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Research Article

# STABILITY-INDICATING REVERSE-PHASE HIGH- PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD DEVELOPMENT FOR SIMULTANEOUS ESTIMATION OF SOFOSBUVIR AND DACLATASVIR IN PURE AND PHARMACEUTICAL FORMULATION

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**ABSTRACT**

**Objective:** The objective of the study was to develop a novel, simple, sensitive, accurate, precise, and stability-indicating reverse-phase (RP) liquid chromatographic method for simultaneous estimation of sofosbuvir and daclatasvir in pure and pharmaceutical formulation.

**Methods:** In the present work, chromatographic separation was done using stationary-phase discovery column (250 mm×4.6 mm; 5 µm particle size) and mobile phase consisting of 0.1N potassium dihydrogen orthophosphate buffer:acetonitrile (55:45, v/v), with the flow rate of 1 ml/min, and the detection of column effluents was achieved with photodiode array at 260 nm.

**Results:** The retention time of sofosbuvir and daclatasvir was found to be 2.32 min and 3.06 min, respectively. Stability-indicating studies were conducted according to the guidelines of International Conference on Harmonization (ICH) Q1A R2, and validation of the method was done as per the ICH guidelines Q2R1. The linearity of the method was in the concentration range of 100–600 µg/ml and 15–90 µg/ml for sofosbuvir and daclatasvir, respectively. The correlation coefficients were found to be 0.9996 and 0.9996 for sofosbuvir and daclatasvir, respectively. The limit of detection and limit of quantification were found to be 0.19 µg/ml and 0.59 µg/ml for sofosbuvir and 0.02 µg/ml and 0.05 µg/ml for daclatasvir, respectively.

**Conclusion:** The stability-indicating RP high-performance liquid chromatographic (RP-HPLC) method was novel, simple, precise, accurate, and sensitive for simultaneous estimation of sofosbuvir and daclatasvir in pure and pharmaceutical formulations. Hence, the developed method was adopted for qualitative and quantitative analysis of sofosbuvir and daclatasvir in pure and pharmaceutical formulations.

**Keywords:** Sofosbuvir, Daclatasvir, Reverse-phase high-performance liquid chromatographic, Stability indicating, Validation, International Conference on Harmonization.

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## INTRODUCTION

Hepatitis is a kind of liver disease, and various forms of liver diseases are hepatitis A virus, hepatitis B virus, hepatitis C virus (HCV), hepatitis D virus, hepatitis E virus, and liver cirrhosis [1,2]. Sofosbuvir (GS-7977/PSI-7977) is a nucleotide HCV NS5B polymerase inhibitor. It is a prodrug of 2-deoxy-2-fluoro-2-C-methyluridine, which is converted to its active form within hepatocytes. Oral sofosbuvir was well tolerated and demonstrated significant advance in the treatment of patients with chronic hepatitis C genotypes (GT) 2–3 infection. Sofosbuvir chemically called as (S)-isopropyl-2-{[(S)-{[(2R,3R,4R,5R)-5-{2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl) methoxy–(phenoxy) phosphoryl amino) propanoate [3-5]. Daclatasvir is the first HCV NS5A replication complex inhibitor and has potent antiviral effect and clinical efficacy across multiple HCV GT 1–5. Daclatasvir was administered as a part of triple therapy in combination with other directly acting antiviral drugs to increase antiviral activity and to decrease the drug resistance. Daclatasvir is chemically called as methyl N-[(2S)-1-[(2S)-2-[5-[4-[4-[2-[(2S)-1-[(2S)-1-(2S)-2-(methoxy carbonyl amino)-3-methyl butanoyl] pyrrolidin-2-yl]-1H-imidazol-4-yl]-4-biphenylyl]-1H-imidazol-2-yl] pyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl]carbamate. Daclatasvir and Sofosbuvir combination had shown 90% SVR (Sustained virological response) rates for 12 weeks in patients without cirrhosis [6-9].

Literature survey revealed that spectrophotometric methods [10-16], spectrofluorimetry [17], high-performance liquid chromatographic (HPLC) methods [18-25], ultra-performance liquid chromatography (UPLC) methods [26,27], and UPLC-tandem mass spectrometer [28] have been reported for simultaneous estimation of sofosbuvir and daclatasvir alone or in combination with other drugs. From literature, it was confirmed that two HPLC methods were reported for simultaneous estimation of sofosbuvir and daclatasvir, but no forced degradation studies were performed in one method [24]. Another method, i.e., separation of sofosbuvir and daclatasvir, was very slow [25]. Hence, an attempt was made to develop a simple, rapid, precise, accurate stability-indicating reverse-phase HPLC (RP-HPLC) method for simultaneous estimation of sofosbuvir and daclatasvir in pure and pharmaceutical formulation. The method was validated as per the International Conference on Harmonization (ICH) guidelines [29], while forced degradation studies were conducted according to the ICH guidelines [Figs. 1 and 2] [30].

## METHODS

### Selection of wavelength

Sofosbuvir and daclatasvir solutions in diluent were scanned in the range of 200–400 nm, individually to scan ultraviolet (UV) spectrum. The overlain UV spectra of sofosbuvir and daclatasvir had shown 260 nm as a suitable wavelength for performing current RP-HPLC method, and both drugs show appropriate absorption at this wavelength.

### Chromatographic conditions

The chromatographic separation of sofosbuvir and daclatasvir was achieved with stationary-phase discovery column (250 mm×4.6 mm,

5 µ) and mobile phase consisting of 0.1 N potassium dihydrogen orthophosphate buffer and acetonitrile (55:45, v/v). The flow rate was 1 ml/min. Mobile phase was degassed using an ultrasonic water bath for 10 min, and then, vacuum was filtered through a 0.45 µ membrane filter. The column effluents were detected at a wavelength of 260 nm using photodiode array (PDA) as a detector.

**Preparation of 0.1N potassium dihydrogen orthophosphate buffer (pH-4.8)**

1.36 g of potassium dihydrogen orthophosphate was accurately weighed and dissolved in 900 ml of water, 1 ml of triethylamine was added, and then, pH was adjusted to 4.8 with dilute orthophosphoric acid solution. Finally, buffer was degassed using an ultrasonic water bath for 10 min and filtered through the 0.45 µ membrane filter.

**Preparation of standard solution**

40 mg of sofosbuvir and 6 mg of daclatasvir standard were accurately weighed and transferred into 10 ml calibrated volumetric flasks. 7 ml of diluent was added and sonicated to remove dissolved gases for 5 min, and final volume was made up to the mark with diluent and working standard solutions were also prepared. 1 ml from above two stock solutions was pipetted out in 10 ml volumetric flasks, and volume was made up to mark with diluent.

**Sample preparation**

10 tablets were weighed and powdered, and the average weight of total tablets was calculated. Powder equivalent to sofosbuvir 400 mg and daclatasvir 60 mg was transferred into 100 ml volumetric flask, 50 ml diluent was added and sonicated to dissolve gases for 25 min and filtered using 0.22 µ membrane filters to get clear solution, and final volume was made up with diluent. After suitable dilution, above stock solution of the sample was used for routine analysis.

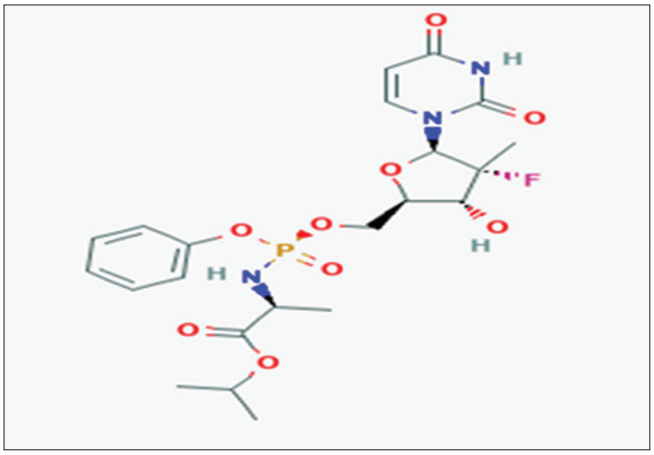

**Fig. 1: Structure of sofosbuvir [5]**

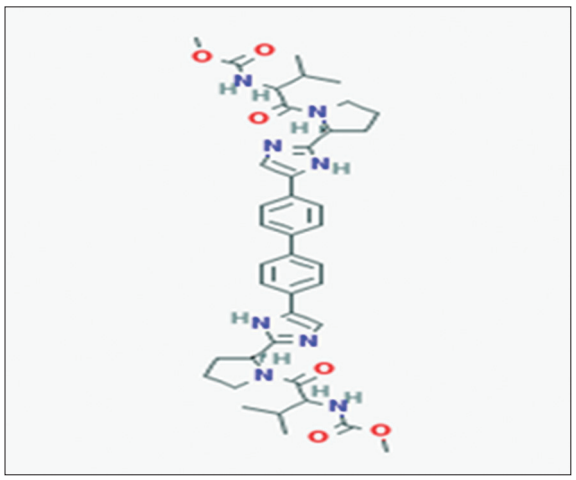

**Fig. 2: Structure of daclatasvir [8]**

**Method validation**

The method was validated as per the ICH guidelines for validation of analytical procedures. Validation parameters include system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, robustness, and specificity.

**System suitability**

System suitability tests were performed to ensure that system is working properly. System suitability parameters, including tailing factor, plate count, retention time, and resolution, were analyzed using the proposed HPLC method. System suitability was evaluated by injecting six replicates of standard mixture solution.

**Linearity**

Linearity of the proposed method was evaluated by constructing calibration curve between concentrations against corresponding peak area using the least square method. The slope and correlation coefficient ($r^2$) values were calculated for both sofosbuvir and daclatasvir.

**Precision**

Precision of the developed method was estimated by six replicate injections of sample solution at working concentration, on the same day (intra-day precision) and on the 2nd day (inter-day precision) to ensure repeatability of the analytical method.

**Accuracy (recovery)**

Accuracy of the developed method was estimated by spiking the analyte sample solution with standard solution at 50%, 100%, and 150% levels. Percentage recovery and percent relative standard deviation (% RSD) were calculated to determine the accuracy.

**Specificity**

Specificity of the proposed method was carried out by comparing chromatograms of blank, standard solution, and sample solution at working concentration. The chromatograms reveal no peak at retention time of sofosbuvir and daclatasvir, and there was a good correlation between standard and sample. It was observed no interference of excipients in blank with drug. Hence, it can be concluded that the proposed method is said to be specific.

**LOD and LOQ**

LOD and LOQ were estimated based on the standard deviation of the response and slope of calibration curve of sofosbuvir and daclatasvir. The following formula is used to calculate LOD and LOQ.

- LOD=3.3σ/s
- LOQ=10σ/s
- σ is standard deviation of response
- s is slope of calibration curve.

**Robustness**

Robustness of the developed was estimated by subjecting minor changes in chromatographic conditions, such as flow rate change (±0.1 ml), temperature change (±5°C), and mobile-phase change (±10%). There was no marked change in the present developed method, which indicates reliability of the method. The data are given in Tables 1 and 2.

**Stability-indicating studies**

Stability-indicating studies were conducted at different stress conditions, to demonstrate intrinsic stability of drug substance and to aid in establishing recommended storage conditions. Stability-indicating studies include acid hydrolysis, base hydrolysis, peroxide hydrolysis, neutral hydrolysis, thermal degradation, and photodegradation.

**Acid hydrolysis**

1 ml of standard solution of sofosbuvir 400 µg/ml and daclatasvir 60 µg/ml and 1 ml of 1N HCl had been taken in 10 ml volumetric flask

and refluxed at 60°C for 30 min. The solution was neutralized with sufficient volume of 1N NaOH. The flask was diluted up to 10 ml with diluent. The resultant solution had been filtered using 0.22 µ syringe filter, and the respective chromatograms were recorded by injecting the solution into HPLC system.

**Base hydrolysis**

1 ml of standard solution of sofosbuvir 400 µg/ml and daclatasvir 60 µg/ml and 1 ml of 1N NaOH had been taken in 10 ml volumetric flask and refluxed at 60°C for 30 min. The solution was neutralized with sufficient volume of 1N HCl. The flask was diluted up to 10 ml with diluent. The resultant solution had been filtered using 0.22 µ syringe filters, and the respective chromatograms were recorded by injecting the solution into HPLC system.

**Peroxide hydrolysis**

1 ml of standard solution of sofosbuvir 400 µg/ml and daclatasvir 60 µg/ml and 1 ml of 20% $H_2O_2$ had been taken in 10 ml volumetric flask and refluxed at 60°C for 30 min. The solution was diluted up to 10 ml with diluent. The resultant solution had been filtered using 0.22 µ syringe filters, and the respective chromatograms were recorded by injecting the solution into HPLC system.

**Thermal degradation**

1 ml of standard solution of sofosbuvir 400 µg/ml and daclatasvir 60 µg/ml had been taken in 10 ml volumetric flask and placed in an oven at 105°C for 6 h. The flask was diluted up to 10 ml with diluent. The resultant solution had been filtered using 0.22 µ syringe filters, and the respective chromatograms were recorded by injecting the solution into HPLC system.

**Photodegradation**

1 ml of standard solution of sofosbuvir 400 µg/ml and daclatasvir 60 µg/ml had been taken in 10 ml volumetric flask and kept in UV chamber at 200 $Wh/m^2$. The resultant solution was diluted up to 10 ml with diluent and filtered using 0.22 µ syringe filters, and the respective chromatograms were recorded by injecting the solution into HPLC system.

**Neutral degradation**

1 ml of standard solution of sofosbuvir 400 µg/ml and daclatasvir 60 µg/ml and 1 ml of water had been taken in 10 ml volumetric flask and refluxed at 60°C for 30 min. The resultant solution was diluted up to 10 ml with diluent and filtered using 0.22 µ syringe filters, and the respective chromatograms were recorded by injecting the solution into HPLC system.

## RESULTS AND DISCUSSION

**Method development**

To develop an optimized method for the estimation of sofosbuvir and daclatasvir simultaneously, several trails were conducted. The development trails were conducted based on the chemical nature of sofosbuvir and daclatasvir. During optimization of the method, selection of column has been done based on reasonable retention time, number of theoretical plates, tailing factor, peak shape. Mobile phase was selected based on polarity of functional groups present in sofosbuvir and daclatasvir. By analyzing the above factors, chromatographic separation was done on discovery column (250 mm×4.6 mm, 5 µ particle size) using mobile phase of buffer:acetonitrile (65:35 v/v), flow rate of 1 ml/min was favorable for good separation and injection volume 10 µL, and the column eluents were detected with a PDA detector at 260 nm. Sofosbuvir and daclatasvir were eluted at retention time of 2.32 min

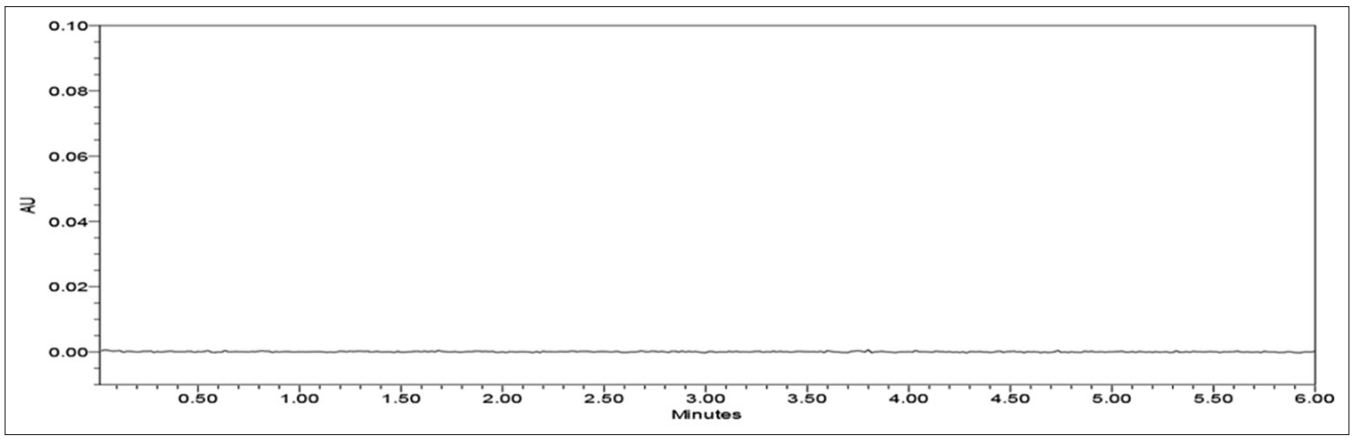


**Fig. 3: Chromatogram of blank**

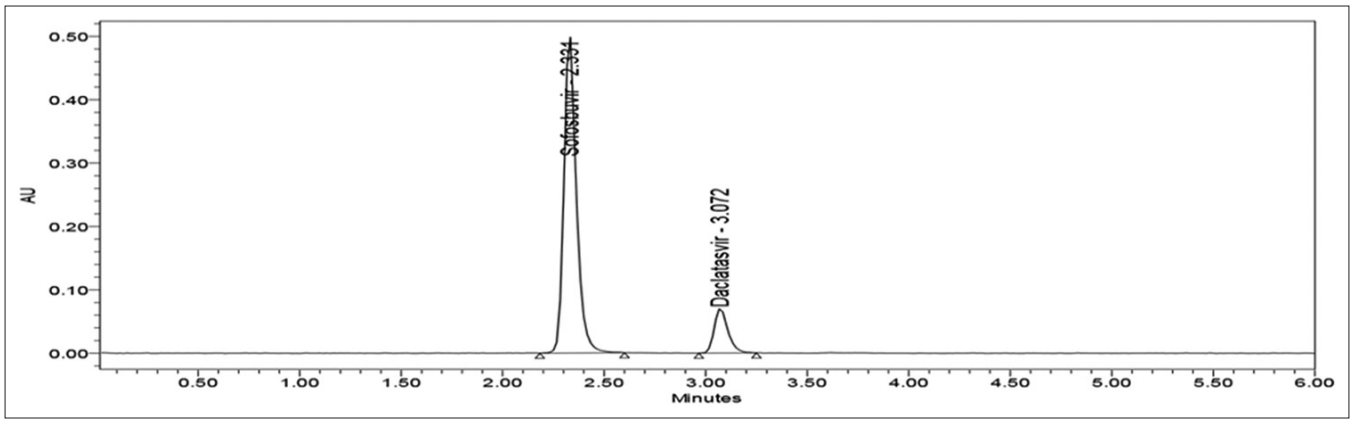


**Fig. 4: Chromatogram of standard sofosbuvir and daclatasvir**

and 3.06 min, respectively. The optimized chromatographic conditions are shown in Table 3, and the typical HPLC chromatograms of blank, standard, and sample are represented in Figs. 3-5.

**Table 1: Robustness of sofosbuvir (n=6)**

| Parameter | Peak area (mean±SD) | % RSD |
|---|---|---|
| Flow rate minus | 2449712±11206.6 | 0.5 |
| Flow rate plus | 2070125±20873.8 | 1.0 |
| Mobile phase minus | 2164500±8956.1 | 0.4 |
| Mobile phase plus | 2135946±28019.5 | 1.3 |
| Thermal minus | 2229699±16814.9 | 0.8 |
| Thermal plus | 2224090±3477.0 | 0.2 |

SD: Standard deviation, RSD: Relative standard deviation

**Method validation**

*System suitability*

Six replicate injections of sofosbuvir and daclatasvir standard solution were injected, and the chromatograms were recorded. It produced theoretical plate >2000, tailing factor <2, and % RSD <2, which ensure that the system is suitable for analysis of sofosbuvir and daclatasvir. The results of system suitability are summarized in Table 4.

*Linearity and calibration curve*

Linearity was estimated at six concentration levels ranging from 100 to 600 µg/ml for sofosbuvir and 15 to 90 µg/ml for daclatasvir in triplicates. The regression line equation was found to be y=5044.6x+2538.6 for sofosbuvir and y=5593.5x+222.6 for daclatasvir,

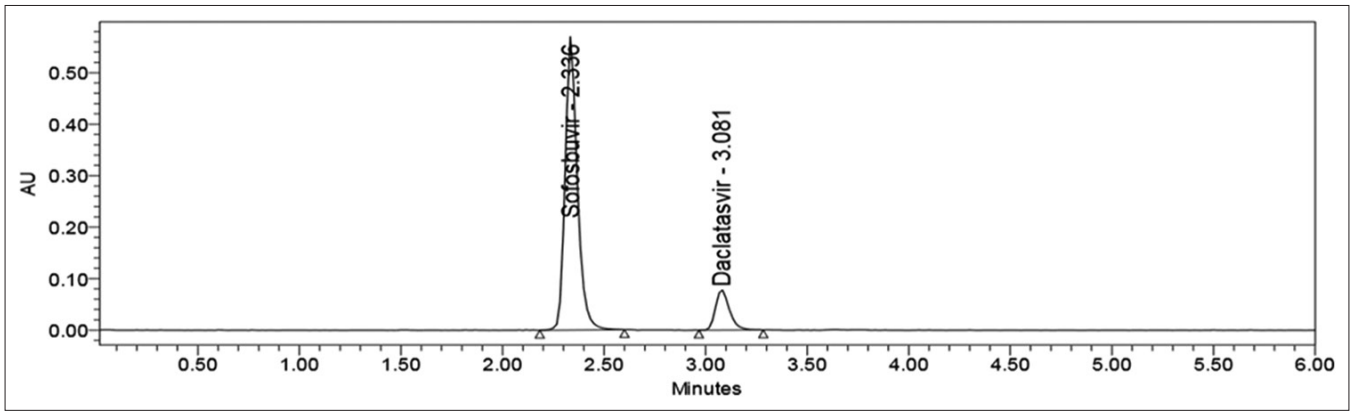


**Fig. 5: Chromatogram of sample sofosbuvir and daclatasvir**

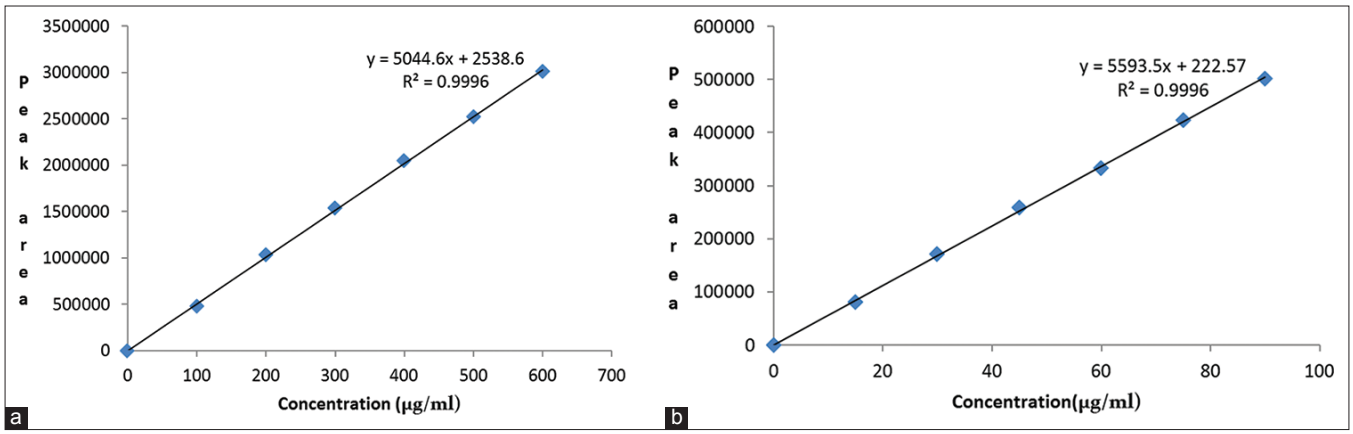


**Fig. 6: Standard calibration curve of (a) sofosbuvir (b) daclatasvir**

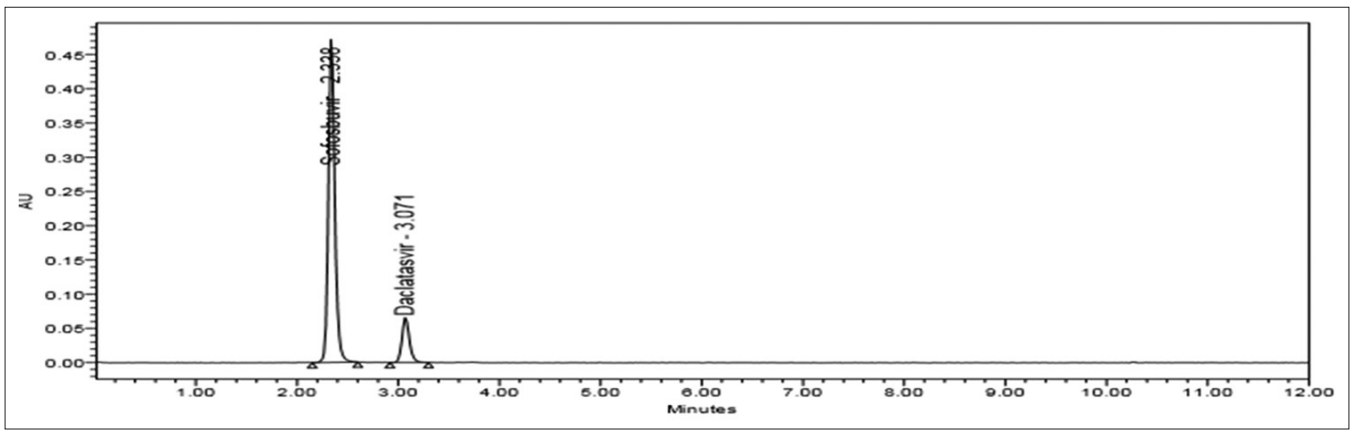


**Fig. 7: Chromatogram of acid degradation**

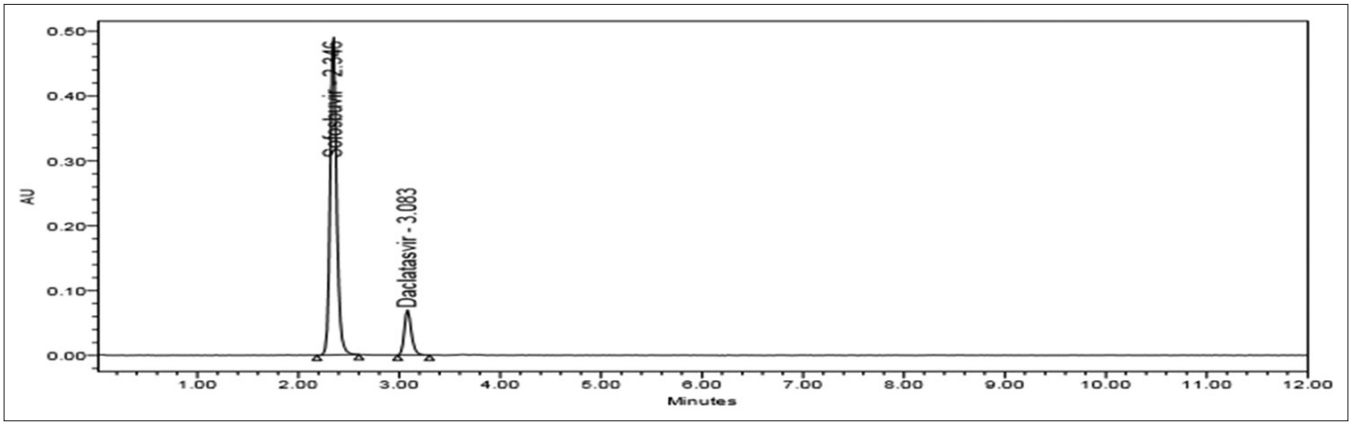


**Fig. 8: Chromatogram of base degradation**

**Table 2: Robustness of daclatasvir (n=6)**

| Parameter | Peak area (mean±SD) | % RSD |
|---|---|---|
| Flow rate minus | 372837±852.6 | 0.2 |
| Flow rate plus | 320314±3874.2 | 1.2 |
| Mobile phase minus | 330986±3123.4 | 0.9 |
| Mobile phase plus | 337958±1294.2 | 0.4 |
| Thermal minus | 346640±3621.7 | 1.0 |
| Thermal plus | 338941±1877.6 | 0.6 |

SD: Standard deviation, RSD: Relative standard deviation

**Table 3: Optimized chromatographic conditions for estimation of sofosbuvir and daclatasvir**

| Parameter | Condition |
|---|---|
| Mobile phase | Buffer:acetonitrile (55:45, v/v) |
| Diluent | Water:acetonitrile (50:50, v/v) |
| Column | Discovary (250 mm×4.6 mm, 5 µ paricle size) |
| Detector | PDA |
| Column temperature | 30°C |
| Detection wavelength | 260 nm |
| Injection volume | 10 µL |
| Flow rate | 1 ml/min |
| Run time | 6 min |

**Table 4: System suitability of developed method**

| Parameters | Sofosbuvir | Daclatasvir | Acceptance criteria |
|---|---|---|---|
| Retention time (min) | 2.343 | 3.079 | |
| Theoretical plates | 6939.8 | 9786.5 | >2000 |
| Tailing factor | 1.21 | 1.288 | <2 |
| Resolution | 6.1 | 6.1 | >2 |

which meet the acceptance criteria. The regression data for calibration curve had shown a good relationship over respected concentration range for sofosbuvir and daclatasvir. Therefore, the proposed method is found to be linear. The results are presented in Tables 5 and 6, and the data are shown in Fig. 6.

*Precision*

The sample solution containing sofosbuvir 400 µg/ml and daclatasvir 60 µg/ml was injected for six times on same day (intra-day precision) and after twenty four hours (inter-day precision) and mean area of six replicate injections was calculated. The % RSD of sofosbuvir and daclatasvir for both intra-day and inter-day precision was found to be <2. The results of the precision study are summarized in Tables 7 and 8.

**Table 5: Linearity and range of sofosbuvir (n=3)**

| S. No. | Concentration (µg/ml) | Peak area (mean±SD) |
|---|---|---|
| 1. | 100 | 477193±1921.5 |
| 2. | 200 | 102503±6489.2 |
| 3. | 300 | 1538714±3815.1 |
| 4. | 400 | 2043897±17370.1 |
| 5. | 500 | 2519156±18471.3 |
| 6. | 600 | 3007332±5788.6 |
| Slope | | 5044.6 |
| Y-Intercept | | 2538.6 |
| Correlation coefficient | | 0.9996 |

n is number of determination. SD: Standard deviation

**Table 6: Linearity and range of daclatasvir (n=3)**

| S. No. | Concentration (µg/ml) | Peak area (mean±SD) |
|---|---|---|
| 1. | 15 | 80708±181.2 |
| 2. | 30 | 169314±840.5 |
| 3. | 45 | 258395±1612.1 |
| 4. | 60 | 331700±793.9 |
| 5. | 75 | 421855±1782.5 |
| 6. | 90 | 501523.7±718.1 |
| Slope | | 5593.5 |
| Y-intercept | | 222.6 |
| Correlation coefficient | | 0.9996 |

n is number of determination. SD: Standard deviation

Acceptance criteria: %RSD should be <2.

*Accuracy*

The accuracy of the method was determined by calculating the mean percentage recovery of each component in the sample mixture at three distinct levels in triplicates. The % recovery of sofosbuvir was found to be in the range of 99.4–99.9% and for daclatasvir 98.8–100.6%. This indicates that recovery values are within acceptable limits, confirming the accuracy of the developed method.

Acceptance criteria: 98–102%

The results are tabulated in Tables 9 and 10.

*LOD and LOQ*

LOD and LOQ were found to be 0.19 and 0.59 µg/ml for sofosbuvir and 0.02 and 0.05 µg/ml for daclatasvir, respectively.

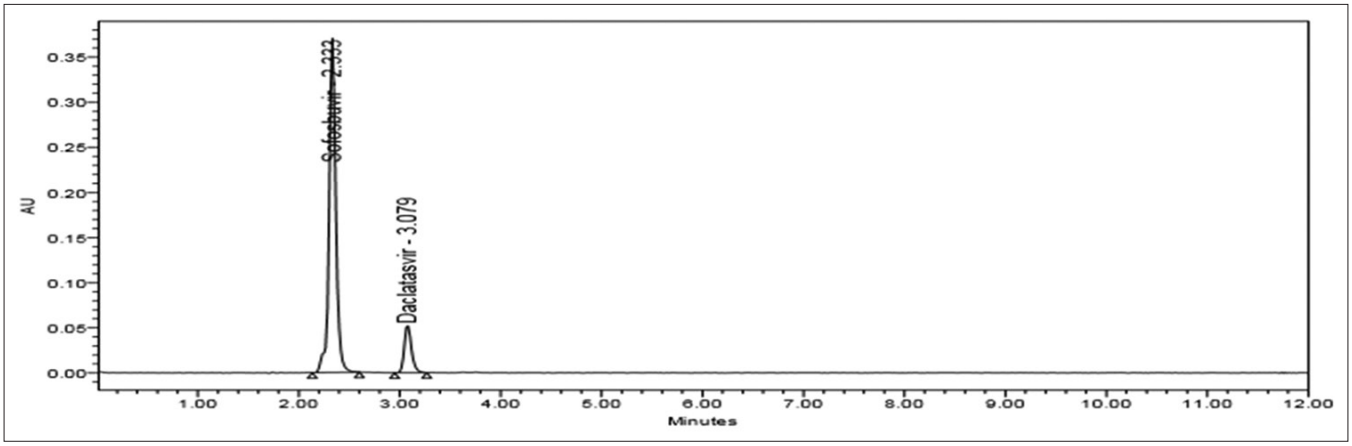


**Fig. 9: Chromatogram of oxidative degradation**

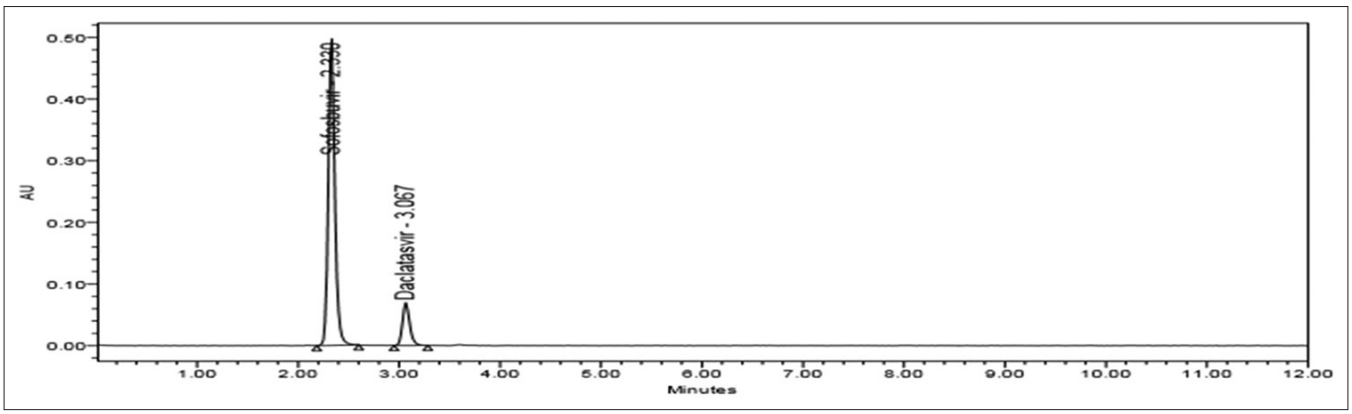


**Fig. 10: Chromatogram of thermal degradation**

**Table 7: Intra-day and inter-day precision for sofosbuvir (n=6)**

| Drug | Concentration (µg/ml) | Intra-day precision | | Inter-day precision | |
|---|---|---|---|---|---|
| | | mean±SD | % RSD | mean±SD | % RSD |
| Sofosbuvir | 400 | 2022909±9780.9 | 0.5 | 2057580±15714.2 | 0.8 |

n is number of determination. SD: Standard deviation, RSD: Relative standard deviation

**Table 8: Intra-day and inter-day precision for daclatasvir (n=6)**

| Drug | Concentration (µg/ml) | Intra-day precision | | Inter-day precision | |
|---|---|---|---|---|---|
| | | Mean±SD | % RSD | Mean±SD | % RSD |
| Daclatasvir | 60 | 333806±2733.3 | 0.8 | 335196±2736.4 | 0.8 |

n is number of determination. SD: Standard deviation, RSD: Relative standard deviation

**Table 9: Accuracy of the developed method for sofosbuvir (n=3)**

| Drug name | Level of addition (%) | Amount added (mg) | Drug found (mg/ml) mean±SD | % Recovery mean±SD | Average % recovery mean±SD |
|---|---|---|---|---|---|
| Sofosbuvir | 50 | 200 | 198.8±1.15 | 99.4±0.57 | 99.6±0.57 |
| | 100 | 400 | 397.5±2.65 | 99.4±0.66 | |
| | 150 | 600 | 599.7±2.65 | 99.9±0.44 | |

n is number of determinations. SD: Standard deviation

*Stability-indicating studies*

Stability-indicating studies were performed to know the stability of drug substance under specified stress conditions. Stability-indicating studies were performed under different stress conditions (acid/base/neutral hydrolysis, dry heat, oxidation, and photolysis). Under each condition, both working standard solution and blank were subjected to stress conditions. The degradation conditions were optimized by optimizing the concentration of degrading reagent and time of exposure. The percentage assay of sofosbuvir and daclatasvir was decreased under all these stress conditions, but there was no predictable degradation peak

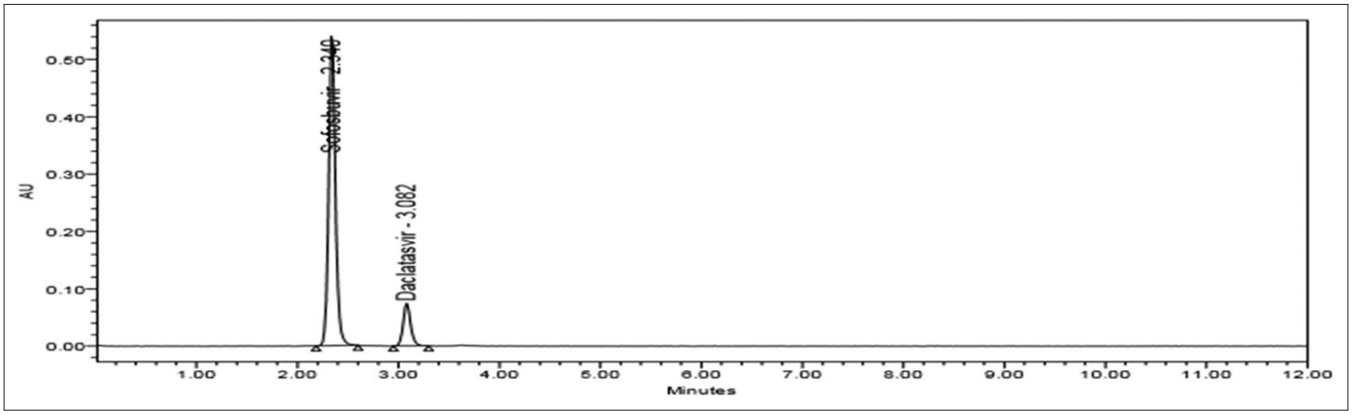


**Fig. 11: Chromatogram of photodegradation**

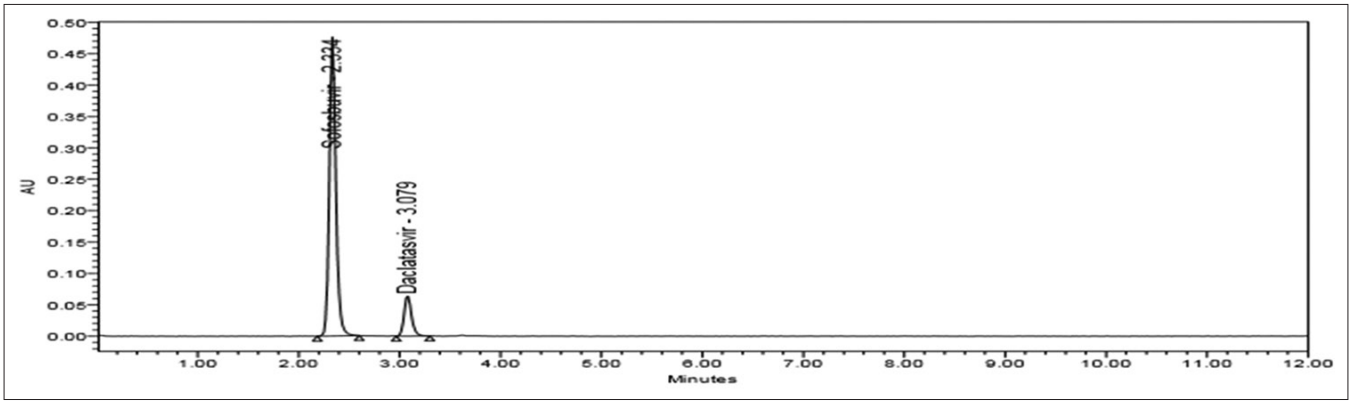


**Fig. 12: Chromatogram of neutral degradation**

**Table 10: Accuracy of the developed method for daclatasvir (n=3)**

| Drug name | Level of addition (%) | Amount added (mg) | Drug found (mg/ml) mean±SD | % recovery mean±SD | Average % recovery mean±SD |
|---|---|---|---|---|---|
| Daclatasvir | 50 | 30 | 29.6±0.25 | 98.8±0.78 | 99.49±0.94 |
| | 100 | 60 | 59.4±0.17 | 99.1±0.26 | |
| | 150 | 90 | 90.5±0.24 | 100.6±0.27 | |

n is number of determinations. SD: Standard deviation

**Table 11: Stability-indicating data of sofosbuvir**

| Degradation parameter | Peak area of sample | Peak area of standard | % Assay | % Degradation |
|---|---|---|---|---|
| Acid degradation | 1931668 | 2024221 | 95.33 | 4.67 |
| Alkali degradation | 1968587 | 2024221 | 97.15 | 2.85 |
| Oxidative degradation | 1989549 | 2024221 | 98.19 | 1.81 |
| Dry heat degradation | 2006587 | 2024221 | 99.03 | 0.97 |
| Photo degradation | 2008725 | 2024221 | 99.14 | 0.86 |
| Neutral degradation | 2012604 | 2024221 | 99.33 | 0.67 |

observed at these degradation conditions, which is shown in Figs. 7-12. The results of stress degradation studies are given in Tables 11 and 12.

## CONCLUSION

The proposed RP-HPLC-PDA method for the estimation of sofosbuvir and daclatasvir in bulk and pharmaceutical dosage was simple, accurate, precise, and reliable method. The present analytical method has been validated completely according to the Q2B ICH guidelines, and the statistical analysis also proved that method was linear, accurate, precise, and specific for analysis of sofosbuvir and daclatasvir in the pharmaceutical dosage form. The forced degradation studies conducted under different stress conditions demonstrated the degradation behavior of sofosbuvir and daclatasvir and stability-indicating property of the current analytical method, and it was proved that the present analytical method can be effectively resolute degradation peak from analytic peak. In the present developed method, PDA detector was used to confirm the peak purity. Therefore, the present RP-HPLC-PDA method was found to be stability indicating and can be used for quantification of sofosbuvir and daclatasvir in bulk and pharmaceutical dosage form.

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**Table 12: Stability-indicating data of daclatasvir**

| Degradation parameter | Peak area of sample | Peak area of standard | % Assay | % Degradation |
|---|---|---|---|---|
| Acid degradation | 320127 | 335125 | 95.43 | 4.57 |
| Alkali degradation | 325462 | 335125 | 97.02 | 2.98 |
| Oxidative degradation | 329195 | 335125 | 98.13 | 1.87 |
| Dry heat degradation | 332481 | 335125 | 99.11 | 0.89 |
| Photo degradation | 333432 | 335125 | 99.4 | 0.6 |
| Neutral degradation | 332828 | 335125 | 99.22 | 0.078 |

## AUTHOR CONTRIBUTION

The first author Swetha Addanki had done all steps in the present research work, and the second author was a mentor of the study.

## CONFLICT OF INTERESTS

Authors confirmed that there was no conflict of interest to publish the present article.

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