RP-HPLC METHOD DEVELOPMENT, VALIDATION AND FORCED DEGRADATION STUDIES FOR THE QUANTITATIVE ESTIMATION OF SOFOSBUVIR IN API AND PHARMACEUTICAL DOSAGE FORM

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Abstract : A simple, rapid, precise, accurate and sensitive reverse phase liquid chromatographic method has been developed for the determination of Sofosbuvir in API and pharmaceutical dosage form dosage form. The chromatographic method was standardized using Develosil ODS HG-5 RP C18, 5µm, 15cm x 4.6mm i.d. column with UV detection at 264 nm and Methanol : Acetonitrile (55:45) ratio at a flow rate of 1.0 ml/ min. The proposed method was successfully applied to the determination of Sofosbuvir in bulk and pharmaceutical dosage form. The method was linear over the range of 0 µg/ml to 16 µg/ml. The recovery was in the range of 98% to 102% and limit of detection was found to be 0.09 µg/ml and quantification was found to be 0.27 µg/ml. Different analytical performance parameters such as precision, accuracy, limit of detection, limit of quantification and robustness were determined according to International Conference on Harmonization (ICH) guidelines.

Keywords: *RP-HPLC, Sofosbuvir, Method development and validation, ICH Guidelines.*

1. INTRODUCTION:

Sofosbuvir (trade name Sovaldi) is a direct acting antiviral medication used as part of combination therapy to treat chronic Hepatitis C, an infectious liver disease caused by infection with Hepatitis C Virus (HCV). HCV is a single-stranded RNA virus that is categorized into nine distinct genotypes, Depending on the genotype, sofosbuvir is often used in combination with other anti-virals such as Ledipasvir, Velpatasvir, Daclatasvir, Simeprevir, Elbasvir, Grazoprevir, Ribavirin, Peginterferon alfa-2a, or Peginterferon alfa-2b with the intent to cure, or achieve a sustained virologic response (SVR), after 12 weeks of daily therapy. SVR and eradication of HCV infection is associated with significant long-term health benefits including reduced liver-related damage, improved quality of life, reduced incidence of Hepatocellular Carcinoma, and reduced all-cause mortality. Treatment with direct acting anti-virals such as sofosbuvir is associated with very minimal side effects, with the most common being headache and fatigue .

The IUPAC Name of Sofosbuvir is propan-2-yl (2S)-2-{[(S)-{[(2R, 3R, 4R, 5R)-5-(2, 4-dioxo-1, 2, 3, 4-tetra hydro Cpyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl] methoxy} (phenoxy) phosphoryl] amino} propanoate.

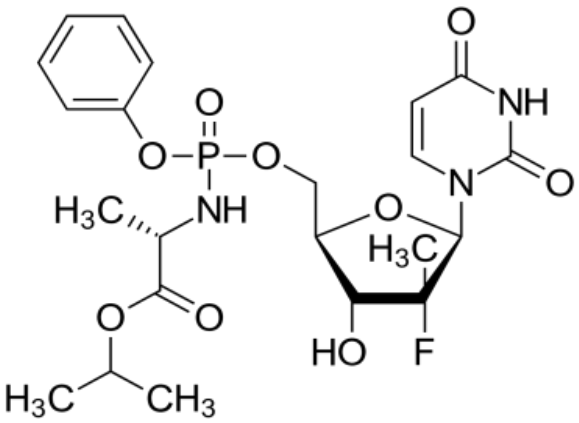


Fig 1: Chemical Structure of Sofosbuvir

2.MATERIALS AND METHODS

2.1 HPLC Instrumentation & Conditions:

The HPLC system employed was HPLC with Empower 2 Software with Isocratic with UV-Visible Detector.

2.2 Standard & sample preparation for UV-spectrophotometer analysis:

25 mg of Sofosbuvir standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.2 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase. The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Sofosbuvir, so that the same wave number can be utilized in HPLC UV detector for estimating the Sofosbuvir. While scanning the Sofosbuvir solution we observed the maxima at 264nm. The UV spectrum has been recorded on ELICO SL-159 make UV – Vis spectrophotometer model UV-2450.

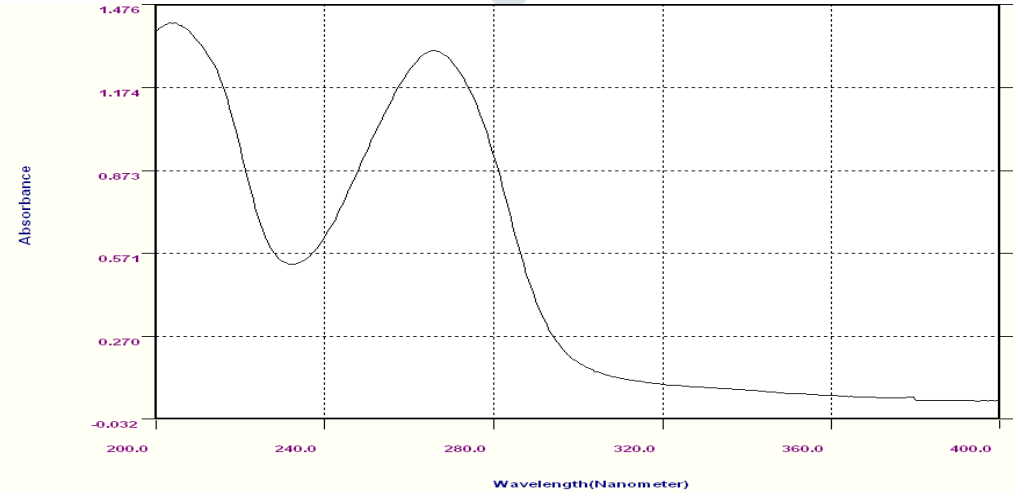


Fig 2: UV Spectrum

2.3 Optimized Chromatographic Conditions:

Column: Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm.

Mobile Phase : Methanol : Acetonitrile (55:45 v/v).

Flow Rate : 1.0ml/minute

Wave length : 264 nm

Injection volume : 20µl

Run time : 08 mins.

Column Temperature : Ambient

Sampler Cooler : Ambient

2.4 MOBILE PHASE PREPARATION

Mobile phase was prepared by taking Methanol: Acetonitrile (55:45 v/v). Mobile phase was filtered through 0.45 µm membrane filter and degassed under ultrasonic bath prior to use. The mobile phase was pumped through the column at a flow rate of 1.0 ml/min.

2.5 SAMPLE & STANDARD PREPARATION FOR THE ANALYSIS

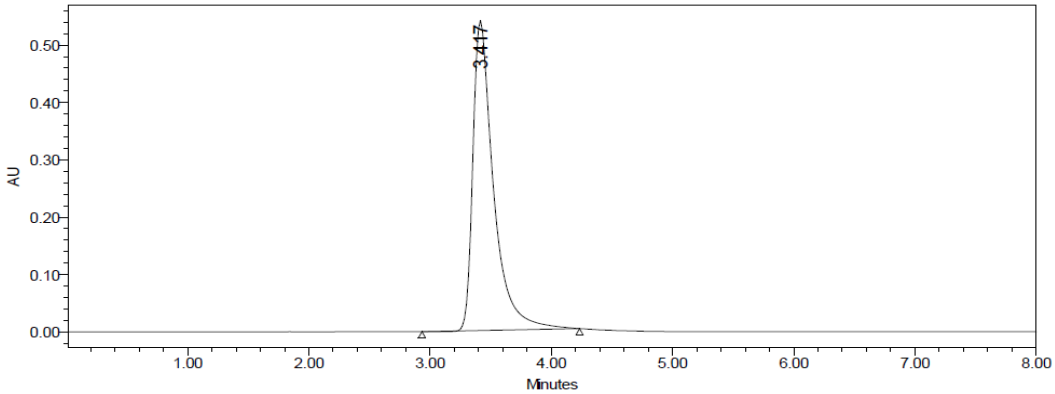
25 mg of Sofosbuvir standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

3.RESULT AND DISCUSSION:

Table-1: Trials for Method Development

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm	Methanol : Acetonitrile = 60 : 40	0.8 ml/min	264 nm	Broad Peak	Method rejected
Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm	Acetate Buffer: Acetonitrile = 40 : 60	1.0 ml/min	264 nm	Splitting of peak	Method rejected
Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm	Phosphate Buffer: Acetonitrile = 30 : 70	1.0 ml/min	264 nm	Tailing peak	Method rejected
Waters ODS (C18) RP Column, 250 mm x 4.6	Phosphate Buffer: Acetonitrile	1.0 ml/min	264 nm	Splitting of peak	Method rejected

mm. 5µm	= 40 : 60				
Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm	Methanol : Acetonitrile = 55:45	1.0 ml/min	264 nm	Good Peak	Method accepted



OPTIMIZED CONDITION

Name	Rt	Peak Area	Theoretical Plates	Tailing Factor
Sofosbuvir	3.417	1114246	3265	1.12

Table 2: Peak Results

4.METHOD VALIDATION

4.1 Accuracy: ***Recovery study:*** The accuracy of the proposed developed method the % recovery studies were carried out by adding different quantities (80%, 100%, and 120%) of pure drug of SOFOSBUVIR was taken and added to the prepared pre-analyzed formulation of concentration 10µg/ml. From that % recovery values were measured. The results were shown in Table-3.

Table-3: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Statistical Analysis
	Amount Added	Amount Found			
S_1 : 80 %	8	8.105	93435	101.312	Mean= 100.0163%
S_2 : 80 %	8	7.898	91287	98.725	S.D. = 1.293505
S_3 : 80 %	8	8.001	92356	100.012	% R.S.D.= 1.293294
S_4 : 100 %	10	10.195	115135	101.95	Mean= 101.4033%
S_5 : 100 %	10	10.152	114687	101.52	S.D. = 0.613379

S_6 : 100 %	10	10.074	113879	100.74	% R.S.D.= 0.60489
S_7 : 120 %	12	12.171	135647	101.425	Mean= 100.6053%
S_8 : 120 %	12	12.044	134324	100.366	S.D. = 0.730041
S_9 : 120 %	12	12.003	133897	100.025	% R.S.D. = 0.725649

4.2 PRECISION :

4.1.1 Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Sofosbuvir (API) the percent relative standard deviations were calculated for Sofosbuvir is presented in the Table-4.

Table-4: Repeatability Results of Precision

HPLC Injection Replicates of Sofosbuvir	**Area Under the Curve**
Replicate – 1	1013546
Replicate – 2	1025824
Replicate – 3	1012351
Replicate – 4	1036584
Replicate – 5	1015419
Replicate – 6	1008572
Average	**1018716**
Standard Deviation	**10495.73**
% RSD	**1.03029**

4.1.2 Intra day & Inter day: The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Sofosbuvir revealed that the proposed method is precise.

Table-5: Results of Intra day & Inter day

Conc. Of Sofosbuvir (API) (µg/ml)	Observed Conc. Of Sofosbuvir (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	8.03	0.25	9.95	0.21
10	10.49	0.36	10.02	0.32
12	11.14	0.14	12.30	0.19

4.2 Linearity and Range

Linearity range was found to be 0-16µg/ml for Sofosbuvir. The correlation coefficient was found to be 0.999, the slope was found to be 10380 and intercept was found to be 9304 for Sofosbuvir.

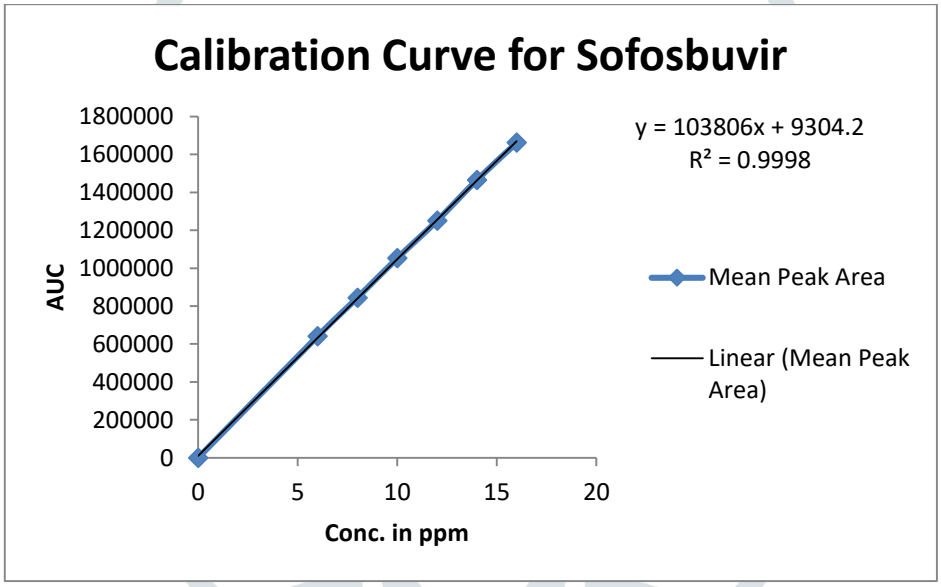


Fig-3: Calibration curve of Sofosbuvir (API)

S. No.	Conc. (µg/ml)	Mean Peak Area
1	0	0
2	6	641233
3	8	844610
4	10	1052647
5	12	1250435
6	14	1465354
7	16	1662043

Table-6: Linearity Results of Sofosbuvir

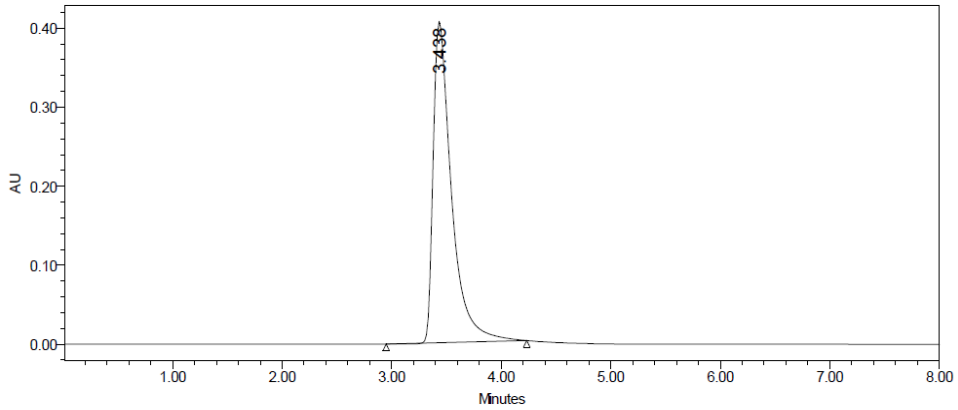


Fig 4: Calibration of Sofosbuvir concentration in 6 ppm

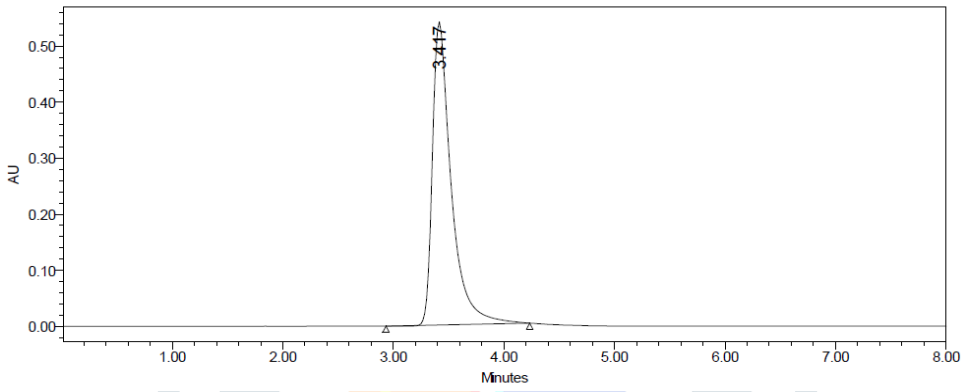


Fig 5: Calibration of Sofosbuvir concentration in 8 ppm

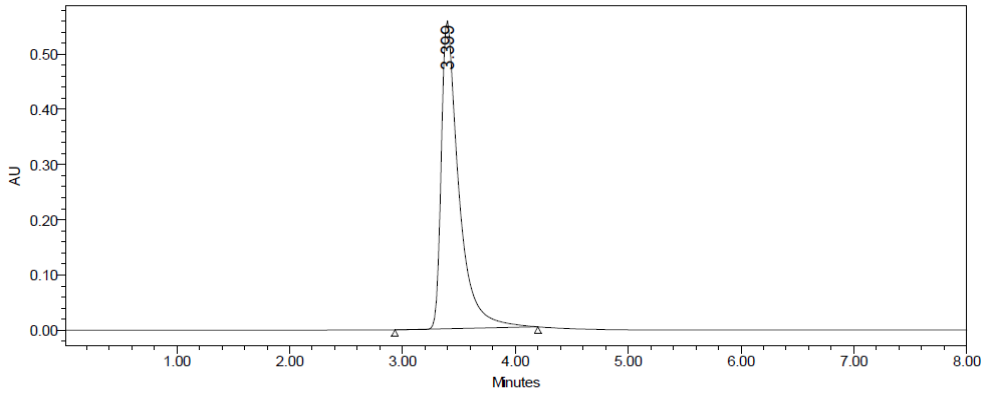


Fig 6: Calibration of Sofosbuvir concentration in 10 ppm

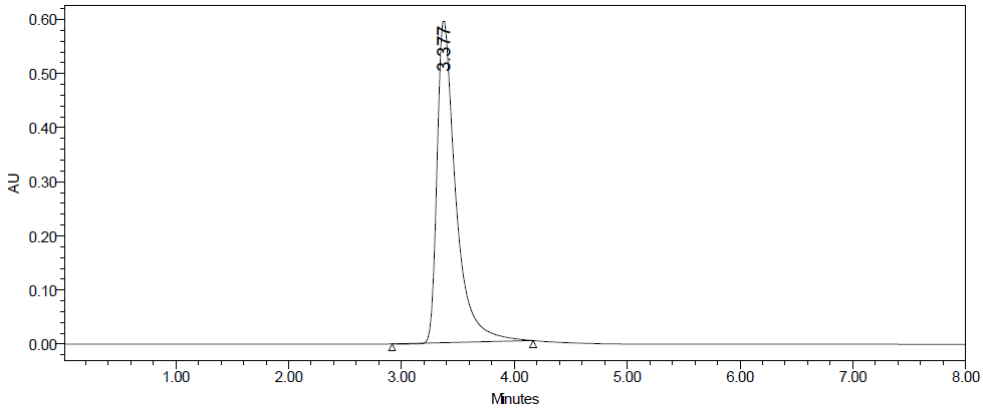


Fig 7: Calibration of Sofosbuvir concentration in 12 ppm

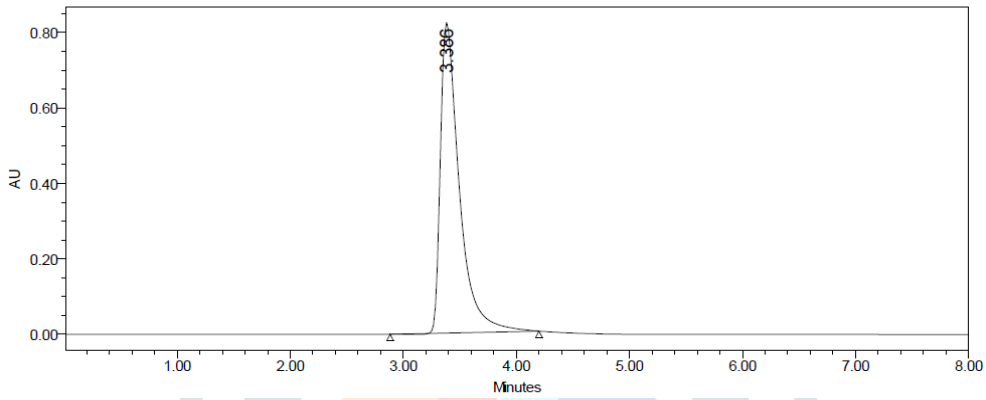


Fig 8: Calibration of Sofosbuvir concentration in 14 ppm

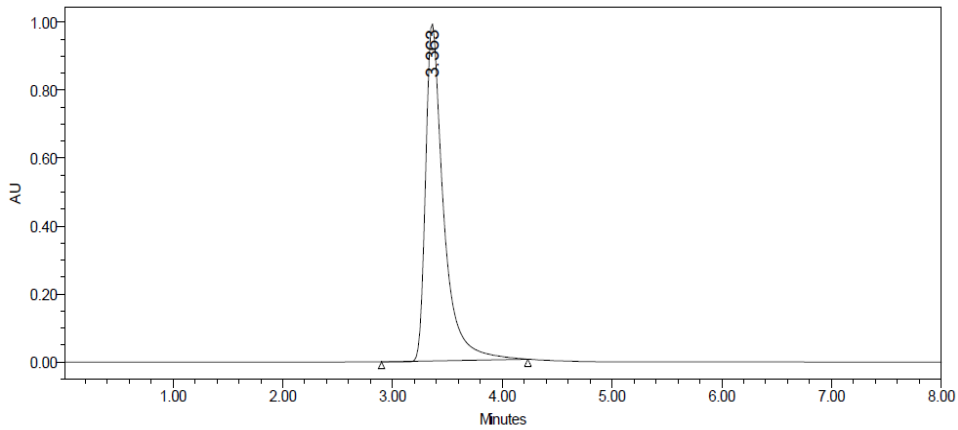


Fig 9: Calibration of Sofosbuvir concentration in 16 ppm

4.3 LOD & LOQ: The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.003 & 0.009 µg/ml respectively.

5 STABILITY STUDIES

5.1 ACID DEGRDATION

A precisely measured 10 mg of unadulterated medication was exchanged to a clean and dry round base jar. 30 ml of 0.1 N HCl was added to it and it was refluxed in a water shower at 600C for 4 hours.

Permitted to cool to room temperature. *The sample was then neutralized using dilute NaOH solution & final volume of the sample was made up to 100ml with water to prepare 100 µg/ml solution. It was injected into the HPLC system against a blank of mobile phase (after optimizing the mobile phase compositions). This experiment was repeated several times using same concentration of HCl (0.1N) and observed its degradation profile. The typical chromatogram shown below is the degradation profile of* Sofosbuvir *in 0.1N HCl.*

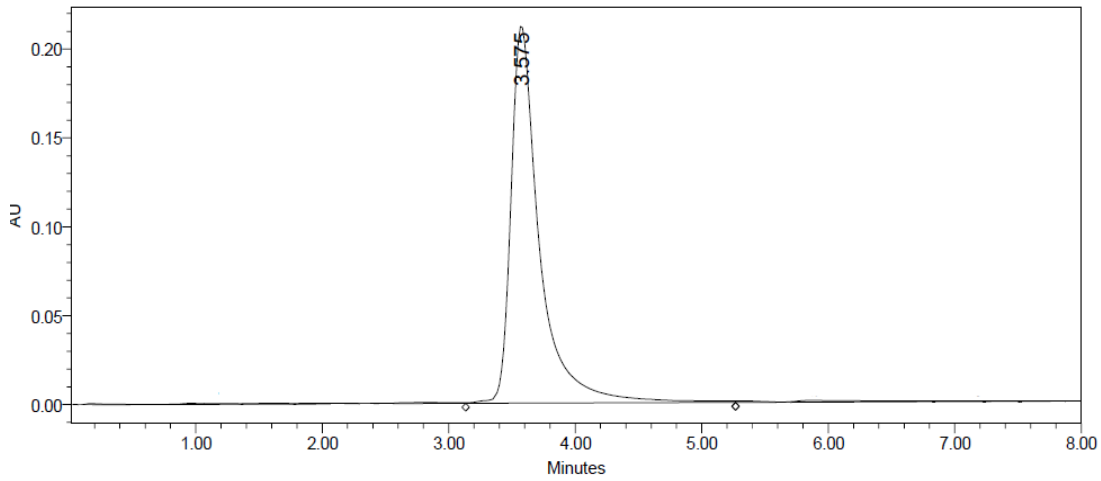


Fig-10: Acidic Degradation

5.2 BASIC HYDROLYSIS:

A precisely measured 10 mg of unadulterated medication was exchanged to a clean and dry round base carafe. 30 ml of 0.1N NaOH was added to it. & it was refluxed in a water bath at 60^{0}C for 4 hours. Allowed to cool to room temperature. The sample was than neutralized using 2N HCl solution & final volume of the sample was made up to 100ml to prepare 100 µg/ml solution. It was injected into the HPLC system against a blank of mobile phase after optimizing the mobile phase compositions. This experiment was repeated several times using same concentration of NaOH such as 0.1N to observe its degradation profile. The chromatogram shown below is the degradation profile of Sofosbuvir *in 0.1N NaOH.*

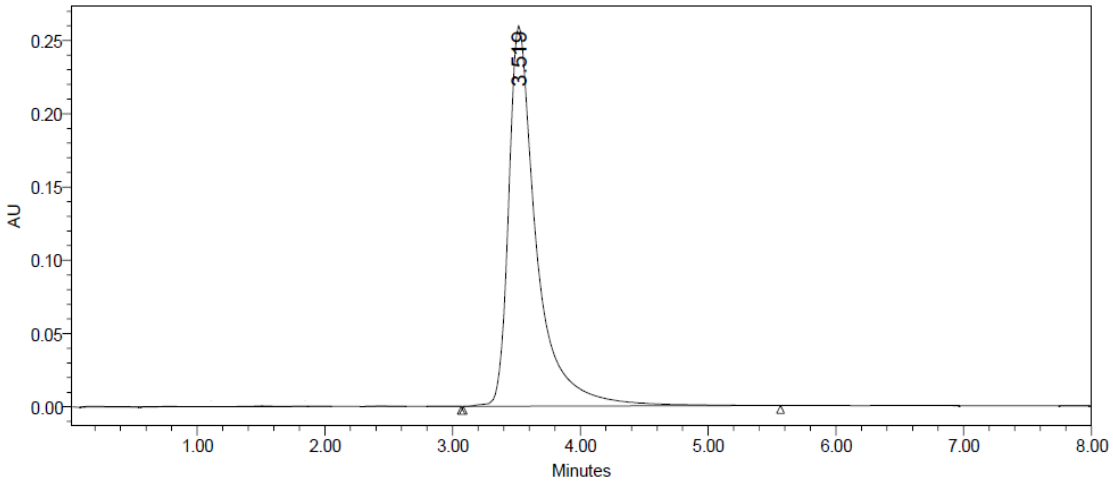


Fig-11: Basic Degradation

5.3 WET HEAT DEGRADATION:

Accurately weighed 10 mg of pure drug was transferred to a clean & dry round bottom flask. 30 ml of HPLC water was added to it. Then, it was refluxed in a water bath at 60^0C for 6 hours uninterruptedly. After the reflux was over, the drug became soluble and the mixture of drug & water was allowed to cool to room temperature. Final volume was made up to 100 ml with HPLC water to prepare 100 µg/ml solution. It was injected into the HPLC system against a blank of mobile phase.

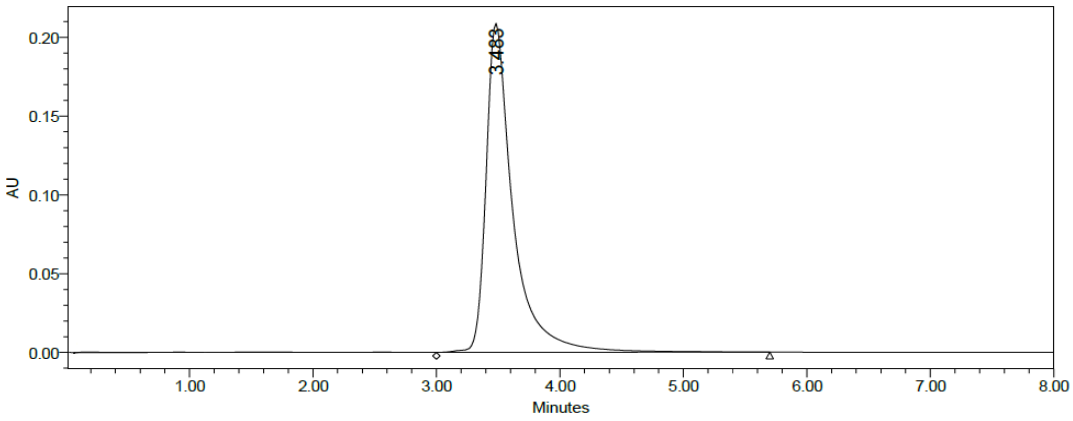


Fig-12: Wet Heat Degradation

5.4 PHOTOLYTIC DEGRADATION:

Approximately 10 mg of pure drug was taken in a clean & dry Petri dish. It was kept in a UV cabinet at 254 nm wavelength for 24 hours without interruption. Accurately weighed 1 mg of the UV exposed drug was transferred to a clean & dry 10 ml volumetric flask. First the UV exposed drug was dissolved in methanol & made up to the mark with mobile phase *to get 100 µg/ml solution.* Finally this solution was injected into the HPLC system against a blank of mobile phase and *chromatogram was obtained.*

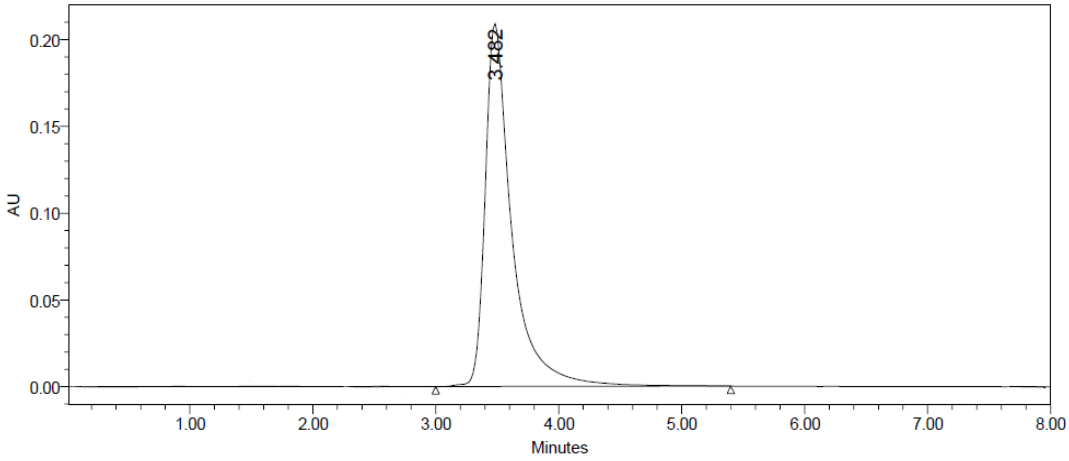


Fig-13: Photolytic Degradation

5.5 OXIDATION WITH (3%) H_2O_2:

Accurately weighed 10 mg. of pure drug was taken in a clean & dry 100 ml volumetric flask. 30 ml of 3% H_2O_2 and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours. Final

volume was made up to 100 ml. using water to prepare 100 µg/ml solution. The above sample was injected into the HPLC system.

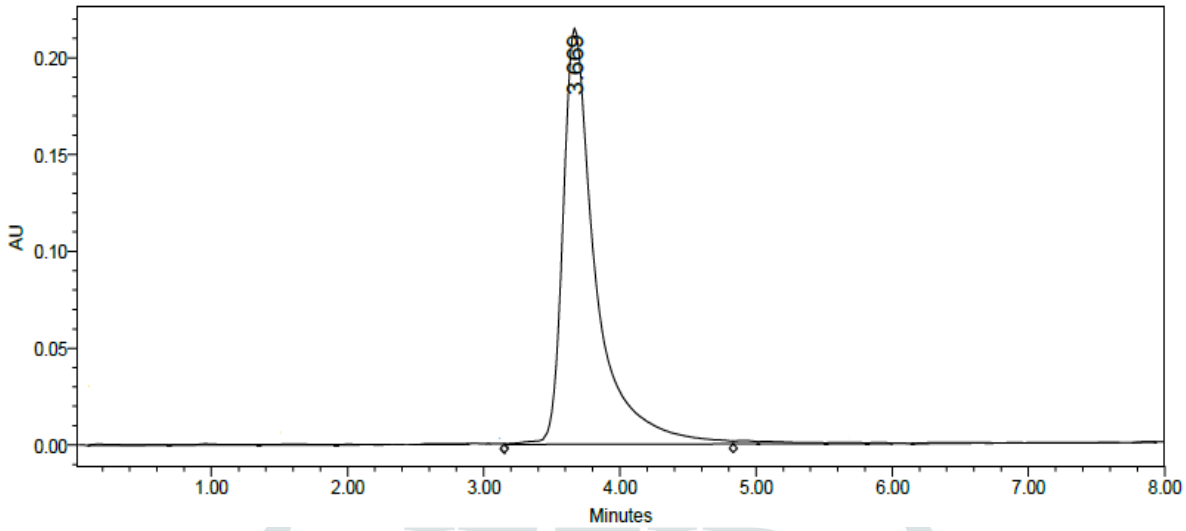


Fig-14: Oxidation Degradation

Table-7: Results of forced degradation studies of Sofosbuvir API.

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	85.69	14.31	100.0
Basic Hydrolysis (0.I M NaOH)	24Hrs.	83.47	16.53	100.0
Wet heat	24Hrs.	79.86	20.14	100.0
UV (254nm)	24Hrs.	87.92	12.08	100.0
3 % Hydrogen peroxide	24Hrs.	80.81	19.19	100.0

6 CONCLUSION

A delicate and specific, sensitive RP-HPLC strategy has been created and approved for the investigation of Sofosbuvir API.

Facilitate the proposed RP-HPLC strategy has amazing affectability, exactness and reproducibility.

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