent as a solvent. Each solution was scanned between 200-400 nm using diluent as a blank. The point at which both drug shows absorbance was selected as wavelength for estimation (245nm).

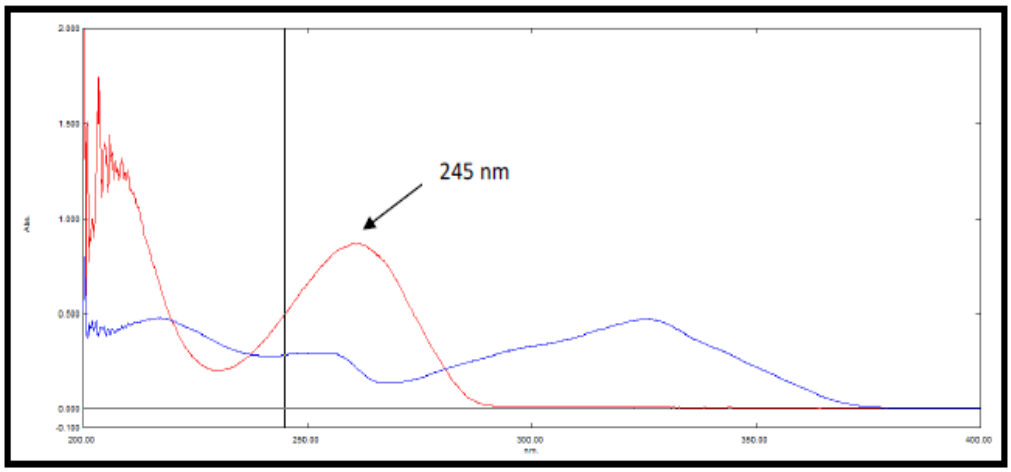

Figure 3: Spectra of standard Sofosbuvir and Ledipasvir for wavelength selection

Table 1: Optimized chromatographic condition

Parameters	Condition
Stationary phase	Inertsil ODS C18 column (150mm X 4.6 mm, 5 µm particle size)s
Mobile Phase	0.1% TFA : Acetonitrile (70:30%v/v)
Pump mode	Isocratic
Flow rate (ml/min)	1.0
Run time (min)	7.0
Volume of Injection (µl)	20
Detection wavelength	245
Retention time (min)	Sofosbuvir : 2.39
	Ledipasvir : 5.51

Table 2: System suitability parameters for final optimized chromatographic conditions

Parameters	Data obtained	
	Sofosbuvir	Ledipasvir
Retention time (Rt)	2.39min	5.51 min
Resolution	7.22	
Theoretical plates (N)	4025	8977
Tailing factor (T_f)	1.05	1.11

So, from all the above explained parameters, it was concluded that that the most efficient resolution and peak symmetry for Sofosbuvir and Ledipasvir were achieved with the above chromatographic conditions and with a mobile phase composed of 0.1%TFA :Acetonitrile (70 : 30 %v/v)

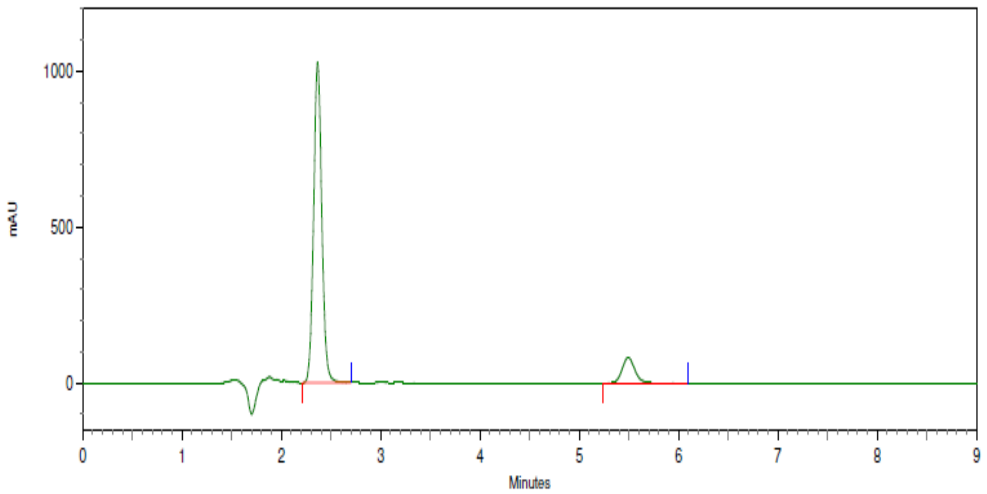

Figure 4: Chromatogram of standard showing separated peaks of Sofosbuvir and Ledipasvir

3. Method Validation

The described method has been validated for linerity, accuracy, limit of detection, precision, and robustness, as per ICH guidelines.[14]

3.1. Linearity and Range:

Linear relation was obtained between mean peak area and concentration of the drug in the range of 100-600 µg/ml for Sofosbuvir and 22.5-135 µg/ml for Ledipasvir. The data of the peak areas obtained with the respective concentration in µg/ml are shown in Table 3 and 5 for Sofosbuvir and Ledipasvir respectively. The linearity curves for Sofosbuvir and Ledipasvir are shown in Fig.5 and 6 respectively.

Table 3: Data of peak areas of Sofosbuvir(100-600µg/ml)

Sr. No.	Concentration (µg/ml)	Peak area of Sofosbuvir
1	100	6018619
2	200	12053329
3	300	18074368
4	400	24115257
5	500	30130479
6	600	36151273

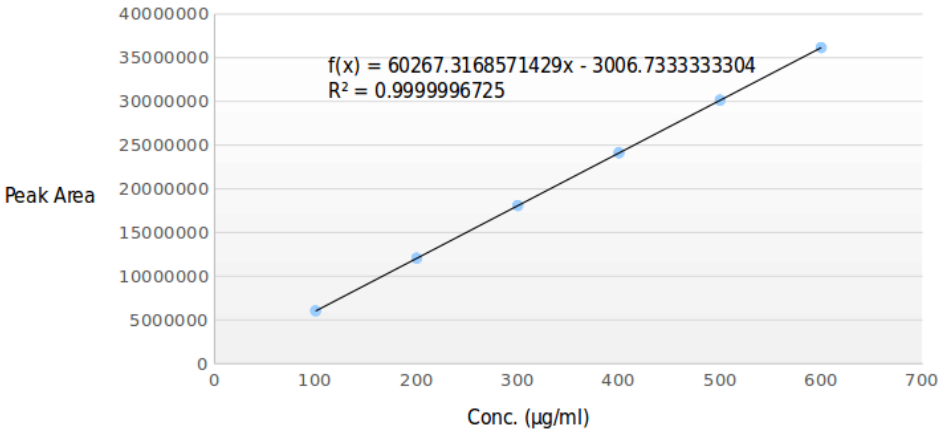

Fig. 5: Calibration curve for Sofosbuvir

Table 4: Data showing regression characteristics of Sofosbuvir

Regression equation	Y = 60267.3169x – 3006.7333
Regression co-efficient	1.0000

Table 5: Data of peak areas of Ledipasvir (22.5-135 µg/ml)

Sr. No.	Concentration (µg/ml)	Peak area of Ledipasvir
1	22.5	806846
2	45	1618653
3	67.5	2427643
4	90	3237175
5	112.5	4043608
6	135	4855008

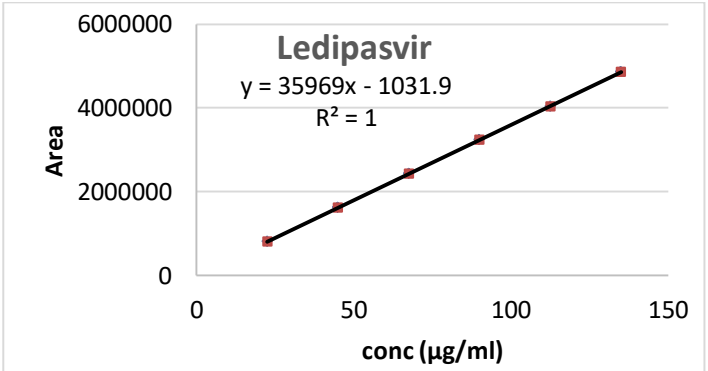

Fig. 6 : Calibration curve for Ledipasvir

Table 6: Data showing regression characteristics of Ledipasvir

Regression equation	Y = 35968.52x-1031.8667
Regression co-efficient	1.0000

3.2. Accuracy

Accuracy refers to closeness of the test results obtained by the method to the true value. Accuracy was performed by the standard addition methods. To a fixed amount of the pre-analysed mixture add a 50%, 100% and 150% of the standard solution and % recovery was calculated. The results are shown in Table 7

Table 7: Recovery data for Sofosbuvir

Amount of Sofosbuvir Present (µg/ml)	Amount of Std Sofosbuvir Added (µg/ml)	Total Amount of Sofosbuvir (µg/ml)	Amount Recovered (µg/ml)	% Recovery
200	300	500	499.830	**99.92**
			501.982	
			496.986	
200	200	400	401.139	**99.72**
			396.993	
			398.532	
200	100	300	300.381	**100.31**
			300.357	
			302.039	

Table 8: Recovery data for Ledipasvir

Amount of Ledipasvir Present (µg/ml)	Amount of Std Ledipasvir Added (µg/ml)	Total Amount of Ledipasvir (µg/ml)	Amount Recovered (µg/ml)	% Recovery
45	67.5	112.5	112.575	**100.45**
			113.023	
			113.423	
45	45	90	90.652	**99.84**
			89.607	
			89.307	
45	22.5	67.5	67.035	**100.55**
			68.129	
			68.453	

3.3. Precision

3.3.1. Repeatability

It was performed by 100% test concentration level and % RSD was calculated. The data for repeatability for combined solution of Sofosbuvir and Ledipasvir is presented in Table 9

Table 9: Repeatability of Sofosbuvir and Ledipasvir

Sr. No.	Sofosbuvir (200 µg/ml)	Ledipasvir (45µg/ml).
1	12363864	1532054
2	12264518	1528040
3	12288474	1530889
4	12289526	1530928
5	12303266	1532196
6	12245790	1552151
Mean	**12292573**	**1534376**
SD	**40493.37**	**8835.02**
% RSD	**0.33**	**0.58**

3.3.2. Interday and Intraday Precision of Sofosbuvir:

The data for Intraday and Interday precision for Sofosbuvir are presented in Table 10 for Intraday Sample solutions containing 200µg/ml of Sofosbuvir and 45µg/ml Ledipasvir were prepared six times and measured on the same day and % RSD was calculated. For Interday Sample solutions containing 200µg/ml of Sofosbuvir and 45µg/ml Ledipasvir were prepared six times and measured on the different days and % RSD was calculated.

Table 10: Intraday and Interday precision data for Sofosbuvir

Concentration (µg/ml)	Intraday		Interday	
	Mean % Estimation ± SD	% RSD	Mean % Estimation ± SD	% RSD
200	100.10 ± 0.5462	0.55	100.24 ± 0.7162	**0.71**

3.3.3. Intraday and Interday Precision of Ledipasvir :

The data for Intraday and Interday precision for Ledipasvir are presented in Table: 10 The % RSD for Intraday precision was found to be 0.53 for Ledipasvir. The % RSD for Interday precision was found to be 0.67 for Ledipasvir.

Table 11: Intraday and Interday precision data of Ledipasvir

Concentration (µg/ml)	Intraday		Interday	
	Mean % Estimation ± SD	% RSD	Mean % Estimation ± SD	% RSD
45	100.05 ± 0.5281	**0.53**	100.03 ± 0.6682	**0.67**

3.4. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Table 12: Limit of detection

Sofosbuvir	Ledipasvir
LOD = 3.3 x (SD/Slope) LOD = 3.3 x (7214/60267.3169) LOD = 0.395 µg/ml	LOD = 3.3 x (SD/Slope) LOD = 3.3 x (1443/35968.5168) LOD = 0.132 µg/ml

Table 13: Limit of Quantification

Sofosbuvir	Ledipasvir
LOQ = 10 x (SD/Slope) LOQ = 10 x (7214/60267.3169) LOQ = 1.197 µg/ml	LOQ = 10 x (SD/Slope) LOQ = 10 x (1443/35968.5168) LOQ = 0.401 µg/ml

3.5. Specificity

The specificity was determined by the comparison of the chromatograms of:

- Blank (mobile phase) (Fig 7)
- Standard sample solutions of Sofosbuvir and Ledipasvir(Fig.8 and 9 for Sofosbuvir and Ledipasvir respectively),
- Sample solution of Sofosbuvir and Ledipasvir (Fig. 10).

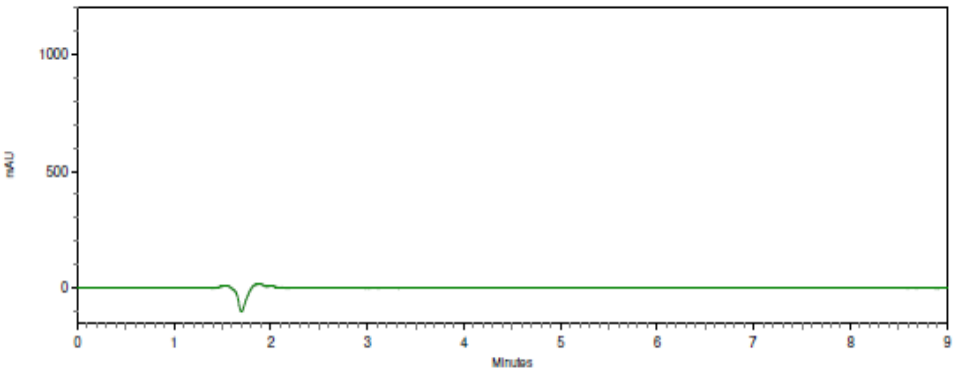

Fig.7: Chromatogram of Blank (mobile phase)

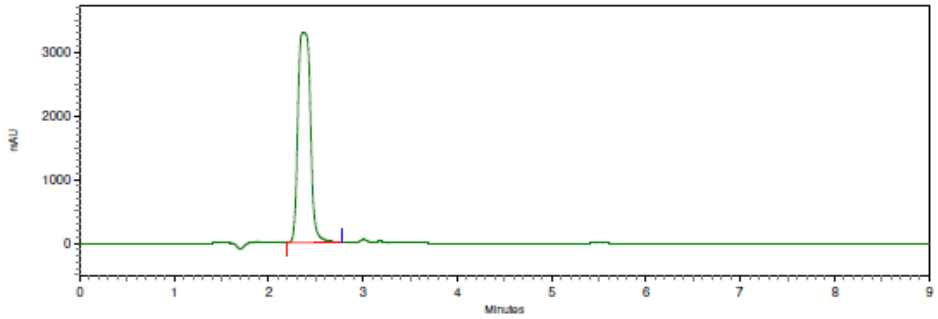

Fig. 8: Chromatogram of standard Sofosbuvir

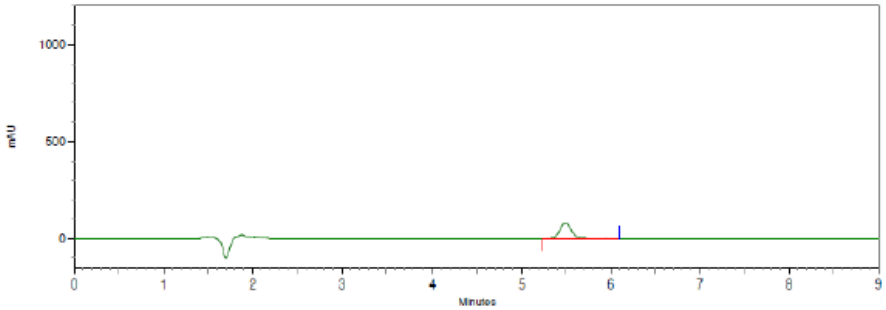

Fig. 9: Chromatogram of standard Ledipasvir

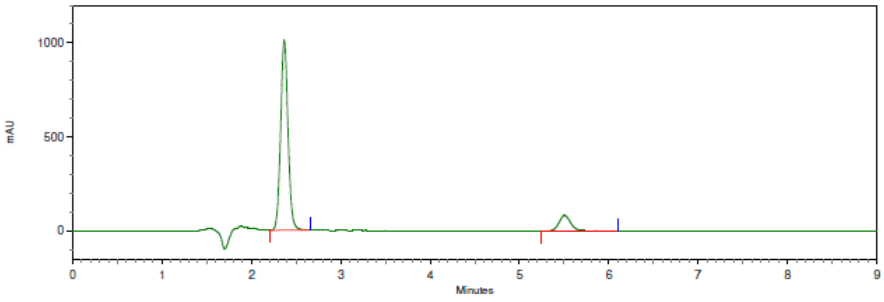

Fig. 10: Chromatogram of sample solution containing Sofosbuvir(200µg/ml) and Ledipasvir (45µg/ml)

3.6. Robustness

Robustness study was performed in following altered chromatographic condition:

Variation in the flow rate (± 0.2 mL/min)

Variation in mobile phase composition (± 2%)

Table 14: Robustness data for ± 0.2 mL/min variation in Flow rate

Flow rate	Peak area		Mean±SD		% RSD	
	SOFOS	LEDI	SOF	LED	SOF	LED
0.8 mL/min	14313927	1757607	14350196 ± 51292.1117	1757224 ± 540.9367	**0.36**	**0.03**
	14386465	1756842				
1.2 mL/min	11094624	1380696	11096462 ± 2599.3245	1380890 ± 275.0645	**0.02**	**0.02**
	11098300	1381085				

Table 15: Robustness data for ± 2% variation in mobile phase

Mobile phase	Peak area		Mean±SD		% RSD	
	SOFOS	LEDI	SOF	LED	SOF	LED
72:28	12441394	1540933	12450838± 13355.1258	1542977 ± 2889.9454	**0.11**	**0.19**
	12460281	1545020				
68:32	12371514	1547725	12371612± 137.8858	1547848 ± 173.9483	**0.00**	**0.01**
	12371709	1547971				

4. Assay Formulation analysis

Table 16: Estimation of Sofosbuvir and Ledipasvir in their Pharmaceutical Dosage Form (Tablet)

Labelled Value (mg)	Amt of Sofosbuvir found (mg)	% Assay	Labelled Value (mg)	Amt of Ledipasvir found (mg)	% Assay
400	402.16	100.54	90	90.20	100.23
	398.92	99.73		89.66	99.62
	402.05	100.51		90.00	100.01
	403.38	100.84		90.26	100.29
	401.36	100.34		90.41	100.46
	402.93	100.73		90.00	100.00
Mean	**401.80**	**100.45**	**Mean**	**90.09**	**100.10**
SD	**1.5791**	**0.3948**	**SD**	**0.2625**	**0.2917**
% RSD	**0.39**	**0.39**	**% RSD**	**0.29**	**0.29**

5. Force degradation studies

5.1. Preparation of sample stock solution

Accurately weighed Sofosbuvir (200.00 mg) and Ledipasvir (45.00 mg) were transferred into 100 ml volumetric flask, sonicated to dissolve and diluted up to mark with diluent to give a stock solution (2000µg/ml) of Sofosbuvir and (450µg/ml) Ledipasvir. Designate it as a combine stock solution. Stock solution (5.0 ml) was transferred to 50 ml volumetric flask and diluted up to mark with diluent to obtain working standard solution (200µg/ml) of Sofosbuvir and (45µg/ml) Ledipasvir.

5.2. Acid degradation study (5NHCL)

From the test stock solution 1ml was taken in 10 ml volumetric flask, add 1ml of 5N HCL and heated at 60˚ for 30 min on a water bath. The flask was removed from the water bath and allows to cool at room temperature. Add 1ml of 5N NaoH to neutralize the solution and diluted to volume with diluents and mixed. 10µl solution were injected in to the system and the chromatograms were recorded to assess the stability of sample.

5.3. Alkali degradation studies (5N NaoH)

From the test stock solution 1ml was taken in 10ml volumetric flask, add 1ml of 5N NaoH and heated at 70˚c for 1h on a water bath. The flask was removed from the water bath and allowed to cool at room temperature. Add 1ml of 5 N HCL to neutralize the solution and diluted to volume with diluents and mixed.10µl solution were injected in to the systeam and chromatograms were recorded to asses the stability of sample.

5.4. peroxide (Oxidation) degradation studies (30%v/v of H2O2)

From the test stock solution 1ml Was taken in 10ml volumetric flask add 1ml of 30% H2O2 and heated at 70˚ c for 1hour on a water bath. The flask was removed from the water bath and allowed to cool at room temperature and diluted to volume with diluents and mixed. 10µl solutions were injected in to the systeam and the chromatograms were recorded to acess the stability of sample.

5.5. Thermal degradation studies (105˚c/ 48hr)

For the thermal degradation 200mg Sofosbuvir and 45mg lLedipasvir drug samples were weighed accurately and transfer to petridish heat the sample in oven for 48hr at 105˚c and transfer the sample into a 100ml volumetric flask dissolve and dilute to volume with diluents. Filter the solution using 0.45µ Nyllon filter. Transfer 5ml of above stock solution to 50ml volumetric flask and make up the volume with diluents to get the concentration of (200µg/ml) of Sofosbuvir and (45µg/ml) Ledipasvir. 10 µl solutions was injected into the system and the chromatogram recorded to access the stability of sample.

5.6. Photolytic degradation (2600lux for 24hr)

For the thermal degradation 200mg Sofosbuvir and 45mg lLedipasvir drug samples were weighed accurately and transfer to petridish. The sample was exposed to UV light in a photolytic chamber at 1.2 millon lux hours for 24h and transfer the sample into a 100ml volumetric flask dissolve and dilute to volume with diluents. Filter the solution using 0.45µ Nyllon filter. Transfer 5ml of above stock solution to 50ml volumetric flask and make up the volume with diluents to get the concentration of (200µg/ml) of Sofosbuvir and (45µg/ml) Ledipasvir. . 10µl solutions was injected into the systeam and the chromatogram recorded to access the stability of sample.

6. RESULT AND DISCUSSION

6.1. Optimized chromatographic conditions

The optimized chromatographic conditions fig3. The best peak shape and maximum separation was achieved with mobile phase ACN : 0.1% TFA in the proportion of 30:70 %v/v, peak symmetry and reproducibility obtained on inetsil ODS C18 column (150X4.6mm, 5µm), optimum Wave length To detecting analyte was found to be 245 nm , a flow rate of 1ml/min yield optimum separation and peak symmetry. Chromatogram of Sofosbuvir and Ledipasvir fig 4 and optimized chromatographic condition is shown in table 1.

6.2. Linearity

Linear relation was obtained between mean peak area and concentration of the drug in the range of 100-600 µg/ml for Sofosbuvir and 22.5-135 µg/ml for Ledipasvir. Shown in the fig 5 and 6.

6.3. Accuracy

The percentage recovery for Sofosbuvir were 99.72-100.31% and for Ledipasvir 99.84-100.55%.shown in the Table 6 and 7.

6.4. Precision

6.4.1 Repeatability

The data for repeatability for combined solution of Sofosbuvir and Ledipasvir is presented in Table: 8 and % RSD was found to be 0.33 for Sofosbuvir and 0.58for Ledipasvir.

6.4.2 Intraday and interday precision

The data for Intraday and Interday precision for Sofosbuvir are presented in Table: 9. The % RSD for Intraday precision was found to be 0.55 for Sofosbuvir. The % RSD for Interday precision was found to be 0.71 for Sofosbuvir.

The data for Intraday and Interday precision for Ledipasvir are presented in Table: 10. The % RSD for Intraday precision was found to be 0.53 for Ledipasvir. The % RSD for Interday precision was found to be 0.67 for Ledipasvir.

6.5. LOD and LOQ

The LOD for Sofosbuvir and Ledipasvir were found to be 0.395µg/ml and 0.132µg/ml respectively, shown in table no 11.

LOQ for Sofosbuvir and Ledipasvir were found to be 1.197µg/ml and 0.401µg/ml respectively, shown in table 12.

6.6. Specificity

The specificity of the method was evaluated with regard to interferance due to presence of any othe excipient. The figure show that the selected drugs were clearly separated. Fig 7 and 10 show that the chromatogram of blank , standard. solution. There were no interfering peak at retention time of Sofosbuvir and Ledipasvir.

6.7.Robustness

Result of the robustness Table 13 & 14. The elution order and resolution for all components were not significantly affected. RSD of peak area were found to be well within the limit of 2.0%.

6.8. Degradation study

All the stability results were shown in table 16 and fig 11 (a-e)

Table 17: stability results

Stress Condition	Sofosbuvir % Degradation	Ledipasvir % Degradation
Acid	0.44	1.18
Basic	0.02	0.05
Oxidizing	0.41	1.73
Thermal	0.04	0.05
Photolysis	0.01	0.07

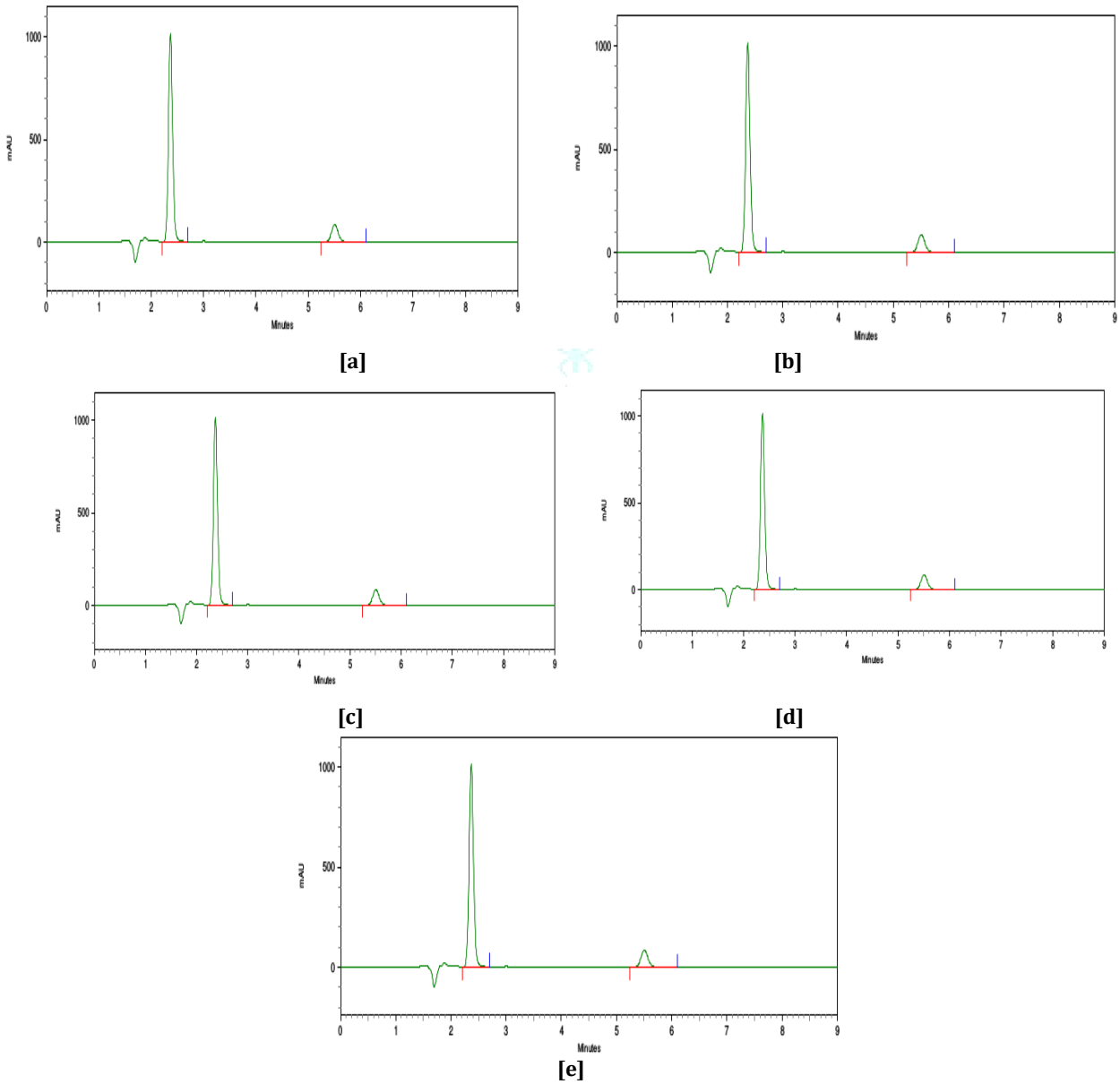

Fig 11: (a) Acid degradation (b) alkali degradation (c) peroxide degradation (d) thermal degradation (e) photodegradation

7. CONCLUSION

A simple, fast, accurate and precise stability- indicating HPLC analytical method has been developed and validated for the Quantitative analysis of Sofosbuvir and Ledipasvir in combined tablet dosage forms. The result of stress testing undertake according to the ICH guidelines reveal that the method is specific and stability indicating. The proposed method has the ability to separate these drugs from their degradation products in tablet dosage forms and hence can be applied to the analysis of routine quality control samples obtained from stability studies.

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