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Research Article

STABILITY-INDICATING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE SIMULTANEOUS ESTIMATION OF SELECTED DRUGS IN BULK AND FORMULATIONS WITH GREENNESS ASSESSMENT USING GREEN ANALYTICAL PROCEDURE INDEX

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ABSTRACT

Objectives: A comprehensive, verified, and dependable reversed-phase high-performance liquid chromatography method was created to assess velpatasvir and sofosbuvir in bulk and fixed-dose combo tablets.

Methods: The analysis was performed using a Waters Alliance 2695 high-performance liquid chromatography (HPLC) system equipped with an autosampler, a 2998 PDA detector, and Empower 2 software. Chromatographic separation was achieved on a BDS Hypersil C8 column (4.6 × 50 mm, 5 µm; Thermo Scientific). The mobile phase consisted of a 50:50 (v/v) mixture of potassium dihydrogen phosphate buffer (pH 3.5, adjusted with 0.1% orthophosphoric acid) and HPLC-grade acetonitrile. The detection wavelength was set at 288 nm, and the flow rate was maintained at 1.0 mL/min.

Results: In the concentration range of 2.5–15 µg/mL for velpatasvir and 20–120 µg/mL for sofosbuvir, the methodology consistently exhibits high correlation coefficients (R^2) of 0.999, indicating exceptional linearity. Sofosbuvir exhibited a higher degree of precision than velpatasvir, with a recovery rate of 100.11% in contrast to 99.31%. To verify the precision and robustness (with a %RSD of <2%), the International Council for Harmonization Q2 (R1) principles were applied. Velpatasvir's limits of detection/limit of quantification values were 0.01/0.04 µg/mL, whereas sofosbuvir's were 0.13/0.40 µg/mL.

Conclusion: The observed degradation levels state the method's specificity and applicability for quality control and stability testing, as they are within acceptable limits. The green analytical procedure index (GAPI) was employed to assess the method's environmental impact. Applicability for quality control and stability testing was also assessed. The method's environmental impact was evaluated using the GAPI.

Keywords: Green analytical procedure index, ICH guidelines, RP-HPLC, Sofosbuvir, Velpatasvir.

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INTRODUCTION

A combination of sofosbuvir and velpatasvir is employed to treat chronic hepatitis C virus (HCV) infection. The chemical formula for sofosbuvir is $C_{22}H_{29}FN_3O_9P$, and the structure is shown in Fig. 1 [1].

Velpatasvir ($C_{49}H_{54}N_8O_8$), a potent non-structural protein 5A (NS5A) inhibitor shown in Fig. 2, is used in combination with Sofosbuvir for the treatment of chronic hepatitis C [2].

Velpatasvir acts as a substrate for the transporter proteins P-glycoprotein and ATP-binding cassette superfamily G member 25, and inhibits NS5A. A literature review reveals that few methodologies for quantifying sofosbuvir and velpatasvir, both separately and in combination with other pharmaceuticals, have been reported [3-5].

A successful validation was performed to confirm that the developed methodology adhered to ICH Q2 R1 criteria. Limited research has reported the use of high-performance liquid chromatography (HPLC) for the simultaneous evaluation of these combination dosage formulations. The current approach showed excellent linearity, accuracy, precision, and stability following the application to specific stress conditions [6-10]. Green analytical chemistry is an essential aspect of pharmaceuticals. Recently, GAC has become the preferred method for studies. Initiatives for environmental sustainability designed to reduce or eliminate the adverse impacts of chemical processes are essential to GAC's goal. Many industrial sectors are presently prioritizing chemical processes due to their environmental sustainability. This is comprehensible considering the significant advantages they often provide in terms of decreased expenses, enhanced production, and conservation of time and resources. The objective of the GAC guidelines was to reduce or eliminate the utilization of hazardous solvents in chemical testing and procedures, ensuring that no residues were produced as a result of these activities. The study employs methods for assessing greenness, adhering to the 12 principles of green chemistry. Assessment utilizing the green analytical procedure index (GAPI). The GAPI was employed to assess the method's environmental impact. Although analytical methods for the simultaneous estimation of sofosbuvir and velpatasvir have been reported previously [11,12], these studies primarily focused on conventional chromatographic conditions with longer run times and lacked a systematic green analytical evaluation. The present work addresses this gap by developing and validating a rapid, eco-friendly reversed-phase HPLC (RP-HPLC) method that employs an optimized mobile phase and a specific column, which offers improved peak resolution, reduced solvent consumption, and shorter analysis time. Furthermore, the greenness of the method was quantitatively assessed using tools such as GAPI, demonstrating its environmental advantages over existing approaches.

METHODS

Instruments required

The analysis employed a 2998 PDA detector, an autosampler, and Empower 2 software on a Waters Alliance 2695 HPLC System. A BDS

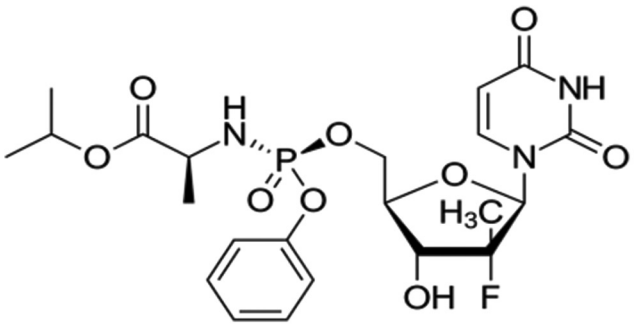

Fig. 1: Structure of sofosbuvir

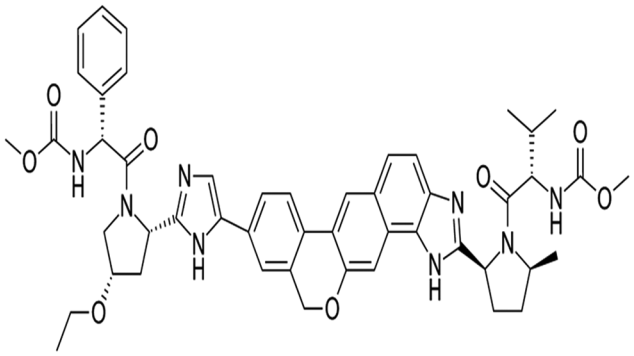

Fig. 2: Structure of velpatasvir

C8 column, measuring 4.6 × 50 mm with a particle size of 5 µm, was employed for separation. An electronic balance from Denver, Whatman No. 41 filter paper, and an ultrasonic bath sonicator (Frontlines FS 4, Mumbai, India) were employed. The HPLC method was effectively utilized to achieve the required chromatographic run time with sufficient sensitivity and selectivity.

Reagents used

Received free samples of sofosbuvir and velpatasvir from Hetero Drugs Limited in Hyderabad, India. The research utilized Milli-Q water and HPLC-grade acetonitrile sourced from SD Fine Chemicals, India. Rankem Ltd. provided potassium dihydrogen orthophosphate and orthophosphoric acid.

Mobile phase and diluent

To prepare the buffer, 1.36 g of potassium dihydrogen phosphate was dissolved in 900 mL of Milli-Q water. The pH was subsequently adjusted to 3.5 using 0.1% OPA, and the volume was increased to 1000 mL. A 50:50 (v/v) mixture of this buffer and acetonitrile was prepared, filtered, and degassed. The diluent comprised a buffer and acetonitrile combination in a 50:50 ratio, accurate, linear, and robust for routine analysis.

Standard and sample solutions

Standard solution

By dissolving 40 mg of sofosbuvir and 5 mg of velpatasvir in a diluent, a standard stock solution was formed, which was subsequently sonicated. After the final volume was adjusted to 50 mL, sofosbuvir and velpatasvir concentrations of 800 µg/mL and 100 µg/mL, respectively, were attained. This sample produced a working solution that contained 80 µg/mL of sofosbuvir and 10 µg/mL of velpatasvir [10-12].

Sample solution

Powdered tablets were transferred to a 50 mL vial that contained 10 mL of diluent, and an amount that corresponded to the labeled dose was added. The mixture was centrifuged, filtered, and diluted to a stock solution with concentrations of 800 µg/mL Sofosbuvir and 100 µg/mL velpatasvir after being sonicated for 25 min. A working solution of 80 µg/mL sofosbuvir and 10 µg/mL velpatasvir was subsequently produced using this stock [13-15].

RESULTS AND DISCUSSION

Chromatographic conditions

Chromatographic separation was achieved on a BDS Hypersil C8 column (4.6 × 50 mm, 5 µm; Thermo Scientific) using a mobile phase of 0.01 M potassium dihydrogen phosphate buffer and acetonitrile (50:50, v/v). The column was maintained at 25 ± 2°C, with a 1.0 mL/min flow rate and 288 nm detection wavelength. Before use, the mobile phase was filtered through a 0.45 µm membrane and degassed. Standard solutions were then accurately transferred into autosampler vials for analysis.

Method validation

The International Council for Harmonization's (ICH) recommended HPLC method was verified against Q2 (R1) criteria for analytical method validation [16-19].

System suitability study

The system suitability investigation is displayed in Table 1. Fig. 3 shows the good peak areas and theoretical plates of >5000 and a good tailing factor of <2.

Specificity

This technique indicated that sofosbuvir and velpatasvir exhibited retention time (RT) of 2.136 and 2.871 min (Fig. 4), respectively, with no interfering peaks from either the blank or the placebo.

Linearity

The standard solutions of sofosbuvir and velpatasvir were prepared in linear concentration ranges of 20–120 µg/mL and 2.5–15 µg/mL, respectively, to establish the calibration curves shown in Figs. 5 and 6. The results demonstrated excellent linearity across the tested ranges, with correlation coefficients (R^2) of 0.999 for both analytes, confirming the reliability of the developed RP-HPLC method. The regression equations derived from the calibration data were y = 25574x + 4971 for sofosbuvir and y = 74488x + 551.9 for velpatasvir, indicating a strong linear relationship between concentration and peak response, as shown in Table 2.

Precision

Evaluations were conducted utilizing system precision, repeatability, and intermediate precision to meticulously assess the proposed HPLC method's precision in accordance with the criteria outlined in ICH Q2 (R1). System precision (%RSD: sofosbuvir 0.80%, velpatasvir 0.9%; Table 3) and repeatability (%RSD: sofosbuvir 0.5%, velpatasvir 0.3 %, Table 4) confirmed consistent instrument and method performance. Intermediate precision conducted on a different day also showed acceptable %RSD values (%RSD: Sofosbuvir 0.9%, Velpatasvir 0.6 %, Table 5).

System precision

From a single volumetric flask of working standard solution six injections were given and the obtained results were mentioned in Table 3.

Method precision

Six replicate working standard solutions were prepared from a single stock and injected to determine the method precision and shown in Table 4.

Intermediate precision

Six replicate solutions were prepared from a single stock and analyzed in the very next day and summarized in Table 5.

Table 1: System suitability of sofosbuvir and velpatasvir

S. No.	Sofosbuvir			Velpatasvir			
Inj.	Retention time	Theoretical plate (TP)	Tailing	Retention time	Theoretical plate (TP)	Tailing	Resolution
1	2.136	3967	1.27	2.871	4916	1.05	4.7
2	2.138	3902	1.27	2.884	4927	1.11	4.9
3	2.141	4049	1.28	2.887	4989	1.04	4.8
4	2.141	4031	1.28	2.888	4844	1.05	4.7
5	2.152	4154	1.28	2.897	5099	1.06	4.9
6	2.156	4146	1.30	2.907	5166	1.07	4.8

Table 2: Linearity, concentration, and peak area of sofosbuvir and velpatasvir

Sofosbuvir		Velpatasvir	
Concentration	Peak area	Concentration	Peak area
20	518858	2.5	151547
40	1047581	5	297597
60	1502795	7.5	447465
80	2085606	10	592112
100	2542313	12.5	751020
120	3078920	15	892625

Table 3: System precision

Concentration (µg/mL)	Sofosbuvir 80 (µg/mL)	Velpatasvir 10 (µg/mL)
Area	2083227±16811.5	591153±5084.8
%RSD	0.8	0.9

All values are expressed as mean±SD, n=6. RSD: Relative standard deviation

Table 4: Repeatability

Concentration (µg/mL)	Sofosbuvir 80 (µg/mL)	Velpatasvir 10 (µg/mL)
Area	2082039±11176.8	590954±1971.9
%RSD	0.5	0.3

All values are expressed as Mean±SD, n=6. RSD: Relative standard deviation

Table 5: Intermediate precision

Concentration (µg/mL)	Sofosbuvir 80 (µg/mL)	Velpatasvir 10 (µg/mL)
Area	2058367±18272.0	585593±3376.8
%RSD	0.9	0.6

All values are expressed as mean±SD, n=6. RSD: Relative standard deviation

Table 6: Accuracy table of Sofosbuvir

Level %	Spiked	Recovered	% Recovery	Mean % recovery
50%	40	40.13	100.31	100.11%
	40	39.83	99.56	
	40	39.82	99.56	
100%	80	80.44	100.55	
	80	79.76	99.70	
	80	79.99	99.99	
150%	120	122.77	102.30	
	120	119.29	99.40	
	120	119.51	99.59	

Table 7: Accuracy table of Velpatasvir

Level %	Spiked	Recovered	% Recovery	Mean % recovery
50	5	4.97	99.37	99.31%
	5	4.93	98.56	
	5	4.97	99.50	
100	10	9.94	99.39	
	10	9.86	98.60	
	10	9.96	99.64	
150	15	14.89	99.27	
	15	14.98	99.88	
	15	14.94	99.58	

Table 8: Sensitivity

Drugs	LOD	LOQ
Sofosbuvir	0.13	0.40
Velpatasvir	0.01	0.04

LOD: Limits of detection, LOQ: Limit of quantification

Accuracy

The conventional addition procedure was used to create samples with three levels of precision. For every accuracy level and mean %, three shots were administered. Velpatasvir and sofosbuvir had respective recovery rates of 99.31% and 100.11% shown in Tables 6 and 7.

Sensitivity

Using the calibration curve's slope and standard deviation, limits of detection (LOD) and limit of quantification (LOQ) were determined in accordance with ICH Q2(R1). Sofosbuvir had the values (LOD: 0.13 µg/mL; LOQ: 0.40 µg/mL), whereas velpatasvir had the sensitivity (LOD: 0.01 µg/mL; LOQ: 0.04 µg/mL), indicating lower sensitivity. The method effectively detects and quantifies two drugs within the tested range (Table 8).

Robustness

The robustness of the developed RP-HPLC method was evaluated by introducing deliberate, minor variations in analytical parameters to assess the method's reliability under slightly altered conditions. The changes included variations in flow rate (±0.1 mL/min), mobile phase composition (±10% organic phase), and column temperature (±5°C) relative to the optimized chromatographic conditions (mobile phase: 50:50 v/v potassium dihydrogen phosphate buffer [pH 3.5] and acetonitrile). Here, component A refers to the phosphate buffer, and component B refers to acetonitrile. After each modification, parameters such as retention time (RT), tailing factor (TF), theoretical plates (N), and resolution (Rs) were determined for both sofosbuvir and velpatasvir. The results are summarized in Table 9, showing that all variations produced %RSD values below 2%, with negligible shifts in RT and consistent system suitability metrics. This confirms that the method is robust and unaffected by small operational variations. The data indicate that small changes in flow rate, mobile phase composition, and temperature do not significantly affect chromatographic performance. All key parameters remain within acceptable limits (%RSD < 2%), confirming the robustness and reproducibility of the proposed RP-HPLC method.

Assay procedure

Six replicate injections of standard and sample solutions (10 µL each) were tested under optimized chromatographic settings (Figs. 7 and 8). The assay was developed by comparing the peak regions of the sample to

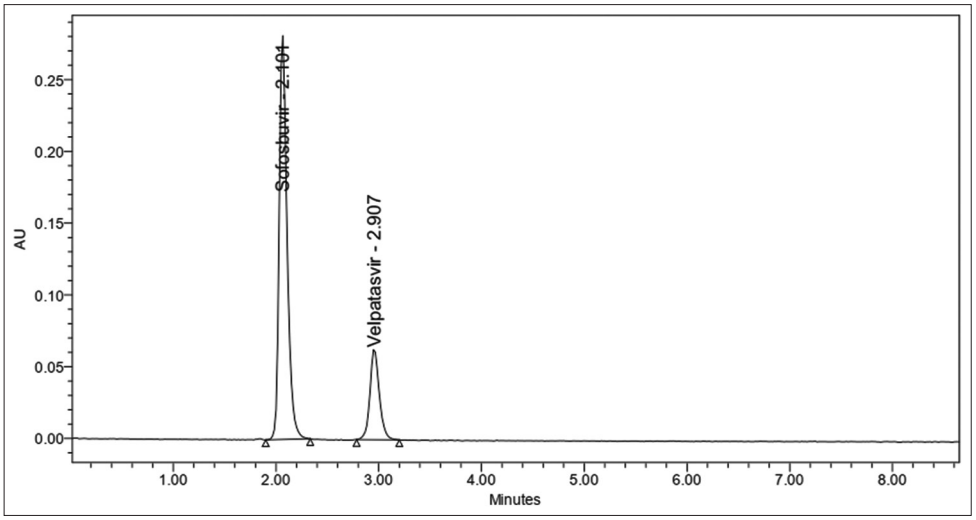

Fig. 3: Optimized chromatogram

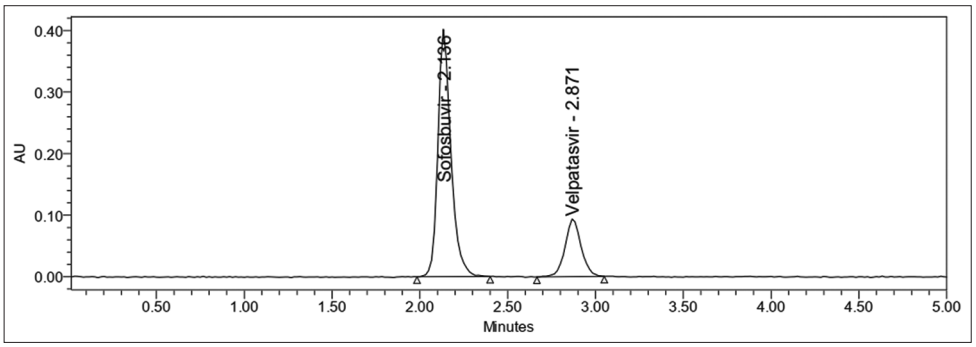

Fig. 4: System specificity chromatogram

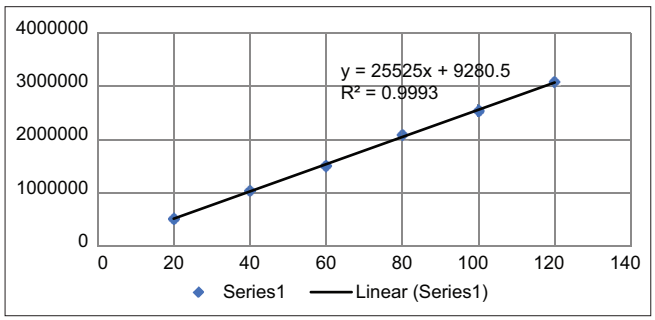

Fig. 5: Calibration curve of sofosbuvir

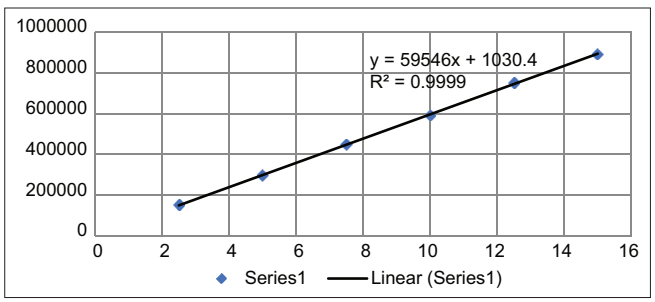

Fig. 6: Calibration curve of velpatasvir

Table 9: Robustness data

Condition	Parameter	Sofosbuvir	Velpatasvir	%RSD
Flow Rate (–) 0.9 mL/min	Retention time (min)	2.241	2.990	0.4
	Tailing factor	1.21	1.06	0.3
	Theoretical plates	3920	4845	-
	Resolution	-	4.6	-
Flow Rate (+) 1.1 mL/min	Retention time (min)	2.021	2.751	0.6
	Tailing factor	1.28	1.09	0.5
	Theoretical plates	4052	4978	-
	Resolution	-	4.8	-
Mobile Phase (–) 45A: 55B (–10% organic)	Retention time (min)	2.185	2.936	0.5
	Tailing factor	1.22	1.04	0.4
	Theoretical plates	4005	4955	-
	Resolution	-	4.7	-
Mobile Phase (+) 35A: 65B (+10% organic)	Retention time (min)	2.091	2.801	0.3
	Tailing factor	1.24	1.07	0.2
	Theoretical plates	3988	4902	-
	Resolution	-	4.8	-
Temperature (–) 25°C	Retention time (min)	2.153	2.881	0.2
	Tailing factor	1.26	1.05	0.3
	Theoretical plates	4027	4963	-
	Resolution	-	4.7	-
Temperature (+) 35°C	Retention time (min)	2.118	2.845	0.4
	Tailing factor	1.25	1.08	0.4
	Theoretical plates	4048	4985	-
	Resolution	-	4.9	-

RSD: Relative standard deviation

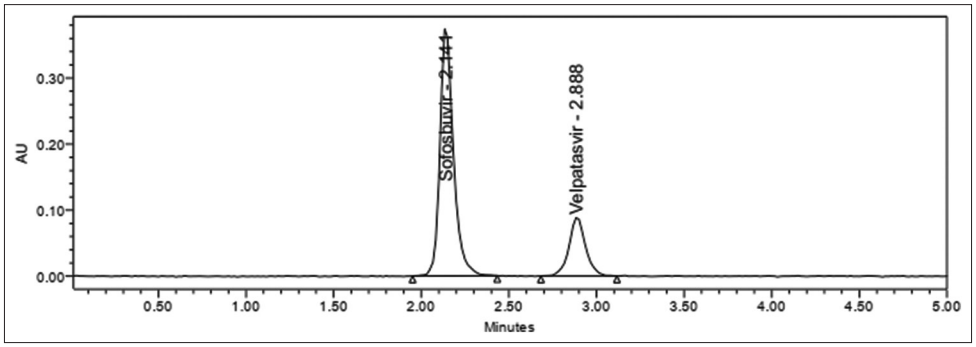

Fig. 7: Working standard solution chromatogram

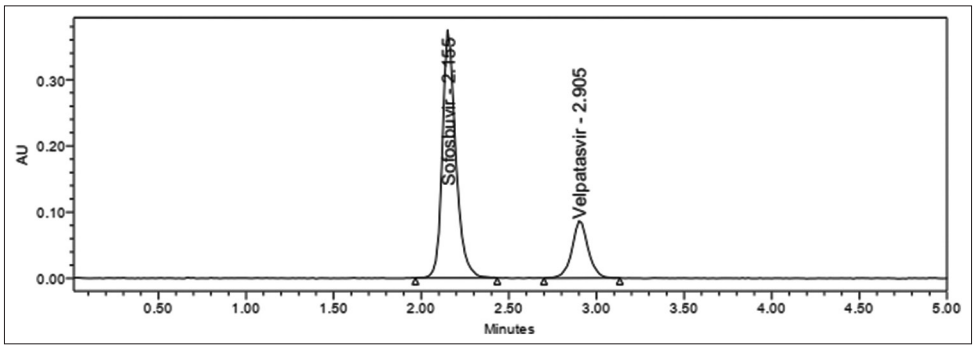

Fig. 8: Working sample solution chromatogram

those of the standard. The method demonstrated remarkable accuracy and precision. The mean assay values were 99.87% for sofosbuvir and 99.84% for velpatasvir, both according to ICH criteria (Table 10).

Stability studies and forced degradation analysis

The stability studies of sofosbuvir and velpatasvir in the combined dosage form, under various stress conditions as per ICH guidelines. The forced degradation study was conducted for predetermined time intervals under different stress environments to simulate potential degradation pathways.

Acidic and basic hydrolysis were performed by exposing the drug to 1 N HCl and 1 N NaOH, respectively, at 60°C for 2 h. Oxidative degradation was carried out using 3% hydrogen peroxide at room temperature for 2 h. For photolytic degradation, the samples were exposed to ultraviolet (UV) and visible light for 24 h, achieving a cumulative exposure of 200 W·h/m^2 and 1.2 million lux·h/m^2, as recommended by ICH. Both sofosbuvir and velpatasvir exhibited controlled degradation under these stress conditions, confirming the stability-indicating nature of the developed RP-HPLC method. The highest degradation was observed under acidic conditions – 7.28% for sofosbuvir and 5.26% for velpatasvir – whereas the lowest degradation occurred under aqueous (neutral) stress, at 1.62% and 0.43%, respectively (Table 11).

The forced degradation chromatograms (Fig. 9) clearly demonstrate the formation of distinct degradation products under different stress conditions. Under acidic hydrolysis, two degradation peaks (single degradation peak [DP-I] and double degradation peak [DP-II]) appeared at earlier retention times, attributed to the cleavage of sofosbuvir's phosphoramidate and ester linkages, whereas velpatasvir showed partial amide hydrolysis. Alkaline stress generated a DP-I, indicating susceptibility of sofosbuvir's ester moieties to base-catalyzed nucleophilic substitution. Oxidative degradation resulted in the formation of DP-I, associated with the oxidation of heteroaromatic rings present in both analytes. In thermal stress, a distinct degradant (DP-III) appeared, suggesting thermally induced structural rearrangement. Conversely, UV photolysis and neutral (water) stress produced no significant degradants, confirming strong intrinsic stability under these conditions.

Table 10: Assay studies

Drug	Label claim	Amount found	Label Claim %	RSD %
Sofosbuvir	400	399.36	99.84	0.54
Velpatasvir	100	99.87	99.87	0.33

RSD: Relative standard deviation

Table 11: Degradation studies of sofosbuvir and velpatasvir

S. No.	Degradation condition	% Degraded (Sofosbuvir)	% Degraded (Velpatasvir)
1	Acid	7.28	5.26
2	Alkali	4.46	5.00
3	Oxidation	3.34	3.71
4	Thermal	2.81	2.70
5	UV	1.62	1.57
6	Water	1.62	0.43

The consistent retention times of the parent peaks, baseline stability, and absence of co-eluting degradants further validated the stability-indicating nature and specificity of the developed RP-HPLC method.

Green assessment

The GAC guidelines were designed to minimize or eliminate the use of hazardous solvents in chemical testing and procedures, thus ensuring that no residues are generated as a consequence of these activities. The study adheres to the twelve principles of green chemistry and employs methodologies for evaluating greenness. Utilization of the GAPI for assessment. The method's environmental impact was evaluated using the GAPI.

Acetonitrile (ACN) was utilized as the organic solvent in the suggested analytical approach, and its greenness was assessed using the Complex GAPI index, which is shown in Fig. 9. A comprehensive instrument designed to quantify the environmental effect of a whole analytical process, from sample collection to final assessment, is the GAPI. It is

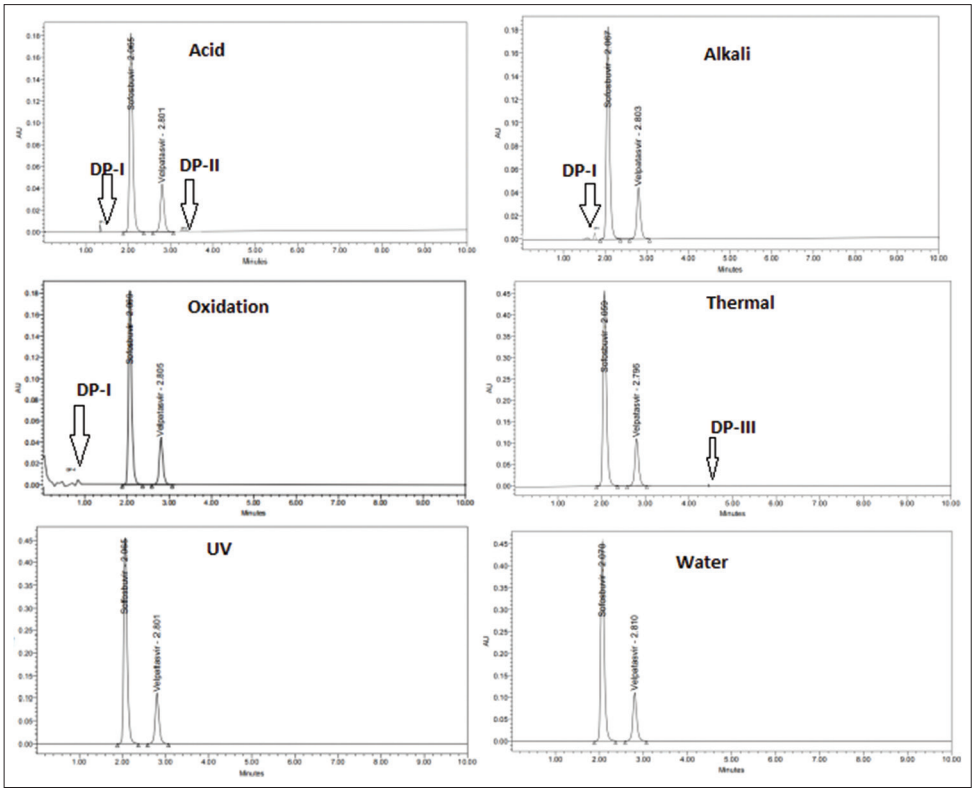

Fig. 9: Degradation chromatogram

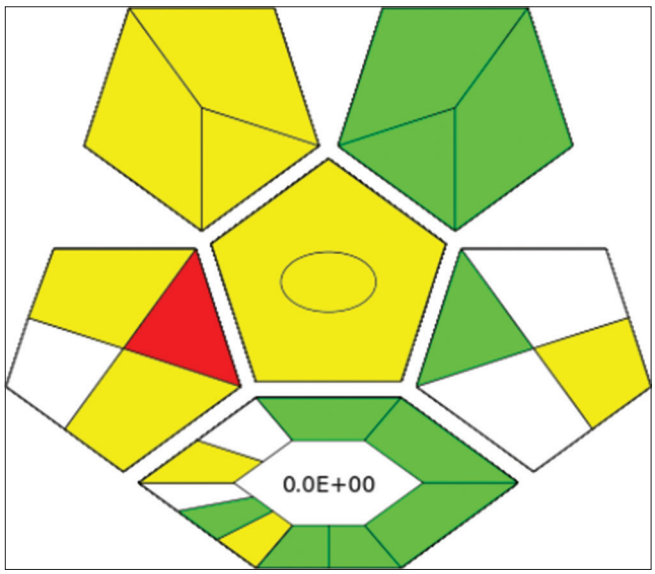

Fig. 10: GAPI assessment

represented graphically by a pentagram with five segments, each of which represents a crucial step in the analytical process: Waste or energy production, chemicals and solvents, instruments, sample collection, and sample processing. To facilitate a rapid visual assessment of the method's greenness, each segment is color-coded as green (low environmental effect), yellow (moderate impact), or red (high impact).

In the evaluated method, the GAPI diagram displays 8 green, 7 yellow, and 1 red segment (Fig. 10). The green zones reflect environmentally favorable practices in areas such as low energy consumption, minimal solvent volume, efficient instrumentation, and micro-scale extraction techniques. The yellow segments highlight moderate concerns in sample handling, waste generation, and the scale of chemical usage. The one red segment is linked to the use of ACN, which is categorized as hazardous due to its flammability and toxicity, indicating a notable safety and environmental risk.

Although the method uses acetonitrile, with approximately 5 mL consumed per analysis, greener alternatives such as ethanol and methanol did not provide acceptable chromatographic performance. Therefore, the use of ACN is acknowledged as a limitation, and future work will focus on developing an eco-friendlier method using safer solvent systems. The GAPI tool effectively illustrates the balance of these factors, confirming the method's overall classification as an environmentally conscious analytical approach.

CONCLUSION

The RP-HPLC method, characterized by its simplicity, precision, and accuracy, enables the simultaneous testing of velpatasvir and sofosbuvir in tablet dosage form. The retention times of 2.871 and 2.136 min for velpatasvir and sofosbuvir, respectively, suggest efficient separation with a shorter run time. The system accuracy produced %RSD values of 0.8% and 0.9% for sofosbuvir, and 0.5% and 0.3% for velpatasvir, respectively, indicating remarkable repeatability and consistency. The method demonstrated exceptional accuracy, achieving mean recovery rates of 100.11% for sofosbuvir and 99.31% for velpatasvir. The LOD for velpatasvir and sofosbuvir were 0.01 µg/mL and 0.13 µg/mL, respectively, whereas their LOQ were 0.04 µg/mL and 0.40 µg/mL. The regression equations obtained from the calibration curves exhibited outstanding linearity and sensitivity. Degradation tests were conducted on the formulation, and all degraded samples remained within permissible limits, demonstrating the method's efficacy in indicating stability. The proposed method is efficient, cost-effective, and suitable for routine quality control assessments in pharmaceutical companies, as evidenced by the reduced retention times and robust validation metrics. Furthermore, by minimizing solvent consumption, analysis duration, and ecological footprint, the method advocates for the tenets of green analytical chemistry.

AUTHORS' CONTRIBUTIONS

Greeshma Sivarajan: Organizing, generating ideas, gathering information, and producing papers. Saravanan Gopal: Literature review and interpretation of data. After reading the manuscript, each contributor consented to its publication.

CONFLICTS OF INTEREST

Not applicable.

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