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ECO-ENGINEERED STABILITY-INDICATING RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF EMPAGLIFLOZIN AND VILDAGLIPTIN WITH AGREE–GAPI VALIDATION

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ABSTRACT

Background: A novel and reliable reverse-phase high-performance liquid chromatography (RP-HPLC) method was established and validated to allow for the simultaneous determination of empagliflozin (EMPA) and vildagliptin (VILDA) in combination.

Methods: The chromatographic separation was carried out on a C18 column (250 mm × 4.6 mm, 5 µm) using a mobile phase consisting of water (pH 4.5, adjusted with 0.01% orthophosphoric acid) and acetonitrile in a 45:55 (v/v) ratio. Detection was performed at 212 nm, and the retention times for EMPA and VILDA were observed to be approximately 4.6 and 5.4 minutes, respectively.

Results: The method demonstrated good linearity for EMPA and VILDA in the ranges of 1–150 ppm and 2–300 ppm, correspondingly, with correlation coefficients (r^2) of 0.999 for both analytes. The regression equations for EMPA and VILDA were $y = 7688.6x - 215$ and $y = 5749.7x - 30204$, respectively. Precision, expressed as %RSD, was found to be below 2%. The method also exhibited suitable accuracy, with recovery rates between 98.5% and 101.3% for EMPA and 98.7% and 101.2% for VILDA. The detection and quantification limits (LOD and LOQ) were 2.9 ppmand 1.9 ppmfor EMPA, and 8.82 ppmand 5.76 ppmfor VILDA, respectively. The method was robust and resilient to minor variations in conditions.

Conclusion: Forced degradation studies revealed that EMPA and VILDA were stable under different stress environments, with no interference from degradation products at their respective retention times. This developed RP-HPLC method is suitable for the routine quality control of EMPA and VILDA in combined pharmaceutical preparations, confirming its specificity and reliability. The greenness of the developed method was assessed using AGREE and GAPI tools, yielding a high sustainability score of 0.73.

KEYWORDS: Empagliflozin; Vildagliptin; RP-HPLC; Stability-Indicating; Method Development; Validation; Forced Degradation

INTRODUCTION

Type 2 diabetes mellitus is often managed with combination therapy targeting different physiological pathways. Empagliflozin (EMPA) is an oral antidiabetic drug of the sodium–glucose co-transporter 2 (SGLT2) inhibitor class,[1]which lowers blood glucose by promoting urinary glucose excretion. Vildagliptin (VILDA) is an oral antihyperglycemic agent that acts as a dipeptidyl peptidase-4 (DPP-4) inhibitor[2], increasing incretin hormone levels to stimulate insulin release and suppress glucagon, thereby improving glycemic control These two drugs have complementary mechanisms and can be used concurrently to achieve better glycemic control in patients. Fixed-dose combinations (FDCs) of SGLT2 inhibitors with DPP-4 inhibitors are of growing interest for improving patient compliance and therapeutic efficacy.

Although several analytical methods are available for the separate estimation of empagliflozin and vildagliptin in pharmaceutical products[3], there is currently no documented stability-indicating HPLC method for[4] their combined analysis. Establishing a stability-indicating technique is critical for the accurate measurement of active ingredients even in the presence of impurities or degradation products[5], which is vital for maintaining drug quality and safety throughout its shelf life[6]. The primary objective of this work was to create a straightforward, efficient, and robust reverse-phase HPLC (RP-HPLC) method capable of simultaneously quantifying EMPA and VILDA in combined Synthetic mixture, represented here by a synthetic laboratory-prepared mixture.[7] The method was systematically validated according to the ICH Q2(R1) guidelines for analytical method validation[8]. Furthermore, forced degradation experiments were conducted under various stress conditions—acidic, alkaline, oxidative, thermal, and photolytic—as

outlined in ICH Q1A(R2) to confirm the method's ability to indicate stability.[9] The validated method is intended to support routine quality control and stability assessment of pharmaceutical formulations containing both antidiabetic agents.[10] Although several HPLC methods exist for the individual estimation of empagliflozin and vildagliptin, no validated, stability-indicating method has been reported for their combined analysis. Additionally, prior studies lack comprehensive greenness evaluation using tools like AGREE and GAPI.[11-21]

MATERIALS AND METHODS

Reagents and Materials

Reference standards of empagliflozin (EMPA) and vildagliptin (VILDA) were supplied from internal laboratory sources (analytical grade). Methanol and acetonitrile suitable for HPLC were obtained from Finar and Rankem (Ahmedabad, India) The greenness of the developed method was assessed using the GAPI (Green Analytical Procedure Index) tool, which provides a visual representation of the method's environmental impact.

Instrumentation

HPLC measurements were performed on a Shimadzu Prominence system featuring an LC-20AD quaternary gradient pump and an SPD-M40 photodiode array (PDA) detector, all managed with LabSolutions software. Sample injection was done manually with a Rheodyne injector and a 20 µL loop, while the injection volume for analysis was fixed at 10 µL. Chromatographic separation was accomplished on a reverse-phase C18 column (250 mm × 4.6 mm, 5 µm particle size)[18]. For weighing, an electronic analytical balance (Shimadzu AUW-220D) was employed. A sonicator (Trans-O-Sonic, model D120/1H) was used to assist in dissolving the samples.

Preparation of Standard and Sample Solutions

Standard Stock Solutions: Separate stock solutions of EMPA and VILDA were prepared by dissolving 10 mg of each drug in 10 mL of methanol in volumetric flasks (giving 1000 µg/mL). The flasks were sonicated for ~10 minutes to ensure complete dissolution before bringing to volume. From each stock, a working standard solution of 100 ppmwas prepared by pipetting 1 mL stock into a 10 mL flask and diluting to volume with methanol.

Synthetic Mixture Preparation:

A synthetic tablet mixture was formulated to simulate a combined dosage form containing 25 mg EMPA and 100 mg VILDA per tablet. The mixture (total weight ~460 mg) included common excipients: microcrystalline cellulose (filler, ~300 mg), croscarmellose sodium (disintegrant, ~30 mg), and magnesium stearate (lubricant, ~5 mg), The powder blend was mixed thoroughly to ensure homogeneity.

Sample Preparation:

A synthetic mixture equivalent to 25 mg empagliflozin and 100 mg vildagliptin was weighed into a 100 mL volumetric flask, extracted with methanol by sonication for 5–10 minutes, then diluted to volume and filtered. The resulting stock (250 ppm EMPA, 1000 ppm VILDA) was further diluted: 2 mL was brought to 10 mL with methanol, yielding a final solution of 50 ppm EMPA and 200 ppm VILDA for HPLC analysis.

Chromatographic Conditions:

Isocratic separation was achieved at ~25 °C using a mobile phase of water (pH 4.5, 0.01% OPA), methanol, and acetonitrile (35:35:30, v/v/v) at 1.0 mL/min. Detection was performed at 212 nm with a 10 µL injection volume.[16] Retention times were approximately 4.6 min for empagliflozin and 8.4 min for vildagliptin. The system was equilibrated and conditioned prior to analysis, with a total run time of 20 minutes to ensure complete elution.

Method Development and Optimization

Optimal separation was achieved on a C18 column with a mobile phase of water (pH 4.5, 0.01% OPA): methanol: acetonitrile (35:35:30, v/v/v). Raising the pH to 4.5 was critical for clear, symmetric peaks of both EMPA and VILDA, overcoming issues with peak suppression and splitting at lower pH.

Specificity: No interference observed at EMPA and VILDA retention times in blank, placebo, or standard; peak purity confirmed by PDA.

Linearity: Calibration curves were linear across 25–150 ppm (EMPA) and 100–600 ppm (VILDA), with regression analysis applied.

Precision: Repeatability (%RSD) assessed by six replicates at assay concentration; intra- and interday precision evaluated at three concentration levels, all within acceptable %RSD.

Accuracy: Recovery studies at 50%, 100%, and 150% of assay levels showed 98–102% recovery.

LOD/LOQ: Determined by standard deviation and slope method; values confirmed experimentally.

Robustness: Minor variations in mobile phase, flow rate, and detection wavelength had negligible impact on results (%RSD < 2%).

Solution Stability: No significant change in peak area after 24 h at room temperature.

Results and Discussion

Specificity:

The developed method demonstrated good specificity. In chromatograms of the blank mobile phase and a placebo (excipients only) sample, no peaks appeared at the retention times corresponding to EMPA (~4.6 min) or VILDA (~8.4 min. In the combined standard/ sample (Figure 1), the two active ingredients produced distinct peaks that were

baseline-resolved. Furthermore, peak purity analysis using the PDA detector indicated purity indices > 0.999 for both EMPA and VILDA peaks in all samples, even in the presence of degradants. These results confirm that the method can selectively analyze EMPA and VILDA without interference from excipients or degradation products, fulfilling ICH requirements for specificity.

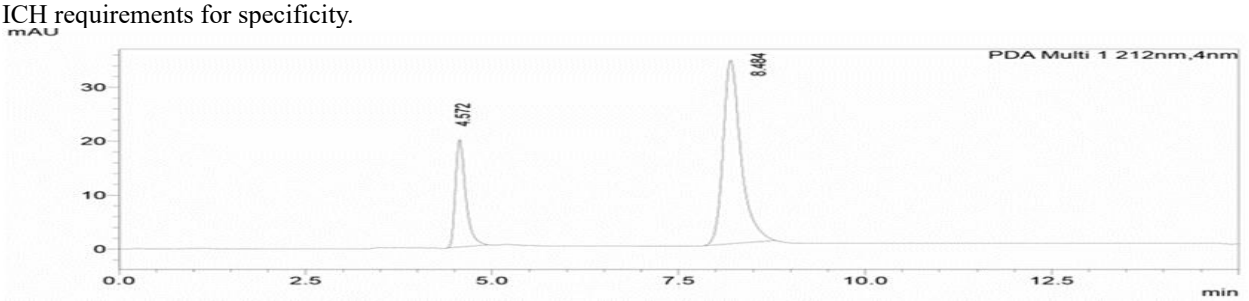

Figure 1: Chromatogram of EMPA + VILDA standard mixture at 212 nm under optimized conditions (EMPA at 4.57 min and VILDA at 8.48 min, baseline-resolved, with no interfering peaks).

Linearity and Range:

The HPLC method showed excellent linearity for both analytes. EMPA was linear over 25–150 µg/mL (R^2 = 0.998), while VILDA exhibited linearity over 100–600 µg/mL (R^2 = 0.999). The regression equations were y = 7688.6x − 215 (EMPA) and y = 5749.7x − 30204 (VILDA). The high correlation coefficients confirm a strong concentration–response relationship, demonstrating the suitability of the method for accurate quantitative analysis. (figure 2-3)

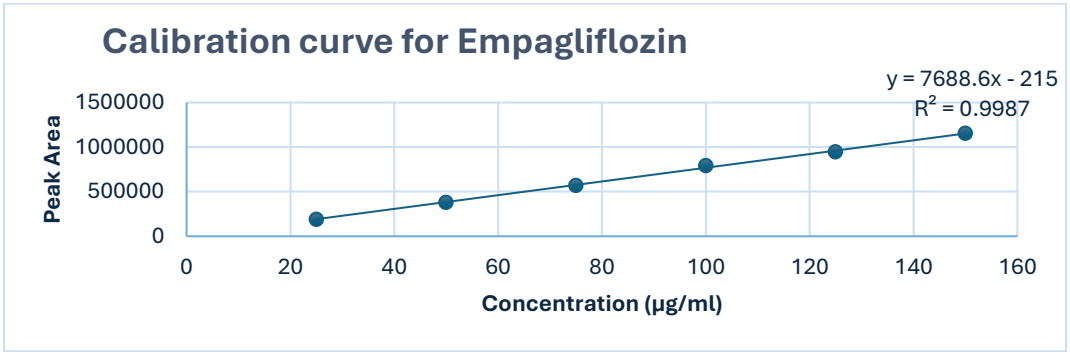

Figure 2: Calibration curve of empagliflozin (EMPA) at 212 nm (plot of peak area vs. concentration, showing linear fit with R^2 = 0.998 over 25–150 µg/mL).

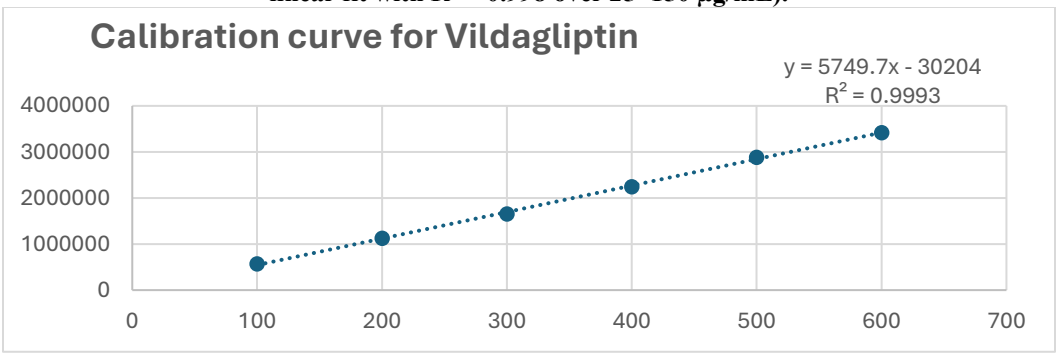

Figure 3: Calibration curve of vildagliptin (VILDA) at 212 nm (linear fit with R^2 = 0.999 over 100–600 µg/mL)

4.3 Precision:

The method's precision was evaluated in terms of both repeatability and intermediate precision, and the results are summarized in Table 1 and 2.

4.4 Repeatability

Six replicate injections of a standard mixture (EMPA 75 ppm+ VILDA 300 µg/mL) yielded %RSD values for peak areas of 1.05% for EMPA and 1.09% for VILDA (Table 1). This indicates a high degree of consistency in the HPLC system response for multiple injections of the same sample.

Table 1: Repeatability results for EMPA and VILDA (six replicates at assay concentration, showing peak areas and %RSD).

Drug	EMPA	VILDA	
Con. (µg/ml)	Area	Con. (µg/ml)	Area
75	572120	300	1679502
75	579641	300	1671229
75	579355	300	1670236
75	578339	300	1646160
75	577334	300	1654517
75	582296	300	1673067
AVG	578181	AVG	1663042
SD	3403.57	SD	12009.98
%RSD	0.58	%RSD	0.72

Intraday Precision:
At three concentration levels within the linear range, the intraday %RSD for peak areas were found to be in the range of 0.5–1.5% for EMPA and 0.7–1.8% for VILDA, all well below the 2% threshold.
Interday Precision:
The method's reproducibility on different days was also excellent. The %RSD of measurements for the same three concentration levels performed on three consecutive days remained < 2% for both drugs. This included different analysts and a different column of the same type on one of the days, to rigorously challenge intermediate precision. the method's performance is not significantly influenced by small day-to-day factors.

Table 2: Intraday and Interday Precision of Empagliflozin (EMPA) and Vildagliptin (VILDA) at Three Concentration Levels

Sr. No.	Drug Name	Concentration (µg/mL)	Intraday – Mean Area ± SD	Intraday %RSD	Interday – Mean Area ± SD	Interday %RSD
1	EMPA	25	189588.41 ± 1433.48	0.76	190979.45 ± 2000.90	1.05
		75	572406.09 ± 1372.80	0.24	574989.13 ± 8719.85	1.52
		150	1143332.28 ± 14432.98	1.26	1140031.80 ± 20603.89	1.81
2	VILDA	100	567032.54 ± 7052.72	1.24	572931.88 ± 10010.08	1.75
		300	1683153.56 ± 15602.05	0.93	1669645.84 ± 27477.12	1.65
		600	3410421.69 ± 39743.29	1.17	3428684.59 ± 46614.72	1.36

Accuracy:
The accuracy of the method, expressed as percent recovery of known added amounts of analyte, was within the acceptable range for both EMPA and VILDA. Results of the recovery studies are given in Table 3.

Table 3: Accuracy (Recovery) of EMPA and VILDA at 0%, 50%, 100%, and 150% Spiking Levels

Level	Concentration of Drug (µg/mL)	Amount of API Added (µg/mL)	Total Concentration (µg/mL)	Amount Recovered (Mean ± SD)	% Recovery
0%	EMPA: 50	0	50	50.78 ± 0.18	101.56
50%	EMPA: 50	25	75	73.94 ± 0.79	98.59
100%	EMPA: 50	50	100	101.50 ± 0.79	101.50
150%	EMPA: 50	75	125	123.17 ± 0.63	98.54
0%	VILDA: 200	0	200	201.80 ± 0.52	100.90
50%	VILDA: 200	100	300	296.85 ± 4.22	98.95

100%	VILDA: 200	200	400	403.03 ± 3.95	100.76
150%	VILDA: 200	300	500	500.68 ± 3.71	100.14

Limit of Detection and Quantitation

The LOD and LOQ for EMPA and VILDA were determined using the calibration curve method. For EMPA, the LOD was approximately 2.9 ppm, and the LOQ was about 8.8ppm,. For VILDA, the LOD was around 1.9 ppm and LOQ 5.8 ppm.These low concentration values indicate the method's high sensitivity, which is attributed to the UV absorption of both analytes at 212 nm and the efficient chromatographic separation with minimal noise. The method can thus detect and quantify very small amounts of EMPA and VILDA, which is useful for analysis of trace levels, such as impurities or degradation products, if needed.

Practical verification involved injecting diluted solutions near the LOD levels and confirming a signal-tonoise ratio > 3 for LOD and > 10 for LOQ, which was observed. The results confirm that the method has adequate sensitivity for the intended purposes.

Robustness

The robustness of the developed method was confirmed for both empagliflozin (EMPA) and vildagliptin (VILDA) by introducing minor variations in flow rate (±0.1 mL/min), detection wavelength (±3 nm), and mobile phase composition (±5%). For EMPA, %RSD values remained within 1.41 across all conditions, indicating consistent performance, with peak areas ranging from 568098.2 to 576694.71. Similarly, VILDA showed reliable assay values, with %RSD not exceeding 1.81 and peak areas between 1670200.21 and 1727721.67. These results demonstrate that the method remains unaffected by small, deliberate changes in analytical parameters, confirming its robustness.

Assay of Synthetic Mixture

The validated method was applied to the assay of EMPA and VILDA in the prepared synthetic tablet mixture (simulating a combined Synthetic Mixture). The sample solution (50 ppm EMPA and 200 ppm VILDA nominal) was injected in six replicates.

The assay results indicated that the synthetic mixture contained 99.8% of the labeled amount of EMPA and 100.5% of the labeled amount of VILDA, on average. The %RSD for assay results of each drug was < 2%. These values are well within acceptable pharmaceutical limits (typically 98–102% of label claim), demonstrating that the method can accurately quantify the active ingredients in a combined formulation. The small deviation from 100% may be attributed to minor weighing or dilution errors, but no significant bias was observed. This also effectively demonstrates that common excipients present in the synthetic mixture did not interfere with the quantitation of EMPA and VILDA.

Forced Degradation Studies:

A key feature of this method is its stability-indicating nature, confirmed through forced degradation studies. Both EMPA and VILDA, alone and in combination, were exposed to various stress conditions in accordance with ICH Q1A(R2) guidelines. The degraded samples were then analyzed to determine the extent of degradation and to verify that degradation products were well-resolved from the main analyte peaks. A summary of the applied conditions and results is provided below.

Acidic Degradation:

Sample solutions were treated with 0.1 N HCl and kept at room temperature for 2 hours. Significant hydrolysis was observed. EMPA degraded by about 11.2%, while VILDA degraded by 13.1%, as determined from the decrease in their peak areas relative to an untreated control. Chromatograms showed additional peaks at retention times ~2.5–2.9 min (for EMPA) and ~6.0 and 11.2 min (for VILDA), corresponding to acid degradation products. Importantly, the main peaks of EMPA and VILDA remained well-resolved and free from interference by these degradants. The resolution between parent peaks and the nearest degradant peak was > 2. The acid degradants were small, well-separated peaks, indicating the method's ability to detect acid-induced breakdown.

Basic Degradation:

Samples were exposed to 0.1 N NaOH (base hydrolysis) for 2 hours. EMPA showed a degradation of approximately 13.1%, whereas VILDA showed a slightly higher degradation of about 15.5%. Chromatograms indicated the formation of degradation products eluting at around 2.0–3.0 min and 10.4 min for the mixture. Again, the parent drug peaks at 4.6 and 8.4 min were baseline-resolved from these degradant peaks. The magnitude of base-induced degradation was the highest among the tested conditions for VILDA (consistent with VILDA's susceptibility to base hydrolysis). The method proved capable of quantifying the remaining drug (approximately 85–87% remaining for VILDA under base stress) while separating the degradant peaks.

Oxidative Degradation:

Oxidative stress was performed using 3% H_2O_2 (hydrogen peroxide) at room temperature for 1 hour. Both drugs exhibited only minor oxidative degradation. EMPA had ~9.7% degradation, and VILDA ~12.3% degradation. The chromatogram of the mixture showed small additional peaks due to oxidation products (e.g., one around 3.5 min for EMPA and another around ~5.8 min likely from VILDA's oxidation). These peaks were well separated from the main peaks. The majority of EMPA and VILDA remained intact (approx. 90% retained), illustrating that both are relatively stable to oxidative conditions. The method effectively differentiated the slight drop in the main peak areas and the emergence of new tiny peaks, confirming specificity under oxidative stress.

Thermal Degradation:
Dry heat (thermal) stress was conducted by exposing the solid drug mixture to 70 °C for 4 hours. This resulted in minimal degradation: EMPA showed ~6.2% degradation and VILDA ~8.1% degradation. Chromatograms did not show any prominent new peaks aside from the slight reduction in main peak areas, indicating that any thermal degradants were either very minor or not strongly UV-absorbing. A small hump or shoulder was observed in the chromatogram for VILDA's thermal stress at ~7.2 min, possibly a minor degradant, but it did not interfere with the VILDA peak. The stability of both drugs under dry heat means the method primarily just measures the slightly reduced content, which it did accurately.
Photolytic Degradation: Photostability was assessed by exposing samples to direct sunlight for 6 hours. Both EMPA and VILDA were found to be quite stable under photolytic conditions, with degradation extents of only 7.8% (EMPA) and 6.9% (VILDA). The chromatograms were nearly identical to those of non-stressed samples, with perhaps a negligible additional peak or increased baseline in some cases. The method easily quantified the slight decrease in the parent peaks and showed no co-elution issues. Table 4

Table:4 Forced Degradation Summary of EMPA and VILDA under Various Stress Conditions

Sr. No.	Degradation Condition	Drug	Conc. Taken (µg/mL)	Conc. Found (µg/mL)	RT of Degradant (min)	% Recovery	% Degradation
1	Acid	EMPA	75	66.6263	2.5, 2.9	88.84	11.16
		VILDA	300	260.551	6.03, 11.2	86.85	13.15
2	Base	EMPA	75	65.1647	2.04, 2.9	86.89	13.11
		VILDA	300	253.623	3.6, 10.4	84.54	15.46
3	Oxidative	EMPA	75	67.6946	3.5, 5.8	90.26	9.74
		VILDA	300	263.209	7.2, 11.3	87.74	12.26
4	Thermal	EMPA	75	70.359	4.5	93.81	6.19
		VILDA	300	275.810	8.3	91.94	8.06
5	Photolytic	EMPA	75	69.1855	4.5	92.25	7.75
		VILDA	300	279.198	8.3	93.07	6.93

AGREE and GAPI assessment
The greenness of the developed analytical method was evaluated using AGREE and GAPI tools. The AGREE tool provided a quantitative score of 0.73, suggesting strong adherence to green chemistry principles. The GAPI pictogram visually represents the method's sustainability across various stages, with predominant green and yellow zones indicating minimal environmental impact. These results confirm the eco-friendly and sustainable nature of the analytical procedure. shows in figure 4

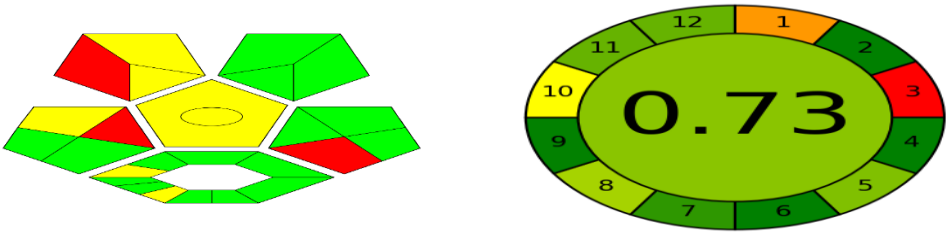

Figure 4. AGREE and GAPI assessment of the analytical method for green chemistry evaluation.

DISCUSSION
The developed RP-HPLC method successfully achieved simultaneous estimation of empagliflozin (EMPA) and vildagliptin (VILDA) with satisfactory chromatographic performance. Well-resolved peaks were obtained at retention times of 4.57 min and 8.48 min for EMPA and VILDA, respectively, without interference from excipients or degradation products, confirming the specificity of the method (Figure 1).
Excellent linearity was observed over the concentration ranges of 25–150 µg/mL for EMPA and 100–600 µg/mL for VILDA, with correlation coefficients exceeding 0.998 (Figures 2 and 3). Precision studies demonstrated %RSD values below 2%, indicating good repeatability and intermediate precision (Tables 1 and 2). Accuracy studies yielded recoveries within 98–102%, confirming the reliability of the proposed method (Table 3).
The method exhibited adequate sensitivity with low LOD and LOQ values and remained unaffected by small deliberate changes in chromatographic conditions, demonstrating robustness. Furthermore, forced degradation studies

revealed that both drugs underwent degradation under stress conditions while maintaining acceptable peak purity and resolution, establishing the stability-indicating nature of the method (Table 4).
The AGREE score of 0.73 and favorable GAPI assessment (Figure 4) further confirmed the environmental sustainability of the analytical procedure. Overall, the validated method is suitable for routine quality control and stability evaluation of EMPA and VILDA in combined pharmaceutical formulations.

CONCLUSION

A robust and reproducible reverse-phase high-performance liquid chromatography (RP-HPLC) method was successfully established and validated for the simultaneous quantification of empagliflozin and vildagliptin in combined (synthetic mixture) formulations. The method utilizes isocratic elution on a C18 column, with a mobile phase composed of water (pH adjusted to 4.5 with 0.01% orthophosphoric acid), methanol, and acetonitrile in a 35:35:30 ratio. The flow rate was maintained at 1.0 mL/min, and detection was performed at 212 nm using a UV detector. Under these chromatographic conditions, empagliflozin and vildagliptin were effectively separated, exhibiting retention times of approximately 4.6 minutes and 8.4 minutes, respectively.
The method is linear over a wide range, with $R^2 \geq 0.998$, and provides high sensitivity (LOD 1.9–2.9 µg/mL). It meets all validation criteria: the results for precision (%RSD < 2%), accuracy (98–102% recovery), and robustness confirm the method's reliability. The specificity is evidenced by the absence of interference from excipients and the clean separation of degradation products in stressed samples.
Forced degradation studies confirmed the method's stability-indicating capability,[21] as it could distinctly resolve parent drugs and degradation products under acid, base, oxidative, thermal, and photolytic conditions. Empagliflozin and vildagliptin exhibited the highest degradation (11–15%) under strong acid/base hydrolysis, with minimal changes (<10%) under other stress conditions. All degradants were well separated from the main analytes, and peak purity analysis verified the absence of co-eluting impurities.
In summary, the validated RP-HPLC method is suitable for routine quality control and stability assessment of empagliflozin and vildagliptin in combined formulations.

Ethical Statement

The present study involved the formulation development and evaluation of Bempedoic acid solid dispersion using pharmaceutical excipients and standard analytical techniques. The study did not involve human participants, animal experimentation, biological samples, or clinical data. Therefore, ethical committee approval was not required. All experimental procedures were carried out in accordance with standard laboratory practices and institutional guidelines.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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Data Availability Statement

The data generated and analyzed during the current study are included within this published article. Additional information supporting the findings of this study is available from the corresponding author upon reasonable request.

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