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HPLC METHOD FOR THE DETERMINATION OF SOFOSBUVIR AND LEDIPASVIR IN TABLET DOSAGE FORM

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ABSTRACT
Sofosbuvir and Ledipasvir are available in solid dosage form to cure hepatitis C. the objective of this study was to develop simple HPLC method for both active components. Preparation of buffer: 1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water. Chromatographic conditions are mobile phase: 50% OPA (0.1%): 50% Acetonitrile, flow rate: 1 ml/min, column: Discovery C8 (4.6 x 250mm, 5µm), detector wave length: 230nm, column temperature: 30°C, injection volume: 10µL, run time: 7 min, diluent: water and acetonitrile in the ratio 50:50 v/v. method validation was carried out and results confirmed the method ruggedness and stability indicating. Optimized method can be used for regular analysis.

Keywords: Sofosbuvir, Ledipasvir, HPLC method development, Stability indicating

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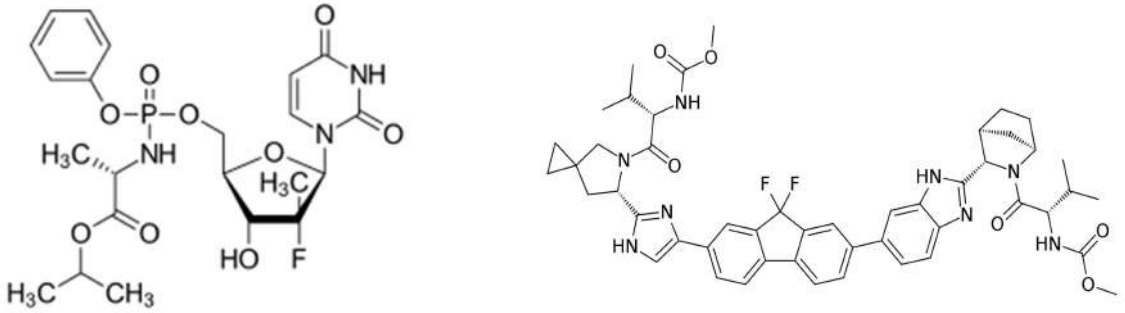
INTRODUCTION

Sofosbuvir is used cure hepatitis C[1-2]. the chemical name of Sofosbuvir is Isopropyl (2*S*)-2-[[[(2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetrahydrofuran-2-yl]methoxy-phenoxy-phosphoryl]amino]propanoate. Sofosbuvir is used along with ribavirin, peginterferon-alfa, simeprevir, Ledipasvir, daclastavir and velpatasvir[3-5]. Combination dosage form will be used for longer treatment durations, depending on specific circumstances, genotype and cost effective based perspective. The side effects of Sofosbuvir are fatigue, headache, nausea, rash, irritability, dizziness, back pain and anemia.

Ledipasvir is used to great hepatitis C[6-7]. The most commonly used combination with Sofosbuvir to treat chronic hepatitis C genotype 1 patients[8-10]. Ledipasvir chemical name is Methyl *N*-[(2*S*)-1-[(6*S*)-6-[5-[9,9-Difluoro-7-[2-[(1*S*,2*S*,4*R*)-3-[(2*S*)-2-(methoxy carbonyl amino)-3-methylbutanoyl]-3-azabicyclo[2.2.1]heptan-2-yl]-3*H*-benzimidazol-5-yl] fluoren-2-yl]-1*H*-imidazol-2-yl]-5-azaspiro[2.4]heptan-5-yl]-3-methyl-1-oxobutan-2-yl] carbamate. The side effects are fatigue and headache. Chemical structure of Sofosbuvir and Ledipasvir was represented in figure-1.



Sofosbuvir Ledipasvir

Figure-1: Chemical structure of Sofosbuvir and Ledipasvir

Literature reports were studied and understand the previous research outcomes. Some of authors were reported with dissolution profile determination[11-12], HPLC methods [13-14] and TLC and spectroscopic methods[15-16] and plasma samples analysis[17].

MATERIALS AND METHOD

Preparation of buffer: 1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water.

Mobile phase	**:**	50% OPA (0.1%): 50% Acetonitrile
Flow rate	**:**	1 ml/min
Column	**:**	Discovery C8 (4.6 x 250mm, 5µm)
Detector wave length	**:**	230nm
Column temperature	**:**	30°C
Injection volume	**:**	10µL
Run time	**:**	7 min
Diluent	**:**	Water and Acetonitrile in the ratio 50:50

Preparation of Standard stock solutions: Accurately weighed 4.5mg of Ledipasvir, 20mg of Sofosbuvir and transferred to 50ml & 50ml volumetric flasks and 3/4th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (90µg/ml of Ledipasvir and 400µg/ml Sofosbuvir)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (9µg/ml of Ledipasvir and 40µg/ml of Sofosbuvir)

Preparation of Sample stock solutions: 5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 100ml volumetric flask, 50ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (900µg/ml of Ledipasvir and 4000µg/ml of Sofosbuvir)

Preparation of Sample working solutions (100% solution): 0.1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (9µg/ml of Ledipasvir and 40µg/ml of Sofosbuvir)

RESULTS AND DISCUSSION

METHOD DEVELOPMENT:

Trial 1:

Chromatographic conditions:

Mobile phase: Water and Methanol taken in the ratio 45:55; Flow rate: 1 ml/min, Column: Altima C18 (4.6 x 150mm, 5µm), Detector wave length: 230nm, Column temperature : 30°C, Injection volume: 10µL, Run time: 10 min, Diluent: Water and Acetonitrile in the ratio 50:50.

Results: Sofosbuvir was eluted but Ledipasvir not eluted so further trial was carried out.

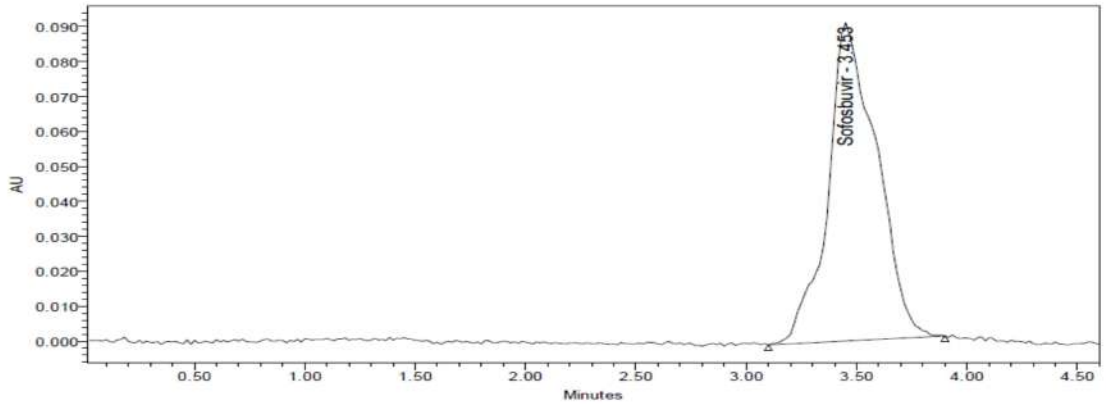


Figure-2: Trial chromatogram 1

Trial 2:

Chromatographic conditions:

Mobile phase: 0.1% OPA: Acetonitrile (40:60), Flow rate: 1 ml/min, Column: Altima C18 (4.6 x 150mm, 5µm), Detector wave length: 230nm, Column temperature: 30°C, Injection volume: 10µL, Run time: 10 min, Diluent: Water and Acetonitrile in the ratio (50:50 V/V).

Results: Sofosbuvir and Ledipasvir were eluted but Ledipasvir shows tailing and baseline disturbances were observed so further trial was carried out

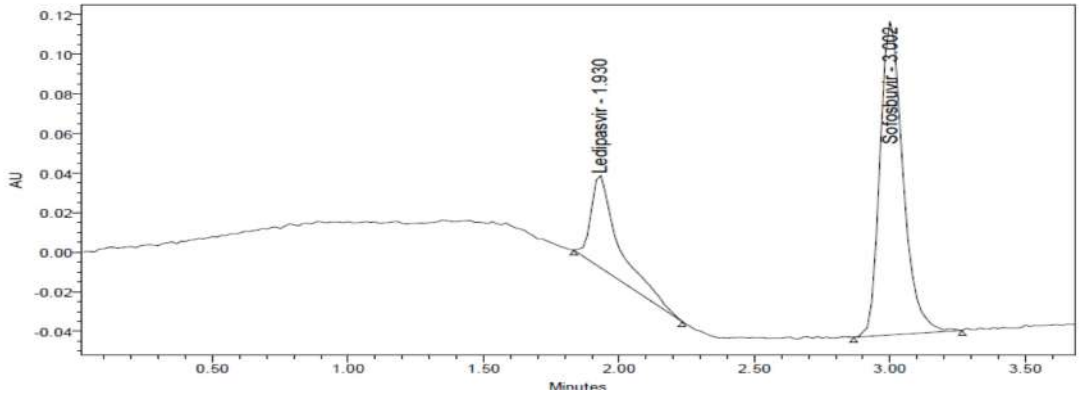


Figure-3: Trial chromatogram 2

Trial 3:

Chromatographic conditions:

Mobile phase: 55% OPA: 45% Acetonitrile, Flow rate: 1 ml/min, Column: Altima C18 (4.6 x 150mm, 5µm), Detector wave length: 230nm, Column temperature: 30°C, Injection volume: 10µL, Run time: 10 min, Diluent : Water and Acetonitrile in the ratio 50:50.

Results: Both peaks shapes were not good and ledipasvir shows fronting so, further trail was carried out.

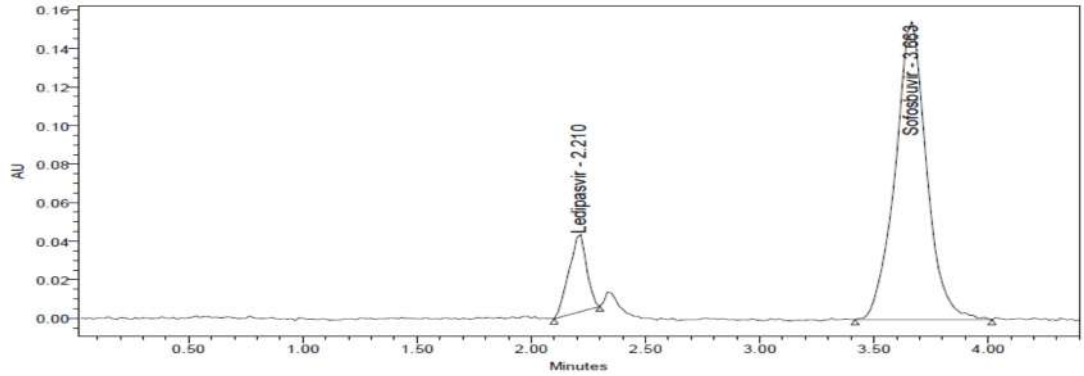


Figure-4: Trial chromatogram 3

Trial 4:

Chromatographic conditions:

Mobile phase: 55% 0.01N Kh2po4: 45% Acetonitrile, Flow rate: 1 ml/min, Column: Altima C18 (4.6 x 150mm, 5µm), Detector wave length: 230nm, Column temperature: 30°C, Injection volume: 10mL, Run time: 10 min, Diluent: Water and Acetonitrile in the ratio 50:50.

Results: Ledipasvir peak shape was not good so, further trail was carried out.

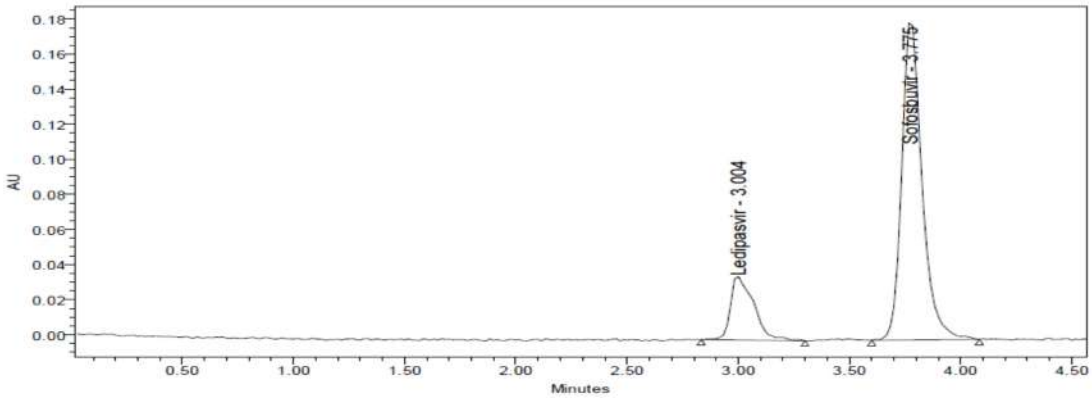


Figure-5: Trial chromatogram 4

Trial 5:

Chromatographic conditions:

Mobile phase: 50% 0.01N Kh2po4: 50% Acetonitrile, Flow rate: 1 ml/min, Column: Discovery C18 (4.6 x 250mm, 5µm), Detector wave length: 230nm, Column temperature: 30°C, Injection volume: 10mL, Run time: 10 min, Diluent: Water and Acetonitrile in the ratio 50:50 v/v.

Results: Both peaks shapes were good but retention time was more so, further trail was carried out.

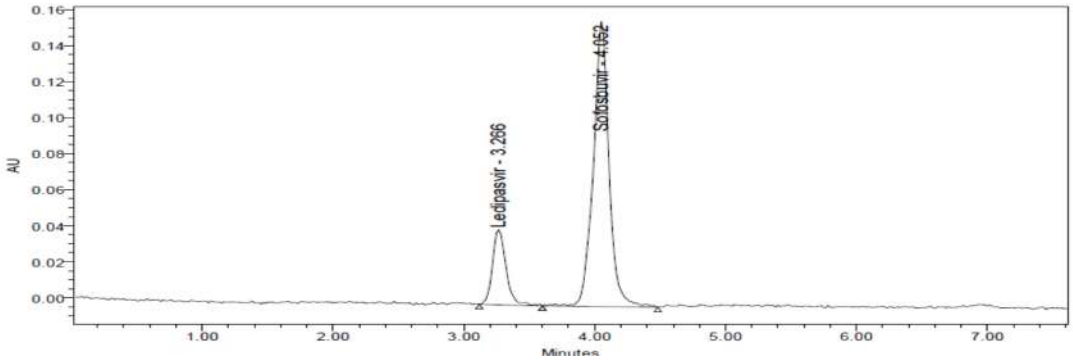


Figure-6: Trial chromatogram 5

Optimized method:

Chromatographic conditions:

Mobile phase: 50% OPA (0.1%): 50% Acetonitrile, Flow rate: 1 ml/min, Column: Discovery C8 (4.6 x 250mm, 5µm), Detector wave length: 230nm, Column temperature: 30°C, Injection volume: 10µL, Run time: 7 min, Diluent: Water and Acetonitrile in the ratio 50:50 v/v.

Results: Both peaks have good resolution, tailing factor, theoretical plate count and resolution.

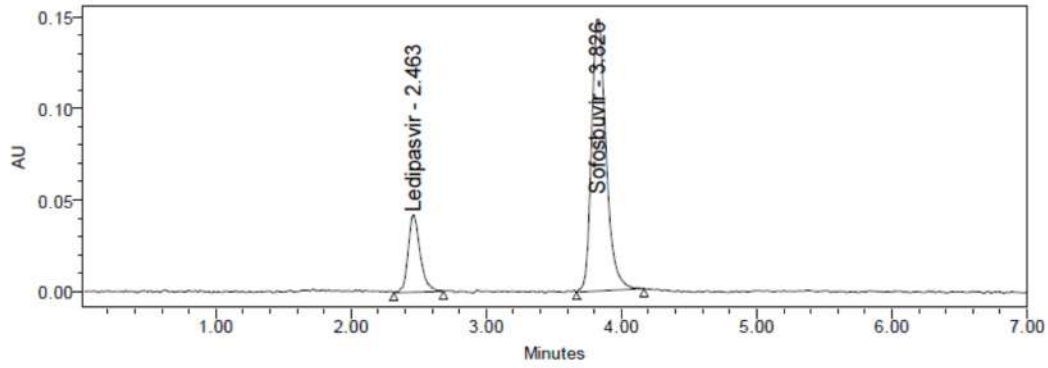


Figure-7: Trial chromatogram 6

Observation: Ledipasvir and Sofosbuvir were eluted at 2.463 min and 3.826 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

METHOD VALIDATION:

System suitability:

HPLC method chromatographic conditions system suitability parameters with six replicate standard solutions. Blank, placebo and standard solution chromatogram were represented figure-8 to 10. System suitability results were tabulated in table-1.

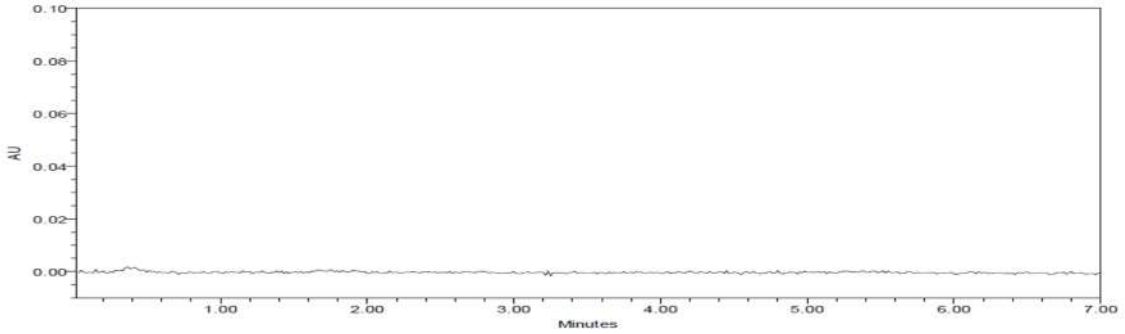


Figure-8: Blank chromatogram

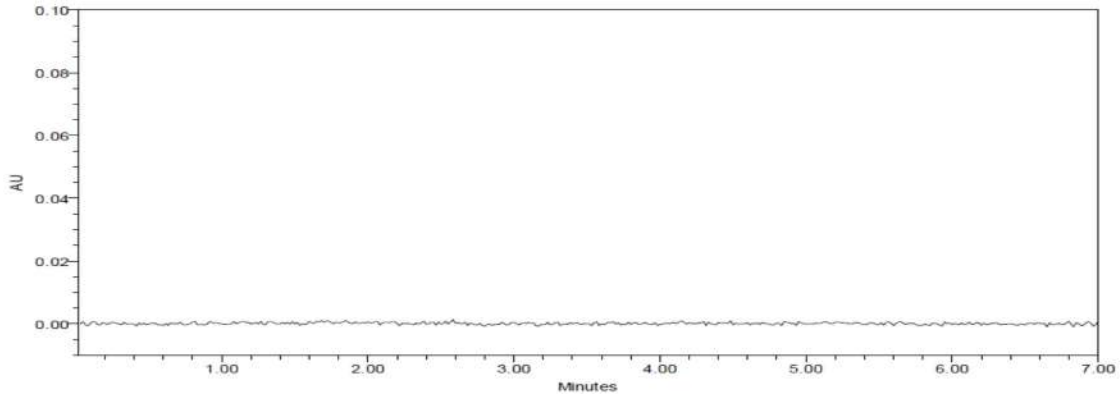


Figure-9: Placebo chromatogram

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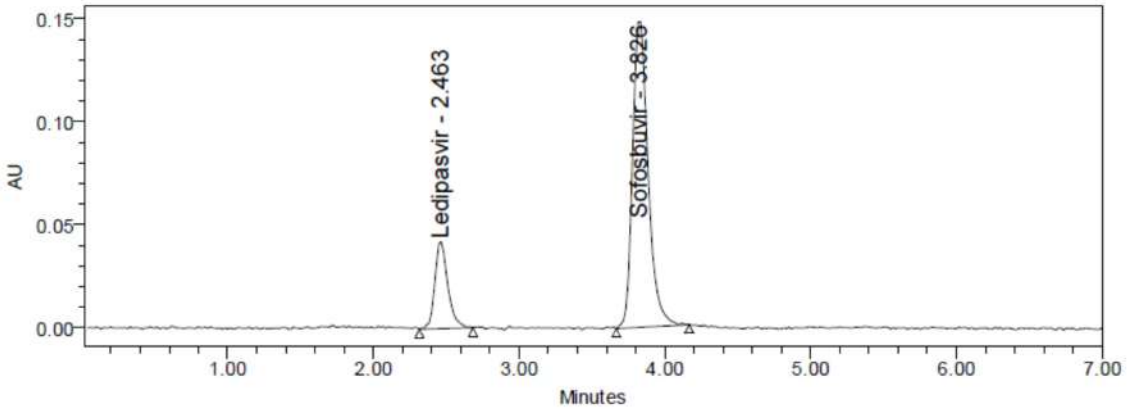

Figure-10: Standard chromatogram

Table-1: System suitability results

System suitability results						
Injection	**Ledipasvir**			**Sofosbuvir**		
	RT (min)	**Area**	**Tailing factor**	**RT (min)**	**Area**	**Tailing factor**
1.	2.450	241795	1.35	3.808	1020204	1.28
2.	2.452	243770	1.29	3.808	1017682	1.30
3.	2.452	246707	1.39	3.814	1014691	1.35
4.	2.453	244954	1.34	3.816	1043798	1.29
5.	2.455	245584	1.34	3.817	1028914	1.31
6.	2.463	245095	1.19	3.826	1020344	1.27
Average	NA	244651	NA		1024272	NA
%RSD		0.7			1.0	

Precision:

Precision of the HPLC method was validated and performed the system precision and method precision. Six replicate test solutions were prepared and injected in the system. Precision samples % assay and % RSD was calculated. Intermediate precision was performed with same method and different HPLC system different column and different analyst. Precision and intermediate precision results were tabulated in table-2.

Table-2: Precision and intermediate results

S.No.	**Precision assay (%)**		**Intermediate precision assay (%)**	
	Sofosbuvir	**Ledipasvir**	**Sofosbuvir**	**Ledipasvir**
1.	99.23	100.02	100.35	99.99
2.	98.79	100.47	100.56	100.21
3.	100.17	101.26	100.26	100.16
4.	100.02	99.85	101.00	100.14
5.	99.45	100.07	100.59	100.24
6.	100.34	99.47	100.24	100.54
Avg.	**99.67**	**100.19**	**100.50**	**100.21**
%RSD	**0.60**	**0.62**	**0.28**	**0.18**

Specificity:

Specificity was performed to confirm the interference from the blank, placebo and degradation studies. Degradation studies were performed with acid, base, peroxide, thermal, UV/visible, water and humidity stress conditions. Degradation samples assay and % of degradation results were calculated. Force degradation studies chromatograms were represented in figure-11 to 17. Specificity results were tabulated in table-3.

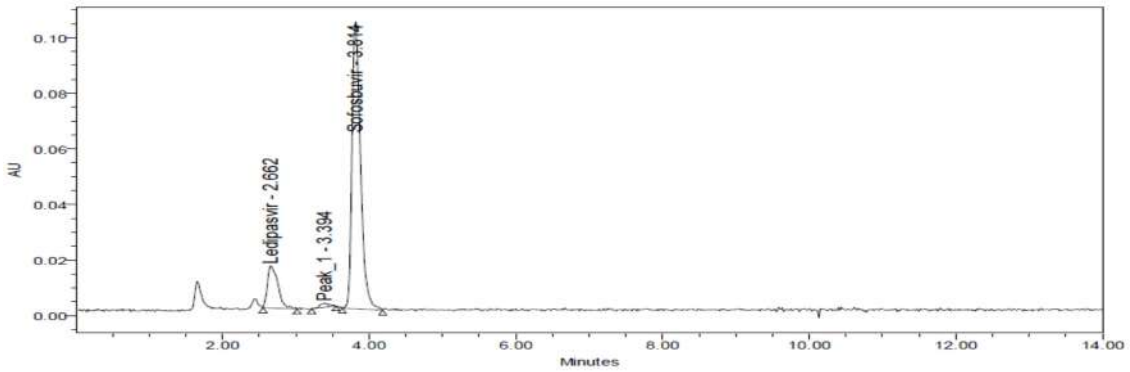

Figure-11: Acid Degradation chromatogram

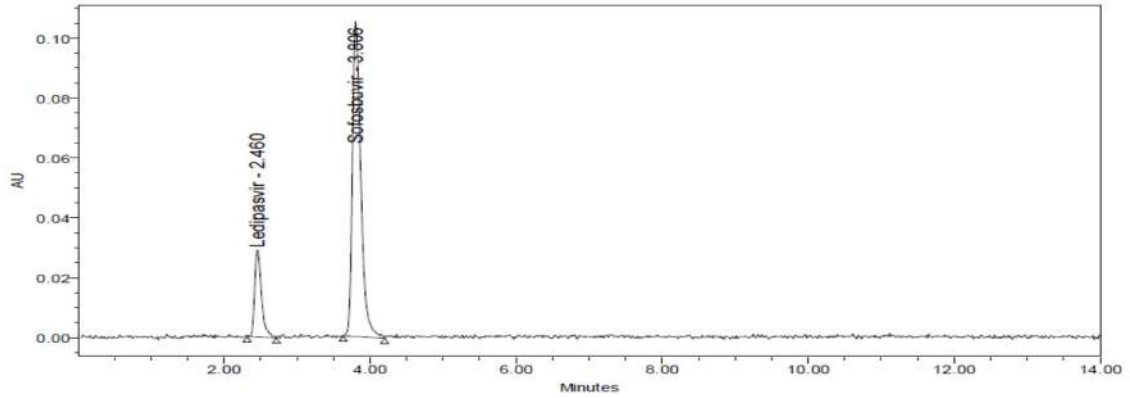

Figure-12: Base Degradation chromatogram

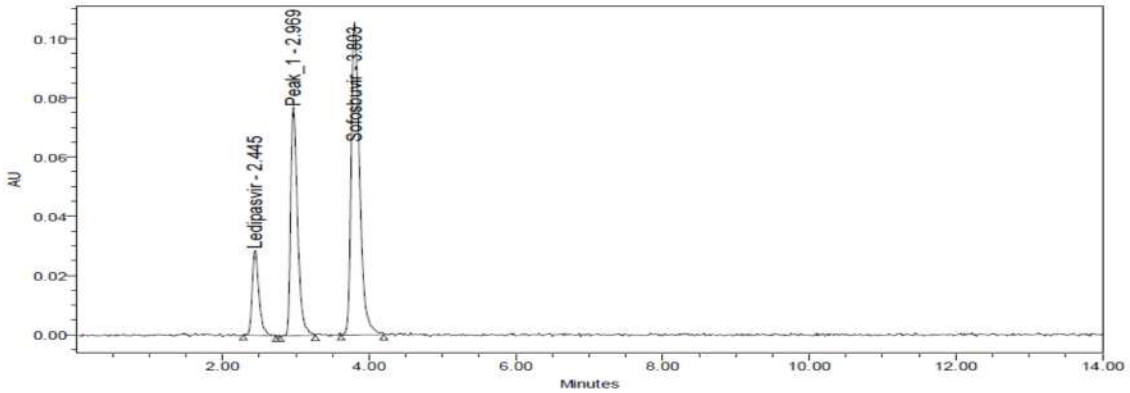

Figure-13: Peroxide Degradation chromatogram

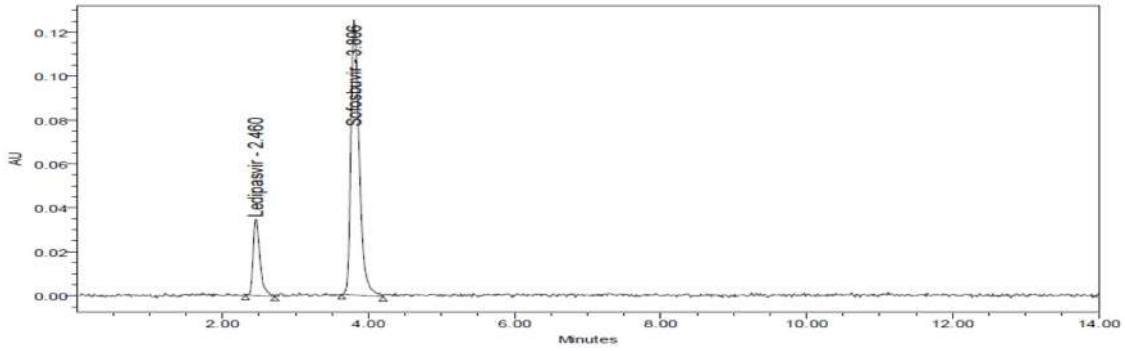

Figure-14: Thermal Degradation chromatogram

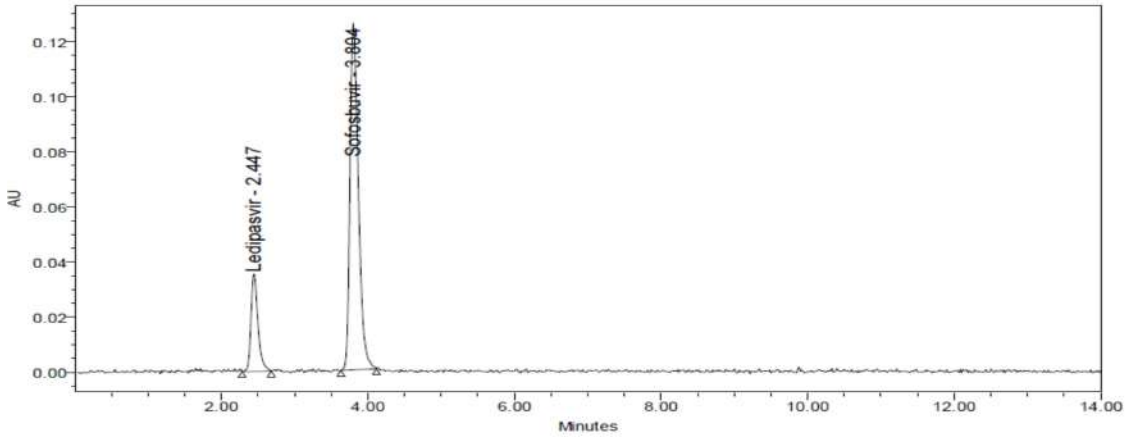

Figure-15: Water Degradation chromatogram

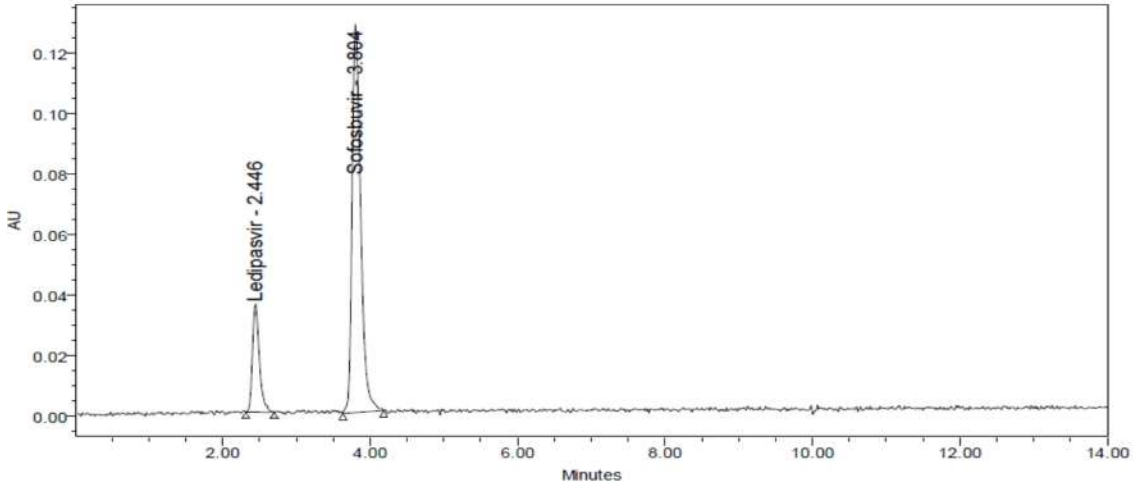

Figure-16: UV/ Visible Degradation chromatogram

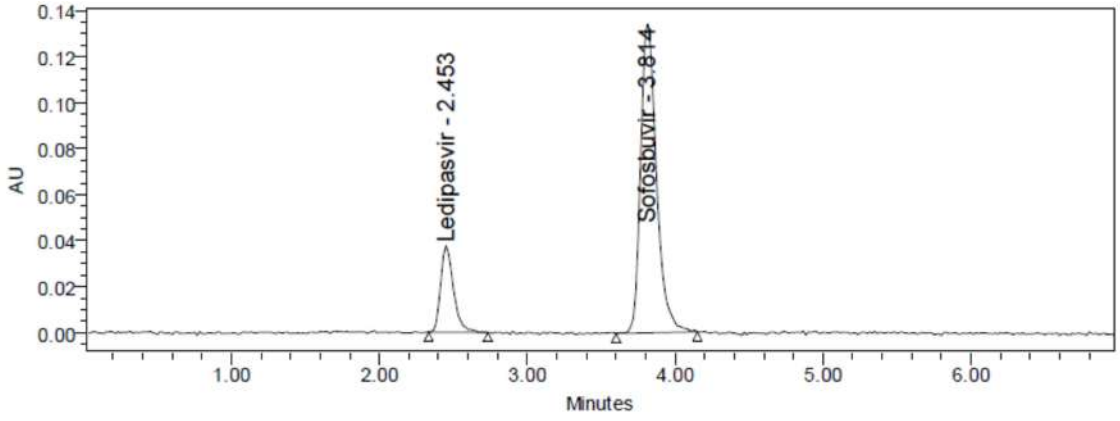

Figure-17: Humidity degradation chromatogram

Table-3: Specificity results

Sofosbuvir degradation results				
S.No.	**Name of Stress and condition**	**% assay**	**% degradation**	**Peak purity**
1.	Acid /2N-60°C/30 min	94.59	5.41	Pass
2.	Base /2N- 60°C/30 min	96.33	3.67	Pass
3.	Peroxide /20%- 60°C/ 30 min	97.12	2.88	Pass

4.	Water -60°C/1 hr	97.31	2.69	Pass
5.	Thermal (105°C for 6 hrs)	98.85	1.15	Pass
6.	UV/visible light	99.13	0.87	Pass
7.	Humidity 75% RH, 1 day	96.25	3.75	Pass
Ledipasvir degradation results				
1.	Acid /2N-60°C/30 min	95.25	4.75	Pass
2.	Base /2N- 60°C/30 min	95.80	4.20	Pass
3.	Peroxide /20%- 60°C/ 30 min	94.05	5.95	Pass
4.	Water -60°C/1 hr	97.4	2.57	Pass
5.	Thermal (105°C for 6 hrs)	98.00	2.00	Pass
6.	UV/visible light	99.00	1.00	Pass
7.	Humidity 75% RH, 1 day	96.35	3.50	Pass

Linearity:

Linearity was performed with different concentration levels 25%, 50%, 75%, 100%, 125% and 150% linearity levels. Linearity levels chromatograms were represented in figure-18 to 23. Linearity results were tabulated in table-4.

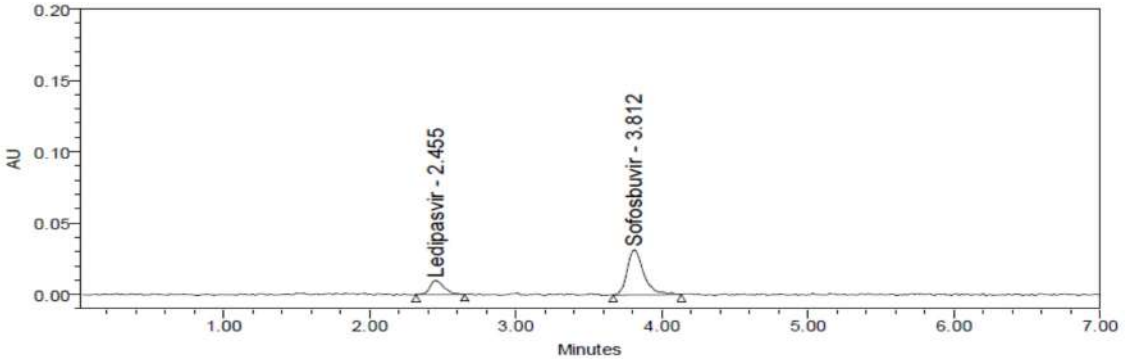

Figure-18: Linearity 25% level chromatogram

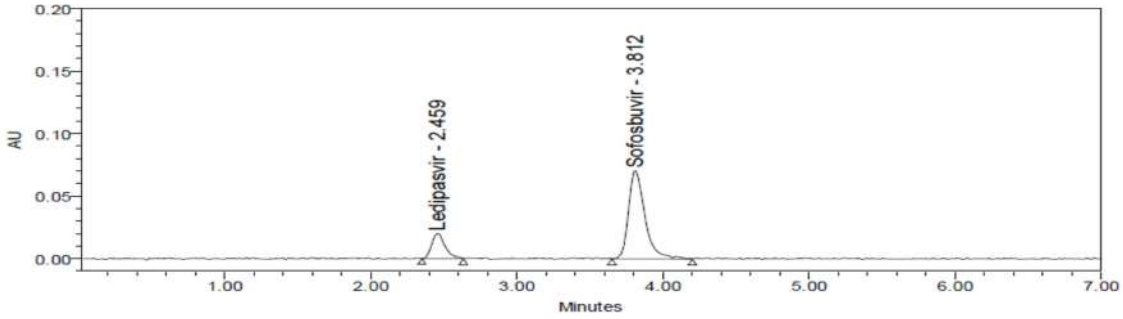

Figure-19: Linearity 50% level chromatogram

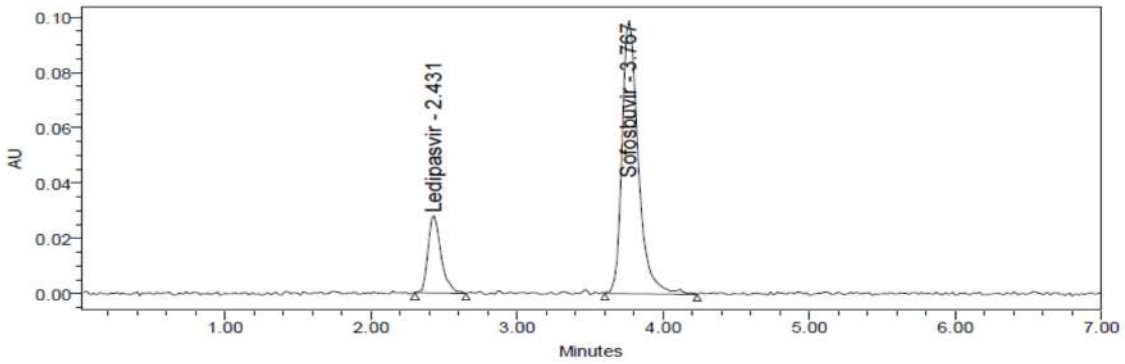

Figure-20: Linearity 75% level chromatogram

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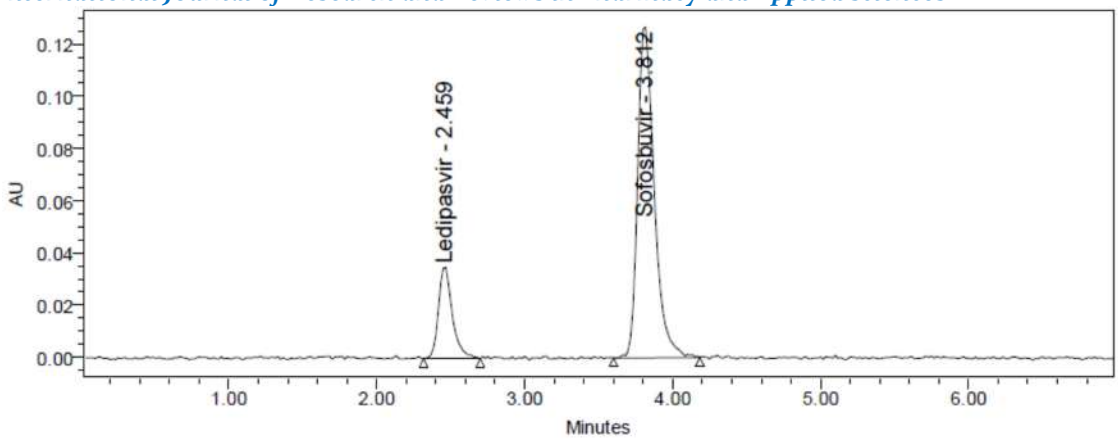

Figure-21: Linearity 100% level chromatogram

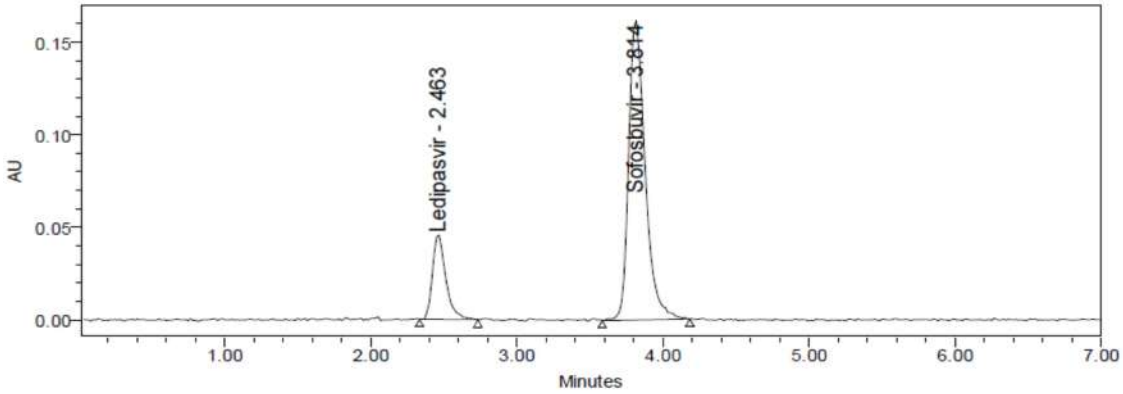

Figure-22: Linearity 125% level chromatogram

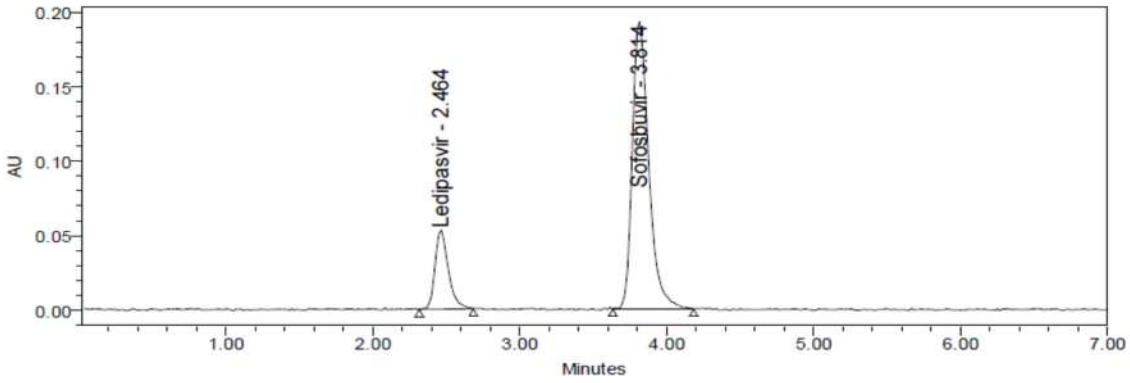

Figure-23: Linearity 150% level chromatogram

Table-4: Linearity results

Linearity level	Sofosbuvir		Ledipasvir	
	Conc.	Area	Conc.	Area
25%	2.25	68277	10	251376
50%	4.5	132541	20	517394
75%	6.75	194007	30	751518
100%	9	245769	40	1013807
125%	11.25	315061	50	1252472
150%	13.5	374769	60	1508267
Corr. Coe.	0.9991		0.9998	

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Accuracy:

Accuracy was validated with 50%, 100% and 150% levels. Placebo and active ingredients were used to prepare three accuracy levels. Accuracy % recovery was performed with three replicates. % recovery results were tabulated in table-5.

Table-5: Accuracy results

Sofosbuvir accuracy results									
Level	50%			100%			150%		
Recovery (%)	99.99	99.69	99.57	100.50	100.85	100.69	98.94	99.44	99.50
Mean (%)	99.75			100.68			99.29		
Ledipasvir accuracy results									
Level	50%			100%			150%		
Recovery (%)	100.46	100.07	100.88	100.09	99.91	100.29	100.06	100.05	99.83
Mean (%)	100.47			100.10			99.98		

Ruggedness:

Ruggedness was performed to confirm the stability of mobile phase, test solution and standard solution. Bench top and refrigerator stability studies were carried out with precision samples 1 and 2. Bench top stability studies were carried out at initial, day-1 and day-3. Refrigerator stability was carried out at initial, day-3 and day-5. Ruggedness results were tabulated in table-6.

Table-6: Ruggedness results

Sofosbuvir ruggedness results							
Time in day	**Bench top stability test solution**				**Tailing factor**	**%RSD**	**Bench top stability standard solution**
	Test-1	Test-2	Difference				Similarity factor
			Test-1	Test-2			
Initial	99.23	100.02	NA	NA	1.3	0.62	0.99
Day-1	99.98	100.62	0.75	0.60	1.4	0.25	0.98
Day-3	100.02	100.34	0.79	0.32	1.3	0.35	0.99
	Refrigerator stability test solution						**Refrigerator stability standard solution**
Initial	99.23	100.02	NA	NA	1.2	0.42	1.00
Day-3	99.96	100.21	0.73	0.19	1.5	0.51	0.99
Day-5	99.94	100.36	0.7	0.34	1.4	0.35	0.98
Ledipasvir ruggedness results							
Time in day	**Bench top stability test solution**				**Tailing factor**	**%RSD**	**Bench top stability standard solution**
	Test-1	Test-2	Difference				Similarity factor
			Test-1	Test-2			
Initial	98.79	100.47	NA	NA	1.3	0.52	0.98
Day-1	99.86	100.25	1.07	0.22	1.6	0.23	0.99
Day-3	99.35	100.34	0.56	0.13	1.2	0.62	0.98
	Refrigerator stability test solution						**Refrigerator stability standard solution**
Initial	98.79	100.47	NA	NA	1.3	0.51	0.99
Day-3	99.26	100.16	0.47	0.31	1.5	0.25	1.00
Day-5	99.64	100.31	0.85	0.16	1.1	0.34	0.99

Robustness:

Robustness was performed to confirm the chromatographic conditions variations such as flow rate, column oven temperature, mobile phase organic solvent ration variation and filter validation was performed with PVDF, NYLON filter papers. System suitability results were calculated for each change and filter validation. Results were represented in table-7 and 8.

Table-7: Results of Effect of variations

Variation condition		**Flow rate ml/min**			**Column temperature**		
Variation changes		0.8	1.0	1.2	25°C	30°C	35°C
Sofosbuvir	Tailing factor	1.2	1.4	1.1	1.3	1.2	1.2
	% RSD	0.36	0.25	0.31	0.25	0.24	0.23
Ledipasvir	Tailing factor	1.2	1.5	1.2	1.3	1.3	1.1
	% RSD	0.26	0.24	0.31	0.31	0.26	0.28
Variation condition		**M.P organic solvent ratio**					
Variation changes		55:45	50:50	45:55			
Sofosbuvir	Tailing factor	1.2	1.4	1.3			
	% RSD	0.25	0.24	0.36			
Ledipasvir	Tailing factor	1.3	1.5	1.4			
	% RSD	0.25	0.31	0.24			

Table-8: Filter Variability results

Sofosbuvir filter validation									
Centrifuged		**Nylon filter**				**PVDF filter**			
% assay		% assay		% Difference		% assay		% Difference	
Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2
99.69	99.86	99.89	99.99	0.20	0.13	100.02	100.31	0.33	0.45
Ledipasvir filter validation									
100.21	100.31	100.02	100.21	0.19	0.10	100.26	100.24	0.05	0.07

CONCLUSION

Sofosbuvir and Ledipasvir are hepatitis C medicinal products. These two are available in combination dosage form. Simple HPLC method was developed and validated to determine the both ingredients assay in a single HPLC method. Method validation was performed with specificity, precision, linearity, accuracy, ruggedness and robustness. This method can be applied to determine the both ingredients in regular manufacturing.

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