STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS DETERMINATION OF SOFOSBUVIR AND DACLATASVIR IN TABLET DOSAGE FORM

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ABSTRACT

New stability indicating RP-HPLC method for the simultaneous estimation of sofosbuvir and daclatasvir in its pharmaceutical dosage form was developed and validated. Column used was PhenomenexC18 (150mm x 4.6mm, 5µ) with mobile phase water and acetonitrile (50:50 V/V) in isocratic mode. Flow rate of mobile phase 1.0mL min^{-1} and column oven temperature were maintained at 30°C. Sofosbuvir and daclatasvir were detected at a wavelength 230nm. The retention times for sofosbuvir and daclatasvir were found to be 3.06 min and 4.76 min, respectively. Validation of the method was done according to ICH guidelines. The method was found to be accurate, precise, specific and robust. The method shows good linearity in concentration range of 50 µg mL^{-1} – 500 µg mL^{-1} of sofosbuvir and 7.5 µg mL^{-1} – 75 µg mL^{-1} of daclatasvir, with correlation coefficient of 0.999 for both the drugs. The drugs as well as their degradation products produced in stress study were separated using this developed method.

Keywords: Sofosbuvir, daclatasvir, HPLC, Method development, Validation, Forced degradation

INTRODUCTION

Approximately 5% of the world population (350–400 million people) is chronically infected with HBV; 75% of infected people are Asian[1].Sofosbuvir is a promising drug for chronic Hepatitis C viral infection, as it offers several advantages over the existing drug therapies, particularly in dealing with patients with the history of liver disease and patients who cannot tolerate interferon-containing therapies[2]. Sofosbuvir and daclatasvir are effective in treatment of chronic HCV genotype 4 infections with minimal adverse events[3].

Sofosbuvir (Fig.1) and daclatasvir (Fig.2) in combination are used as antiviral drugs for the treatment of Hepatitis C. Chemically, sofosbuvir is isopropyl (2*S*)-2-[[[(2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxopyrimidin – 1 – yl) – 4-fluoro-3hydroxy – 4 – methyl – tetrahydrofuran – 2 – yl]methoxy – phenoxy-phosphoryl]amino] propanoate. It is a white to off-white colored crystalline solid powder which is slightly soluble in water and having pKa value of 9.38[4,5].

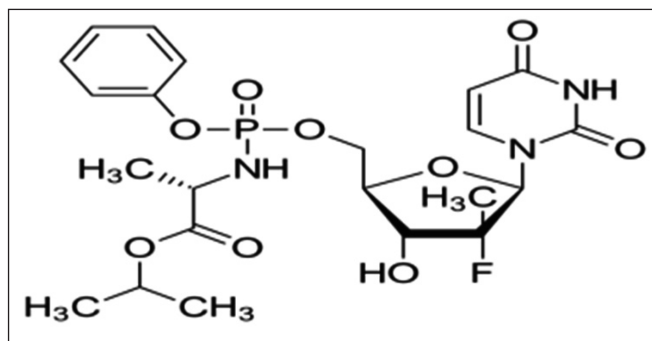

Fig. 1: Sofosbuvir

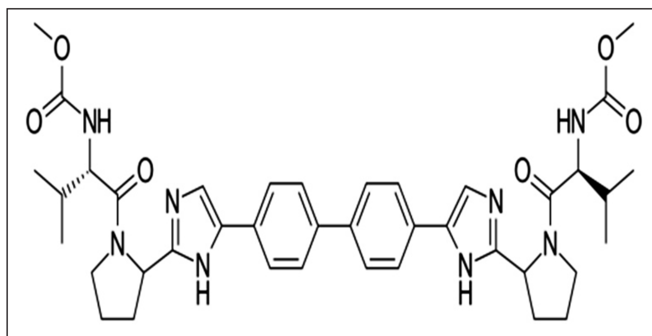

Fig. 2: Daclatasvir

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Chemically, daclatasvir is methyl [(2*S*)-1-{(2*S*)-2-[4-(4-{2-[(2*S*)-1-{(2*S*)-2-[(methoxy carbonyl)amino]-3-methyl butanoyl}-2-pyrrolidinyl]-1H-imidazol-4-yl}-4-biphenyl yl)-1*H*-imidazol-2-yl]-1-pyrrolidinyl}-3-methyl-1-oxo - 2-butanyl] carbamate[6,7].

Various methods are available in the literature for analysis of sofosbuvir and daclatasvir, for example UV & HPLC[8], HPLC method in human plasma[9], UP-HPLC method in human plasma[10] with or without combination with other antiviral medicines, but there is no developed method for stability indicating RP-HPLC for sofosbuvir and daclatasvir. Stability indicating methods are commonly used to check the degradation pathways and degradation products of the active pharmaceutical ingredients that could form during storage, and gives idea of formulation development as well as manufacturing and packaging. Procedures for the preparation of specific degradation products needed for method validation are often developed from these studies[11], so our aim is to establish a new technique for analysis of sofosbuvir and daclatasvir in their pharmaceutical dosage forms. In this study, we used column Phenomenex C18 (150mm x 4.6mm, 5µ) mobile phase acetonitrile and water in a ratio of 50:50 (V/V), at flow rate 1.0 mL min^{-1} and detection was done using PDA detector at 230 nm.

MATERIALS AND METHODS

Instrument and chromatographic conditions

Schimadzu HPLC system with Phenomenex C18 (150mm x 4.6mm, 5µ) column and PDA detection mode was used. An isocratic mode with water and acetonitrile in 1:1 (V/V) as mobile phase at 1.0 mL min^{-1} flow rate was used for separation of drugs. The detection of drugs was done at 230 nm with column oven temperature maintained at 30°C. The other instruments used were digital balance (Raptech), ultrasonic bath (Cyberlab) and hot air oven (Nova).

Chemical and reagents

Standard drugs sofosbuvir and daclatasvir were gifted by Cadila Pharmaceuticals Ltd., Ahmedabad. The sofosbuvir tablets (Hepcinat) contains 400 mg of sofosbuvir (manufactured by Natco Pharma, India) and daclatasvir tablets (Daclahep) contains daclatasvir 60 mg (manufactured by Hetero Labs Ltd, India). They were purchased from local pharmacy. The chemicals used for development of the method were of AR grade and procured from Sigma Aldrich. The solvents used were of HPLC grade and purchased from Merck.

Mobile phase

Acetonitrile and water were taken in the ratio 50:50 (V/V) and sonicated for 20 minutes.

Standard solution preparation

40 mg of sofosbuvir working standard and 6mg daclatasvir working standard were accurately weighed and transferred into a 10 mL clean dry volumetric flask, 7mL of mobile phase added, the sample sonicated for 20 minutes and the final volume made up with mobile phase. From this prepared stock solution, 1 mL was withdrawn and added into a 10mL volumetric flask and then made up to the final volume with mobile phase (sofosbuvir 400 µg mL^{-1} and daclatasvir 60 µg mL^{-1}).

Preparation of sample solution

Average weight of the tablets was calculated by weighing 20 tablets (Hepcinat) and an amount which is equivalent to 40mg of sofosbuvir was taken into 10mL volumetric flask. Same procedure was performed for daclatasvir (Dacliza) amount of tablet equivalent to 6mg of daclatasvir was taken into same flask. The sample was dissolved in 10mL of mobile phase. The above solution was sonicated for 10 minutes and filtered using HPLC filters. 1 mL of this prepared solution was taken into 10 mL volumetric flask and the volume made up with mobile phase acetonitrile and water (50:50 V/V) to obtain 400 µg mL^{-1} of sofosbuvir and 6 µg mL^{-1} of daclatasvir. The solution was sonicated for 10 minutes and injected under above chromatographic conditions and peak area was measured.

METHOD DEVELOPMENT

Trials were performed for the method development and the best peak with least tailing factor was found to be with RT= 3.06 minutes for sofosbuvir and 4.76 minutes for daclatasvir.

Method validation

This developed method was validated as per ICH quality Q2(R1) guidelines[17]. The validation parameters such as specificity, linearity, range, accuracy, precision, detection limit, quantification limit and system suitability studies were studied.

Specificity

Solutions of standard and sample were prepared according to the test method and injected into chromatographic system.

Linearity

From the stock solution, suitable dilutions were prepared using mobile phase acetonitrile and water (50:50 V/V) as solvent at the range of 50, 100, 200, 300, 400 and 500 μg mL^{-1} of sofosbuvir and 7.5, 15, 30, 45, 60 and 75 (μg mL^{-1}) of daclatasvir respectively. 20 μg mL^{-1} amount of every sample of dilution was injected into the column with the rate of 1.0 mL min^{-1} and detection of the drug within the elute was monitored at wavelength 230 nm and the results were recorded. From these results,the mean peak areas of sofosbuvir and daclatasvir were calculated and a plot of concentration Vs peak areas obtained. The regression of the plot was calculated by least square regression methodology. The slope and intercept values for standardization curve was y = 3130.2x – 179405 for sofosbuvir and y = 1354.9x – 9679 for daclatasvir.

Accuracy

Accuracy of the method was determined by injecting the standard solutions of 50%, 100% and 150% and calculated the concentration and measured the individual recovery and mean recovery values. The % recovery for each level must be found within the range of 98.0 to 102.0%.

Precision

Precision of the method was resolved by repeatability (intra-day) and intermediate precision (inter-day). Repeatability study is used to indicate efficiency of the analytical procedure within a laboratory over a short period of time in the same day. Intermediate precision was calculated by comparing the assays of given drugs on different days (3 days).

Acceptance criteria: The % RSD should not be more than 2%

Limit of detection and limit of quantification (LOD and LOQ)

From the linearity data the limit of detection and quantitation were calculated, following formula is used.

$$LOD = \frac{3.3\sigma}{S} \qquad LOQ = \frac{10\sigma}{S}$$

where σ = standard deviation of the response

S = slope of the calibration curve of the analyte

LOD of sofosbuvir and daclatasvir by linearity data were found to be 0.4 and 0.2 μg mL^{-1} and LOQ of sofosbuvir and daclatasvir were found to be 0.9 and 0.5 μg mL^{-1}

Robustness

A study was conducted by changing flow rate of mobile phase 0.9 mL min^{-1},1.0 mL min^{-1}and1.1 mL min^{-1} and also by changing ratio of mobile phase. M1 = Mobile phase acetonitrile: water in a ratio (50:50 V/V), M2 = Mobile phase acetonitrile: water in a ratio (51:49 V/V), M3 = Mobile phase acetonitrile: water in a ratio (49:51 V/V), as shown in Tables V & VI.

System suitability

System suitability tests are based on the concept that the equipment, analytical procedure and sample to be analyzed constitute can be evaluated as such. British pharmacopoeia was taken as a reference for these tests. The observer %RSD was meeting the BP recommendation (RSD(%) <1.0). Other parameters like number of theoretical plates, capacity factor and tailing factor were having good agreement when compared with pharmacopeia specifications.

Stability Indicating analytical method

Stability indicating studies are analytical procedure used to determine the amount of degradation products present in the Active pharmaceutical ingredients. These methods are used to detect the changes of active pharmaceutical ingredient with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference[12,13].

Forced degradation studies were carried out as per ICH Q1A(R2) guidelines and the parameters such as acid hydrolysis, alkali hydrolysis, thermal degradation, oxidative degradation and photolytic degradation were studied[14-16].

Acid hydrolysis

To 1 mL of stock solution of sofosbuvir and daclatasvir, 1 mL of 0.1N HCl was added into separate 10 mL standard flasks and refluxed for 30min at 60° C. The resultant solutions was diluted to obtain 400 μg mL^{-1} sofosbuvir and 60 μg mL^{-1} solution of daclatasvir respectively with mobile phase and 10 μL solution was injected into the system and the chromatogram was recorded to assess the stability of sample.

Alkaline hydrolysis

To 1 mL of stock solution of sofosbuvir and daclatasvir, 1 mL of 0.1M NaOH was added into separate 10mL

standard flasks and refluxed for 30 mins at 60°C. The resultant solutions were diluted to obtain 400 µg mL^{-1} sofosbuvir and 60 µg mL^{-1} of daclatasvir respectively, with mobile phase and 10 µL solution was injected into the system and the chromatogram was recorded to assess the stability of the sample.

Thermal degradation

1 mL of stock solution of sofosbuvir and daclatasvir was added into separate 10mL standard flasks and refluxed for 6h at 105° C. The resultant solution were diluted to obtain 400 µg mL^{-1} sofosbuvir and 60 µg mL^{-1} of daclatasvir respectively, with mobile phase and 10 µL solution was injected into the system and the chromatogram was recorded to assess the stability of sample.

Oxidative degradation

To 1 mL of stock solution of sofosbuvir and daclatasvir, 1 mL of 2% H_2O_2 was added into separate 10mL standard flasks and refluxed for 30 mins at 60° C. The resultant solutions were diluted to obtain µg mL^{-1} sofosbuvir and µg mL^{-1} of daclatasvir, respectively with mobile phase and 10 µL solution was injected into the system and the chromatogram was recorded to assess the stability of sample.

RESULTS AND DISCUSSION

Trials were performed for the method development and the best peak with least tailing factor was found to be with RT= 3.06 min for sofosbuvir and 4.76 min for daclatasvir, as shown in Fig. 3.

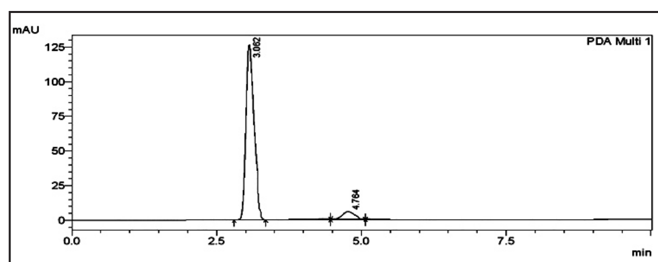

Fig. 3: Chromatogram of sofosbuvir and daclatasvir

Linearity

Results of the statistical data derived from linearity studies showed a good correlation between concentration and peak response (Fig. 4 and Fig. 5). The linear regression equations for linearity of sofosbuvir and daclatasvir were y = 3130.2x – 179405 and y = 1354.9x - 9679, respectively, and the correlation coefficient for both sofosbuvir and daclatasvir was R^2 = 0.9999, as shown in Table I.

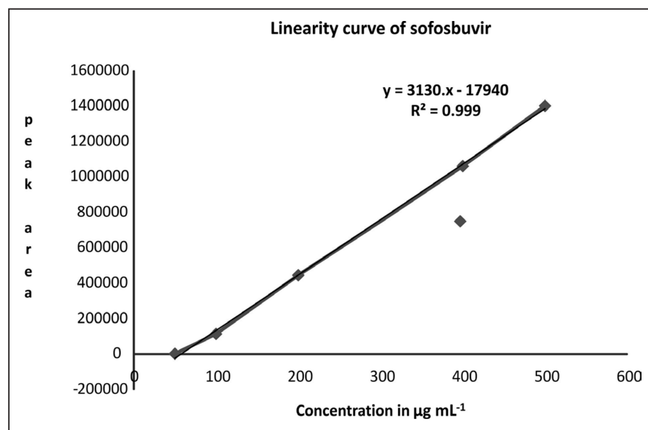

Fig. 4: Linearity curve of sofosbuvir

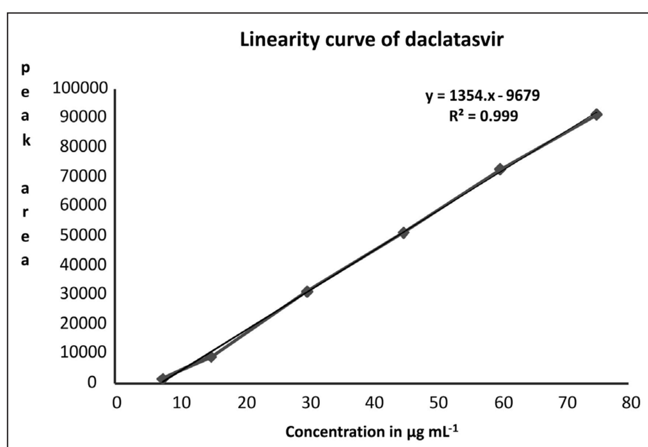

Fig. 5: Linearity curve of daclatasvir

Accuracy

The results of the three concentrations ranging from 50 % to 120 % analyzed (n = 3) for recovery studies are shown in Table II. The overall recovery of sofosbuvir and daclatasvir was 100 ± 1 % at each concentration, and the RSD % of recovery studies were less than 2.0 %. The results show that the method is accurate and suitable for assay of sofosbuvir and daclatasvir, as shown in Table II.

Precision

The results for interday and intraday precision show that the RSD for peak response of both analytes at different concentrations, analyzed replicates (n = 6), on different days is less than 2.0 %. The results indicate that the given method is precise and repeatable within the acceptable limits and criteria which is shown in Tables III and IV.

Robustness

Robustness was performed for flow rate and change in mobile phase ratio and the results showed the minor

Table I: Linearity data

Sr. No.	Sofosbuvir		Daclatasvir	
	Concentration (μg mL^{-1})	Average Area	Concentration (μg mL^{-1})	Average Area
1	50	2079	7.5	1597
2	100	113784	15	8975
3	200	445692	30	31221
4	300	750000	45	51220
5	400	1061827	60	72664
6	500	1402050	75	91264
Slope (a)		3130		1354
Intercept (b)		17940		9679
Correlation coefficient (R^2)		0.999		0.9991

Table II: Accuracy data

Drug	Concentration				% Recovery	% RSD
	Initial tablet sample	Authentic amount added	Claimed total amount	Total amount found ± SD		
Sofosbuvir	40	20	60	60.61±0.33	101.02	0.0586
	40	40	80	79.98±0.55	99.98	1.7504
	40	60	100	100.0 ±0.09	100.09	0.6684
Daclatasvir	6	4	10	10.11±0.42	101.1	1.2301
	6	14	20	20.01±0.61	100.05	0.0586
	6	19	25	24.99±0.03	99.96	1.9932

Table III: Intraday precision

Drug	Conc. taken μg mL^{-1}	Found μg mL^{-1}	% recovery± SD	% RSD
Sofosbuvir	25	24.78	99.12±0.23	1.50
	50	50.01	100.02±0.07	1.87
	100	99.98	99.98±0.37	1.76
Daclatasvir	25	25.7	102.8±0.44	1.58
	50	49.3	98.6±1.23	0.15
	100	100.04	100.04±0.75	1.44

Table IV: Interday precision

Drug	Conc. taken μg mL^{-1}	Found μg mL^{-1}	% recovery± SD	% RSD
Sofosbuvir	25	24.98	99.92±1.15	0.09
	50	50.21	100.42±0.45	0.28
	100	100.33	100.33±0.80	1.16
Daclatasvir	25	24.74	98.96±0.09	0.73
	50	49.43	98.86±1.83	1.36
	100	100.73	100.73±0.04	0.95

Table V: Robustness (Flow rate)

Sr. No.	Sofosbuvir			Daclatasvir		
	Peak area			Peak area		
	0.90	**1.00**	**1.10**	**0.90**	**1.00**	**1.10**
1	1067827	1173834	1098684	72664	81765	74576
2	1091772	1174505	1077364	75893	83569	76836
3	1072071	1187493	1068576	73666	85981	75163
4	1061613	1176370	1050923	73967	82843	75760
5	1051248	1137681	1055038	74764	84960	73773
Mean	1068906	1169977	1070117	74190.8	83823.6	75221.6
SD	13409.16	18881.81	19149.11	1212.71	1674.07	1163.49
RSD	1.25	1.61	1.79	1.63	2.00	1.55

Table VI: Robustness (Mobile phase)

Sr. No.	Sofosbuvir			Daclatasvir		
	Peak area			Peak area		
	M1	**M2**	**M3**	**M1**	**M2**	**M3**
1	1010654	1233981	998799	74313	84556	77659
2	1029760	1239876	986644	72435	84758	75633
3	1021348	1267883	995741	74675	85876	74670
4	1030255	1256732	974678	73769	86223	74758
5	1012136	1287663	992435	71434	84766	77155
Mean	1020831	1257227	989659	73325	85236	75975
SD	9327.117	21688.587	9509.956	1356.813	757.554	1371.982
RSD	0.91	1.72	0.960	1.85	0.88	1.80

M1 = Mobile phase Acetonitrile: Water in a ratio (50:50) *M2 = Mobile phase Acetonitrile: Water in a ratio (51:49)*

M3 = Mobile phase Acetonitrile: Water in a ratio (49:51)

changes of the given values in chromatographic conditions and did not influence the results for sofosbuvir and daclatasvir. The value of RSD for replicates (n = 6) are shown in Tables V and VI.

Limit of detection and limit of quantitation (LOD and LOQ)

From the linearity plot, the LOD and LOQ are calculated:

LOD of sofosbuvir and daclatasvir were found to be 0.4 and 0.2 and LOQ of sofosbuvir and daclatasvir were found to be 0.9 μg mL^{-1} and 0.5 μg mL^{-1}.

System suitability

A set of optimized conditions, i.e., combination of water and acetonitrile 50:50 % V/V as mobile phase at a flow rate of 1.0 mL min^{-1} over a Phenomenex C8 150 mm × 4.6 cm, 5 μm, was selected and system suitability was assessed according to BP guidelines. Statistical data of different parameters like retention time (Rt), theoretical plates (N), capacity factor (k') and Tailing Factor (Tf) of sofosbuvir and daclatasvir were calculated for peak response by LC solution software. The results showed that all the performance parameters of the analytical method comply with BP requirements for system suitability. The RSD for Rt of both analytes was less than 2.0 %, tailing factor (Tf) was between 0.8 to 1.5, capacity factor (k') 0.5–10, and the number of theoretical plates was more than 2000. The method was suitable for simultaneous analysis of sofosbuvir and daclatasvir successfully applied for determination of both analytes in tablet dosage form, as shown in Table VII.

Table VII: System suitability using proposed HPLC method

	Sofosbuvir	Daclatasvir	Recommended values
Retention time (Rt, min)	3.062	4.764	-
Theoretical plates (N)	1688	2348	The more theoretical plates, better separation
Capacity factor (k’)	3.7	5.3	0.5<k’<10
Tailing factor (Tf)	1.2	1.10	0.8<Tf< 1.5

Table VIII: Forced degradation study

Mode of degradation	Sofosbuvir			Daclatasvir		
	Peak Area	% Obtained	% Degradation compared with control	Peak Area	% Obtained	% Degradation compared with control
Control	1402050			91264		
Acid (0.1 N HCl)	1261827	89.99	10.01	78272	85.67	14.33
Base (0.1N NaOH)	1199386	85.54	14.46	87306	95.66	14.02
Peroxide	1306218	93.16	6.84	67337	73.78	26.22
Thermal	1400502	99.88	0.12	81413	89.20	10.8
UV	1303571	92.89	7.11	79271	86.85	13.15

Forced degradation study: Stress degradation studies were established for sofosbuvir and daclatasvir by subjecting it to acid, base, oxidation and thermal as well as UV stress.

Highest degradation was found in alkaline condition in case of sofosbuvir and in peroxide in case of daclatasvir. The stress samples were assayed and results shown were within the range when compared against a reference standard. Therefore, the RP-HPLC methodology developed was considerably suitable for routine analysis, as shown in Table VIII.

DISCUSSION

The developed RP-HPLC method for the estimation of sofosbuvir and daclatasvir in bulk and formulation was found to be simple, precise, accurate and reproducible. The developed method was validated as per the ICH guidelines and the results obtained were well within the limits. The statistical analysis of the developed method confirms minimal deviation and all the validation parameters were well within the specified range. Hence, the proposed method can be successfully applied for analysis of sofosbuvir and daclatasvir in bulk and formulation.

Forced degradation studies of daclatasvir in bulk showed that the drug was mostly degraded in all stress conditions.

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