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STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF SOFOSBUVIR AND SIMEPREVIR BY RP-HPLC IN BULK FORM

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ABSTRACT

A rapid, novel, and efficient stability indicating reverse-phase high performance liquid chromatographic (RPHPLC) analytical method been developed and validated for the combination therapy which are used in the treatment of hepatitis C named sofosbuvir and simeprevir. The seperation were carried out on C18 reversed phase column (inertsil) ODS C18(250mm x 4.6ID, 5µm).In RP-HPLC, the sample analyte was resolved by taking the Mobile phase as a mixture of Acetonitrile and water (20:80% v/v) in a gradient mode of elution and the flow rate is set to 1.0ml/min and the column oven temperature is 25 °C. The detection was done at 260nm for sofosbuvir and 288nm for simeprevir. The retention time of Sofosbuvir was found to be 3.87min and the retention time of simeprevir was found to be 5.49min. Sofosbuvir and simeprevir were Stability studies including acid, alkaline, oxidation, photolysis and also thermal degradation studies. The degraded products were all well resolved with different retention time values. Linearity was found with significantly high value of correlation coefficient. The method conducted found good resolution and suitable retention time The present analysis is validated in terms of accuracy, precision, specificity, linearity, system suitability, limit of detection, limit of quantification, robustness and ruggedness as per International Conference of Harmonization (ICH) guidelines.

KEYWORDS: *Sofosbuvir, simeprevir, Acetonitrile, RP-HPLC, method validation, Forced degradation studies.*

INTRODUCTION

Sofosbuvir is a nucleotide analog and it works by inhibiting the enzyme NS5B polymerase viral protein which is responsible for RNA polymerase that performs the replication process and hence it works by preventing hepatitis C virus (HCV) to replicate itself by inhibiting the RNA polymerase which is used for treating the chronic hepatitis C infection as a combination therapy. The IUPAC name of sofosbuvir is S)-Isopropyl-2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)-(phenoxy)phosphorylamino)propanoate with molecular formula of $C_{22}H_{29}FN_3O_9P$ and its molecular weight is 529.45. Simeprevir is a direct acting anti-viral agent which inhibits NS3/4A protease, thus preventing viral maturation through inhibition of protein synthesis, Genotype 1, most prevailing form of hepatitis C virus (HCV) which includes peg interferon-alfa and ribavirin. It contains a molecular formula of $C_{38}H_{47}N_5O_7S_2$ and with molecular weight 749.942g/mol. The method has been performed by using column Inertsil C18 ODS (250mm×4.6mm×5µ) as a stationary phase and mobile phase as Water : Acetonitrile (80:20) with a flow rate of 1.0ml/min and drug detection was observed at 254nm and the column temperature is maintained ambient. Both the drugs are soluble in Water, methanol, Acetonitrile. The method developed by RP-HPLC was validated according to ICH guidelines for system suitability, LOD, LOQ, linearity, accuracy, precision, intermediate precision, and robustness. The present study proved that the expected RP-HPLC method is uncomplicated, rapid, accurate and precise that can be used for the everyday analysis of sofosbuvir and simeprevir in the pure form[1-7].

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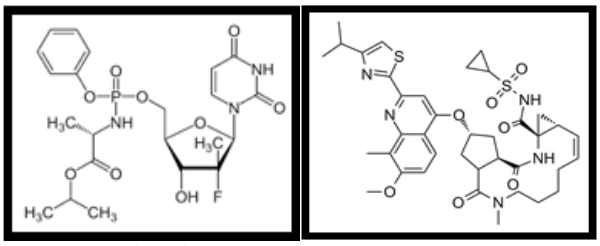

(A) (B)

Fig. 1: Structure of Sofosbuvir (A) andSimeprevir (B)

MATERIALS AND METHOD

Instruments: Waters Alliance 2690, Software: Empower-2, Detector: PDA 996, Inertsil ODS (250×4.6mm×5µ), Fast Clean Sonicator, Lab India pH Meter, Sartorious Electronic Balance.

Chemicals: Sofosbuvir, Simeprevir, HPLC Grade Methanol, Acetonitrile and Water.

HPLC Method Development:

The RP-Hplc method of sofosbuvir and simeprevir was performed by several trials for various parameters like different columns, flow rates, and mobile phases, Finally the following chromatographic conditions were set and method was optimized.

Standard stock preparation:

Weigh accurately about 10 mg of Simeprevir and 10mg of Sofosbuvir and transfer into a 10ml of clean volumetric flasks separately, add small amount of methanol and sonicate for 10mins to dissolve the drug completely and make up the volume with the same solvent.

Preparation of working standard:

Further pipette out about 0.4ml of sofosbuvir and 0.4ml of Simeprevir from the standard stock solutions into a single 10ml volumetric flask, make the volume using methanol.

Validation Parameters:

Linearity:

A Series of solutions were prepared using sofosbuvir and simeprevir working standards at different concentration levels from 20ppm to 80ppm of target concentration. Measure the peak area response of solution at Level1 and Level 6 for six times and also Level 2 and level 5 for two times. Refer Table No.2-3.

Acceptance criteria:Correlation coefficient $r^2 \geq 0.999$.

System suitability:

Standard preparations are injected into the system as per the test method. The system suitability parameters like theoretical plates, resolution and asymmetric factor were recorded. Refer Table No.4-5.

Accuracy:

Accuracy is the method which can be determined by Recovery studies. Assay of drug was conducted in triplicate as per test method with equal amount of Sofosbuvir and Simeprevir into each Volumetric flask for each spiked level to obtain the concentrations of 50%,100%, 150% of the labelled amount. Refer Table No.6-7.

$$\%\text{Recovery} = \frac{Amount\ \ found}{Amount\ \ added} \times 100$$

Acceptance criteria:The %Recovery of sofosbuvir and simeprevir at each spiked level should be in the range of 98-102%.

Precision:

The precision of analytical method was studied between a series of multiple sampling of homogenous sample. The precision is expressed in terms of %RSD. The %RSD was found to be 0.75% in the results of precision. Refer Table No.8-11.

Acceptance criteria:The %RSD values of Sofosbuvir and Simeprevir preparations should be less than 2.0%

1. Repeatability:

a. System precision: Standard solutions were prepared as per test method and injected five times.

b. Method precision: Prepared samples of Sofosbuvir and Simeprevir as per the test method are injected for six times into the column. Refer Table No.8-11.

2. Intermediate precision (Analyst to Analyst Variability)

A study was performed by two different analysts as per test method. Refer Table No.12-15.

Ruggedness of test method:

System to system variability study was performed on different HPLC systems, with similar conditions at different times. Samples were prepared and analysed as per test method. Refer Table No.12-13.

Robustness:

Effect of variation in flow rate: A study was performed to determine the effects of changes in flow rate. Standard solutions were prepared as per test method and was injected into the HPLC system with a flow rate of 1.0ml/min and 1.2ml/min. The system suitability parameters were calculated and found to be within the limits. Refer Table No.14-15.

Limit of Detection:

The limit of detection of an individual analytical procedure is its lowest amount of sample analyte to be detected, but necessarily not quantified.

$$LOD = 3.3*\sigma/S$$

Where σ = standard deviation of the response, S = slope of the calibration curve.

Limit of Quantification:

The limit of quantification of an individual analytical procedure is its lowest amount of sample analyte to be quantitatively determined.

$$LOQ = 10*\sigma/S$$

Where σ = standard deviation of the response, S = slope of the calibration curve.

Forced Degradation/Stability Studies:

1) Oxidation:Take 0.4ml of Standard stock solution of Sofosbuvir and and simeprevir into a single flask, sonicate for 10mins and Add about 1ml of hydrogen peroxide and kept for 30mins at 60°c. From this prepared solutions 10µl solution was injected and chromatograms were recorded.

2) Acid degradation study:Take 0.4ml of Standard stock solution of Sofosbuvir and and simeprevir in a single flask ,sonicate for 10mins and add 1ml of 2N Hydrochloric acid and kept for 30mins at 60°c. Inject 10µlof this solution and chromatograms were recorded.

3) Alkali Degradation Studies:Take 0.4ml of Standard stock solution of Sofosbuvir and and simeprevir in a single flask and add 1ml of 2N Sodium Hydroxide, sonicate for 10mins and kept for 30mins at 60°c. Inject 10µlof this solution and chromatograms were recorded.

4) Dry Heat Degradation Studies:The standard stock solution was placed in an oven at 105°c for 6hrs. The solution as taken and diluted to obtain 40µg/ml for both solutions and inject 10µl of this solution into system and chromatograms were recorded.

5) Photo Degradation Studies:The photochemical stability study was studied by exposing the drug solutions of both containing 40µg/ml to UV light by placing the container in UV chamber for about 7days or by keeping at 200watt hours/m^2 in a photo stability chamber,10µl of this solution is taken and injected into the system and the chromatograms were recorded.

6) Neutral Degradation Studies:Neutral degradation was studied by keeping the drug solution(40µg/ml) in water for 6hrs at 60°c, 10µl of this solution was taken and injected into the system and the chromatograms were recorded.

RESULTS AND DISCUSSION

Optimized Method:

Table No. 1: Chromatographic conditions

Column	Inertsil C18 ODS (250×4.6mm×5µ)
Flow rate	1.0ml/mi
Detector wavelength	254nm
Column Temperature	Ambient
Mobile phase	Water: Acetonitrile (80:20)
Injection volume	20µl
Run time	10mins
Retention time	3.873 for sofosbuvir and 5.491 for simeprevir

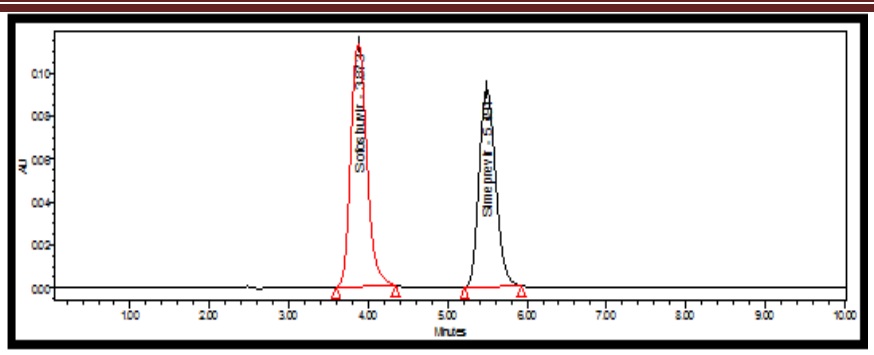

Fig. 2: Standard Optimized Chromatogram

Observation: Optimized Retention time of Sofosbuvir and Simeprevir was found as 3.873mins and 5.491mins.

Validation Data:

Linearity:

Table No. 2: Linearity data and plot of Sofosbuvir

Concentration (ppm)	Average Area	Statistical Analysis	
0	0		
20	1537659	**Slope**	76817
30	2306789		
40	3075642	**y-intercept**	-3691
50	3791987		
0	4612485		
70	5381485	**Correlation coefficient**	0.999
80	6150878		

Table No. 3: Linearity data of Simeprevir

Concentration (ppm)	Average Area	Statistical Analysis	
0	0		
20	231487	**Slope**	11549
30	347187		
40	462878	**y-intercept**	116.6
50	578575		
0	687632		
70	809980	**Correlation coefficient**	0.999
80	925692		

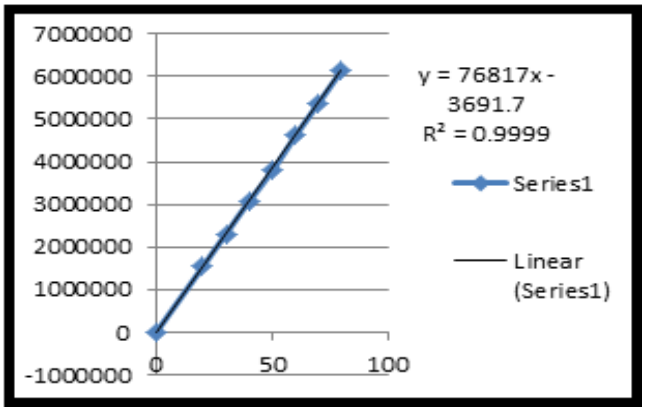

Fig. 3: Linearity plot of Sofosbuvir

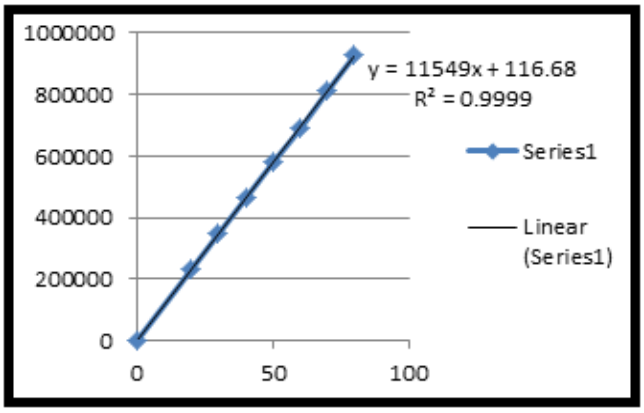

Fig. 4: Linearity plot of Simeprevir

Observation: the correlation coefficient should not be less than 0.999. Hence the expected method is showing linear graph and correlation coefficient (R^2) was found within limits.

System Suitability:

Table No. 4: System suitability data for Sofosbuvir

Injection	RT	Peak Area	USP plate count	USP Tailing
1	3.874	3075451	5023.845712	1.128
2	3.873	3074875	5010.547812	1.139
3	3.874	3075782	5036.874214	1.181
4	3.873	3076321	5027.254178	1.178
5	3.875	3075881	5084.658952	1.115
Mean	3.8738	3075614	5036.825471	1.148
SD	0.000837	495.4337	--------	-------
%RSD	0.021598	0.016	--------	------

Table No. 5: System suitability data for Simeprevir

Injection	RT	Peak Area	USP plate count	USP Tailing
1	5.496	462856	8325.874512	1.101
2	5.495	462732	8384.547862	1.114
3	5.496	462923	8314.875424	1.119
4	5.496	462874	8372.784518	1.096
5	5.499	462648	8392.084512	1.078
Mean	5.496	462818	8358.8754210	1.099
SD	0.001517	105.3561	------	-----
%RSD	0.027592	0.0227	------	-----

Accuracy (%Recovery):

Table No. 6: Accuracy Data of Sofosbuvir

% of spiked level	Area	Amount added	Amount found	%Recovery	Statistical Analysis	
50%	1537486	20	20.06	100.31	**Mean**	100.31
50%	1537948	20	20.06	100.34		
50%	1537145	20	20.05	100.29	**%RSD**	0.026
100%	3075627	40	40.08	100.21	**Mean**	100.22
100%	3075789	40	40.08	100.22		
100%	3076633	40	40.09	100.24	**%RSD**	0.017
150%	4612458	60	60.09	100.15		
150%	4612110	60	60.08	100.14	**Mean**	100.15
150%	4612987	60	60.09	100.16	**%RSD**	0.009

Table No. 7: Accuracy Data of Simeprevir

% of spiked level	Area	Amount added	Amount found	%Recovery	Statistical Analysis	
50%	231420	20	20.03	100.14	**Mean**	100.28
50%	231879	20	20.05	100.33		
50%	231978	20	20.02	100.38	**%RSD**	0.128
100%	462198	40	40.07	100.02	**Mean**	100.05
100%	462278	40	40.06	100.04		
100%	462568	40	40.07	100.10	**%RSD**	0.042
150%	687358	60	59.50	99.17		
150%	687130	60	59.48	99.14	**Mean**	99.09
150%	687978	60	59.56	99.26	**%RSD**	0.063

Observation: The % Recovery of Sofosbuvir and Simeprevir was found between 98-102%, within limits.

Precision:

1)Repeatability:

Table No.8: Data of Repeatability for Sofosbuvir

	Injection	System Precision		Method Precision	
		Peak Areas of Sofosbuvir	%Assay	Peak Areas of Sofosbuvir	%Assay
Concentration 40ppm	1	3075956	100.20	3075184	100.20
	2	3075008	100.19	3074236	100.17
	3	3076871	100.25	3075998	100.22
	4	3075897	100.22	3075564	100.21
	5	3075124	100.19	3075678	100.21
	6	3075480	100.21	3075368	100.20
Statistical Analysis	**Mean**	3075722	100.21	3075338	100.20
	SD	682.868	0.022	606.8436	0.019
	%RSD	0.022	0.022	0.019	0.019

Table No. 9: Data of Repeatability for Simeprevir

	Injection	System Precision		Method Precision	
		Peak Areas of Simeprevir	%Assay	Peak Areas of Simeprevir	%Assay
Concentration 40ppm	1	462848	100.16	462798	100.15
	2	462789	100.15	462874	100.17
	3	462897	100.17	462948	100.18
	4	462804	100.15	462815	100.15
	5	462874	100.17	462637	100.12
	6	462864	100.17	462899	100.17
Statistical Analysis	Mean	462846	100.16	462828	100.16
	SD	41.775	0.009	108.7249	0.023
	%RSD	0.009	0.009	0.023	0.023

2) Intermediate precision:

Table No. 10: Intermediate Precision data of Sofosbuvir

	Injection	Analyst-1		Analyst-2	
		Peak Areas of Sofosbuvir	%Assay	Peak Areas of Sofosbuvir	%Assay
Concentration 40ppm	1	3075184	100.20	3076879	100.25
	2	3074236	100.17	3075487	100.21
	3	3075998	100.22	3074256	100.17
	4	3075564	100.21	3075879	100.22
	5	3075678	100.21	3076987	100.26
	6	3075368	100.20	3075786	100.22
Statistical Analysis	Mean	3075338	100.20	3075879	100.22
	SD	606.8436	0.019	1002.408	0.032
	%RSD	0.019	0.019	0.032	0.032

Table No. 11: Intermediate Precision data of Simeprevir

	Injection	Analyst-1		Analyst-2	
		Peak Areas of Simeprevir	%Assay	Peak Areas of Simeprevir	%Assay
Concentration 40ppm	1	462798	100.15	462798	100.15
	2	462874	100.17	462874	100.17
	3	462948	100.18	462948	100.18
	4	462815	100.15	462815	100.15
	5	462637	100.12	462637	100.12
	6	462899	100.17	462899	100.17
Statistical Analysis	Mean	462828	100.16	462828	100.16
	SD	108.7249	0.023	108.7249	0.023
	%RSD	0.023	0.023	0.023	0.023

Observation: Individual Assays and %RSD should be NMT 2.0% and found within limit and passes the intermediate precision.

Ruggedness:

Table No. 12: System to System variability of Sofosbuvir

	Injection	System-1		System-2	
		Peak Areas of Sofosbuvir	%Assay	Peak Areas of Sofosbuvir	%Assay
Concentration 40ppm	1	3075184	100.20	3074657	100.18
	2	3074236	100.17	3074951	100.19
	3	3075998	100.22	3074961	100.19
	4	3075564	100.21	3074454	100.17
	5	3075678	100.21	3074245	100.17
	6	3075368	100.20	3074158	100.16
Statistical Analysis	Mean	3075338	100.20	3074571	100.18
	SD	606.8436	0.019	608.42	0.011
	%RSD	0.019	0.019	0.011	0.011

Table No. 13: System to System variability of Simeprevir (System-1)

	Injection	System-1		System-2	
		Peak Areas of Simeprevir	%Assay	Peak Areas of Simeprevir	%Assay
Concentration 40ppm	1	462798	100.15	462587	100.11
	2	462874	100.17	462930	100.18
	3	462948	100.18	462005	99.98
	4	462815	100.15	462168	100.01
	5	462637	100.12	462440	100.07
	6	462899	100.17	462229	100.03
Statistical Analysis	Mean	462828	100.16	462393	100.06
	SD	108.7249	0.023	108.97	0.072
	%RSD	0.023	0.023	0.072	0.072

Observation: The % RSD and %Assay was found within the limits.

Robustness:
Data for Robustness:

Table No. 14: Data for Effect of Variability in Flow Rate of Sofosbuvir

	Std Area	Tailing factor		Std Area	Tailing factor		Std Area	Tailing factor
Flow 0.8ml	3064485	1.073	Flow 1.0ml	3075875	1.079	Flow 1.2ml	3086485	1.045
	3064798	1.114		3075114	1.046		3086478	1.048
	3064652	1.062		3075348	1.067		3086689	1.097
	3064951	1.078		3075990	1.075		3086113	1.049
	3064351	1.101		3075784	1.097		3086498	1.064
	3064245	1.108		3078685	1.064		3086874	1.084
Avg	3064580	1.089	Avg	3075632	1.071	Avg	3086522	1.064
SD	269.8293	0.0211	SD	335.2919	0.017	SD	254.1459	0.021
%RSD	0.0088	1.940	%RSD	0.010	1.586	%RSD	0.008	2.027

Table No. 15: Data for Effect of Variability in Flow Rate of Simeprevir

	Std Area	Tailing factor		Std Area	Tailing factor		Std Area	Tailing factor
Flow 0.8ml	460578	1.022	Flow 1.0ml	462846	1.049	Flow 1.2ml	463764	1.009
	460946	1.060		462798	1.064		463487	1.011
	460798	1.075		462520	1.004		463654	1.010
	460699	1.018		462897	1.010		463987	1.045
	460701	1.023		462964	1.021		463378	1.034
	460398	1.054		462400	1.031		463646	1.047
Avg	460686	1.042	Avg	462737	1.029	Avg	463652	1.026
SD	187.1595	0.024	SD	255.1166	0.023	SD	213.1231	0.018
%RSD	0.040	2.308	%RSD	0.048	2.245	%RSD	0.045	1.763

Limit of Detection and Limit of Quantification:

The LOD for this method was found to be and 0.021µg/ml for Sofosbuvir and 0.064µg/ml for Simeprevir.
The LOQ for this method was found to be and 0.021µg/ml for Sofosbuvir and 0.064µg/ml for Simeprevir.

Forced Degradation Studies:
Data for Degradation studies:

Table No. 16: %Degradation of Sofosbuvir

Parameters	Standard Area	Sample Area	%Assay	%Degradation
Acid degradation	3072365	2911631	94.67	5.33
Alkali Degradation	3072365	2941022	95.63	4.37
Oxidation	3072365	3006088	97.74	2.26
Dry Heat Degradation	3072365	3038223	98.79	1.21
Photo Stability	3072365	3031026	98.56	1.44
Neutral Degradation	3072365	3068225	99.77	0.23

Table No. 17: % Degradation of Simeprevir

Parameters	Standard Area	Sample Area	%Assay	%Degradation
Acid degradation	457056	437885	95.71	4.29
Alkali Degradation	457056	435338	95.15	4.85
Oxidation	457056	443470	96.93	3.07
Dry Heat Degradation	457056	447885	97.90	2.10
Photo Stability	457056	436045	95.31	4.69
Neutral Degradation	457056	449078	98.16	1.84

Results: The % Degradation should be not more than 10, hence the results were found within limits.

CONCLUSION

The proposed analytical method was developed and validated for the simultaneous study of sofosbuvir and simeprevir. First of all, the maximum absorbance is detected at the wavelength of 254nm. The separation was achieved by using the column Inertsil C18 ODS (250×4.6mm×5µ) and it gave good resolution and peak areas. The Flow rate is fixed at 1.0ml/min with injection volume of 20µl. The analyte is well resolved by using the mobile phase as Water: Acetonitrile (80:20) and fixed for the study as it is highly economical, and easily available, showing good results with the run time of 10mins because the retention time of sofosbuvir and simeprevir was found to be 3.873 and5.491.%Recovery values are also found to be within limits and %RSD found to be less than 2.0 and acceptable. The method was found to be robust and rugged. Hence it can be used conveniently for routine and every day analysis in pure form.

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