STABILITY INDICATING HPLC METHOD FOR SOFOSBUVIR AND DACLATASVIR IN COMBINATION

Mrinalini C. Damle[a]* and Nivedita B. Pawar[a]

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ABSTRACT

Direct acting fixed dose combination of sofosbuvir and daclatasvir to treat the viral hepatitis C disease is available in the market. So, a precise and robust stability indicating HPLC method for sofosbuvir and daclatasvir was developed. The SunQ C18 column (250 x 4.6 mm) was used for chromatographic separation with mobile phase consisting of 0.03 mM potassium dihydrogen phosphate buffer (pH 7): ACN (50: 50V/V). Optimised method satisfies the system suitability parameters with good resolution with 4.9 min Rt of sofosbuvir and 7.6 min Rt of daclatasvir. The method was validated as per ICH guidelines. Linearity was observed over range of 10-50 (µg mL^{-1}) and 2.25-11.25 (µg mL^{-1}) for sofosbuvir and daclatasvir, respectively. Both drugs were subjected to various stress conditions and high recovery values were found for daclatasvir on photolytic stress. The degradation was more on oxidative and hydrolytic stress for sofosbuvir. This optimised method offers new insight towards stability studies of both drugs.

Keywords: Stability indicating method, sofosbuvir, daclatasvir, HPLC

INTRODUCTION

The hepatitis C virus (HCV) induces both chronic and acute infection and it is a significant cause of morbidity and mortality amongst individuals living with HIV worldwide. Antiviral medicines can cure more than 95 per cent of people with hepatitis C. Pegylated interferon (PEG IFN), in conjunction with ribavirin (RBV), has been the predominant mode of treatment for HCV for the last 15 years. Major advancements have recently been introduced in all oral direct-acting antiviral (DAA) therapies and it improves the treatment rates for HCV genotype[1]. And now, high sustained virological response rates are achieved when DAAs are used in combination[2].

Chemically, sofosbuvir (SOF) is propan-2-yl (2*S*)-2-{[(*S*)-{[(2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2 yl]methoxy}(phenoxy)- phosphoryl] amino}propanoate (Fig. 1). SOF is a nucleotide analogue inhibitor of NS5B (non-structural protein 5B) polymerase - the key enzyme mediating HCV RNA replication.

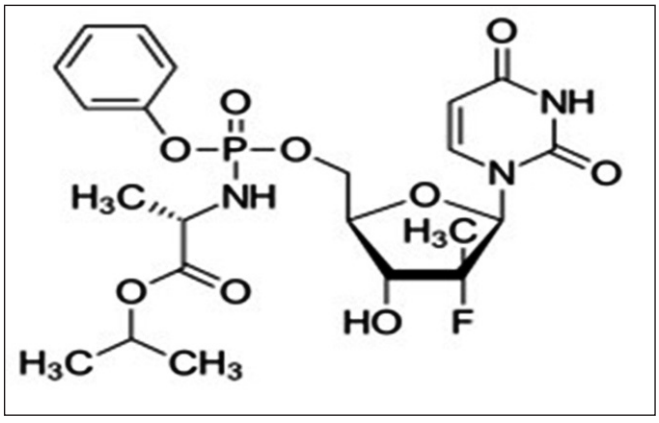

Fig. 1: Structure of sofosbuvir

The drug daclatasvir (DAC) is chemically methyl *N*-[(2*S*)-1-[(2*S*)-2-[5-[4-[4-[2-[(2*S*)-1-[(2*S*)-2-(methoxycarbonylamino)-3-methylbutanoyl]pyrrolidin-2-yl]-1*H*-imidazol-5-yl]phenyl]phenyl]-1*H*-imidazol-2-yl] pyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl]carbamate (Fig. 2). DAC has potent inhibitory activity against all 6 types of HCV genotypes. The cis- and trans- acting functions of NS5A get targeted by DAC and then modulate the NS5A phosphorylation along with HCV replication complexes. This fixed dose combination is highly effective and well tolerated which available as combination of 400: 30,60,90 mg (SOF: DAC).

Literature survey shows some UPLC[3], HPLC[4-6], HPTLC[7] UPLC ms/ms[8,9] methods for estimation of stated

[a] Department of Quality Assurance, AISSMS College of Pharmacy, affiliated to Savitribai Phule Pune University, Pune- 411 001, Maharashtra, India

*For Correspondence: E-mail: damle_mc@aissmscop.com

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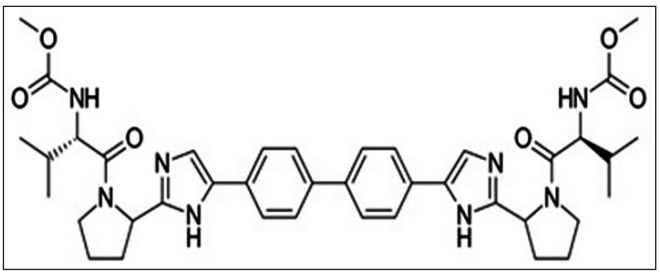

Fig. 2: Structure of daclatasvir

drugs from biological samples. A study of the literature denotes such stability indicating HPLC[10,11] method for individual drugs. Also, some stability indicating HPLC[12,13] methods have been reported but variance is observed between forced condition, percent recovery values, etc. The present research concentrates on the development and validation of stability indicating HPLC method for simultaneous estimation of sofosbuvir and daclatasvir. Method is simple, sensitive and offers new perspective towards stability studies with validation according to the ICH[14,15].

MATERIALS AND METHODS

Reagents and chemicals

NATCO Pharma, Hyderabad, India had cordially provided the APIs. Methanol (HPLC and AR Grade), hydrochloric acid (HCl), potassium dihydrogen phosphate, 30 % hydrogen peroxide (H_2O_2) and sodium hydroxide were acquired from Loba Chemie Pvt. Ltd., Mumbai, India. HPLC grade water was collected with the conductivity below 0.05 µS cm^{-1} using Extrapure Lab Link water purifier system.

Instrumentation and chromatographic condition

The samples were analysed on JASCO HPLC system, model PU 2080 Plus pump with Rheodyne sample injection port (20 µL). The study was performed using Sun Q C18 (250 x 4.6 mm) column and detection was carried with PDA detector (MD 2010) with Borwin chromatography software (version 1.5) at wavelength of 260 and 318 nm (SOF and DAC, respectively). Mobile phase was optimized to contain 0.03 mM potassium dihydrogen phosphate buffer (pH 7): ACN (50:50 V/V) at the flow rate of 1 mL min^{-1}, employed in isocratic mode. The study involved other instruments like UV-Visible spectrophotometer (Jasco V-730), Photo stability chamber (Newtronic), Vacuum pump (JET-VAC-J1) and hot air oven (Kumar Laboratory Oven).

Mobile phase optimisation

In order to achieve optimal chromatographic conditions, various columns were tried out such as HiQsil, Neosphere and SunQ C18 columns. Initially, simple ACN:water (60:40 V/V) system was tried but didn't give significant number of theoretical plates. Here, ACN was selected as organic modifier. Afterwards, ammonium acetate buffer was tried with ACN but gave unresolved peaks. Next, phosphate buffer of pH 5 was used with ACN (60:40 V/V) gave good resolution but retention time of DAC were increased. Finally it was observed that retention time of DAC changes by pH without affecting the retention time of SOF. Significant resolution and desired system suitability parameter were obtained by using 0.03 mM potassium dihydrogen phosphate buffer (pH 7): ACN (50: 50 V/V).

Preparation of mobile phase

The pH of 0.03 mM potassium dihydrogen phosphate was adjusted to 7 by triethylamine. Then it was mixed in equal proportion with ACN and filtered through 0.45 µm membrane filter. Then it was sonicated for 15 min on ultrasonicate bath.

Preparation of standard stock solution (400:90)

Separately 10 mg of sofosbuvir and daclatasvir were dissolved in 10 mL of methanol, so as to get a stock solution with concentration of 1000 µg mL^{-1} each. 4 mL of 1000 µg mL^{-1} of SOF and 0.9 mL from 1000 µg mL^{-1} of DAC stock solution were separately prepared with diluent methanol to get SOF(400 µg mL^{-1}) and DAC (90 µg mL^{-1}). Now 1 mL from each stock was taken and diluted to 10 mL to get SOF (40 µg mL^{-1}) and DAC (9 µg mL^{-1}). Representative chromatograms are shown in Fig. 3 and Fig. 4, and data is summarised in Table I.

Table I: System suitability parameters

Sr. No	Parameter	Sofosbuvir	Daclatasvir
1	Concentration (µg mL^{-1})	40	9
2	Rt (min)	4.973	7.613
3	Area	1504779.917	718686.939
4	Plates	7946.111	3513.578
5	Asymmetry	1.08	1.01

Preparation of sample solution

Sample solution was prepared by spiking method which is used for assay. Excipient blend was prepared in mortar which was equivalent to 900mg (400 mg of SOF, 90 mg of DAC and rest was excipient). The 90 mg of powder blend, equivalent to 40 mg of SOF and 9 mg of

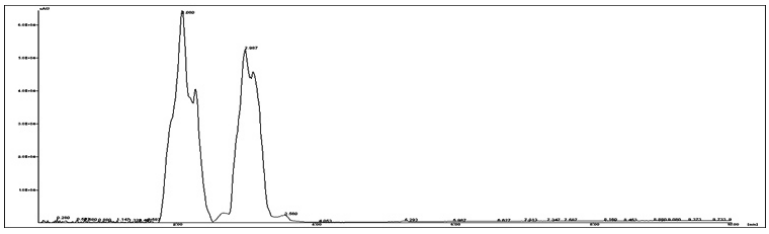

Fig. 3: Mobile phase blank

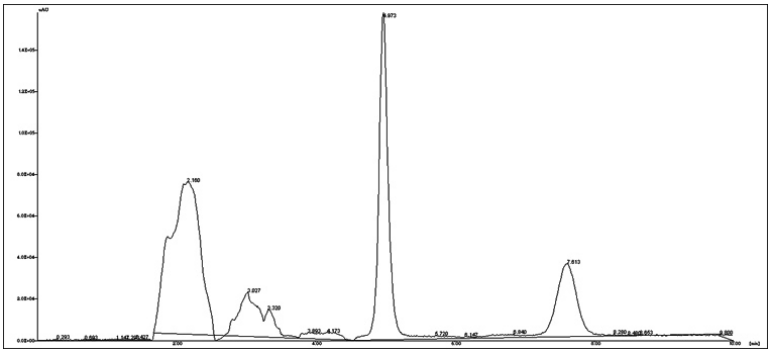

Fig. 4: Representative chromatogram of sofosbuvir (4.973 min) and daclatasvir (7.613 min)

DAC, was weighed, dispersed in methanol and sonicated for 10 minutes. (4000 µg mL^{-1} of SOF and 900 µg mL^{-1} of DAC). The solution was filtered and further dilution was prepared in methanol.

Stress degradation studies

The effects of variety of environmental conditions on stability and quality of drugs have to be tested. So, for various periods of time, the drugs were exposed to different stress conditions using different strengths of reagents. The conditions was tried in order to achieve 70 - 90 percent recovery. The APIs underwent hydrolysis under different pH, oxidation, thermal and photolytic degradation conditions.

Table II: Summary of stressed degradation

Parameter	Condition	% Recovery	
		Sofosbuvir	Daclatasvir
Acid hydrolysis	0.1 N HCl 10 min	76.84724	84.3369
Alkaline hydrolysis	0.1 N NaOH Immediate	74.9413	72.48122
Neutral hydrolysis	Immediate	78.30233	80.5243
Oxidative degradation	30 % V/V H_2O_2, 30 min	73.41108	83.34892
Thermal degradation	80°C, 8 h	81.43826	95.5281
UV degradation	200 Watt hours/square meter	67.36302	96.29745
Fluorescence degradation	1.2 million lux h	94.51048	93.75931

Acid and alkali catalysed hydrolysis

Samples were prepared separately for both drugs by adding 1 mL of stock solution (400 µg mL^{-1} and 90 µg mL^{-1} of SOF and DAC, respectively) to 1 mL of 0.1N HCl. Methanol was added to make up the volume up to 10 mL. Solution was kept in dark for 10 min. Thereafter chromatogram was recorded.

For alkali catalysed hydrolysis, the samples were prepared separately for both drugs by adding 1 mL of stock solution (400 µg mL^{-1} and 90 µg mL^{-1} of SOF and DAC, respectively) to 1 mL of 0.1 N NaOH. Methanol was added to make up the volume up to 10 mL and injected immediately.

Neutral hydrolysis

Samples were prepared separately for both drugs by adding 1 mL of stock solution (400 µg mL^{-1} and 90 µg mL^{-1} of SOF and DAC, respectively) to 1 mL of water. Methanol was added to make up the volume up to 10 mL and injected immediately.

Oxidative degradation

Samples were prepared separately for both drugs by adding 1mL of stock solution (400 µg mL^{-1} and 90 µg mL^{-1} of SOF and DAC, respectively) to 1 mL of 30 % H_2O_2. Methanol was added to make up the volume up to 10 mL. Solution was kept in dark for 30 min. Thereafter chromatogram was recorded.

Thermal degradation

Bulk drug powder was exposed to temperature of 80 °C for 8 h. 10 mg of dried powder was then measured individually and dissolved to 10 mL in methanol. Then final methanolic dilutions (40 µg mL^{-1} and 9 µg mL^{-1} of SOF and DAC) were injected and chromatograms were recorded.

Photolytic degradation

Samples were exposed to cool white fluorescent light providing illumination of not less than 1.2 million lux h and to UV light for not less than 200 watt h $square^{-1}$.

Table III: System precision of sofosbuvir and daclatasvir

Parameter	Drug	Concentration (µg mL^{-1})	Standard deviation	%RSD
Intra day precision	Sofosbuvir	10	1.844079	1.804582
	Daclatasvir	2.25	1.79056444	1.8006583
Inter day precision	Sofosbuvir	10	1.999932	1.949461
	Daclatasvir	2.25	1.898223399	1.88297779

Table IV: Method precision of sofosbuvir and daclatasvir

Parameter	Drug	Concentration (µg mL^{-1})	Standard deviation	%RSD
Intra day precision	Sofosbuvir	10	1.422035	1.421352
	Daclatasvir	2.25	1.128224974	1.13372572
Inter day precision	Sofosbuvir	10	1.623954	1.606307
	Daclatasvir	2.25	2.16359612	2.14791867

Table V: Accuracy data at three different levels

Drug	% level	Initial amount (µg mL^{-1})	Amount added (µg mL^{-1})	% Recovery
Sofosbuvir	80	20	16	100.3991
	100	20	20	102.8291
	120	20	24	99.22395
Daclatasvir	80	4.5	3.6	98.9414
	100	4.5	4.5	101.7642
	120	4.5	5.4	98.03396

Table VI: Robustness study of sofosbuvir and daclatasvir

Parameter	Condition	% RSD of Sofosbuvir	% RSD of Daclatasvir
Mobile phase ratio	Buffer : ACN (48 : 52 V/V)	0.99041	1.576332
	Buffer : ACN (52 : 48 V/V)	2.02671	1.076299
pH of buffer	pH 7.2	2.050556	1.880922
	pH 6.8	0.572638	1.771493

From this final methanolic dilutions (40 µg mL^{-1} and 9 µg mL^{-1} of SOF and DAC) were prepared and injected to get chromatogram.

Method validation

The method development and validation of proposed analytical method includes specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness. It was validated according to ICH Q2(R1).

RESULTS

Some contradictions were observed during literature survey. Researchers used the heating process along with hydrolytic condition. We have developed the method by optimizing exposure time and with strength of reagent like 0.5 N, 0.1 N acid and alkali. The results are summarised in Table II. The method offers short run time than other stated methods. Also, the sample preparation was not so complicated. Standard stock solution was scanned over 200-400nm, whereby maximum absorbance was observed at 260 nm (SOF) and 318 nm (DAC). Spectra are shown in Fig. 5. Further, by following ICH guidelines method was validated.

Linearity and range

The mixtures for linearity were prepared from standard stock solution (400 µg mL^{-1} and 90 µg mL^{-1} of SOF and DAC, respectively). The SOF was found to be linear over range of 10-50 µg mL^{-1}. DAC was linear over range of 2.25- 11.25 µg mL^{-1}. The procedure was repeated for 6 times to get the linear regression equation. Then, concentration was plotted against peak area. Overlain chromatograms are shown in Fig. 6.

Precision

The method was evaluated by intra day and inter day study. The 6 replicates of 10 µg mL^{-1} of SOF and 2.25 µg mL^{-1} of DAC were evaluated within same day and consecutive day to assess the system precision. From

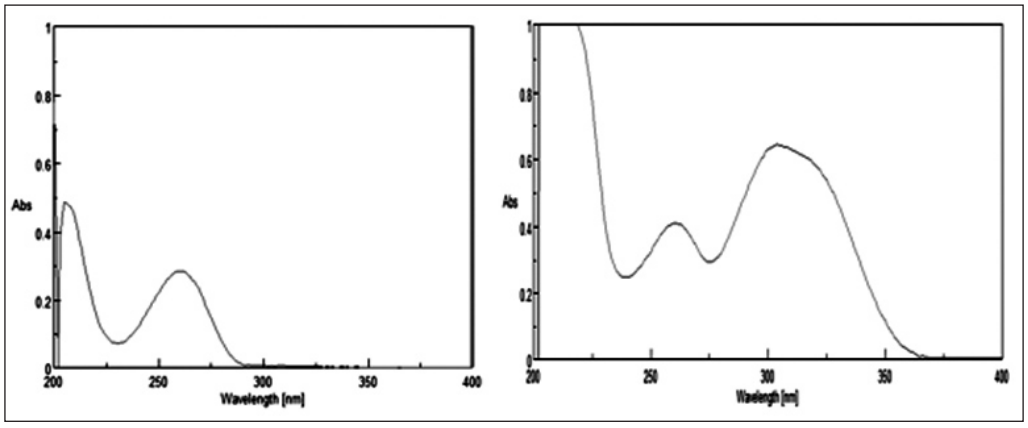

Fig. 5: UV spectra of sofosbuvir (260 nm) and daclatasvir (318 nm)

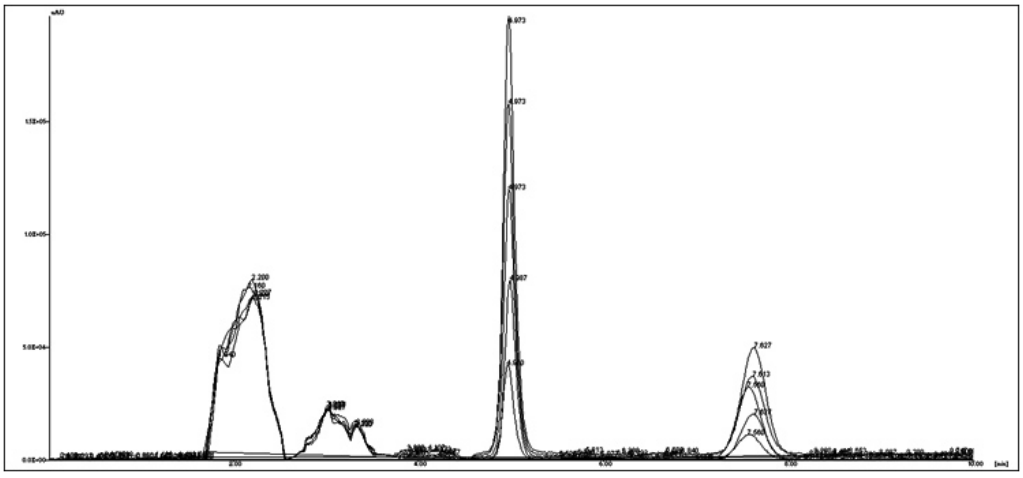

Fig. 6: Linearity of sofosbuvir (10-50 µg mL^{-1}) and daclatasvir (2.25-11.25 µg mL^{-1}) respectively

the solution of tablet blend 6 replicates of both drugs were evaluated within same day and consecutive day to assess the method precision. The % RSD was less than 2% (Table III and IV).

Assay

From methanolic solution of spiked powder blend (40 µg mL^{-1} of SOF plus 9 µg mL^{-1} of DAC) 1 mL was mixed with 1 mL of mobile phase to get solution of concentration 20 µg mL^{-1} of SOF plus 4.5 µg mL^{-1} of DAC. The 3 replicates of same concentration were evaluated to get assay results.

Accuracy

Accuracy was carried out by spiking method. The standard drug was spiked in blend at 3 different levels of 80 %, 100 % and 120 %. The 3 replicates of 3

Table VII: Validation parameters of both drugs

Sr. No.	Parameter		Sofosbuvir	Daclatasvir
1	Linearity range		y = 36436x + 10135 R^2 = 0.9994 10 – 50 (µg mL^{-1})	y = 82907x + 53743 R^2 = 0.9911 2.25-11.25 (µg mL^{-1})
2	System precision (%RSD)	Intra Day	1.804582	1.8006583
		Inter Day	1.949461	1.88297779
	Method precision (%RSD)	Intra Day	1.421352	1.13372572
		Inter Day	1.606307	2.14791867
3	Accuracy (% Recovery)	80 %	100.3991	98.9414
		100%	102.8291	101.7642
		120%	99.22395	98.03396
4	Assay (%RSD)		1.592299	1.356237
5	LOD		1.081613	0.171765
6	LOQ		3.277616	0.5205
7	Robustness		Robust	Robust

concentrations were evaluated to calculate % recovery (Table V).

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

The limits were calculated from values of regression equation. Both the LOD and LOQ were determined using formula 3.3 σ/S and 10 σ/S, respectively. Here, S is slope of calibration curve and σ is standard deviation of area at lowest concentration.

Robustness

Robustness was performed by carrying out small and deliberate changes to developed system. Peak area was checked after doing the changes to mobile phase ratio and pH of mobile phase. The optimised system is robust as %RSD is below 2 % (Table VI).

The results of the validation proved that the established method complied with the validation parameters and data is summarised in Table VII.

DISCUSSION

A simple, accurate and robust stability indicating HPLC method has been developed.

Literature shows different kinds of mobile phases like tertiary and multiple component mobile phases. Here, optimised method consists of binary mobile phase, which gives good resolution between both drugs. The method ensures all system suitability parameter within short run time. Based on percent recovery and the reproducibility of testing, we have optimized the stressed condition. Daclatasvir was found to be relatively unstable in alkaline stressed condition while sofosbuvir shows high percent recovery values for fluorescent light exposure and thermal stress. The method is linear, accurate, precise and robust and hence can be used for routine stability monitoring purposes.

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