International Journal of Farmacia

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Development and Validation of Stability Indicating RP-HPLC Method for Estimation of Sofosbuvir in Bulk and Tablet Dosage Form

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ABSTRACT

A simple, precise and accurate stability indicating RP-HPLC method has been developed and subsequently validated for estimation of Sofosbuvir (SOF) n bulk and from their combination dosage form using a Kromasil C18 (250mm × 4.6 mm, 5µ) at 25^0C. Mobile phase consisted mixture of 0.1% Ortho phosphoric acid buffer and acetonitrile in the ratio 55:45 (v/v) with flow rate was 1 ml/min and detection was carried out by photodiode array detector at 260nm. The retention time for SOF was found to be 2.06 min. SOF and their dosage form were exposed to thermal, photolytic, oxidative, acid-base hydrolytic stress conditions, the stressed samples were analyzed by proposed method. Peak purity results suggested no other co-eluting, interfering peaks from excipients, impurities, or degradation products due to variable stress condition, and the method is specific for the estimation of SOF in presence of their degradation products and impurities. The proposed method has permitted the quantification of SOF over linearity in the range of 100 – 600 µg/ml and its percentage recovery was found to be 99.10 – 101.74 %. The % RSD of intraday and inter day precision were found 0.3% and 0.6% according to International Conference on Harmonization (ICH) Q2B guidelines.

Keywords: Sofosbuvir, Rp-Hplc, Stability Indicates, Stress Conditions

INTRODUCTION

SOF is a prodrug of 2'-deoxy-2'-fluoro-2'-C-methyluridine monophosphate that is phosphorylated intra cellularly to the active triphosphate form. Used for the treatment of chronic Hepatitis C [1]. Chemically, SOF is Propan-2-yl(2S)-2-{[(S)-{[(3R,4R,5R)-5-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl] methoxy} (phenoxy) phosphoryl] amino} propanoate [2] with empirical formula of $C_{22}H_{29}FN_3O_9P$ and molecular weight 529.4525 g/mol. It is a White to Off-white non-hygroscopic crystalline solids [3]. Slightly soluble in water (pH 1.2-7.7), freely soluble in ethanol and acetone, soluble in 2-propanol and insoluble in heptanes [4]. The chemical structure of SOF was shown in Fig.01. SOF is a prodrug nucleotide analog used as part of combination therapy to treat hepatitis C virus (HCV) infection or to treat co-infection of HIV and HCV. After metabolism to the active antiviral agent 2'-deoxy-2'-α-fluoro-β-C-methyluridine-5'-triphosphate (also known as GS-461203), the triphosphate serves as a defective

substrate for the NS5B protein, an RNA-dependent RNA polymerase required for replication of viral RNA. SOF is used in combination therapy to treat chronic hepatitis C virus (HCV) infected patients with HCV genotype 1,2,3, or 4, and to treat HCV and HIV co-infected patients. The combination therapy includes either ribavirin alone or ribavirin and peg-interferon alfa [5]. Sofosbuvir is nucleotide analog inhibitor, which specifically inhibits HCV NS5B polymerase. Sofosbuvir prevents HCV viral replication by binding to the two Mg2+ ions present in HCV NS5B polymerase's GDD active site motif [6].

Stability testing is an important part of the process of drug product development. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and enables recommendation of storage conditions, retest periods, and shelf lives to be established. The two main aspects of a drug product that play an important role in shelf life determination are assay of the active drug and degradation products generated during the stability study. The drug product in a stability test sample needs to be determined using a stability indicating method, as recommended by the International Conference on Harmonization (ICH) guidelines [7] and U.S. Pharmacopoeia (USP) 26 [8]. Although stability indicating methods have been reported for assay of various drugs in drug products, most of them describe assay procedures for drug products containing only one active drug substance. Only few stability indicating methods are reported for assay of drug products containing two or more active drug substances. The objective of this work was to develop a simple, precise, and rapid column liquid chromatography (LC) procedure that would serve as stability indicating assay method for drug product of SOF.

An extreme literature survey revealed that very few analytical methods have been reported such as HPLC [9-12] for SOF in individual and combination with other drugs. In order to minimize the batch –to-batch variation, it is very important to develop suitable analytical methods for day –to- day analysis of drugs. It was found that one attempt has been made to develop stability indicating studies and estimation of SOF by RP-HPLC at the starting of my work. Therefore, it was thought of interest in development and validating an advanced new sensitive, specific, precise, accurate stability indicating RP-HPLC method for estimation of SOF in bulk drug and in pharmaceutical dosage form. We here in report a simple, rapid and reliable HPLC for the estimation of SOF in bulk and pharmaceutical dosage forms as per ICH guidelines [13-16].

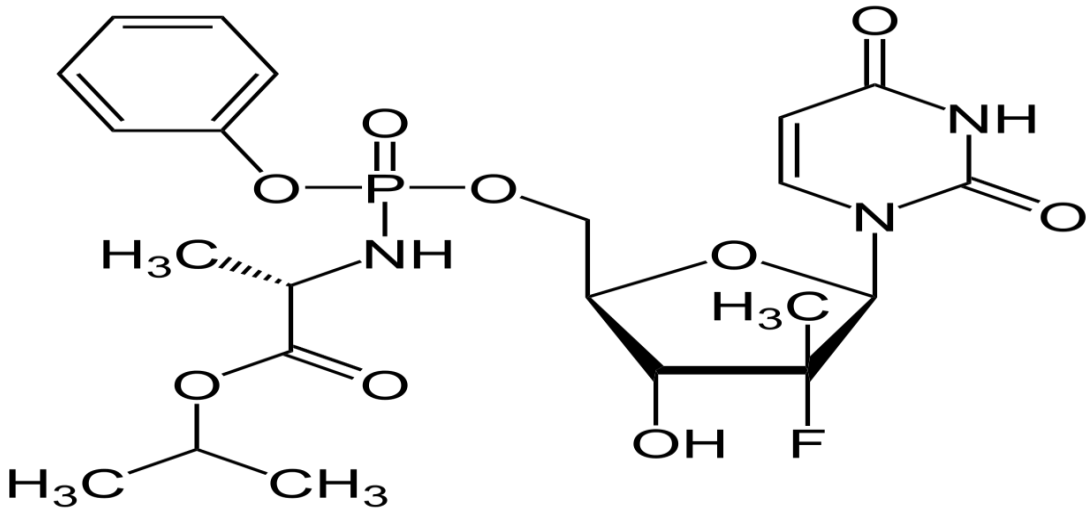

Fig 01: Structure of Sofosbuvir.

EXPERIMENTAL

REAGENTS AND MATERIALS

Pure standard of SOF was obtained as gift sample from Spectrum Pharma Research laboratory in Hyderabad. Acetonitrile, Water HPLC grade (Merck Specialties Pvt Ltd, Mumbai, India), Ortho phosphoric acid HPLC (merck specialities pvt ltd, mumbai), All solvents used in this work are HPLC grade. Sovaldi Tablets (Mylan Pharmaceuticals Private Limited) containing Sofosbuvir Marketed formulation was purchased from local market, High precision weighing balance (wensar instruments, hyderabad), micro pipette (in labs,10-100 µl) were employed in the study. All the glassware employed in the work cleaned with hot water followed acetic anhydride then acetone and dried in hot air oven whenever required. Working environment was maintained in 25^{o}C. However, the chemical structure and purity of the sample obtained were confirmed by melting point, TLC Studies.

HPLC APPARATUS AND CHROMATOGRAPHIC CONDITIONS

The analysis was performed on A Waters 2695 RP-HPLC separation module (Waters Corporation, Milford, USA) equipped with PDA detector having back pressure 5000psi, automatic injector and Kromasil C18 (250mm × 4.6 mm, 5µ). Data acquisition was performed by using Empower 2 software. Single pan Balance (Mettler Toledo), Control Dynamics pH meter (Mettler Toledo), Sonicator (Labindia Instruments). Different mobile phases were tested in order of their polarity to find out the best conditions for the separation of sofosbuvir. An isocratic RP-HPLC system was used for analysis of samples at 25^{o}C column oven temperature. The chromatographic separation was achieved on Kromasil 250 mm x 4.6 mm, 5µ column using 0.1% Ortho phosphoric acid buffer and acetonitrile 55:45 % v/v as mobile phase at a flow rate of 1 ml/min. The mobile phase was filtered through 0.45 µm nylon membrane filter and degassed before use. The injection volume was 10 µl and the total runtime was set as 5 minutes. The determination of analytes was carried out at 260 nm using PDA detector.

PROCEDURE RECOMMENDED

PREPARATION OF MOBILE PHASE

1 ml of Ortho phosphoric acid solution in a 1000ml of Volumetric flask add about 100ml of milli-Q water and final volume make up to 1000ml with milli-Q water and 100% acetonitrile taken in the ratio 55:45 (v/v) were employed as a mobile phase.

PREPARATION OF STOCK SOLUTION

Accurately Weighed and transferred 40mg Sofosbuvir working Standard into a 10ml clean dry volumetric flask, add 5 ml of diluents (first dissolved in methanol and make up Acetonitrile: Water (50:50)), sonicated for 30 minutes and make up to the final volume with diluents. From the above stock solution, 1 ml was pipette out in to a 10ml volumetric flask and then make up to the final volume with diluents.

Table.01: Chromatographic Condition of SOF in API

Parameters	Condition
Column	Kromasil 250 mm x 4.6 mm, 5m.
Column Temperature	25^{0}C
Wavelength	260nm
Diluent	First dissolved in methanol and make up quantity with Acetonitrile: Water in the ratio of (50:50)
Injector volume	10 µl
Flow rate	1 ml/min
Runtime	5 min
Retention time	2.06 min
Theoretical Plates	2613

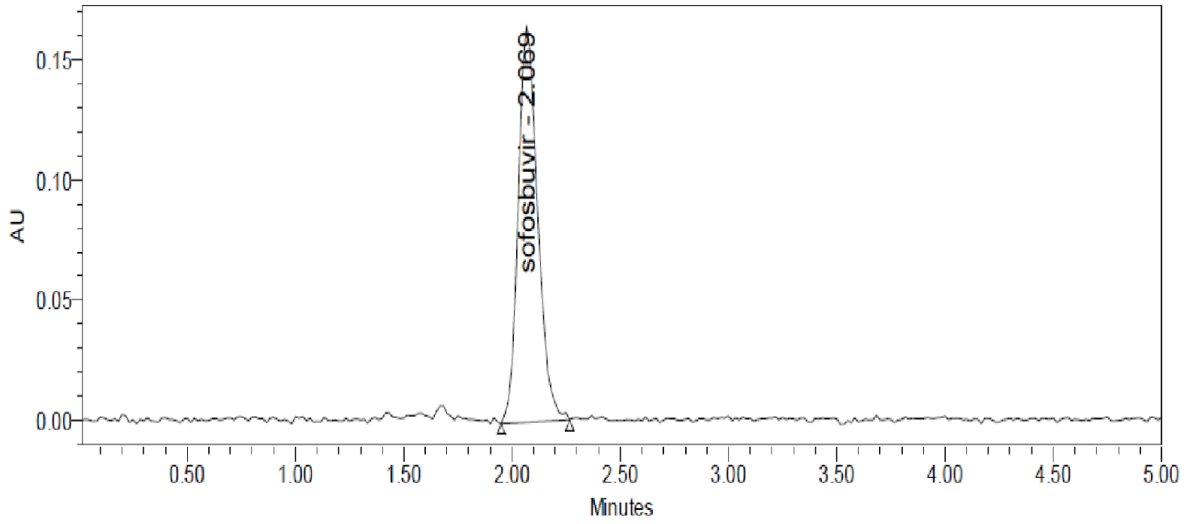

Fig.02; A typical chromatogram of SOF

PREPARATION OF SAMPLE FOR FORCED DEGRADATION STUDIES

PREPARATION OF ACID INDUCED DEGRADATION PRODUCT

1 ml of SOF stock solution was taken into 10 ml Round Bottom (RB) flask and refluxed with 1 ml 2N Hydrochloric acid at 60^0C for 30 minutes. The resultant solution was collected, diluted with diluent to get the concentration of 400 µg/ml and 10 µl solution was injected into HPLC system and chromatograms were recorded.

Preparation Of Alkali Induced Degradation Product

1 ml of SOF stock solution was taken into 10 ml Round Bottom (RB) flask and refluxed with 2N Sodium hydroxide at 60^0C. After 30 minutes the resultant solutions was diluted with diluent to get the concentration of 400 µg/ml and 10 µl solution was injected into HPLC system and chromatograms were recorded.

Preparation Of Hydrogen Peroxide Induced Degradation Product

1 ml of SOF stock solution was taken into 10 ml volumetric flask and 1 ml of freshly prepared 20% H_2O_2 solution was added into volumetric flask and solution were kept at 60^0C for 30 minutes. Then the resultant solution was injected into HPLC system to get the chromatograms.

Photochemical Stability Induced Product

The photochemical stability study of the drug was studied by exposing the sample concentration of 4000 µg/ml to UV light in UV chamber for 7 days or 200 Watt hours/$m^{2.}$ Then the resultant solution was diluted and 10 µl solution was injected in to the HPLC system.

Dry Heat Induced Degradation Product

To study the dry heat degradation studies, the standard drug solution of Sofosbuvir was placed in oven for 6 hours at 105^0C. The resultant solutions were diluted to get the concentration of 400 µg/ml and 10 µl solution was injected into HPLC system and chromatograms were recorded to assess the stability studies.

Construction of Linearity

The concentrations of analyte were prepared from the stock solution by taking suitable volume (0.25 – 1.5 ml) and diluted up to 10 ml to get the desired concentrations for linearity in the range of 100 – 600µg/ml. the prepared solutions were filtered through 0.45µm nylon membrane filter and each of the dilutions was injected three times into the column. The calibration curve for SOF was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

ESTIMATION OF SOF

Analysis of marketed formulation was purchased from local market. 20 tablets were weighed and average weight was calculated. Then from the transferred the equivalent to one tablet to 100ml volumetric flask, 70ml of diluent was added and the mixture was allowed to stand with intermittent sonication for 25 mins to ensure complete solubility of drug. Further the volume made up with diluent and the resulting solution was passed through 0.45µm membrane filtered. From the filtered solution, 1ml was pipette out into 10ml volumetric flask and made upto 10ml with diluent. From the solution, 10µl was injected into HPLC system and peak area was recorded (Fig.3) with detector at 260nm. The % assay was calculated with obtained peak area of detector response. The % assay was found to be 100.50% for Sofosbuvir. This indicates that developed method can be used for routine analysis.

Table.02: % Assay results of Sofosbuvir in formulation

Tablets	Drug	Dosage (mg)	Sample concentration (µg/ml)	Amount found (µg/ml)	% Assay
1	Sofosbuvir	400	400	402	100.50

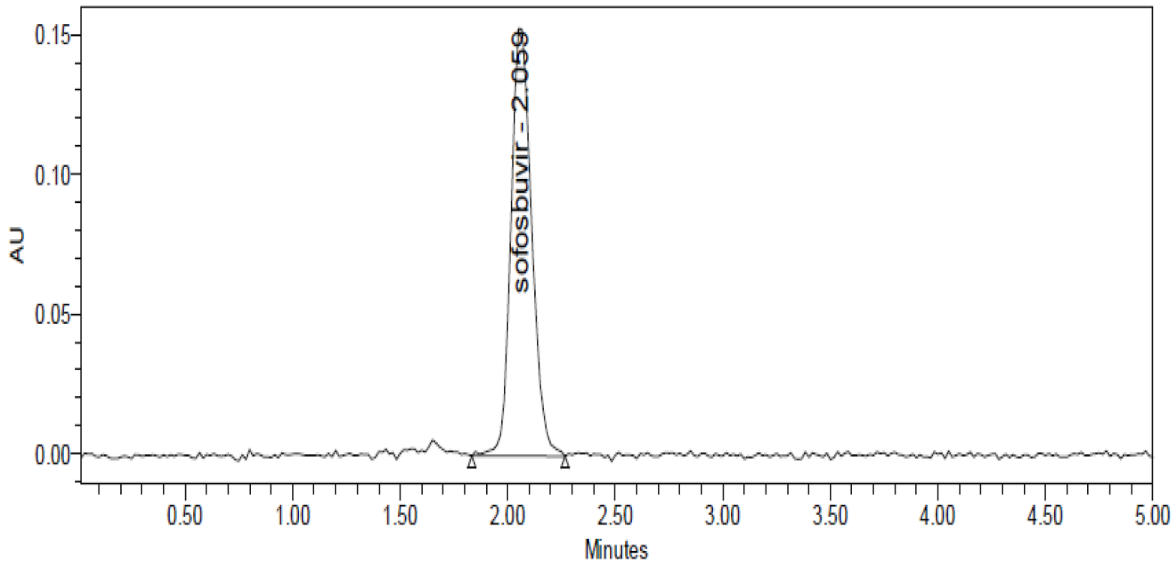

Fig 03: Chromatogram showing assay of Sofosbuvir marketed dosage form (Sovaldi tablet)

METHOD VALIDATION

As per the International Conference on Harmonization (ICH) guidelines, the method validation parameters such as Specificity, Linearity, Precision, Accuracy, Limit of Detection/ Quantification and Robustness were optimized.

RESULTS

The present RP-HPLC method for the quantification of SOF in bulk and pharmaceutical dosage forms, revealed as simple, rapid, accurate and precise method with significant shorter retention time of 2.06.min.

Accuracy

The accuracy of the proposed method was determined by standard addition method. It is the closeness of the analytical results obtained by the

analysis to the true value. A known amount of standard drug was added to the fixed amount of injection solution. Accuracy was expressed as percentage recovery. Recovery test was performed with three different concentrations i.e. 200 µg/ml, 400µg/ml and 600 µg/ml for Sofosbuvir. The % recovery results were calculated and given in Table.03.

Table.03: % Recovery results of Sofosbuvir

Conc.	Sofosbuvir		
	Amount added (µg/ml)	Amount recovered (µg/ml)	% Recovery
50%	200	198.21	99.10
	200	199.77	99.88
	200	198.47	99.24
100%	400	406.98	101.74
	400	406.49	101.62
	400	406.57	101.64
150%	600	599.28	99.88
	600	603.15	100.53
	600	598.95	99.82

Linearity

A series of six concentrations in the range of 100 to 600µg/ml of Sofosbuvir has been prepared and peak areas were recorded at 260nm. A calibration curve was plotted between peak area versus concentration of respective Sofosbuvir and the response of the drug was found to be linear. The linear regression equation ($y = mx + c$) was found to be $y = 2452.2x + 22728$. (Fig.4) for Sofosbuvir. The linearity results were given in Table.04 & 05.

Table.04: Linearity results of Sofosbuvir

Concentration (µg/ml)	Area	Average area	% RSD
	291315		
100	291748		
	291256	291440	0.09
	514225		
200	512112		
	522464	516267	1.10
	763943		
300	769903		
	769294	767713	0.43
	983383		
400	990845		
	991145	988458	0.44
	1262003		
500	1245602		
	1265185	1257597	0.84
	1492919		
600	1482395		
	1486292	1487202	0.36

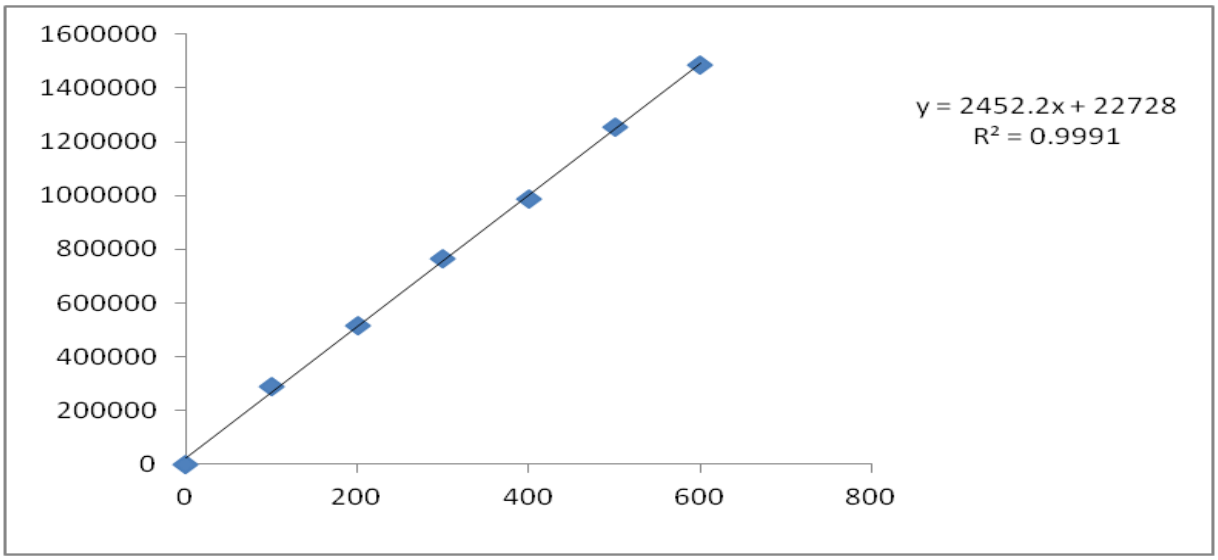

Fig .04: Calibration curve of Sofosbuvir

Table.05: Slope and intercept value of SOF Precision

Linearity curve	**Sofosbuvir**	
	Slope	**Intercept**
Value	2452.2	22728
Correlation coefficient (r^2)	0.9991	

Repeatability or Precision of the method was determined by injecting six replicates of standard solution at 400µg/ml of SOF into HPLC system. From the results obtained it was found that the proposed method was precise given in Table 06.

Table.06: Precision data Robustness

Injection	**Sofosbuvir concentration**	**Area**
1		988616
2		991620
3		983591
4	400µg/ml	984602
5		989132
6		980856
Mean		986403
STDV		**4036.9**
%RSD		**0.4**

Robustness study of the method was determined by changing the parameters such as flow rate, mobile phase ratio and temperature. Drug samples were analyzed under small changed conditions and chromatogram was recorded. It was found that these deliberate changes were not affected the chromatograms of both drug samples and given in Table 07.

Table.07: Actual conditions and proposed conditions of the method
Effect of Flow rate

Parameters	Actual conditions	Proposed variations
Flow rate	1ml/min	0.9, and 1.1ml/min
Mobile phase ration	55:45 % v/v	±10%
Temperature	25 ^{0}C	20 ^{0}C, 30 ^{0}C

By changing the flow rate (1ml/min ±0.1ml) no drastic changes was seen in chromatographic parameters and were given in Table 08 & 09.

Table.08: Robustness data at flow rate 0.9ml/min of SOF

Parameters	RT	Area	Average area	% RSD
Sofosbuvir	2.260	971995	975116	0.3
	2.264	978519		
	2.266	974440		
	2.268	976791		
	2.275	978479		
	2.281	970471		

Table.09: Robustness data at 1.1ml/min of Sofosbuvir

Parameters	RT	Area	Average area	% RSD
Sofosbuvir	1.869	965678	955559	1.0
	1.875	943208		
	1.879	944178		
	1.881	962880		
	1.881	960063		
	1.883	957347		

Effect of Mobile phase

In mobile phase, organic phase was changed to ±10%. It was found that change in mobile phase was not affected the chromatogram parameters and were given in Table 10 & 11.

Table.10: Robustness data at mobile phase ration 45:55% v/v

Parameters	RT	Area	Average area	% RSD
Sofosbuvir	1.887	923741	931686	0.6
	1.890	929021		
	1.892	937085		
	1.892	927732		
	1.897	936415		
	1.903	936119		

Table.11: Robustness data at mobile phase ration 65:35% v/v Effect of Temperature

Parameters	RT	Area	Average area	% RSD
Sofosbuvir	2.228	961352	964502	0.7
	2.228	960588		
	2.229	955685		
	2.230	968539		
	2.232	967730		
	2.242	973119		

Temperature of the column was changed to ±5^{0}C and chromatogram was recorded. From the results, it was found that change in temperature also not affected the chromatogram parameters and were given in Table 12 & 13.

Table.12: Robustness data at temperature 20 ^{0}C

Parameters	RT	Area	Average area	% RSD
Sofosbuvir	2.021	928242	925434	0.5
	2.025	919000		
	2.025	921372		
	2.036	923694		
	2.036	931974		
	2.043	928319		

Table.13: Robustness data at temperature 35 ^{0}C

Parameters	RT	Area	Average area	% RSD
Sofosbuvir	2.031	937150	939030	0.5
	2.037	940122		
	2.037	941376		
	2.037	932061		
	2.038	946158		
	2.045	937314		

Limit of Detection (LOD)

Limit of detection is the known concentration of SOF and establishing minimum concentration at which the SOF can be reliably detected. It was calculated based on the standard deviation of the response and the slope of the standard calibration curve. The LOD was found to be 0.762 µg/ml of SOF.

Limit of Quantification (LOQ)

Limit of quantification is the known concentration of SOF and establishing minimum level at which the SOF can be quantified with acceptable accuracy and precision. The LOQ was found to be 2.308 µg/ml of SOF. The LOD and LOQ results were given in Table.14.

Table.14: LOD and LOQ results of SOF

Sample	LOD	LOQ
Sofosbuvir	0.762 µg/ml	2.308 µg/ml

FORCED DEGRADATION STUDIES

Forced degradation studies (FDS) or stability indicating studies were carried out in presence of degradation products of the samples. These studies were performed to evaluate the specificity of the proposed method. According to the ICH guidelines Q1A (R2) various stress induced degradation conditions such as acid, alkali, oxidation, photolytic and dry heat were used for the stability indicating studies. All the degradation products were resolved from sample peaks. Under each condition the chromatograms were recorded and studied using PDA detector. The sample peaks were tested for its purity using chromatographic software.

EFFECT OF ACID AND ALKALINE HYDROLYSIS

In acid and alkaline hydrolysis, sample solutions were subjected to acid and alkali hydrolysis (2N HCl and 2N NaOH respectively for 30 minutes at 60^0C). In acid induced degradation drug found to undergo minor degradation with degradation product retention time of 2.055 minutes (Fig.05) and in base induced conditions also retention time 2.049min (Fig.06). Results of acid and base degradation were given in Table 15 & Table 16.

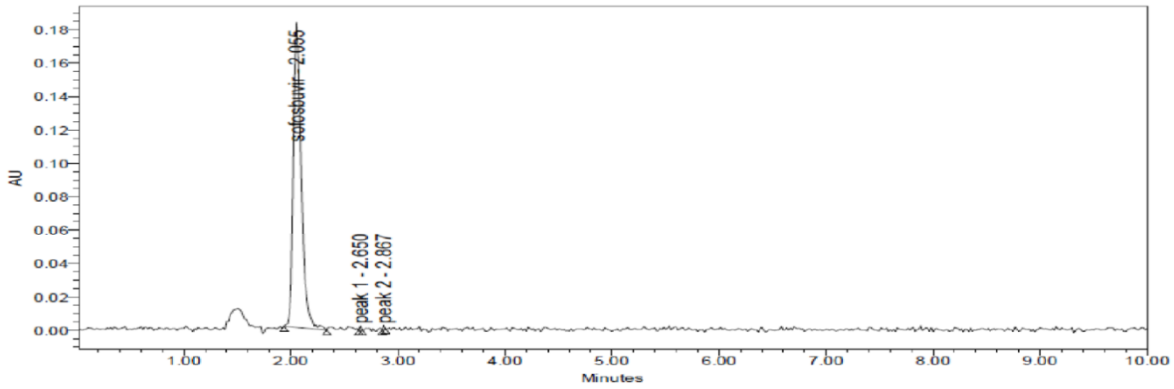

Fig.05: Chromatogram of Acid degradation

Table 15: Results of acid degradation studies

Sample	RT	Purity angle	Purity threshold	USP Plate count
Sofosbuvir	2.055	2.314	2.515	3681
peak 1	2.650	N/A	N/A	101124
peak 2	2.867	N/A	N/A	118335

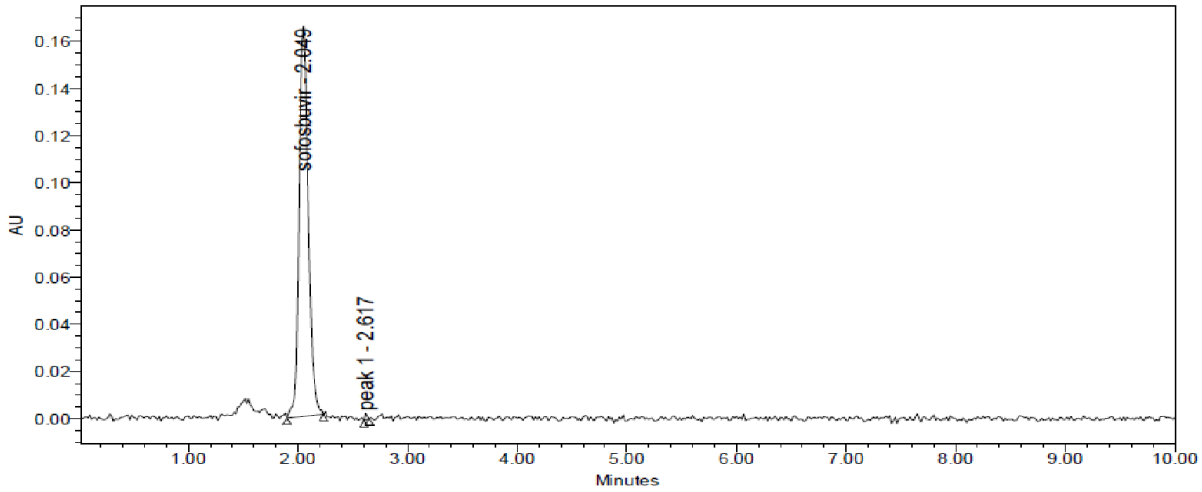

Fig.06: Chromatogram of base degradation

Table 16: Results of base degradation

Sample	RT	Purity of angle	Purity of threshold	USP Plate count
Sofosbuvir	2.049	2.126	2.316	2930
peak 1	2.617	N/A	N/A	56114

EFFECT OF OXIDATIVE DEGRADATION

In oxidative stress conditions sample solutions were added to the hydrogen peroxide solution (20 %H_2O_2 for 30 minutes at 60 ^{0}C) and it was found that sample get degraded with the degradation product retention time of Sofosbuvir 2.058 minutes (Fig.07). The results of oxidative degradation were shown in Table 17.

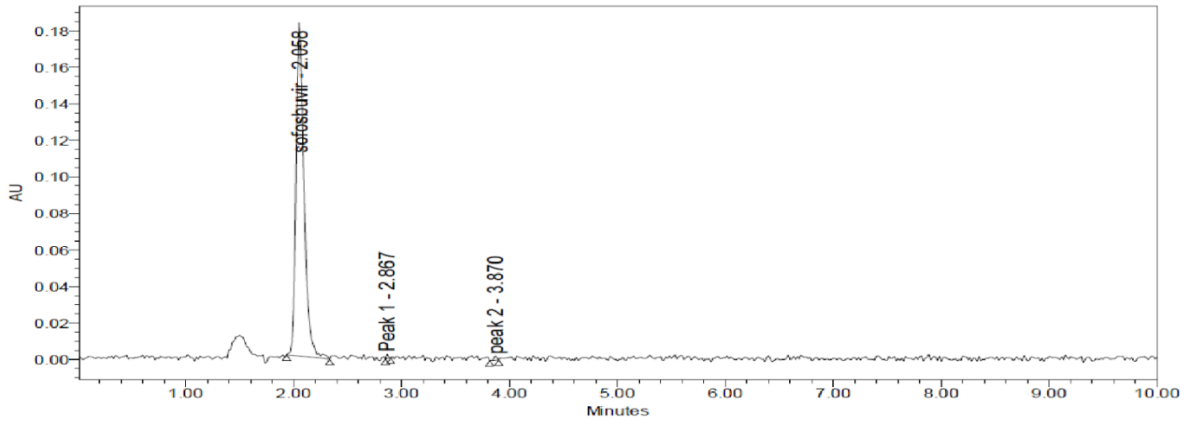

Fig.07: Chromatogram of oxidative degradation

Table 17: Results of oxidative degradation studies

Sample	RT	Purity of angle	Purity of threshold	USP Plate count
Sofosbuvir	2.058	2.214	2.541	3681
peak 1	2.867	N/A	N/A	100827
peak 2	3.870	N/A	N/A	70341

EFFECT OF PHOTOLYTIC AND DRY HEAT DEGRADATION

When samples were subjected to photo and thermal degradation, no degradation was found in both the samples which is indicated that both the samples were very stable in Photolytic conditions (Fig.09) and dry heat (Fig.10). The results of photolytic and dry heat degradation were shown in Table 19 and 20.

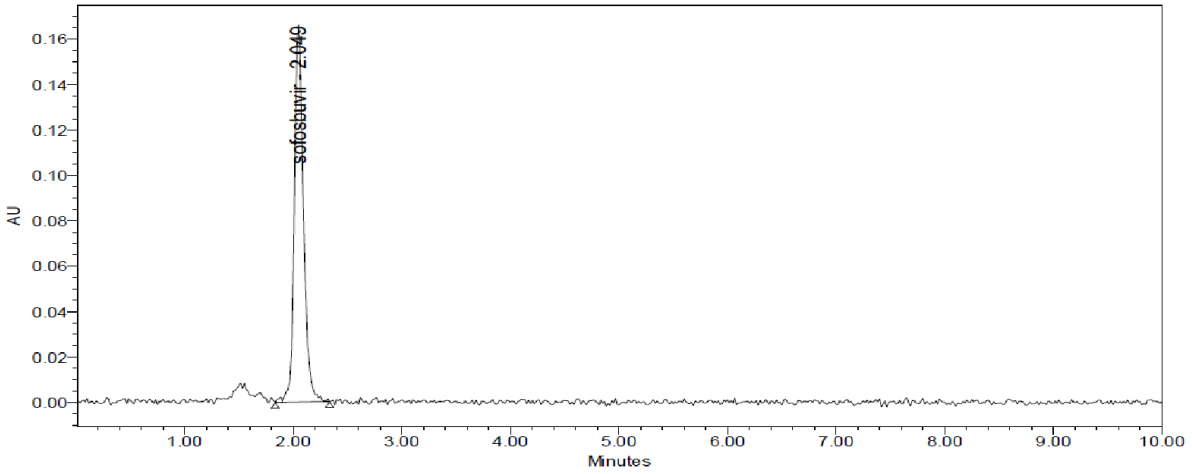

Fig.09: Chromatogram of photolytic degradation

Table 19: Results of photolytic degradation studies

Sample	RT	Purity of angle	Purity of threshold	USP Plate count
Sofosbuvir	2.049	2.571	2.824	2897

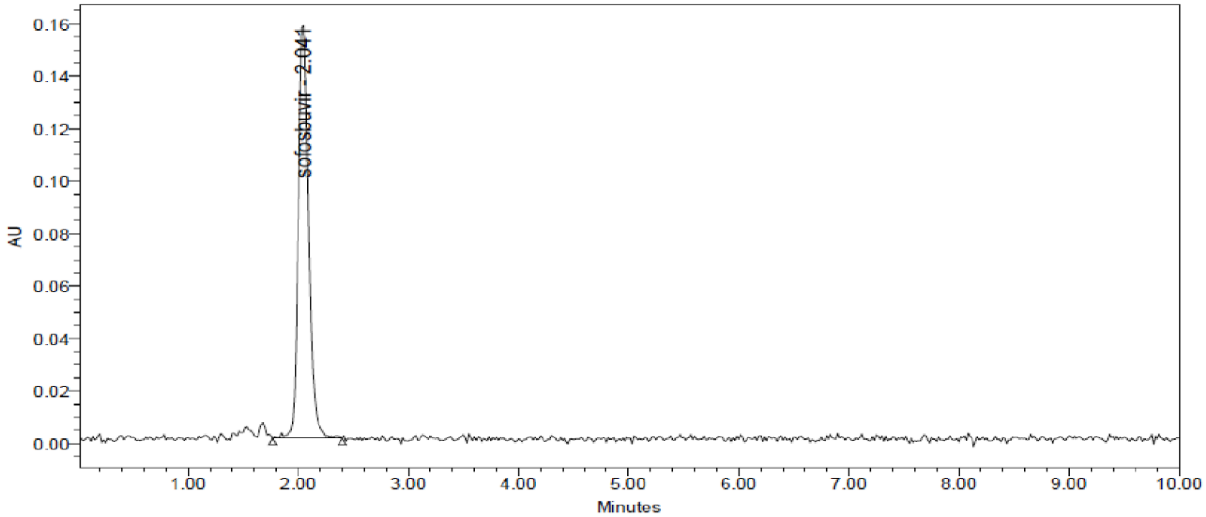

Fig.08: Chromatogram of dry heat degradation

Table .18: Results of dry heat studies

Sample	RT	Purity of angle	Purity of threshold	USP Plate count
Sofosbuvir	2.041	2.793	3.190	2240

CONCLUSION

In the present study, a stability indicating RP-HPLC method was developed and successfully validated according to ICH guidelines for the estimation of SOF. The method was validated for various parameters like specificity, linearity, accuracy, precision, robustness, LOD and LOQ. The forced degradation studies were carried out and degradation peaks were separated with developed stability indicating RP-HPLC method. The validated method was applied for the assay of commercial tablets of SOF in formulation. All the results obtained of various parameters were found to be within the acceptance limits. Thus the developed method in the present work is simple, sensitive, accurate, rapid, precise and robust. Hence the above method can be successfully applied for estimation of SOF in both bulk and tablet dosage form.

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