Original Article

Forced Degradation Study of Sofosbuvir: Identification of Degradation Products by LC-ESI-MS

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ABSTRACT

Introduction: In order to establish stability indicating techniques to comprehend the degradation behavior of drug under stress environments and to establish degradation pathway for drug substance, forced degradation is a potential tool employed more frequently in pharmaceutical developments. The sole need of present research was to establish stability and forced degradation pathway profiling of sofosbuvir. **Methods:** The stability of sofosbuvir has been validated by RP-HPLC method, where mixture of **Methanol:** Water with 0.1 % formic acid (50:50 % v/v) was used as mobile phase at 1.0 mL/min in gradient method. Different stress conditions were employed as per ICH guidelines. **Results:** Total three degradation products (DPs) were observed. In acidic and alkaline conditions hydrolysis product was found to be DP I at a R_t 4.2, with *m/z* 488 and DP II at a R_t 3.6, with *m/z*, 393.3 respectively. In oxidative state with H_2O_2 as oxidizing agent, DP III at RT 3.2, with *m/z* at 393. **Discussion and Conclusion:** Sofosbuvir showed degradation in acidic, basic and oxidative medium. No degradation was observed in thermal, UV light and neutral conditions. 23% degradation was seen in acidic medium and DP I with *m/z* value of 487 could be due to removal of $C(CH_3)_2$ from methoxy end of molecular structure. 50% degradation was observed in alkaline conditions and DP II with *m/z* value of 488 was seen in ESI source in MS. Oxidative degraded products could be formed due to formation of amine oxide which may be formed from tertiary amines by hydrogen peroxide.

Key words: Sofosbuvir, Degradation, HPLC, LC-ESI-MS, Pathway.

INTRODUCTION

Forced degradation comprises of degradation of a novel drug or its product at severe environments than accelerated environments. Forced degradation gives information for the documentation of possible degradants, degradation paths, and characteristic stability of the drug. It also apprises any possible polymorphic or enantiomeric elements and the dissimilarity between drug associated degradation and excipients. A New Drug Application (NDA) registration entails data of forced degradation readings as degradation reaction kinetics, forced degradation products (DPs), mass balance, drug peak purity, structure , etc. Hence, forced degradation is an important work for an NDA recording document.[1]

Sofosbuvir (SOFOS) is developed by Gilead Pharmaceuticals and marketed under the trade name Sovaldi. It is an anti-viral agent acting directly against HCV. Chemically, it is a mono-phosphorylated pyrimidine nucleotide prodrug (Figure 1). Sofosbuvir is approved by the U.S. Food and Drug Administration (FDA) on 2013 for use in patients for treatment of HCV either alone or in combination with ribavirin and ledipasvir. In a joint recommendation revealed in 2016, the authorities recommend Sofosbuvir as first line medical aid together with supplementary antivirals for all six genotypes of Hepatitis C.[2] Sofosbuvir, a nucleotide analog, a highly potent NS5B polymerase inhibitor of the Hepatitis C virus

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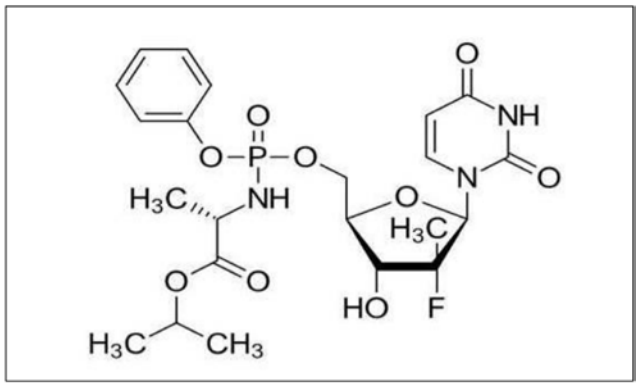

Figure 1: Structure of sofosbuvir.

(HCV), recently approved by U.S FDA and vended under the brand Sovaldi.[3] Amid all the others, it is a treatment used for the management of hepatitis C.[4] It is also used with other combinations and for chronic treatments depending on the type of hepatitis C virus involved, but a few combinations of sofosbuvir give better results in the treatment of HCV for example combinations with some other antiviral drugs like ribavirin, peginterferon-alfa, simeprevir, ledipasvir, or daclatasvir.[5,6] The therapy rates estimated are 30 to 97% conditional on the type of hepatitis C virus. But safety of pregnant women is unclear; while, some of it may also give harmness to the baby or foetus and it is recommended to avoid the treatment of sofosbuvir/ribavarin for pregnant females and their man sexual spouses. Literature review reveals that very less work has been reported for the methods of analysis of SOFOS. There are limited reports as per Literature viz HPLC[7] and UV/ HPLC,[8] UHPLC,[9] UPLC-MS/MS,[8,9] and LC-MS/MS[10] for sofosbuvir. A comprehensive study using the LC and LC-MS for degradation behaviour of SOFOS under various ICH recommended stress conditions has not been reported. So, the work has been performed to develop a selective and validated stability indicating HPLC method and forced decomposition studies according to the ICH recommendations. An integral aim of the study was to identify degradation products and to postulate complete degradation pathway of the drug. The ICH guideline authorized Stability testing of New Drug Substances and Products to perform test that requires testing the stabiltity of active substances, the proposed method was validated as per as ICH Guidelines and its updated international convention.[11]

MATERIALS AND METHODS

Chemicals and reagents

Dr. Reddy's Ltd. (Hyderabad, Andhra Pradesh, India), kindly supplied pure drug sample of sofosbuvir as a gift sample. All Chemicals and reagents viz , methanol, acetonitrile, Sodium hydroxide (NaOH), Hydrochloric acid (HCl), Hydrogen peroxide (H_2O_2) used were of analytical grade and purchased from Merck chemicals, Mumbai, Maharashtra, India.

Instruments

Model Agilent LC-1260 was used for the HPLC studies. The detector consisted of Photo diode array (PDA) (Agilent model no. G1315D 1260 DAD VL with 220 VA). Agilent 5 TC-C_{18} column (150 mm: 4.6 mm id, 5 µm) was used to perform the separation. LC Open Lab Software solution was used to record the chromatogram. The filtration of mobile phase was done through the Millipore glass filter assembly attached with a vacuum pump. Nexera 2 LC system (Shimadzu Corporation, Kyoto, Japan) connected to a triple quadrupole mass spectrometer (LC-MS 8040; Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source was employed for MS analysis. The mass spectra of the degradation products were taken in ESI (Turbo Spray) positive ion mode and analyzed in the triple quadrupole analyser. The columns used were SUPELCOSIL LC-18-S HPLC analytical column (5-µm particle size, 25-cm length × 4.6-mm inner diameter; Sigma-Aldrich). The MS data was integrating using analyst software. The effluent of the column passed into the mass spectrometer through a flow splitter which splits volume of mobile phase and deliver minimum amount of mobile phase in MS. Hydrolytic degradation products (DPs) were generated with the use of Carousel six stage reaction station. High precision hot air oven capable of controlling the temperature within ± 2°C was used to obtain thermal degradation products. The samples for photodegradation study were obtained by keeping samples in a photostability chamber. A pH meter (Equiptronics, Mumbai, India) was used to check and adjust the pH of the buffer solution. Also, sonicator (Citizon, Mumbai, India) and precision analytical balance (Shimadzu Aux 220) were used in the present studies.

Experimental

Selection of analytical wavelength

A stock solution of sofosbuvir (1000 µg/mL) was prepared in methanol and UV spectrum of 10 µg/mL solution of sofosbuvir was taken, it showed maximum absorbance at 261nm (Figure 2).

Selection of solvent

By equal proportion of methanol and water with 0.1% formic acid it was found that there was improvement in resolution. Hence, the mobile phase optimised was methanol and water with 0.1% formic acid (50:50 v/v)

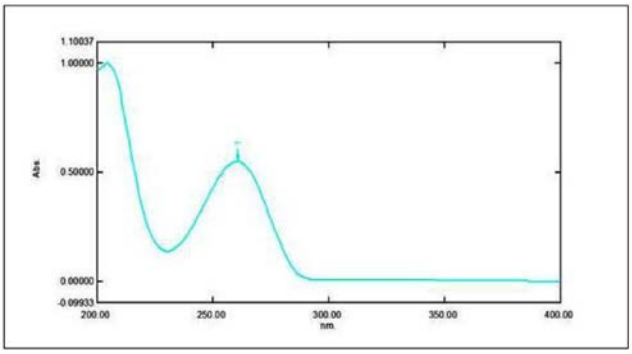

Figure 2: UV Spectrum of sofosbuvir.

with 1 ml/min of flow rate that gave acceptable retention time (tR), theoretical plates and good resolution of the sofosbuvir and degradation products.

Method validation

As per ICH guidelines,[12-14] the proposed method was validated for the stability indicating assay and to establish the stability of Sofosbuvir with forced degradation in different conditions by RP-HPLC method. The developed method was validated to convince the dependability of results of analysis for different limits, i.e. linearity, range, accuracy, precision, robustness, limit of quantification (LOQ), limit of detection (LOD), and specificity. To determine the linearity, the sequential dilutions (5-10 µg/mL) of Sofosbuvir in 50% methanol in triplicate were analysed. The recovery studies were performed to check the accuracy by spiking the standard solution into previously tested samples. 3 replicates of 3 different concentrations (05, 10, 15 µg/ml) of sofosbuvir were analyzed in a day and percentage RSD was calculated. For the inter day variation studies, 3 replicates of different concentrations were analyzed on 3 consecutive days and percentage RSD were calculated. The robustness of the method was planned by change of flowrate (0.9, 1, and 1.1 ml) and content of acetonitrile in organic phase variation by 1% variation. LOD and LOQ were checked by the parameter of signal-to-noise ratio. The specificity was established by stressing the drug sample in prescribed conditions. The stability of samples prepared were checked by scanning six repeats of 10 µg Sofosbuvir standard samples at different time breaks with fresh mobile phase.

Forced degradation studies

Sofosbuvir (100 mg) was weighed accurately and transferred into a 100 mL volumetric flask. Methanol (50 mL) was added and the flask was sonicated for 20 min, and then diluted up to the mark with methanol. An aliquot (5.0 mL) was further diluted to 100 mL with the same solvent. The final solution contained 50 µg/mL of sofosbuvir. In all degradation studies the average peak area of sofosbuvir after injection of 50 µg/mL solution in three replicates was obtained. Acid decomposition studies were carried out by refluxing the solution of sofosbuvir in 0.1 N HCl at 70°C for 6 hr. The studies under alkaline condition were carried out in 0.1 N NaOH and solution of sofosbuvir was refluxed for 10 h at 70°C. The resultant solutions were neutralized and diluted with methanol to obtain 50 µg/mL solutions and 20 µL was injected into the system. To study hydrogen peroxide (H_2O_2) induced degradation, the sofosbuvir solution was exposed to 3 % hydrogen peroxide at room temperature for 7 days and then heated for 10 min in boiling water bath to completely take away the excess of peroxide. The resultant solution was diluted to obtain 50 µg/mL solutions and 20 µL was injected into the system. The photochemical stability of the sofosbuvir was studied by the exposing the stock solution of sofosbuvir (1000 µg/mL) to direct sunlight for a period of 21 days, kept on terrace. The solution was diluted with methanol to obtain a solution 50 µg/mL and then 20 µL was injected into the system. The thermal stability of the sofosbuvir was studied by the exposing the stock solution of sofosbuvir (1000 µg/mL) to a temperature of 500C for a period of 21 days. The solution was diluted with methanol to obtain a solution 50 µg/mL and then 20 µL was injected into the system.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

A simple protocol was performed to determine sofosbuvir. For this determination, various water and methanol combinations were tested. At a final point, methanol: water with 0.1% formic acid in a proportion of 50:50 (v/v) was chosen; the mobile phase was sonicated and filtered. The analytical conditions were preliminarily checked using a C_{18} column and ESI in both positive and negative ionization modes. In general, an adequate ionization was achieved using water with 0.1% formic acid and methanol as mobile phases. A gradient mode was optimized in order to separate impurities or degradants. The selected analytical conditions produced a final backpressure lower than 120 bars.

Validation of the method

The performance of the HPLC was guaranteed by system suitability parameters. Six replicates of Sofosbuvir samples were injected, and column performance parameters like tailing factors, retention time, and number of theoretical plates were observed. Sofosbuvir has enhanced separation in the set of

conditions as the higher theoretical plates (2243) with less than one unit of % relative standard deviation (RSD) and as per ICH guidelines. The tailing factor was 0.68 with acceptable % RSD. The retention factor was 2.30, which indicates that sofosbuvir has optimum chance to bind with the stationary phase, causing good separation. The co-elution of degradants is studied by peak purity using a PDA detector. Therefore, all system suitability parameters were within the acceptance range. The linear regression equation was y = 47052x + 22548 with correlation coefficient R^2 0.9989 and a calibration graph was plotted for concentration versus area found in the chromatogram. The accuracy was studied for the developed method at all levels. Good recoveries of sofosbuvir in the range from 100.02 % to 102.07 % were obtained at various added concentrations, % RSD of 0.04, was the calculated value which met the accuracy criteria. The parameter of precision was studied as follows. The sample injections was measured for repeatability as the determined quantity of Sofosbuvir in six different replicated solutions, which was articulated as 99.89 % with % RSD of 0.008, and the intermediate precision was calculated as interday analysis and by two different analysts. Both the criteria showed values near to 100% (101.77% and 101.71%) with less than unit % RSD (1.044 and 1.055) at three levels of concentration. The robustness of the method was analysed by the varying the amount % Acetonitrile in the mobile phase composition and flow rate. The three variations in organic phase and flow rate were slightly changed at three levels (-1, 0, and +1) had no significant effect of change in parameters on the results. These were found to be robust and insignificant differences in peak areas and less variability in retention time were observed. The LOD and LOQ were determined by signal-to-noise ratio. The above experimental parameters confirmed that the developed method was specific for sofosbuvir.

Degradation Behavior

The rate of degradation in acid is considerable. The sofosbuvir was highly labile to acid degradation. After reaction in 0.1 N HCl at 70°C for 6 hr, one major degradation products was found which showed peak at 4.2 min as shown by the chromatogram in (Figure 3). Sofosbuvir degraded in alkaline medium. 50% degradation was observed by refluxing sofosbuvir solution with 0.1 M NaOH at 70°C for 10 hr forming degradation product at retention time 3.6 min in HPLC as shown by the chromatogram (Figure 4). More than 10% of degradation was observed on exposure to 3% hydrogen peroxide at room temperature for a period of 7 days, which indicated that sofosbuvir as unstable against oxidative stress. The chromatogram as shown in (Figure 5) had a single degradation product peak at 3.2 min. Sofosbuvir was found to be stable to photochemical degradation and thermal degradation as no degradation was seen after exposing sofosbuvir to 50°C temperature for 21 days and after exposing sofosbuvir to sunlight for 21 days. The chromatogram had no degradation product peaks.

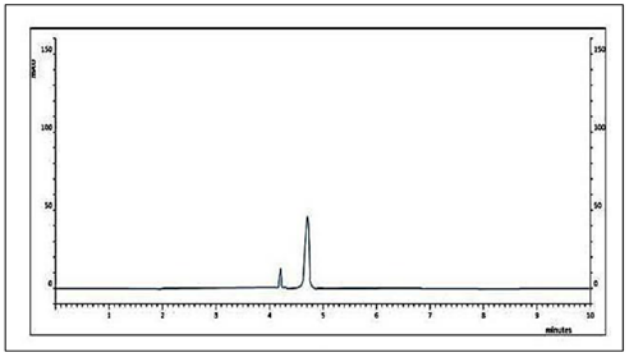

Figure 3: Chromatogram of sofosbuvir in acid induced degradation, Condition: 0.1N HCl at 70°C, 6 hrs.

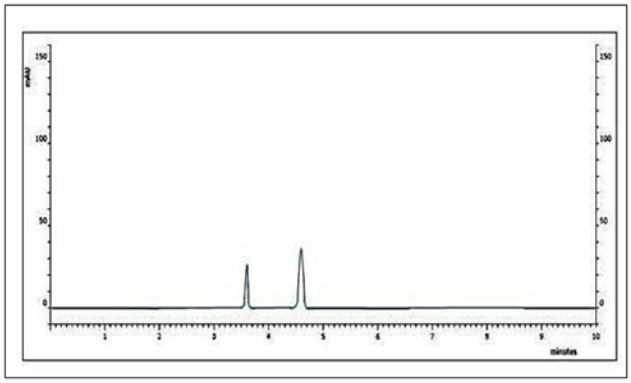

Figure 4: Chromatogram of sofosbuvir in base induced degradation, Condition: 0.1N NaOH at 70°C, 10 hrs.

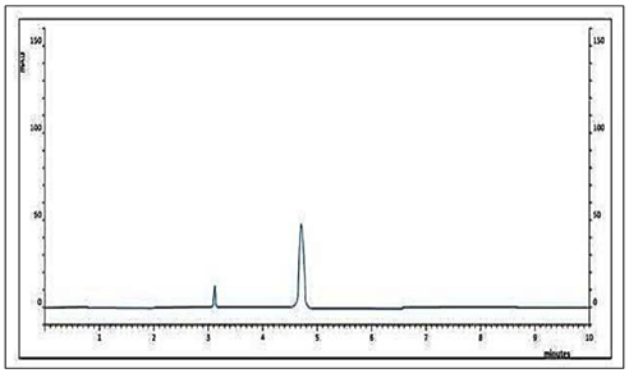

Figure 5: Chromatogram of sofosbuvir in hydrogen peroxide induced degradation, Condition: 3% H_2O_2 at 70°C, 7 days.

The mass spectra of sofosbuvir and its degradation product were taken in ESI (Turbo Ion Spray) positive mode in mass range of 100 - 600 amu and analyzed in the triple quadrupole analyzer. Mass spectra of standard sofosbuvir solution showed protonated ions at *m/z* 530.48 amu (Figure 6). The major degradation product showed peaks at *m/z* 488 amu for DP I in acidic medium (Figure 7). A single peak for one major degradation product DP II showed peaks at *m/z* 393.3 amu in alkaline medium (Figure 8). The major degradation product showed peaks at *m/z* 393 amu for DP III in oxidative state (Figure 9). The proposed fragmentation pathway for the hydrolysis (acidic and basic) and oxidative degradation products of Sofosbuvir is depicted in (Figure 10 and Figure 11) respectively. The chromatographic and spectral data of degradation products of sofosbuvir is summarized in (Table 1). An attempt has made to characterize major degradation products using LC-MS and to identify possible degradation pathway. Mass values for acid and base hydrolysis product was found to be DP I, RT 4.2, *m/z* 488 and DP II, RT 3.6, *m/z* at 393.3 respectively. From mass data, the major pathways for acid degradation products were proposed. As shown in the proposed pathway and the mass values obtained, it is assumed that the loss of - 42 amu and *m/z* value of 487 could be due to the removal of C $(CH_3)_2$ from the methoxy end of

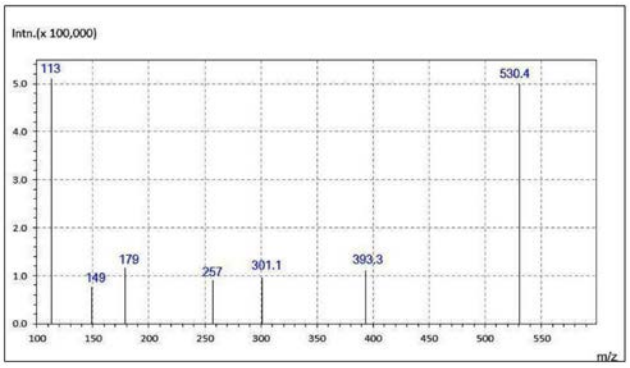

Figure 6: The positive ion mass spectra of sofosbuvir by ESI scanning *m/z* 100 to 600 amu (*m/z* 530.48 amu).

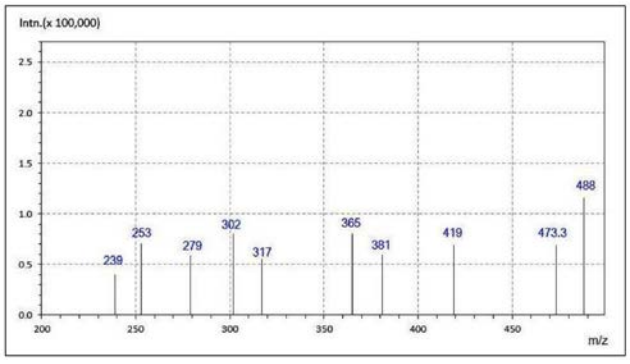

Figure 7: The positive ion mass spectra of sofosbuvir in acid induced degradation by ESI scanning *m/z* 200 to 500 amu (DP I: 4.2 min; *m/z* 488 amu).

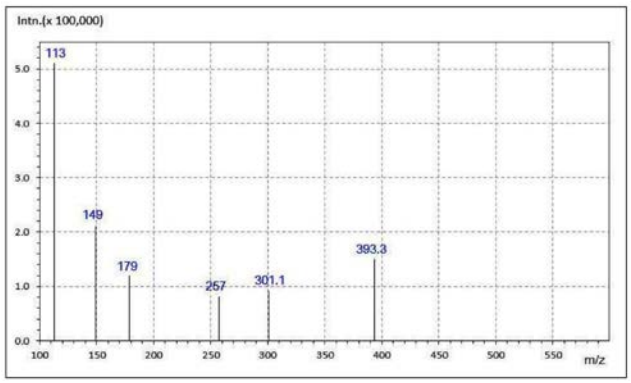

Figure 8: The positive ion mass spectra of sofosbuvir in base induced degradation by ESI scanning *m/z* 100 to 600 amu (DP II:3.6 min; *m/z* 393.3 amu).

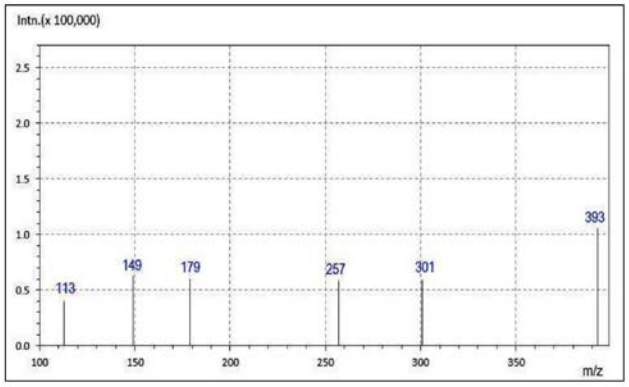

Figure 9: The positive ion mass spectra of sofosbuvir in H_2O_2 induced degradation by ESI scanning *m/z* 100 to 600 amu (DP III: 3.2 min; *m/z* 393 amu).

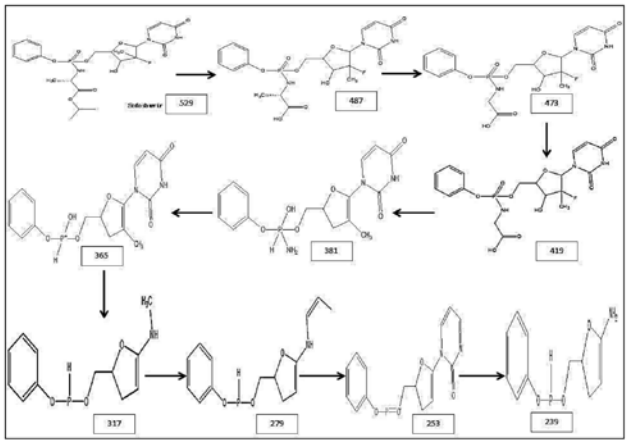

Figure 10: Possible degradation pathway and structure of acid and base degradation products.

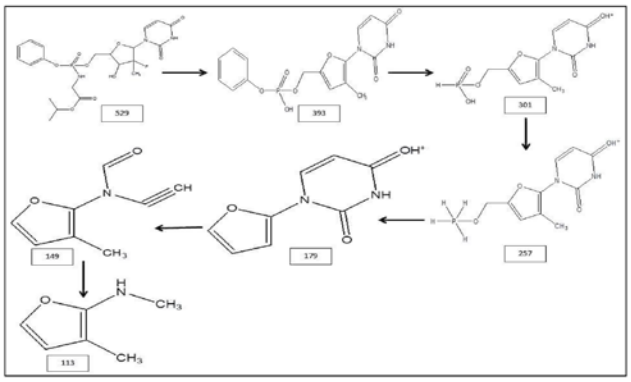

Figure 11: Possible degradation pathway and structure of oxidative degradation products.

Table 1: Chromatographic and spectral data of degradation products of sofosbuvir.

Peak	tR	Measured *m/z*	Calculated *m/z*	Error (ppm)	% Area Degradation	% Area Remaining Drug
Control	4.7	530.48	529.45	- 1.03	0.3	99.7
Acid 1	4.2	488	487.01	- 0.99	23	77
Base 1	3.6	393.3	391.9	- 1.4	50	50
Peroxide 1	3.2	393	391.82	- 1.18	19.02	80.98
Thermal	4.6	-	-	-	0.19	99.81
Photolytic	4.6	-	-	-	0.2	99.8

the molecular structure, loss of -14 amu and *m/z* value of 473 is due to loss of $–CH_3$ group from the same end. -36 amu and *m/z* value of 419 is due to the simultaneous loss of OH and F groups 4-methyloxolano-2-yl part of the structure. - 38 amu and *m/z* value of 381 could be due to the loss of CH, CH, O and H moieties. - 16 amu and *m/z* value of 365 is due to the loss of NH_2 from the phospharyl end. Simultaneous removal of susceptible groups like O, CH_3, H, OH attached to phosphorus lead to - 49 amu and *m/z* value of 317. In the last two steps as depicted in the pathway are due to -26 amu and *m/z* value of 279 from loss of CH_3 and CH and the *m/z* peak of 239 is observed due to the loss of CH_3.

Mass value for oxidative hydrolysis product with H_2O_2 was found to be DP III, RT 3.2, *m/z* at 393. From mass data, the major pathway for oxidative degradation products was proposed. The oxidative degraded products could be formed due to the formation of Amine oxide which may be formed from tertiary amines by hydrogen peroxide.

CONCLUSION

The presence of degradation compounds or impurities may affect the efficacy and safety of pharmaceuticals. The quality of pharmaceutical products is of importance for patient's well-being. In this work, the forced degradation study on sofosbuvir has been carried out as per ICH proposed guidelines as to study its degradation nature and to interpret the degradation pathway mechanism. A comprehensive stress testing of sofosbuvir, a newly approved antiviral drug was carried out according to ICH guideline Q1A (R^2). Stress degradation by hydrolysis under acidic condition was done by using 0.1N HCl, degradation after 6 hrs, was found to be 23 %. Stress degradation by hydrolysis under alkaline condition was done by using 0.1N NaOH, degradation after 10 hrs, was found to be 50 %. Oxidative degradation was done by using 3% hydrogen peroxide and degradation after 7 days, was found to be 19.02 %. No thermal degradation at 50°C temperature and after 21 days. No photolytic degradation was observed after 21 days.

The proposed method for stability study shows that there is appreciable degradation found in the applied stress conditions for Sofosbuvir. Higher degradation was found in alkali stress condition for the drug as compared to other stress conditions. The suggested method can be used for routine analysis of sofosbuvir in quality control laboratories.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

RP-HPLC: high performance liquid chromatography method; **LC-ESI-MS:** liquid chromatographic–electrospray ionization mass spectrometric method; **LPR:** Ledipasvir; **tR:**retention time; **HCV:** hepatitis C virus; **DAA:** directly acting antivirals; **ICH:** International Conference on Harmonization.

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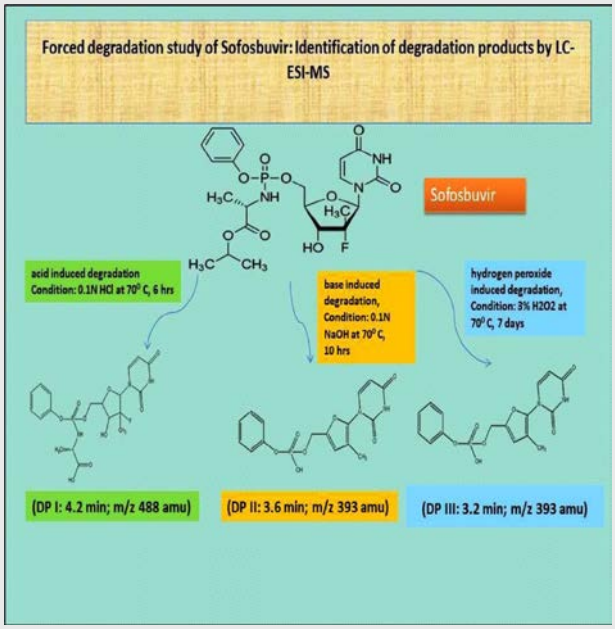
PICTORIAL ABSTRACT



SUMMARY

The stability indicating RP-HPLC method was developed and validated for the accurate and precise determination of the sofosbuvir. The forced degradation studies were also performed to demonstrate the stability indicating nature of the developed method as per the ICH guidelines. The results of the forced degradation studies identified the stressed conditions in which drug is unstable. Further, LC-ESI-MS was used for the identification and characterization of the degradants. The possible structures of the degradants were proposed and the possible degradation pathways were sketched out. Therefore, the current method can be successfully applied for the determination of the drug in bulk and dosage forms. As well as it will find the applications in routine analysis, quality control and research and development.

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