STABILITY-INDICATING REVERSE PHASE-HPLC METHOD DEVELOPMENT AND METHOD VALIDATION FOR QUANTITATIVE DETERMINATION OF DEGRADATION IMPURITIES IN SOFOSBUVIR

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Abstract:
Sofosbuvir is used to treat specific hepatitis C viruses and it is a direct acting antiviral agent. In this study, the HPLC method for the quantification of Sofosbuvir and its three degradation impurities (DIs) were developed and validated for Sofosbuvir drug substance. The specificity of the method was achieved in analytical column Zorbax XDB C18 (150mm X 4.6 mm, 5.0 µm) using a suitable mobile phase 10 mM Ammonium acetate buffer (pH 4.0 with diluted acetic acid) and Acetonitrile in the gradient programme. The flow rate is 1.0 mL/min. the injection volume is 1 µL, detection at 210 nm in UV and total run time is 70.0 minutes. The samples were made for forced degradation under hydrolysis, oxidation, thermal and photolytic conditions. The method was validated for specific, selective, sensitive, linear, rugged, robust and accurate as per the ICH guidelines. The linearity of the method for Sofosbuvir and its three DIs were found from QL level to 400 % concentration level with the correlation coefficient (r^2) > 0.999. The accuracy for Sofosbuvir and its three DIs were performed from QL to 150% level concentration, and mean recovery was found from 98-102%. The degradation and validation study results indicate its unstable nature in acidic, basic, peroxide conditions and stable nature in thermal, neutral and photolytic and mass balance was achieved in all the stress conditions. Therefore, this method could be used in routine stability studies and quality control analysis.

Keywords: Sofosbuvir, HPLC, Degradation impurities, ICH guidelines, Forced degradation, Stability Indicating method.

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1. Introduction:

Sofosbuvir (SFB) is an antiviral agent for treating specific hepatitis C virus (HCV). SFB is prescribed along with other antiviral agents. As a prodrug nucleotide analogue, sofosbuvir undergoes a transformation into its active form, 2'-deoxy-2'-fluoro-C-methyluridine-5'-triphosphate, which acts as a broken substrate for NS5B (non-structural protein 5B). Synthesis[1-2].

The American Association for the Study of Liver Diseases (AASLD) and the Infectious Diseases Society of America (IDSA) suggest Sofosbuvir as first-line therapy in conjunction with other antivirals for all six genotypes of hepatitis C. Their recommendation was issued in a joint statement in 2016.[3]. Since 2014, a fixed-dose combination medication containing sofosbuvir and other antiviral drugs has been marketed for treating chronic hepatitis C [4]. Sofosbuvir, which the FDA approved in October 2014, is also offered as the commercially available drug Epclusa as a fixed-dose combination product with Velpatasvir. initially accepted in June 2016. The chemical formula is $C_{22}H_{29}FN_3O_9P$. The average molecular weight is 529.458. Its physicochemical properties include the water solubility 0.824 mg/mL, Log P Values is 1.63 and pKa is 9.7. It is chemically 4'-({2-butyl-4-oxo-1,3-diazaspiro [4.4] non-1-en-3-yl}methyl)-N-(4,5-dimethyl-1,2-oxazol-3-yl)-2'-(ethoxymethyl)-[1,1'-biphenyl]-2-sulfonamide and has the following chemical structure in **Figure 1A.** The three related compounds (RC) formed under stability conditions of SFB are presented in **Figure** 1B,1C and 1D as SFB-RC01, SFB-RC02 and SFB-RC03 respectively**.** (RC – Related Compound)

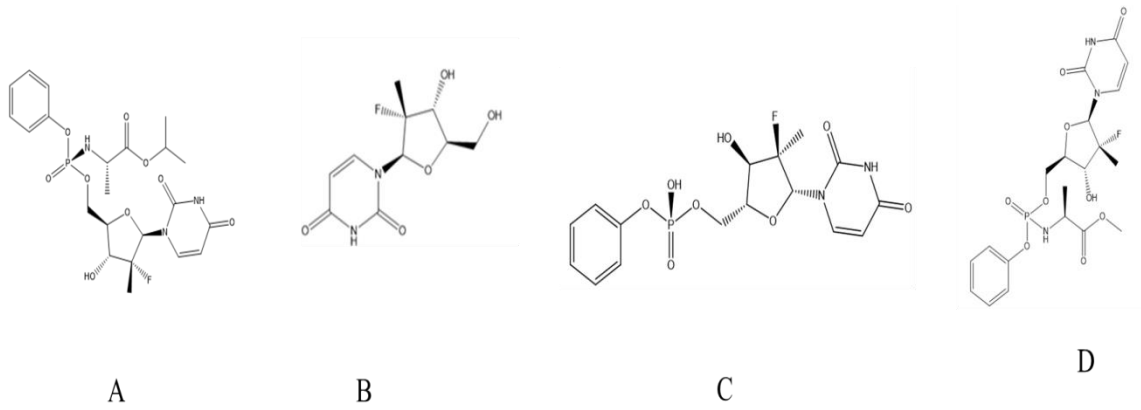

Figure 1: Representative Structure of Sofosbuvir (A), SFB-RC01 (B), SFB-RC02 (C) and SFB-RC03 (D) literature survey indicated no chromatographic methods for determining degradation impurities presented in the SFB[5–8].

The objective of the current study was to create a simple, precise, linear, accurate, rugged, robust and stability-indicating method for identifying degrading impurities that are present in SFB [9–13]. A proven quantitative analytical process called the stability-indicating assay and degradation impurity method typically involves forced degradation and validation experiments.[14].

2. Materials and Methods:

2.1. Instrumentation:

The experiment was performed on a Shimadzu LC-Prominence HPLC equipped with Binary pumps, a PDA detector, an auto injector, a sample cooler, column heater. Electronics Balance from Denver, India, was used. Vacuum microfiltration unit was used with 0.22µm PVDF filters from Millipore.

2.2. Chemicals, reagents and standards:

AR grade Ammonium acetate was procured from Sigma Aldrich, and HPLC grade Acetonitrile was procured from J.T. Baker. Milli-Q water was obtained from Millipore.

2.3. Chromatographic conditions:

The 10mM ammonium acetate buffer was prepared and adjusted pH to 4.0 with diluted acetic acid in milli-Q water and filtered through a 0.22µm membrane filter. HPLC Mobile phase was composed of 10 mM ammonium acetate buffer is in A channel and Acetonitrile is in B channel in gradient programme as in T (min)/%B (0/10, 10/10, 12/15, 50/85, 60/85, 61/10, 70/10). The selectivity was achieved using Zorbax XDB C18 (150mm X 4.6mm, 5.0µm). the flow rate of 1.0 mL/min was employed. The HPLC column temperature and sample temperatures were set at 40°C and 25°C respectively. The analytes were detected at 210 nm.

The injection volume is 1.0 µL and the total run time is 70.0 minutes. Water and Acetonitrile in the ratio of 20:80 v/v is used as diluent.

2.4. Preparation of the SFB Standard Stock Solution:

Weighed 100 mg of SFB Standard accurately and transferred into 100 ml cleaned dry volumetric flask, added about 70ml of diluent, sonicated for 10 minutes, dissolved and made up to the volume with diluent and mixed well. (1000µg/ml SFB).

2.5. Preparation of the SFB Standard Solution:

Transferred 1ml of SFB standard stock solutions into a 100ml volumetric flask and made up to the volume with diluent and mixed well (Stock-II). Further 5 mL of the Stock-II solution was transferred into 25 mL clean and dry volumetric flask and made up to the diluent and mixed well. (2µg/ml SFB)

2.6. Preparation of the impurity stock solution:

Weighed accurately 10mg each of SFB-RC01 impurity, SFB-RC02 impurity and SFB-RC03 impurity and transferred into 200 ml cleaned dry volumetric flask, added about 100ml of diluent, sonicated for 10 minutes and made up to the volume with diluent and mixed well. (50µg/ml impurity).

2.7. Preparation of the impurity spiked solution:

Transferred 1ml from the impurity stock solution and 5 ml of SFB standard stock II solutions into a 25 ml volumetric flask and made up to with diluent and mixed well.

2.8. Preparation of the Sample solution:

Weighed accurately and transferred about 100 mg of SFB drug substance into 100ml cleaned and dry volumetric flask and added about 60 ml of diluent, sonicated for 10 minutes and made up to the volume with diluent and mixed well. (1000µg/ml SFB).

2.9. Preparation of the Spiked Sample solution:

Weighed accurately and transferred about 100 mg of SFB drug substance into 100ml cleaned and dry volumetric flask and added about 60 ml of diluent, sonicated for 10 minutes, added 4 ml of impurity stock solution and make up to the final volume with diluent and mixed well. (1000µg/ml SFB and impurities at 2 µg/ml).

2.10. Preparation of Oxidative degradation sample solution:

Weighed accurately about 100 mg of SFB drug substance and transferred into 100 mL cleaned and dry volumetric flask and added about 60 ml of diluent and sonicated for 10 minutes to dissolve and added 5 mL of 3% Hydrogen peroxide (H_2O_2) solution to the sample containing solution. The resultant solution was kept for 30 minutes at 60°C on a hot water bath. Finally, made up to the volume with diluent and mixed well. (1000µg/ml SFB). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

2.11. Preparation of Acid degradation sample solution:

Weighed and transferred 100 mg of SFB drug substance in 100 mL of volumetric flask and added about 60 ml of diluent and sonicated for 10 minutes to dissolve and added 5 mL of 1N Hydrochloric acid (HCl) solution to the sample containing solution. The resultant solution was kept for 30 minutes at 60°C on a hot water bath. Finally, made up to the volume with diluent and mixed well. (1000µg/ml SFB). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

2.12. Preparation of Alkali degradation sample solution:

Weighed accurately about 100 mg of SFB drug substance and transferred into 100 mL of volumetric flask and added about 60 ml of diluent and sonicated for 10 minutes to dissolve and added 5 mL of 1N sodium hydroxide (NaOH) solution to the sample containing solution. The resultant solution was kept for 30 minutes at 60°C on a hot water bath. Finally, made up to the volume with diluent and mixed well. (1000µg/ml SFB). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

2.13. Preparation of Thermal degradation sample solution:

SFB placed on the Petri dish and kept in a hot air oven at 105°C for 6hrs. After 6 hrs weighed accurately about 100 mg of SFB drug substance and transferred into a100 mL of volumetric flask and added about 60 ml of diluent and sonicated for 10 minutes to dissolve, and finally made up to the volume with diluent and mixed well. (1000µg/ml SFB). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

2.14. Preparation of Photo stability degradation sample solution:

The SFB drug substance was placed in the Photo stability chamber exposing UV light and Visible light at 1.2 million Lux hours and 200-watt hours/minutes respectively. After exposed, weighed and transferred 100 mg of SFB drug stances into 100 mL of volumetric flask, added about 60 mL of diluent and sonicated for 30 minutes with intermittent shaking and makeup to the volume with diluent and mixed well and made up to the volume with diluent and mixed well (1000µg/ml SFB). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

2.15. Preparation of Neutral degradation sample solution:

Weighed about 100 mg of SFB drug substance and made refluxing in 20 mL of water for 6 hrs at a temperature of 60°C, transferred into 100 mL cleaned and dry volumetric flask, added about 60 mL of diluent, sonicated for 10 minutes and made up to the volume with diluent and mixed well (1000µg/ml SFB). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

3.0 Results and Discussions:

3.1. Method Development:

This study aimed to develop the separation and quantification of degradation impurities and sofosbuvir in sofosbuvir drug substance. Waters Allience HPLC system equipped with DAD (Liquid Chromatography equipped with a Diode array detector) and UV as detector, the method was developed to provide the suitability of routine stability studies and Quality control analysis. The method was optimized to improve symmetrical peak shape, the resolution between SFB, Degradation impurities. To achieve the criteria multiple experiments were performed to optimize the column, diluent and mobile phases. The initial HPLC method development was initiated using a gradient method using mobile phase with 0.1% orthophosphoric acid and acetonitrile using the Symmetry C18 column (4.6mm x 150mm, 3.0µm) with a flow rate of 1.0 mL/min. The analyte SFB peak shape was distorted. For the second trial a gradient programme using mobile phase with 10 mM ammonium acetate buffer and methanol using the Zorbax XDB C18 (4.6 x 150mm, 5.0µm) with a flow rate of 1.0 mL/min. The analyte peak shape was distorted. For the third trial a gradient programme using mobile phase with 10mM ammonium acetate adjusted pH to 4.0 with dilute acetic acid and acetonitrile in the using the Zorbax XDB C18 (150mm X 4.6mm, 5.0µm). with a flow rate of 1.0 mL/min. The analyte and degradation impurities peak shape was improved, and the plate count was above 5000. Hence the method was optimized and above conditions are considered as final method. The final optimised chromatograms of spiked sample solution are shown in the **Figure 2.**

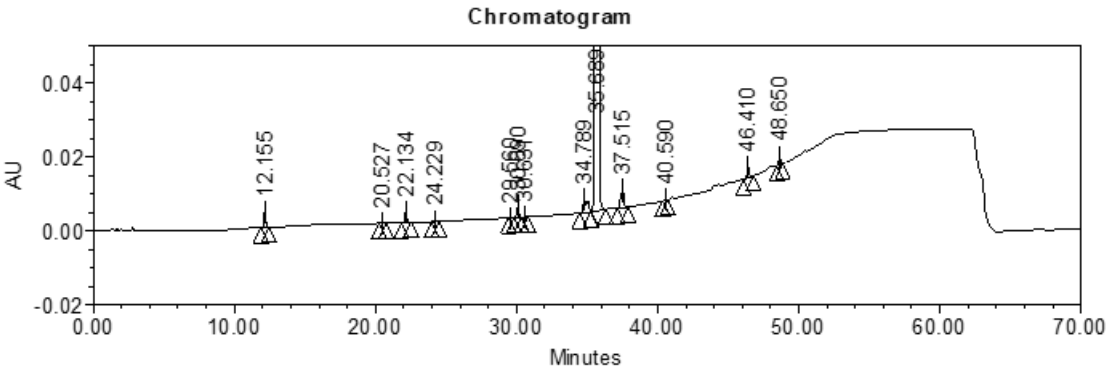

Figure 2: Representative chromatogram of spiked sample solution

3.2. Method Validation

The analytical method validation on HPLC method was performed (in terms of System suitability, Specificity, Sensitivity, Accuracy, Precision, Linearity, Range, Robustness, and Solution stability) in accordance with ICH guidelines[15–17].

3.3. System Suitability:

It is evaluated by injecting six replicate injections of SFB standard solution according to the United States Pharmacopeia (USP) recommendations. The peak asymmetry, theoretical plates, and %RSD for main peak areas were calculated. The results are shown in **Table 1**.

3.4. Specificity and Forced degradation studies: The analytical method was evaluated for the specificity by injecting the blank and as such sample prepared at the specified concentration (100µg/mL) and Standard solution. The method was found specific as there is no interference observed in blank chromatograms at the main peak and degradation impurities retention time, The representative chromatogram of blank, impurity spiked, unspiked and spiked sample were shown in **Figure 3**. The Purity angle and Purity threshold at various degradation conditions are given for Sofosbuvir in **Table 4**. The specificity of the method is also evaluated using forced degradation studies following ICH Q1A and Q1B guideline. The sample degradation was performed as per the below experimental conditions.

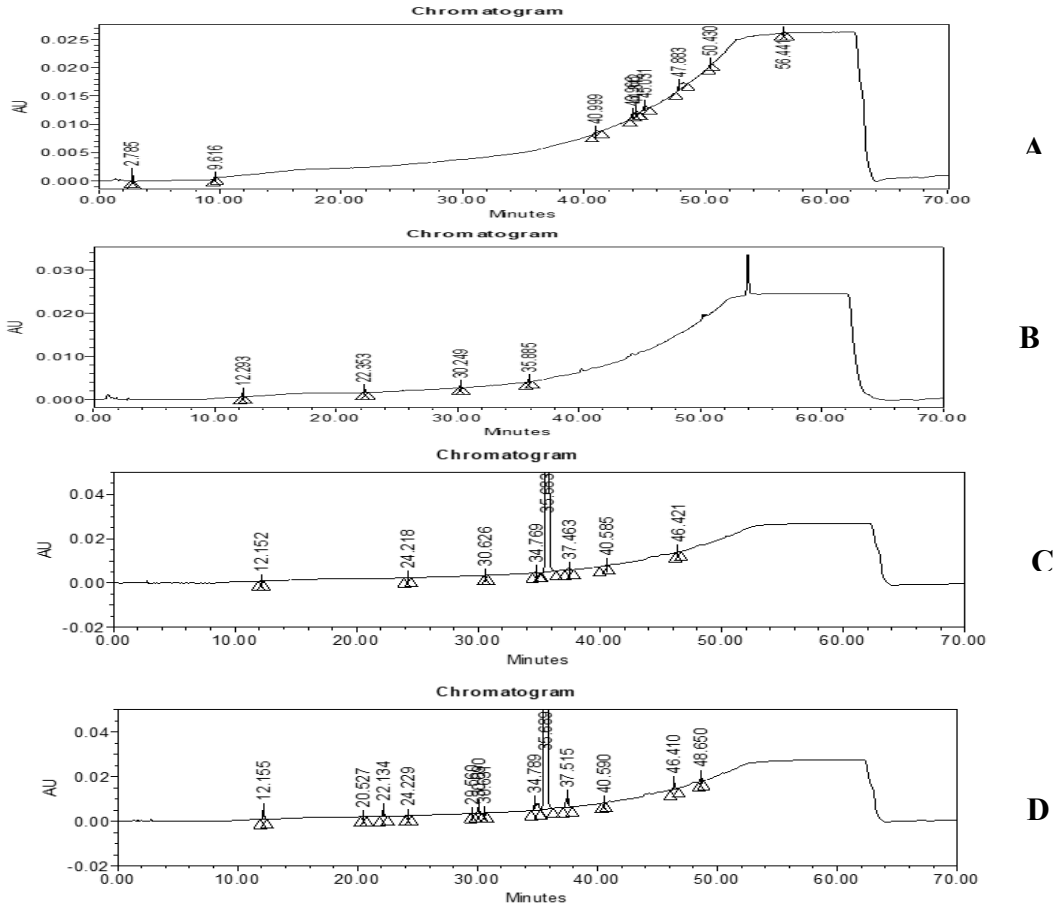

Figure 3: The representative chromatogram of Blank (A), Impurity Spiked (B), Unspiked sample (C) and Spiked Sample (D)**.**

3.4.1 Acid Degradation:
The obtained chromatogram shows significant degradation under the acidic condition. The representative chromatogram shown in **Figure 4**. The results of the percentage assay, percentage degradation, mass balance and peak purity of SFB are in **Table 3**.

3.4.2 Base Degradation:
The obtained chromatogram shows significant degradation under the basic condition. The representative chromatogram shown in **Figure 4**. The results of the percentage assay, percentage degradation, mass balance and peak purity of SFB are in **Table 3**.

3.4.3 Hydrolysis (Neutral):
The obtained chromatogram shows no significant degradation under the hydrolytic condition. The representative chromatogram shown in **Figure 4**. The results of the percentage assay, percentage degradation, mass balance and peak purity of SFB are in **Table 3**.

3.4.4 Thermal Degradation:
The obtained chromatogram shows no significant degradation under the thermal condition. The representative chromatogram shown in **Figure 5**. The results of the percentage assay, percentage degradation, mass balance and peak purity of SFB are in **Table 3**.

3.4.5 Photo Degradation:

The obtained chromatogram shows no significant degradation under the Photo degradation condition. The representative chromatogram shown in **Figure 5**. The results of the percentage assay, percentage degradation, mass balance and peak purity of SFB are in **Table 3**.

3.4.6 Peroxide Degradation:

The obtained chromatogram shows significant degradation under the oxidative degradation condition. The representative chromatogram shown in **Figure 4**. The results of the percentage assay, percentage degradation, mass balance and peak purity of SFB are in **Table 3**.

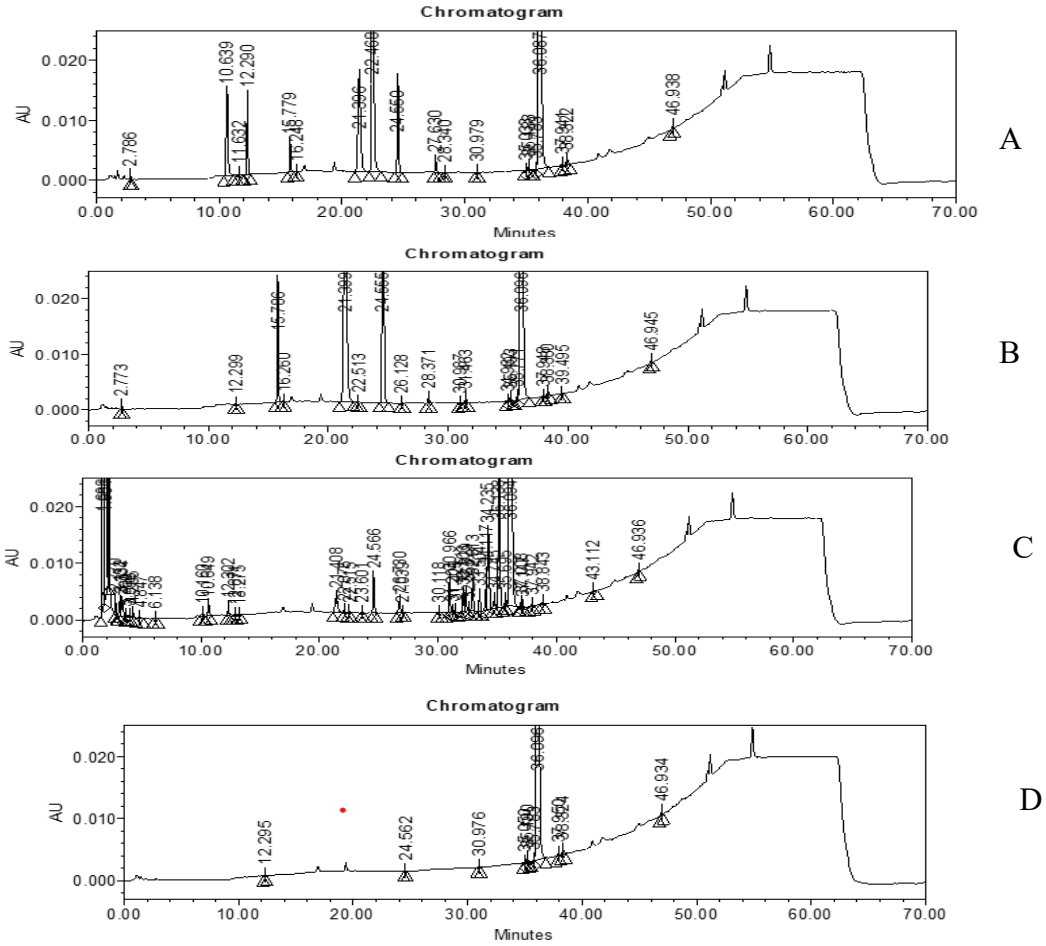

Figure 4: Representative chromatogram Acidic (A), Basic (B), Peroxide (C) and Neutral (D) degradation

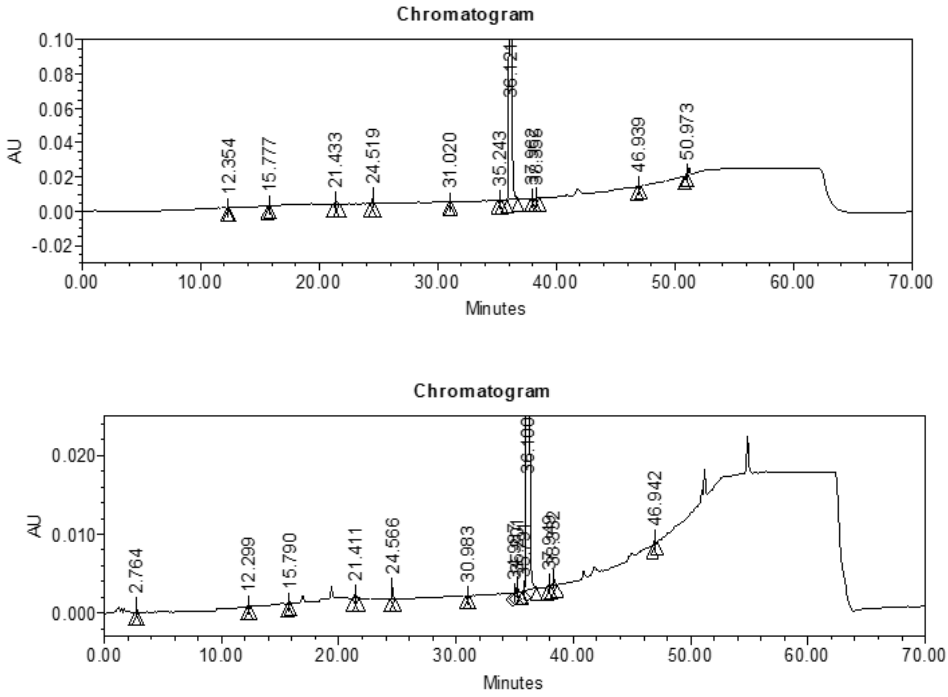

Figure 5: Representative chromatogram Photo Stability (A), Thermal (B).

3.5. Linearity:

The analytical method was evaluated for the linearity by injecting the spiked standard solutions of SFB at concentrations ranging from QL to 400% for more than 6 levels and 3 sets were prepared individually. The calibration curve was obtained by plotting a graph between the average peak areas of 3 sets and the concentrations of SFB. The obtained calibration curve showed a correlation coefficient greater than 0.9998 for SFB and the method is found to be linear**.** The results are tabulated shown in **Table 4**. The linearity solutions were prepared as below. for the preparation of stock solution: Accurately weighed and transferred about 10mg of SFB standard into 200 ml clean dry volumetric flasks, add 70ml of diluent, sonicated for 10 minutes and make up to the final volume with diluents. (50µg/ml SFB). Further dilution preparations ranging from QL to 400% linearity level refer **Table 4.**

3.6. DL and QL:

The DL and QL are defined as the lowest concentration of the analyte, where DL stands for Detection Limit, and QL stands for Quantification Limit. These was evaluated by using the Calibration plot. The calculated DL and QL for SFB are 0.406 ppm and 0.134 ppm respectively and injected the sample into the HPLC similarly calculated for the degradation impurities. The results of QL and DL are shown in **Table**. The % RSD of the peak areas of each analyte is not more than 10.0%.

3.7. Precision and intermediate precision:

3.7.1 Method Precision:

The analytical method was evaluated for method precision by analysing 6 different preparations of SFB sample solution, the %RSD for the %recoveries of SFB and its degradation impurities was calculated. The results confirm that the method is precise for determining SFB by HPLC.

3.7.2 Intermediate Precision:

The analytical method was evaluated for intermediate precision by performing analysis on different days on different equipment. The %RSD for the %recoveries of six different sample preparation of SFB and degradation impurities were calculated. The results confirm the rugged for determination of SFB by HPLC.

3.8. Accuracy:

The analytical method was evaluated to determine the accuracy of the method by using the standard addition method. The experiment was performed in triplicate at QL, 100%, and 150% levels and the % recoveries were calculated. The % recovery values were in the range of 98.0 to 101.1 for SFB which are within the acceptance criteria and similarly calculated to the degradation impurities. The %RSD values of the recoveries obtained for all impurities were less than 1.0. The results are shown in **Table 2.**

3.9. Solution Stability:

The analytical method was evaluated for the solution stability of SFB was determined by storing

the samples in tightly capped volumetric flasks at 25°C and 2-8°C for 48 hrs. The % recovery of samples was calculated against freshly prepared sample solution. The results were found that SFB were stable at 2-8°C and 25°C after 48 hrs.

3.10. Robustness:

The analytical method was evaluated for the robustness by deliberate change in the experimental conditions and the system suitability data were recorded. The variables evaluated in the study were column temperature from 30°C to 40°C as Temperature Minus (TM) and Temperature Plus (TP) respectively, the Flow rate from 0.9 to 1.1 mL/min as Flow Minus (FM) and Flow Plus (FP) respectively and change in detection at 208nm and 212 nm. The results met the acceptance criteria, and the results are shown in **Table 6.**

4. Conclusion:

The optimized experimental and validated results confirm that the analytical method on HPLC can quantify both degradation impurities and SFB using suitable stationary and mobile phases. The proposed analytical method was validated according to ICH Q2 guidelines. The SFB is found susceptible to the peroxide, acidic, basic degradation conditions but remained stable under thermal, photolytic and neutral, forced degradation conditions. The methodology appears to be a specific, linear, accurate, precise robust, and stability-indicating method, according to the degradation and analytical method validation. By employing HPLC, it is possible to quantify degradation impurities and SFB.

5. Acknowledgement

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6. Conflict of Interest

The author has no conflict of interest to declare.

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Table 1: System suitability parameters and retention time results

Injection No.	**Peak Area of Sofosbuvir**	**Statistical Analysis**	
1	32742		
2	32705	Mean	32808
3	32682		
4	32914	SD	110.5
5	32925		
6	32881	% RSD	0.3
USP Theoretical Plate Count		389255	
USP Tailing Factor		1.1	
Sample and impurity ID		**Retention time (min)**	
SFB-RC01		12.296 min	
SFB-RC02		22.353 min	
SFB-RC03		30.249 min	
Sofosbuvir		35.885 min	

Table 2: Accuracy results

Sample #	**Sofosbuvir**			**SFB-RC01 impurity**		
	Recovery (%)	**% Mean**	**% RSD**	**Recovery (%)**	**% Mean**	**% RSD**
QL level Sample 1	97.8			100.7		
QL level Sample 2	97.9	98	0.2	101.9	101.4	0.6
QL level Sample 3	98.2			101.5		
100% Sample 1	100.2			100.6		
100% Sample 2	100.7	100.5	0.3	100.8	100.8	0.3
100% Sample 3	100.5			101.1		
150% Sample 1	101.1			100.2		
150% Sample 2	100.9	101.1	0.2	101.1	100.9	0.7
150% Sample 3	101.3			101.5		

Sample #	**SFB-RC02 impurity**			**SFB-RC03 impurity**		
	Recovery (%)	**% Mean**	**% RSD**	**Recovery (%)**	**% Mean**	**% RSD**

QL level Sample 1	96.1			101.1		
QL level Sample 2	97.3	97.1	0.9	104.1	102.6	1.5
QL level Sample 3	97.8			102.6		
100% Sample 1	96.4			102.8		
100% Sample 2	96.1	96.1	0.3	101.6	102.1	0.6
100% Sample 3	95.9			101.8		
150% Sample 1	97.1			101.9		
150% Sample 2	97.5	97.4	0.2	102.3	102.1	0.2
150% Sample 3	97.5			102.2		

Table 3: Forced degradation conditions for SFB and Peak Purity data

Sample	% Active remaining	% Total Impurities	% Total found (% w/w)	% Mass balance	Peak Purity
Unstressed sample	99.3	0.167	99.467	-	Pass
Light, solution, exposed, ($1.2X10^6$ lux hours and 200.25-watt hours/square meter of UV energy)	98.2	0.513	98.715	99.3	Pass
Light, solution, control, ($1.2X10^6$ lux hours and 200.25-watt hours/square meter of UV energy)	99.1	0.265	99.365	99.9	Pass
1N HCl at 60°C for 30 min.	88.9	9.6791	98.5791	99.1	Pass
1N NaOH at 60°C for 30 Mins.	86.4	12.4776	98.8776	99.4	Pass
3% Hydrogen Peroxide at 60°C for 30 min	91.5	12.947	104.447	105	Pass
Heat 105°C for 6 Hours.	99.1	0.3244	99.4244	100	Pass

Table 4: Linearity dilutions ranging from QL to 400%

% Concentration	Concentration (µg/mL)	Sofosbuvir peak area
QL	0.406	6718
60	1.217	20100
80	1.622	26255
100	2.028	32865
140	2.839	45750
180	3.65	58832
200	4.055	66526
300	6.083	99526
400	8.11	130914
	Slope	16198
	y-intercept	154.952
	r^2	0.9998442

Table 5: DL and QL concentration, results and signal to noise ratio

Peak Name	QL Concentration				DL Concentration			Signal to noise ratio		
	µg/mL	% w/w	Mean Peak Area	% RSD	µg/mL	% w/w	Mean Peak Area	Min.	Max.	Mean
Sofosbuvir	0.406	0.04	6718	1.9	0.134	0.013	2099	10	17	12
SFB-RC01	0.409	0.04	7872	1.1	0.135	0.014	2616	14	24	18
SFB-RC02	0.4	0.04	6912	2.2	0.132	0.013	2267	10	18	13
SFB-RC03	0.398	0.04	6102	1.1	0.131	0.013	2004	10	17	12

Table 6: Robustness Data

Chromatographic condition		Standard		Retention time (min) from spiked sample			
		Tailing	Plate counts	SFB-RC01	SFB-RC02	SFB-RC03	Sofosbuvir
As per method	Inj.1	1	435640	12.965	23.036	30.951	36.583
	Inj.2	1	433162				
Variation in Wavelength							
208 nm	Inj.1	0.9	427336	12.964	23.023	30.934	36.568
	Inj.2	0.9	426551				
212 nm	Inj.1	1	426184	12.958	23.018	30.925	36.555

	Inj.2	1	427157				
Variation in Flow rate							
0.9 mL/min	Inj.1	0.9	472629	13.504	23.644	31.608	37.239
	Inj.2	0.9	467529				
1.1 mL/min	Inj.1	1	388878	12.484	22.492	30.327	35.947
	Inj.2	1	389376				
Variation in Column temperature							
30℃	Inj.1	1	427158	13.384	23.407	31.248	36.806
	Inj.2	1	425268				
40℃	Inj.1	0.9	398207	12.494	22.612	30.564	36.259
	Inj.2	0.9	399674				