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Research Article

A Stability Indicating Reverse Phase High Performance Liquid Chromatography Method for Related Substances of Sofosbuvir in Tablet Dosage Form

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ABSTRACT

Sofosbuvir is an antiviral drug that is used to treat hepatitis C. A present investigation deals with simple, sensitive, rapid, precise and accurate reverse phase high-performance chromatography (RP-HPLC) method developed and validated for related substances of sofosbuvir in tablet dosage form. The chromatographic separation was achieved on a Kromasil 100 C_{18} (250 × 4.6 mm, 5 µ) column. A sofosbuvir and its impurities were extracted by composed mixture of Mobile Phase A: buffer solution: acetonitrile (97.5:2.5% v/v) and Mobile Phase B: acetonitrile, isopropyl alcohol, methanol and purified water (60:20:10:10 % v/v/v/v) using with flow rate was 1-mL/min, column temperature was 25°C, injection volume was 10 µL, Vial thermostat temperature was 10°C. The UV detection was carried out at 263 nm. The retention time of sofosbuvir, methyl ester and ethyl ester were 54.28, 36.31 and 43.77, respectively. The method shows linearity with correlation coefficient of sofosbuvir and its impurity was 0.999 over the 0.5–7.5 ppm range. The average recovery was found to be 90.2–113.9%. The LoD and LoQ for sofosbuvir and its impurities were found to be 0.1 and 0.5 µg/mL, respectively. The method was validated as per ICH guidelines. The developed method was precise, accurate, novel and detectable towards sofosbuvir and its impurity. This method is efficient in separating the sofosbuvir and its impurity. Hence, the proposed method can be utilized for the determination of related substances in routine analysis in quality control department of pharmaceutical Industry.

Introduction

Sofosbuvir is a uridine analogue prodrug converted into a triphosphate nucleotide within the hepatocytes and inhibits the HCV non-structural protein 5B (NS5B), HCV-RNA polymerase. Sofosbuvir is an antiviral drug that is used to treat hepatitis C. Hepatitis C disease is a disease of the liver that is caused by the hepatitis C virus. Sofosbuvir is used for the treatment of chronic hepatitis C infection. Sofosbuvir is a NS5B polymerase inhibitor drug. The NS5B polymerase inhibitor is an RNA-dependent RNA polymerase. Sofosbuvir is a prodrug of 2'-deoxymethyl uridine monophosphate that is phosphorylated. Sofosbuvir is 400 mg to be taken once in a day.

Sofosbuvir's empirical formula is $C_{22}H_{29}FN_3O_9P$ and its molecular weight is 529.5 g/mol.[1-3] Sofosbuvir's IUPAC name is propan-2-yl (2S)-2 [[[(2R, 3R, 4R, 5R)-5-(2,4 dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl] methoxy phenoxy phosphoryl] amino] propanoate. It is a white to off-white, crystalline, non-hygroscopic powder. Slightly soluble in water and freely soluble in ethanol, methanol and acetone, soluble in 2 propanol and insoluble in heptane.[3] Sofosbuvir structure shown in Fig. 1.

Sofosbuvir is not official drug in IP, BP, USP. The extensive literature survey reveals several individual and combined analytical methods available for estimating sofosbuvir in tablet dosage form.[3-48] But no method has been reported

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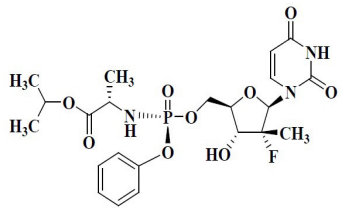

Fig. 1: Chemical structure of sofosbuvir

for stability indicating reverse phase high performance liquid chromatography method for related substances of sofosbuvir in tablet dosage form.

The aim of this present work is to develop and validate a simple, specific, precise, accurate and robust stability indicating reverse phase high performance liquid chromatography method for related substances of sofosbuvir in tablet dosage form as per ICH Q_2 (R_1) guidelines.[49] Pharmaceutical product stability studies and related substances are the most critical parameters for developing novel medications and formulations. The shelf-life prediction plays an important role in the development of all dosage forms of pharmaceutical products, as well as in determining specific storage conditions and recommending label instructions. Stability studies of pharmaceutical product are required for the acceptance and approval of any pharmaceutical product to ensure the maintenance of product quality, safety, and efficacy during the shelf life.

Material and Methods

Materials

Chemicals and reagents

Disodium hydrogen phosphate dihydrate (Merck), potassium dihydrogen orthophosphate (Merck), 1-hexansulfonic acid monohydrate (Spectrochem), orthophosphoric acid (Merck), acetonitrile (Spectrochem), isopropyl alcohol (Spectrochem), methanol (Spectrochem) and water (Spectrochem). Sofosbuvir's working standard and impurities (such as methyl uridine and methyl ester) were obtained from Century Pharmaceuticals Limited.

Instruments

The HPLC Shimadzu Prominence-i LC-2030 PLUS Instrument with PDA Detector (Software- Lab Solution), Digital Analytical Balance- Mettler Toledo (Model-MT204T), pH meter- Lab India (India), Sonicator- Frontline (India), melting point Apparatus (DBK), Column: Kromasil 100 C_{18} (250 mm x 4.6 mm), 5µ.

Methods

Preparation of buffer

Take 10 g of disodium hydrogen phosphate dihydrate, 6 g of potassium dihydrogen orthophosphate and 2 g of 1-hexansulfonic acid monohydrate in 1000 mL of purified water and dissolve them. Adjust the pH 3.0 ± 0.05 using orthophosphoric acid. Filter the solution through a 0.45 µm filter.

Preparation of mobile phase a

Prepare a filtered and degassed mixture of buffer solution, and acetonitrile in a ratio of (97.5:2.5) % v/v.

Preparation of mobile phase b

Prepare a mixture of acetonitrile, isopropyl alcohol, methanol and purified water in a ratio of (60:20:10:10) % v/v/v/v. The gradient programme for a standard solution, sensitivity solution, mobile phase, diluent, placebo, and sample preparation was tabulated in Table 1.

Preparation of diluent

Prepare a filtered mixture of methanol and purified water in the ratio of (50:50) %v/v.

Preparation of standard solution (10 µg/mL)

An accurately weighed quantity of about 25 mg of Sofosbuvir working standard into a 100 mL volumetric flask. Add about 70 mL of diluent and sonicate to dissolve. Make volume up to mark with diluent and mix. Dilute 2 mL of this solution to 50 mL with diluent and mix well.

Preparation of sensitivity solution (0.5 µg/mL)

Transfer a 5 mL of standard solution into a 100 mL volumetric flask, dilute to volume with diluent and mix well.

Preparation of placebo solution

An accurately weighed quantity of placebo powder equivalent to 100 mg of sofosbuvir in a 100 mL volumetric flask. Add 70 mL of diluent and sonicate for 20 minutes, shaking at intervals of 5 minutes. Allow it to stand at room temperature for 10 minutes. Make volume up to the mark with diluent and mix. Centrifuge the sample. Filter the solution through a 0.45 µm Millipore PVDF filter; collect the filtrate by discarding 5 mL of the filtrate.

Table 1: Gradient program for standard solution, sensitivity solution, mobile phase, diluent, placebo and sample preparation

Time	*%Mobile phase A*	*%Mobile phase B*
0	100	0
5	100	0
10	96	4
20	80	20
30	70	30
40	67	33
50	65	35
65	40	60
85	35	65
100	100	0
120	100	0

Preparation of sample solution (1000 µg/mL)

Weigh 20 tablets accurately and calculate the average weight. Crush the tablet into a fine powder. Transfer an accurate Weigh quantity of tablet powder equivalent to about 100 mg of sofosbuvir (approximately 308.75 mg of sample) in a 100 mL volumetric flask. Add 70 mL of diluent and sonicate for 20 minutes with shaking at intervals of 5 minutes. Allow it to stand at room temperature for 10 minutes. Allow to stand at room temperature for 10 minutes and make volume up to mark with diluent. Centrifuge the sample. Filter the solution through a 0.45 µm Millipore PVDF filter; collect the filtrate by discarding 5 mL of the filtrate. Chromatogram of the sample solution is presented in Fig. 2.

Preparation of impurity stock solution

Weigh accurately 10.485 mg of methyl uridine and 10.419 mg of methyl ester in a 50 mL volumetric flask. Add 30 mL of diluent and sonicate to dissolve. Male volume up to the mark with diluent and mix.

Chromatographic system

Column: Kromasil 100 C18 (250 x 4.6 mm), 5 µ
Flow rate: 1 mL/min
Wavelength: 263 nm
Injection volume: 10 µL
Column temperature: 25°C
Vial thermostat temperature: 10°C
Needle wash: Diluent

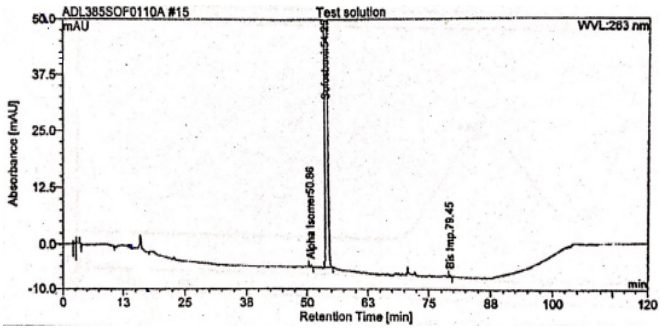

Fig. 2: RP-HPLC chromatogram of sample (1000 µg/ mL)

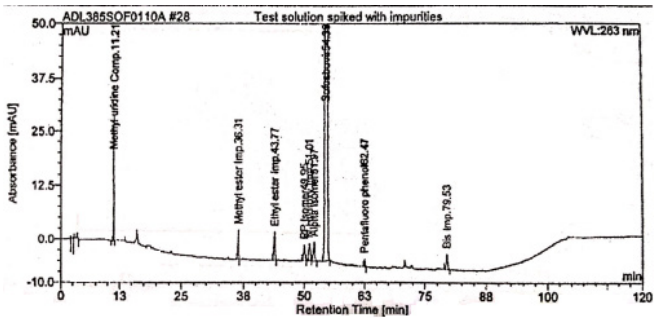

Fig. 3: RP-HPLC chromatogram of sample spike with known impurities

Method Development

- *Method optimization parameters*

An understanding of the nature of API, its synthesis route, impurities, degradation path and degradation product is needed to successfully develop the reverse phase HPLC method. The result of a successful development method should be robust and accurate.

- *Selection of wavelength*

The HPLC method is sensitive to the selection of the detection wavelength. An ideal wavelength is that which gives a good response for drugs and allows impurities to be detected. The wavelength was selected from the spectrum at 263 nm.

- *Selection of stationary phase*

According to the literature review, it was found that different C_{18} columns could be used for the separation of impurities for sofosbuvir.

- *Selection of mobile phase*

Different stationary and mobile phases were used to develop a reverse phase liquid chromatography method for the quantitative determination of impurities in sofosbuvir. Different compositions were tried as a mobile phase to get good peak shape and impurity selectivity in sofosbuvir. After a number of trials were taken, a gradient mobile phase was finalised, as mentioned in the preparation of the mobile phase. The system suitability parameter was achieved for the drug and its impurities.

Method Validation Study

Specificity

- *Interference from placebo with analyte and known impurities*

Specificity was represented by injected diluent solution, placebo solution, standard solution, sample solutions spiked with impurities and individual impurities. That analysis was performed as per the method. There is no interference from the placebo with the analyte and known impurities, the peak purity of the analyte in the sample solution and known impurities is more than 990. A chromatogram of the sample with impurity spikes of methyl uridine and methyl ester is presented in Fig. 3.

- *Interference from degradation products*

A study was organised to reveal the effective separation of degradants/impurities from sofosbuvir. Sample and placebo solutions were exposed to various stress conditions to degrade. Stressed and unstressed samples were injected into the HPLC system with the photodiode array detector by the following test method. All degrading peaks were resolved from the sofosbuvir peak in the chromatogram of all samples and placebo, and we did not

Table 2: Forced degradation study

Stress method	*Stress condition*	*Peak purity*	*% degradation*
Acid hydrolysis	Heated on boiling water bath for 30 minutes, after treated with 3.0 mL 0.5N Hydrochloric acid.	1000	16.6
Alkali hydrolysis	Heated on boiling water bath for 1 hour, after treated with 5 mL of 0.01N Sodium hydroxide.	1000	17.6
Peroxide oxidation	Heated on boiling water bath for 2 Hour, after treated with 5 mL of 3% hydrogen peroxide.	1000	11.8
Thermal degradation	Sample Kept at 100 °C in oven for 6 days.	1000	3.0
UV light degradation	Sample exposed under UV light in a photo stability Chamber for 22 hours.	1000	8.0
Humidity degradation	Sample exposed in Humidity chamber at 40°C and 75% RH for 6 days.	1000	0.0

find any interference at the retention time of sofosbuvir and its impurities under the different conditions. The observation is tabulated in Table 2. The peak purity of the analyte peak is greater than 990.

Sample preparation of force degradation study

Weigh accurately 20 tablets and calculate the average weight. Crush the tablets in to fine powder.

Acid hydrolysis

Transfer an accurately weighed quantity of tablet powder equivalent to 100 mg of sofosbuvir into a 100 mL volumetric flask. Add 70 mL of diluent and sonicate it. Then add 3 mL of 0.1 N hydrochloric acid and then kept on water bath at 80°C for 30 minutes then allow to stand at room temperature for 10 minutes and add 3 mL of 0.1 N Sodium hydroxide. Make volume up to the mark with diluent and mix well. Filter the solution through 0.45 µm Millipore PVDF filter by discarding 5 mL of the filtrate.

Alkali hydrolysis

Transfer an accurately weighed quantity of tablet powder equivalent to 100 mg of sofosbuvir into a 100 mL volumetric flask. Add 70 mL of diluent, sonicate it, and add 5 mL of 0.01 N sodium hydroxide and then kept on a water bath at 80°C for 1-hour. Then allow to stand at room temperature for 10 minutes and add 5 mL of 0.01 N Hydrochloric acid. Make volume up to the mark with diluent and mix well. Filter the solution through 0.45 µm Millipore PVDF filter by discarding 5 mL of the filtrate.

Peroxide oxidation

Transfer an accurately weighed quantity of tablet powder equivalent to 100 mg of sofosbuvir into a 100 ml volumetric flask. Add 70 mL of diluent, sonicate it, and add 5 mL of 3% Hydrogen peroxide and then kept on a water bath at 80°C for 2 hours, then allow to stand at room temperature for 10 minutes. Make volume up to the mark with diluent and mix well. Filter the solution through 0.45 µm Millipore PVDF filter by discarding 5 mL of the filtrate.

Thermal degradation

Weight quantity of tablet powder equivalent to 100 mg of sofosbuvir and exposure to thermal chamber at 100°C for 6 days. Then that transfer to 100 mL volumetric flask. Add 70 mL of diluent and sonicate it. Allow to stand at room temperature for 10 minutes. Filter the solution through 0.45 µm Millipore PVDF filter; collect the filtrate by discarding 5 mL of the filtrate.

UV degradation

Weight quantity of tablet powder equivalent to 100 mg of sofosbuvir and exposure to UV light in a photostability chamber for 22 hours. Then that transfer 100 mL volumetric flask. Add 70 mL of diluent and sonicate it. Allow to stand at room temperature for 10 minutes. Filter the solution through 0.45 µm Millipore PVDF filter; collect the filtrate by discarding 5 mL of the filtrate.

Humidity degradation

Weight quantity of tablet powder equivalent to 100 mg of sofosbuvir and exposure to thermal chamber at 40°C-75% RH for 6 days. Then that transfer 100 mL volumetric flask. Add 70 mL of diluent and sonicate it. Allow to stand

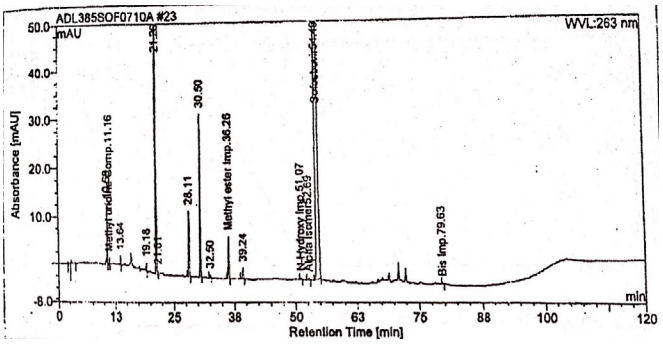

Fig. 4: RP-HPLC chromatogram of sample in alkali degradation

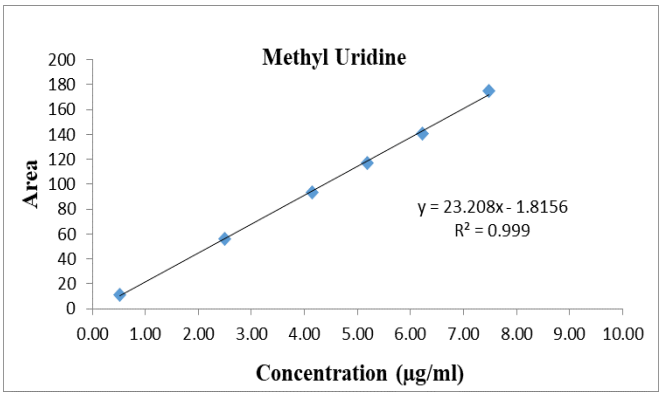

Fig. 5: Calibration curve of methyl uridine

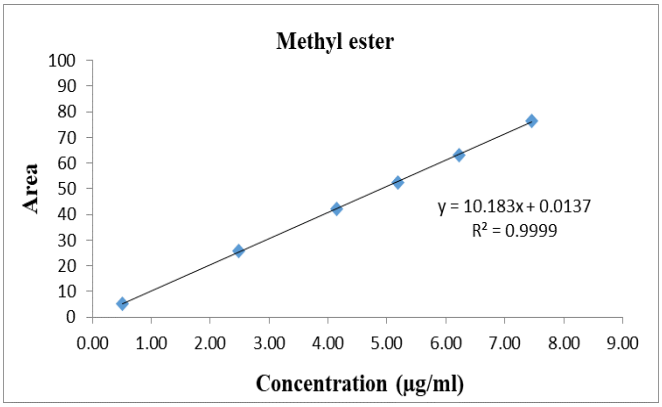

Fig. 6: Calibration Curve of Methyl ester

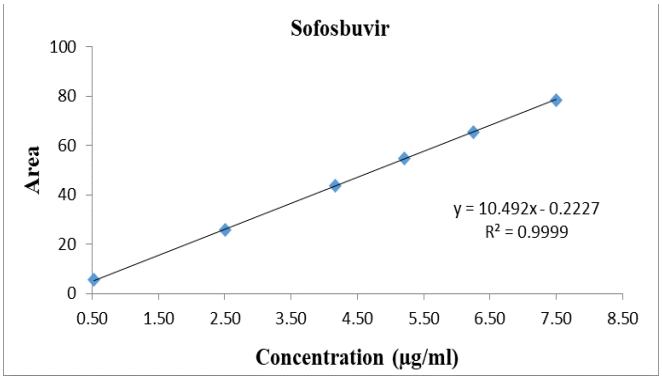

Fig. 7: Calibration Curve of Sofosbuvir

at room temperature for 10 minutes. Filter the solution through 0.45 µm Millipore PVDF filter; collect the filtrate by discarding 5 mL of the filtrate.

Sofosbuvir was sensitive to stress conditions like alkali hydrolysis. The results proved that the developed method has good selectivity and specificity and is also suitable for the determination of impurities in sofosbuvir tablet dosage form. A chromatogram of the sample in alkali hydrolysis was presented in Fig. 4.

Precision

- *System precision*

Precision prepared a standard solution as per the test method and injected it six times into the HPLC system. The retention time and area of the analyte peak were recorded. The observations are tabulated in Table 3. The RSD of the peak area for sofosbuvir standard was found to be 0.1%.

- *Method precision*

In the method precision prepared six control samples and six samples by spiking impurities at specification level and analysed them as per the test method. The samples were prepared as per the method, and the precision study results are tabulated in Table 4.

LoD and LoQ

LoD and LoQ for sofosbuvir and its impurities were predicted by a linearity curve using standard deviation and slope. The precision of predicted LoQ was established by performing six replicate analysis of standard solution at the LoQ level. %relative standard deviation was calculated. The observation is tabulated in Table 5.

LoD = 3.3 X σ/s and LoQ = 10 × σ/s

Where, σ = the standard deviation of the response

s = the slope of the calibration curve

Linearity

The linearity of the developed test procedure was performed from LoQ to 120% of the specification level of two impurities with sofosbuvir. The calibration curve was obtained by plotting peak area against the concentration of impurities and sofosbuvir. The slope, Y-intercept and correlation coefficient were calculated. The correlation coefficient was within the acceptance criteria is not less than 0.999. The calibration curve of methyl uridine, methyl ester and sofosbuvir shown in Figs. 5-7.

Accuracy

The accuracy of the test procedure was determined by spiking the impurities in the pre-analyzed sofosbuvir drug substance. The actual % amount recovered was calculated.

Table 3: System precision data for sofosbuvir

Injection No	*Peak area of analyte*
1	105.712
2	105.385
3	105.629
4	105.410
5	105.329
6	105.580
Mean area	105.508
Theoretical plates	24219
Tailing factor	1.01
% RSD	0.1
Signal to noise ratio	30.1

Table 4: Method precision data

Method Precision				
% Known impurity				
	Methyl uridine	*Methyl ester*	*Alpha isomer*	*Bis impurity*
Mean	0.25	0.26	0.095	0.061
SD	0.0054	0.012	0.0054	0.0040
%RSD	2.147	4.598	5.765	6.620

Table 5: LoD and LoQ Level for sofosbuvir and its impurities

Name of Impurity	*LoD (µg/mL)*	*LoQ (µg/mL)*
Methyl uridine	0.1730	0.5190
Methyl ester	0.1728	0.5183
Sofosbuvir	0.1738	0.5213

Table 6: Accuracy of Methyl uridine and Methyl ester

Methyl uridine								
%Level of recovery	*Set No*	*Wt. of sample*	*Amount of impurity spiked (mg)*	*Total amount recovered (mg)*	*% Recovery*	*Mean % Recovery*	*SD*	*%RSD*
LoQ	1	308.75	0.0125	0.0142	113.6	113.9	1.22	1.07
	2	308.77	0.0125	0.0144	115.2			
	3	308.74	0.0125	0.0141	112.8			
50%	1	308.77	0.1245	0.1239	99.5	98.7	1.77	1.80
	2	308.78	0.1245	0.1245	100.0			
	3	308.75	0.1245	0.1204	96.7			
100%	1	308.78	0.2490	0.2448	98.3	98.4	1.55	1.57
	2	308.76	0.2490	0.2414	96.9			
	3	308.79	0.2490	0.2489	100.0			
150%	1	308.75	0.3757	0.3626	96.5	97.5	0.87	0.89
	2	308.77	0.3757	0.3672	97.7			
	3	308.79	0.3757	0.3688	98.8			
Methyl ester								
LoQ	1	308.74	0.0126	0.0111	88.1	90.2	3.03	3.35
	2	308.77	0.0126	0.0118	93.7			
	3	308.75	0.0126	0.0112	88.9			
50%	1	308.76	0.1263	0.1257	99.5	98.9	1.87	1.89
	2	308.78	0.1263	0.1268	100.4			
	3	308.75	0.1263	0.1223	96.8			
100%	1	308.78	0.2526	0.2492	98.7	98.6	1.50	1.52
	2	308.77	0.2526	0.2450	97.0			
	3	308.75	0.2526	0.2527	100.0			
150%	1	308.75	0.3796	0.3684	97.0	98.1	0.95	0.95
	2	308.79	0.3796	0.3737	98.4			
	3	308.77	0.3796	0.3751	98.8			

Table 7: Stability of sample solution

Time (in hours)	*% Known impurities*				
	Methyl uridine	*Methyl ester*	*Alpha isomer*	*Bis impurity*	*%Total impurities*
Initial	0.26	0.25	0.10	0.06	0.67
12	0.25	0.26	0.09	0.07	0.67
20	0.26	0.26	0.10	0.06	0.68
29	0.26	0.26	0.09	0.06	0.67
45	0.26	0.27	0.09	0.06	0.68
53	0.25	0.27	0.10	0.07	0.69
66	0.26	0.28	0.09	0.06	0.69
80	0.25	0.28	0.09	0.06	0.68

The accuracy was performed at different levels of LoQ, 50, 100, and 150% of the specification limit. The results of accuracy for methyl uridine and ethyl easter are tabulated in Table 6. From the results, it can be concluded that the method is accurate.

Solution stability of analytical solution

Standard solution and sample solution with spiked impurity solution were kept in vials. Thermostat temperature at (10°C). The stability of a standard and a sample spiked with impurities was determined by % difference in the content of sofosbuvir in the standard and samples, calculated against the initial injection. The results are tabulated in Table 7.

Robustness

The robustness of the method expresses the resistance of chromatographic conditions to small changes in the analytical conditions. To estimate the robustness of the analytical method chromatographic conditions like temperature, pH of the mobile phase, flow rate and mobile phase composition were changed. The results are tabulated in Table 8.

Results and Discussion

The sofosbuvir drug and its related substances were found stable under all stress conditions, such as acid hydrolysis,

Table 8: Data for Robustness

Condition	*Parameters*			
	Retention time of Sofosbuvir (min)	*%RSD of replicate standard injection*	*Tailing factor*	*Theoretical plate*
Normal condition	56.35	0.1	1.1	24219
Change in column temperature -5 and +5°C				
Change in column temperature -5 (i.e. 20°C)	56.93	0.1	1.2	23884
Change in column temperature +5 (i.e. 30°C)	55.86	0.1	1.2	24983
Flow rate was changed by -10% and + 10%				
Flow rate was changed by -10% (i.e. 0.9 mL/min)	57.46	0.2	1.1	25483
Flow rate was changed by+10 (i.e. 1.1 mL/min)	55.43	0.1	1.1	23506
Organic phase of Mobile phase was changed by -2 and +2% absolute.				
Organic phase ratio of mobile phase was changed by -2%	56.60	0.1	1.1	24658
Organic phase ratio of mobile phase was changed by +2%	55.96	0.1	1.1	24304
pH of buffer solution was changed by-0.1 until and +0.1.				
pH of buffer solution -0.1 units (i.e. pH 2.9)	56.39	0.1	1.2	24611
pH of buffer solution -0.1 units (i.e. pH 3.1)	56.40	0.2	1.2	24665

alkali hydrolysis, peroxide oxidation, thermal degradation, UV light degradation and humidity degradation. The degradation impurities are generated in alkali hydrolysis. The degradation condition, peak purity and %result of sofosbuvir were determined.

In the specificity study, all degradation products of the analyte and all known impurities were separated, and no interference of the degradation product was observed. The retention time of sofosbuvir was found to be 54.5 minutes, the tailing factor was 1.01 and the theoretical plate was found to be 24219. The peak purity of the analyte in all stress conditions was observed 1000.

The equation of calibration curve for sofosbuvir is Y = 10.492 x -0.2227, methyl uridine is Y = 23.208x-1.8156 and methyl ester is Y = 10.183x + 0.0137, and the correlation coefficient is 0.9999 for sofosbuvir, methyl uridine and methyl ester.

The LoD for sofosbuvir and its impurities such as methyl uridine and methyl ester are 0.1730, 0.1728 and 0.1738 µg/mL, respectively. The LoQ for sofosbuvir and its impurities such as methyl uridine and methyl ester are 0.5190, 0.5183 and 0.5213 µg/mL, respectively.

The accuracy studies were shown as %recovery for sofosbuvir and its impurities at the specification level. The limit of %recovered shown is in the range of 80 and 120% and the results obtained were found to be within the limits. Hence, the method was found to be accurate.

For precision studies, six replicate injections were performed. %RSD was determined from the peak area of sofosbuvir and its impurities. The acceptance limit should be less than 10 and the results were found to be within the acceptance limits.

The analytical results yielded useful and new information yet not reported in the literature on the simultaneous separation of sofosbuvir and its impurities as well as their degradation products by the reverse phase liquid chromatography (RP-HPLC) method. The developed HPLC method was suitable for separating and resolving all impurities and degradation products formed under specific stress conditions. In line with the reported method, we have developed simultaneous separation and validation of related substances and degradation products of sofosbuvir in tablet dosage form for the first time. The advantage of this method over existing reported methods is that it can be used in quality control departments and stability studies of drug substances and pharmaceutical drug products.

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