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Research article

Evaluation of analytical greenness metric for an eco-friendly method developed through the integration of green chemistry and quality-by-design for the simultaneous determination of Nebivolol hydrochloride, Telmisartan, Valsartan, and Amlodipine besylate

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ABSTRACT

In recent years, the field of analytical chemistry has witnessed a notable shift towards the adoption of greener chromatographic methods, aiming to minimize the environmental impact. An effective strategy involves substituting conventional harmful organic solvents with environmentally friendly alternatives, reducing the use of hazardous chemicals that contribute to environmental concerns. However, separating drug substances without the use of buffers and organic solvents presence is a big challenge. To overcome this challenge, a combination of quality-by-design (QbD) and green analytical chemistry (GAC) was employed in this study for method development. A high-performance liquid chromatography (HPLC) method was successfully developed and validated for the simultaneous determination of Nebivolol hydrochloride, Telmisartan, Valsartan, and Amlodipine besylate. The method utilized a mobile phase composed of a mixture of 0.1 % formic acid in water (pH: 2.5) and ethanol. A regular octadecyl silica (ODS) column was employed, and UV detection at 220 nm was utilized. The method exhibited linearity within the concentration range of 25–75 μg/mL for Telmisartan and 150–450 μg/mL for Nebivolol Hydrochloride, Valsartan, and Amlodipine besylate and the correlation coefficient was greater than 0.999 for all the analytes. Limits of detection (LOD) and quantification (LOQ) were determined as 0.01 and 0.04 μg/mL for Telmisartan, 0.06 and 0.20 μg/mL for Nebivolol Hydrochloride, 0.08 and 0.25 μg/mL for Amlodipine besylate, and 0.14 and 0.46 μg/mL for Valsartan, respectively. The developed method underwent thorough validation, encompassing various parameters such as linearity, accuracy, precision, LOD, LOQ, robustness, and ruggedness. The mean recovery values were observed to range between 98.86 % and 99.89 %. The accuracy demonstrated was consistently above 98.98 % for both intra-day and inter-day precisions were with the relative standard deviations less than 2 %. To establish its robustness, a quality-by-design-based experimental design (DoE) approach was implemented. Additionally, the method's environmental friendliness was evaluated using the Analytical Greenness metric (AGREE) an analytical eco scale, both confirming its alignment with sustainable practices and reduced ecological impact. The sustainability of the solvent used in the current study was

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evaluated by Green Solvents Selecting Tool (GSST) Further, the developed method greenness was evaluated with the green analytical tools such as Analytical method greenness score (AMGS) and using the recently released White Analytical Chemistry (WAC) using RGB assessment tool. By employing this greener approach to chromatography method, this study contributes to the ongoing efforts in analytical chemistry to promote sustainable practices and minimize the environmental footprint of analytical methods.

1. Introduction

The primary objective of this study is to develop a chromatographic method that is both environmentally friendly and reliable for the simultaneous estimation of four essential drug substances. Nebivolol Hydrochloride (NEB) and Amlodipine besylate (AML), which fall under the beta and calcium channel blocker classes, along with Telmisartan (TEL) and Valsartan (VAL) that serve as angiotensin II receptor antagonists. These medications are frequently prescribed for various cardiovascular conditions, such as high blood pressure, increased heart rate, and low heart rate. AML, widely used for hypertension, acts as a calcium channel blocker, whereas NEB, a preferred choice for heart failure patients, not only functions as a beta-blocker but also stimulates the production of nitric oxide. Combination therapy involving AML and NEB is commonly employed in hypertension treatment to achieve optimal blood pressure control. Moreover, TEL and VAL are prescribed for hypertension and cardiovascular-related ailments as they counteract the hormone responsible for narrowing blood vessels and elevating blood pressure. The anticipated expansion of the market for these drugs is attributed to factors like aging populations and rising obesity rates. The current literature studies highlight the absence of a single or universally accepted eco-friendly method for simultaneously estimating these four compounds. Previous studies often relied on high concentrations of organic solvents and inorganic salts [1–10]. Hence, this research focuses on the development of a high-performance liquid chromatographic (HPLC) method that excels in terms of speed, reliability, accuracy, and efficiency.

The field of green analytical chemistry (GAC) has emerged in response to the need for analytical methods that prioritize the reduction of hazardous material usage, waste generation, and energy consumption. GAC aims to mitigate the environmental impact of analytical techniques while maintaining the integrity of results. In the current context of increasing environmental concerns, the development of green analytical methods has gained utmost importance. These innovative methods involve the use of alternative solvents such as water, ethanol, and non-toxic, non-volatile substances. They also emphasize the optimization of solvent usage and waste generation. The benefits of implementing green analytical methods are diverse, encompassing reduced environmental impact, enhanced safety for laboratory personnel, and diminished waste disposal costs. Moreover, the utilization of such methods leads to more precise and accurate results by mitigating the formation of interfering compounds associated with hazardous solvents. Within field of the analytical science, the concept of green chemistry holds substantial significance as it enables the reduction or elimination of hazardous organic solvents in laboratory practices [11,12]. Extensive efforts have been made to advocate for the adoption of green solvents or chemicals in analytical methods, resulting in the development of eco-friendly approaches that decrease analysis expenses. The minimization of hazardous solvents and reagents usage contributes not only to environmental preservation but also to the safeguarding of human health [13–15]. Green chemistry, defined as the application of a set of principles to diminish or eradicate the use or generation of hazardous substances in chemical product design, manufacture, and application, serves as the guiding force in this transformative endeavor [15,16]. A green solvent is a chemical solvent that aligns with principles of green chemistry. Providing a concise definition is challenging, qualified experts generally agree that a certified green solvent should exhibit the characteristics such as Low Health Risk, High Safety and Minimal Environmental Impact.

In order to get the more information about sustainable solvents Green Solvent Selection Tool (GSST), a free online tool available at this link: http://green-solvent-tool.herokuapp.com/. The composite sustainability score, G, represents the overall sustainability of the solvent. The G value serves as an indicator of a solvent's environmental impact. A lower G value suggests that the solvent's features are not sustainable, while a higher G value indicates that the solvent is more environmentally friendly. The scale for G spans from 1 to 10 [17,18].

Design of Experiments (DOE) stands as a necessary statistical tool in the field of analytical science, enabling the optimization of analytical methods through the systematic variation of experimental conditions. Its fundamental purpose lies in identifying the ideal combination of factors that yield desired outcomes. By the controlling power of DOE, critical process parameters and their interactions, that will have significant influence on method performance, can be elucidated. This empowers analysts to fine-tune the method, thereby enhancing the reliability and accuracy of the obtained results, while concurrently reducing the number of experiments required. The importance of DOE is evident in the guidance provided by esteemed organizations such as the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) and the United States Pharmacopeia (USP). These entities offer valuable recommendations for the development and validation of analytical methods in the pharmaceutical industry, underlining the significance of DOE in achieving robust and reliable methods. The effectiveness of DOE has been extensively documented in various studies and publications [19–26], showcasing its efficacy in evaluating the robustness of analytical methods and explaining their development processes.

The combination of green chemistry principles and Design of Experiments (DOE) provides a comprehensive and collaborative approach to the development of sustainable chemical processes [26–29]. The integration of DOE methods brings further advantages by streamlining the process optimization journey. Through systematic experimentation and analysis, DOE minimizes the number of trials required, effectively conserving valuable resources, time, and energy. In this study, the developed method underwent rigorous

validation encompassing critical parameters such as linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness, and ruggedness. To establish the method's robustness, a Quality by Design (QbD)-based experimental design approach was meticulously employed. This approach ensures that the method can withstand variations in experimental conditions and still yield reliable and consistent results. Beyond its technical performance, the environmental friendliness of the method was also a important point of evaluation. This assessment was carried out using the Analytical Greenness metric (AGREE) which independently affirmed the method's eco-friendly nature. By satisfying these criteria, the method not only fulfills its analytical objectives but also contributes to the broader objective of sustainability in chemical processes. The AMGS calculator, accessible on the American Chemical Society (ACS) website [30–40], served as the second tool for assessing the environmental impact of the methods. For each method, relevant data such as flow rate, analysis run time, mobile phase composition, and the type and quantity of solvents used in sample preparation were input into the AMGS calculator. Furthermore, the recently released White Analytical Chemistry (WAC) was designed to investigate the whiteness properties of the developed method. White Analytical Chemistry (WAC) serves as both a supplement and an extension to Green Analytical Chemistry (GAC). WAC combines environmental, analytical, and practical viewpoints, offering 12 principles as an alternative to the 12 GAC principles. An easy-to-use algorithm based on the RGB (Red-Green-Blue) model evaluates analytical procedures. The three colors represent different aspects, Red (R) Analytical efficiency (scope, accuracy, precision), Green (G) Ecological impact (waste, energy consumption), Blue (B)Practical economic efficiency (cost-effectiveness). By integrating the effects of the three color groups (RGB), WAC illustrates the analytical method's whiteness. This representation considers the uniformity and combined impact of the analytical process, incorporating environmental and practical aspects [41–46]. Overall, the combination of green chemistry and DOE presents a powerful strategy for the development of sustainable analytical methods, which is demonstrated by the successful validation, robustness assessment, and environmental evaluation of the method employed in this study.

2. Materials and methods

2.1. Reagents, samples and standards

For the mobile phase, diluent, and sample solution preparations, analytical reagent-grade absolute ethanol with a purity of 99.8 %, manufactured by Honeywell Specialty Chemicals Seelze GmbH, Hannover Germany, was utilized. Additionally, formic acid manufactured by Merck KGaA, Darmstadt, Germany, was employed. Milli-Q water from Millipore systems (division of Merck KGaA, Darmstadt, Germany) was obtained for the required aqueous component. All the standards used in the study were provided by United States Pharmacopeia India Private Limited and the samples AML, NEB, VAL, and TEL were obtained from United states pharmacopeia India Pvt Ltd, having an assay value of 99.95 %, 100.21 %, 99.85 %, 99.79 % respectively in agreement with the compendial methods.

2.2. Marketed formulation

Amip-5 manufactured by Cipla, Telma 40 manufactured by Glenmark pharmaceuticals, Nebicard 5 mg and VALZAR 80 mg manufactured by Torrent pharma are used.

2.3. Instrumentation

An Agilent Infinity HPLC system was employed in this study. The system comprised an Agilent 1260 (Santa Clara, CA, United States) Infinity model quaternary pump, capable of operating within a range of 0–600 bar. A sample manager with a flow-through needle and a Photo-Diode Array (PDA) detector were also integrated into the system for all chromatographic separations. The instrument was controlled using Empower 3 software (Waters.

Alliance, Milford, MA, United States). During method development, columns with various dimensions and chemistries, including C18 and C8, were utilized. Ultimately, a Waters Xbridge Shield RP18 column with dimensions of 100 × 4.6 mm and a particle size of 3.5 μm (Waters Corporation, Milford, MA, United States) was chosen for the development and subsequent analysis. To study the impact of chromatographic parameters such as buffer strength, column temperature, and flow rate on the developed method, Design Expert version 8.07.1 software from Stat-Ease, Inc., Minneapolis, USA, was employed. This software facilitated the investigation and optimization of the chromatographic parameters.

2.4. Chromatographic conditions

Chromatographic separations were performed using an HPLC system equipped with a Photo-Diode Array (PDA) detector, which had a wavelength range of 190–400 nm with a focus on 220 nm. The stationary phase employed was a Waters X-Bridge Shield RP18 column with dimensions of 100 × 4.6 mm and a particle size of 3.5 μm. During the analyses, the flow rate was maintained at approximately 1.0 mL/min, while the column temperature was set to 40 °C to ensure optimal separation. The mobile phase consisted of a mixture of 0.1 % formic acid and ethanol, with a ratio of 40:60. This mobile phase composition facilitated the elution and separation of the target compounds.

3. Mobile phase preparations

5.1. Mobile phase: 0.1 % formic acid in water (pH:2.5) and ethanol in the ratio 40:60

Table 1
Design summary and results.

Factor	Name		Units	Minimum	Maximum	Mean	Std Dev	
A	Flow rate		mL/min	0.8	1.2	1	0.2	
B	Column Temp		°C	30	50	40	5	
C	Mobile phase		%	35	45	40	10	
Std	ID	Run	Flow rate	Mobile Phase	Column temp	Resolution (TEL, AML)	Resolution (AML, NEB)	Resolution (NEB, VAL)
9	5	1	0.8	35	50	7.97	5.38	14.38
12	6	2	1.2	35	50	7.15	4.87	13.46
7	4	3	1.2	45	30	3.97	4.46	7.47
1	1	4	0.8	35	30	11.45	8.15	11.45
3	2	5	1.2	35	30	10.51	7.61	10.22
13	7	6	0.8	45	50	3.05	2.93	9.16
17	0	7	1.0	40	40	6.83	5.56	11.14
11	6	8	1.2	35	50	7.13	4.90	13.50
14	7	9	0.8	45	50	3.04	2.92	9.18
6	3	10	0.8	45	30	4.78	5.13	8.36
15	8	11	1.2	45	50	2.56	2.54	8.26
16	8	12	1.2	45	50	2.54	2.53	8.25
4	2	13	1.2	35	30	10.53	7.64	10.21
10	5	14	0.8	35	50	8.01	5.38	14.39
5	3	15	0.8	45	30	4.61	4.93	8.25
2	1	16	0.8	35	30	11.42	8.11	11.31
8	4	17	1.2	45	30	3.96	4.44	7.44

5.2. Diluent: Prepared a Mixture of Ethanol and water in the ratio 80:20

6. **System suitability and standard solution:** To prepare the standard stock solutions, suitable quantities of AML, NEB, VAL, and TEL standards were accurately weighed i.e. A weight of 10 mg of TEL and 60 mg each of AML, VAL, and NEB were transferred into a 20 mL volumetric flask dissolved, diluted upto the mark. Subsequently, 1 mL of this stock solution was further diluted to 10 mL to achieve a concentration of approximately 50 μg/mL for TEL and 300 μg/mL for each of AML, NEB, and VAL. The diluent used was compatible with the standards and the chosen analytical method and ensured proper dissolution and homogeneity of the solutions before proceeding with further analyses.
7. **Assay Preparations:** In order to conduct the assay, appropriate amounts of AML, NEB, VAL, and TEL standards were accurately weighed. These weighed standards were then dissolved in a diluent to achieve a solution with a concentration of approximately 50 μg/mL for TEL and 300 μg/mL for each of AML, NEB, and VAL. Ensure complete dissolution and uniformity of the solutions before proceeding with the assay.
8. **Assay preparation for marketed formulation** To Evaluate content of TEL, AML, NEB, VAL in the commercially available tablets (Amlip-5 from Cipla, Telma 40 from Glenmark pharmaceuticals, Nebicard-5, Valzaar-80 from Torrent pharma), twenty tablets were weighed separately to determine the average weight and finely powdered. Weighed equivalent to 50 mg of TEL into a into a 100 mL volumetric flask containing 70 mL diluent, sonicated for 30 min and diluted upto 100 mL with diluent, centrifuged at 3000 rpm for 5 min. Collected 1 mL of the Supernatant solution and further diluted to 10 mL in diluent, the resultant solution contains 50 μg/mL for TEL. Similarly weighed equivalent to 30 mg of AML, NEB and VAL into a into a separate 100 mL volumetric flask containing 70 mL diluent, sonicated for 30 min and diluted upto 100 mL with diluent, centrifuged at 3000 rpm for 5 min. Used the supernatant solution which contains 300 μg/mL for each of AML, NEB, and VAL used for the analysis. Hence this was successful for marked formulation.
9. **Method validation:** Validation of an analytical method is a crucial step in ensuring its reliability, accuracy, and precision. In this particular study, the developed method underwent a comprehensive validation process, including assessments for system suitability, linearity, accuracy, specificity, limit of detection (LOD), limit of quantification (LOQ), and robustness. Robustness evaluation involved deliberate modifications of experimental conditions, with a focus on key parameters such as flow rate, temperature, and gradient composition that were observed to significantly influence the separations during preliminary method development. To systematically investigate robustness and the interaction effects of these variables on the method, a multi-dimensional design space was constructed using Design Expert software. A full factorial design approach was employed and obtained results were tabulated in Table 1, to establish mathematical models and define an acceptable range of method performance based on experimental results. Critical performance parameters, including resolution and tailing factor, were used to set limits within the design space. A total of 17 experiments were conducted based on the design, and essential performance parameters such as resolution and tailing were calculated for each experiment. To examine the impact of flow rate, it was varied by ±10 % from the original 1.0 mL/min, resulting in flow rates of 0.8 mL/min and 1.2 mL/min. Similarly, the temperature range of 30 °C–50 °C was explored, and the mobile phase composition was adjusted from 35 to 45 to evaluate the effect of column temperature and mobile phase on the developed method. The results of these robustness studies offer valuable insights into the performance of the analytical method and can guide future optimization efforts for different applications. By

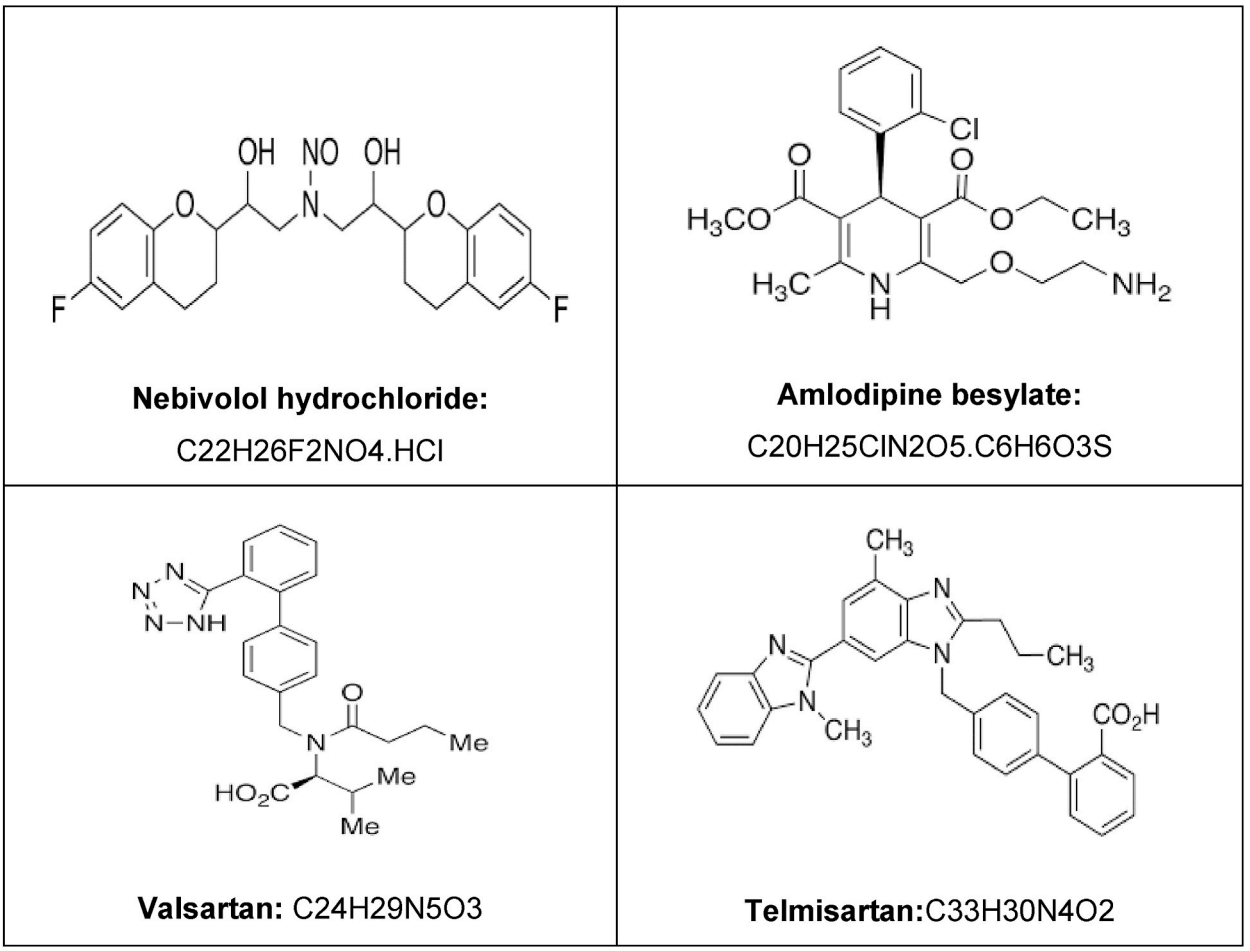

Fig. 1. Structure, chemical name, and molecular formula.

systematically assessing and understanding the impact of various parameters, the developed method can be fine-tuned to ensure consistent and reliable results in diverse analytical scenarios.

4. Results and discussions

4.1. Method development

The objective of this research is to develop a robust and environmentally friendly HPLC method for the simultaneous determination of AML, NEB, VAL, and TEL. Official reported methods having environmental incompatible mobile phases with the longer run times therefore aimed to develop a single method with lesser runtime using sustainable solvents to determine the selected compounds. These compounds possess ionizable groups, AML with a basic nitrogen atom that can be protonated to form a positively charged ammonium ion. NEB contains a tertiary amine group that can also be protonated to form a positively charged ammonium ion. VAL and TEL, on the other hand, contain carboxylic acid groups that can be ionized to produce negatively charged carboxylate ions. The pKa values of all analytes fall within the range of 4.7–9.2. Initial screening experiments were conducted using different buffers, including ammonium acetate, formic acid, and triethylamine, covering pH regions from 2.0 to 7.0, based on the pKa values of the analytes. After careful evaluation, formic acid was selected as the preferred buffer due to its characteristics as a weak organic acid with a pKa value of approximately 3.75. Various reverse phase columns such as C18, C8, and Cyno were evaluated for the separation of analytes. Based on observations, the C18 column was deemed the most suitable, as it is a commonly used reversed-phase column known for its ability to achieve good separation of compounds, including those with ionizable groups. Consequently, the method development continued using the C18 column (see Fig. 1).

To align with the goal of developing a green HPLC method, ethanol was chosen as the organic solvent. Ethanol is considered generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA) and is widely used in the pharmaceutical industry as a solvent and excipient. In comparison to other organic solvents like methanol or acetonitrile, ethanol exhibits lower toxicity and presents fewer health and environmental risks. Moreover, ethanol has high solubility for a wide range of polar and ionizable

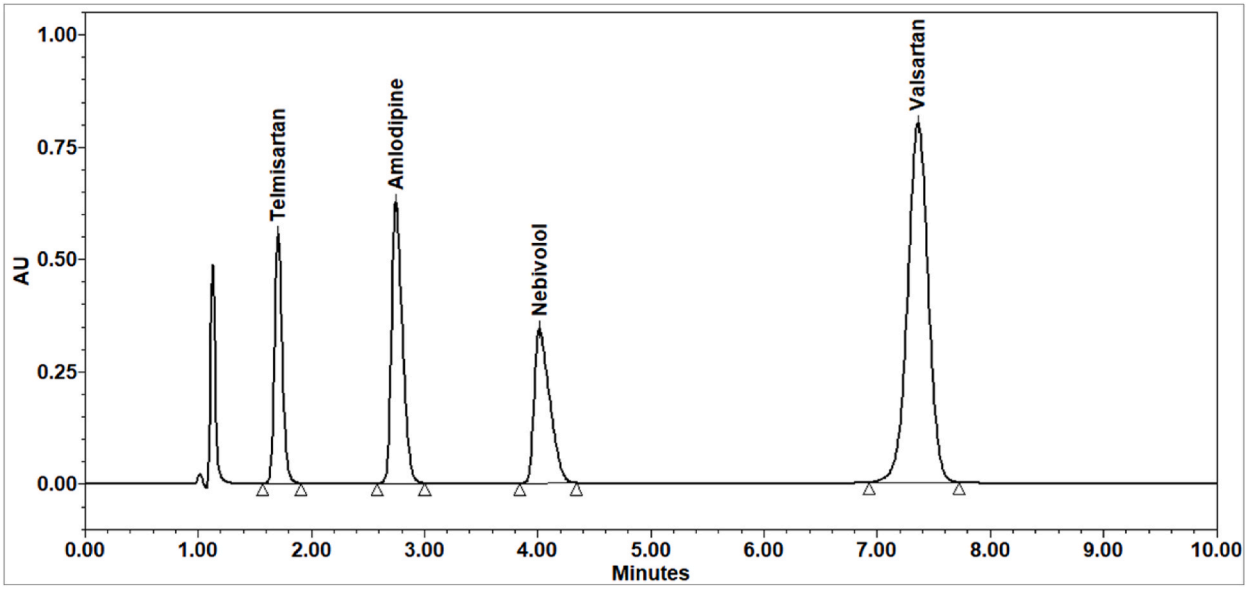

Fig. 2. Standard solution chromatogram from final conditions.

Table 2
System suitability results from standard solution.

Name	Retention Time	USP Resolution	USP Tailing	USP Plate Count	%RSD
Telmisartan	1.707		1.19	5125	0.28 %
Amlodipine	2.747	6.67	1.39	5785	0.22 %
Nebivolol	4.018	5.62	1.63	6297	0.19 %
Valsartan	7.362	11.05	0.98	7965	0.46 %

Table 3
Summary of validation results.

Parameter	TEL	AML	NEB	VAL
Linearity and Range (%)	50–150 %	50–150 %	50–150 %	50–150 %
Slope	31097		32655	100507
Intercept	−349207	−31337	−2332.7	−17603
Correlation-coefficient	0.9995	0.9991	0.9994	0.9994
Accuracy at 50 (%) Level				
% Recovery	98.86	99.65	99.89	99.48
% RSD	0.42	0.22	0.28	0.35
Accuracy at 100 (%) Level				
% Recovery	99.42	99.58	98.98	99.68
% RSD	0.21	0.33	0.48	0.71
Accuracy at 150 (%) Level				
% Recovery	99.81	99.24	99.67	99.39
% RSD	0.35	0.22	0.42	0.51
Intermediate Precision				
% Assay	99.52	99.68	99.51	99.79
%RSD	0.37	0.39	0.51	0.22
Specificity				
Purity1 Threshold	2.015	2.011	2.027	2.016
Purity1 Angle	0.079	0.157	0.946	0.388
LOD and LOQ				
Quantitation Limit (μg/mL)	0.01	0.06	0.08	0.14
Detection Limit (μg/mL)	0.04	0.20	0.25	0.46

compounds, making it a suitable solvent. When added to the mobile phase, ethanol can also act as an ion-pairing agent, aiding in the retention and separation of ionizable analytes on a reversed-phase column. Ethanol, water and formic acid were used in the mobile phase preparation. The composite G-score for water, ethanol and formic acid are 7.3, 6.6 and 5.9 respectively, higher value of G (closer to 10) suggests that the solvent is desirable in terms of sustainability and greenness This score is calculated by assessing several

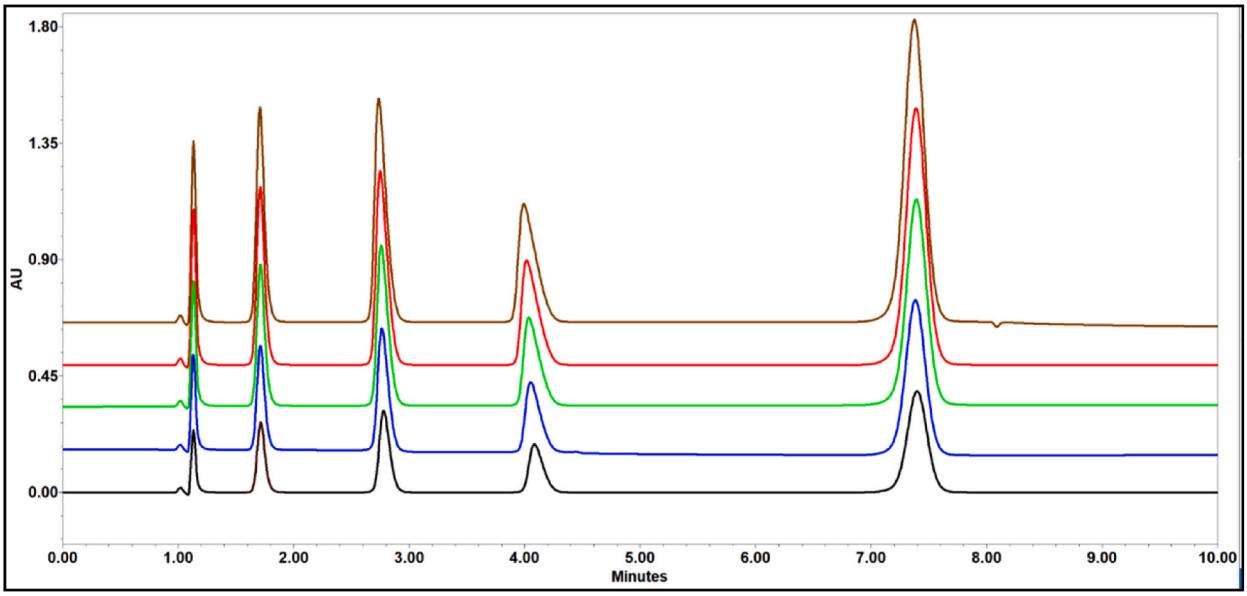

Fig. 3. Chromatograms from linearity standard solution.

parameters, such as biodegradability, toxicity, and potential for bioaccumulation. The method conditions described in the chromatographic conditions section were finalized based on the initial screening findings. The chromatogram presented in Fig. 2 demonstrates well-separated analytes with satisfactory peak shape and resolution. The method was further optimized to achieve these results within a 10-min runtime.

4.2. Method validation

4.2.1. System suitability

To verify the performance and consistency of the chromatographic system, system suitability measurements were conducted. Six replicates of standard solutions were analyzed, and the obtained results were tabulated in Table 2. Various parameters, including resolution between adjacent peaks, tailing factor, theoretical plates, and the percentage of relative standard deviation (% RSD), were calculated. The summarized outcomes of these measurements are presented in Table 2.

4.2.2. Specificity

To verify the specificity of the chromatographic method, peak purity analysis was conducted using a PDA detector. The UV spectra of all analytes were examined, and it was observed that the purity threshold exceeded the purity angle, indicating that all peaks were pure. A summary of these results was compiled and presented in Table 3.

4.2.3. Precision and intermediate precision

The precision of developed analytical method is evaluated using %RSD (relative standard deviation), a widely used metric. %RSD is calculated by dividing the standard deviation of replicate measurements by the mean and expressing it as a percentage. In this study, % RSD was determined by performing six replicate injections of the standard solution. The calculated %RSD values for AML, NEB, VAL, and TEL were below 2.0 %, which is generally considered acceptable for analytical methods. Additionally, from the intermediate precision studies %RSD values around 0.25 % for the peak areas of AML, NEB, VAL, and TEL. These results demonstrate that the developed method exhibits precision and reproducibility. All the results were tabulated in Table 3.

4.2.4. Accuracy

The accuracy of the developed method was evaluated through recovery studies, which involve measuring the method's ability to accurately recover known amounts of analyte from a sample matrix. In this study, the recovery percentage of AML, NEB, VAL, and TEL was determined and found to be within the range 100 ± 2.0 % These values fall well within the acceptance criteria, indicating that the method exhibits good accuracy. Additionally, the low percent RSD values obtained in the recovery studies demonstrate the method's reproducibility and further support its accuracy. A detailed summary of these findings can be found in Table 3.

4.2.5. Linearity

As linearity studies play a crucial role in method validation as they assess the relationship between the concentration of analytes and corresponding response of the analytical method. the linearity of the current assay method was evaluated using a standard solution

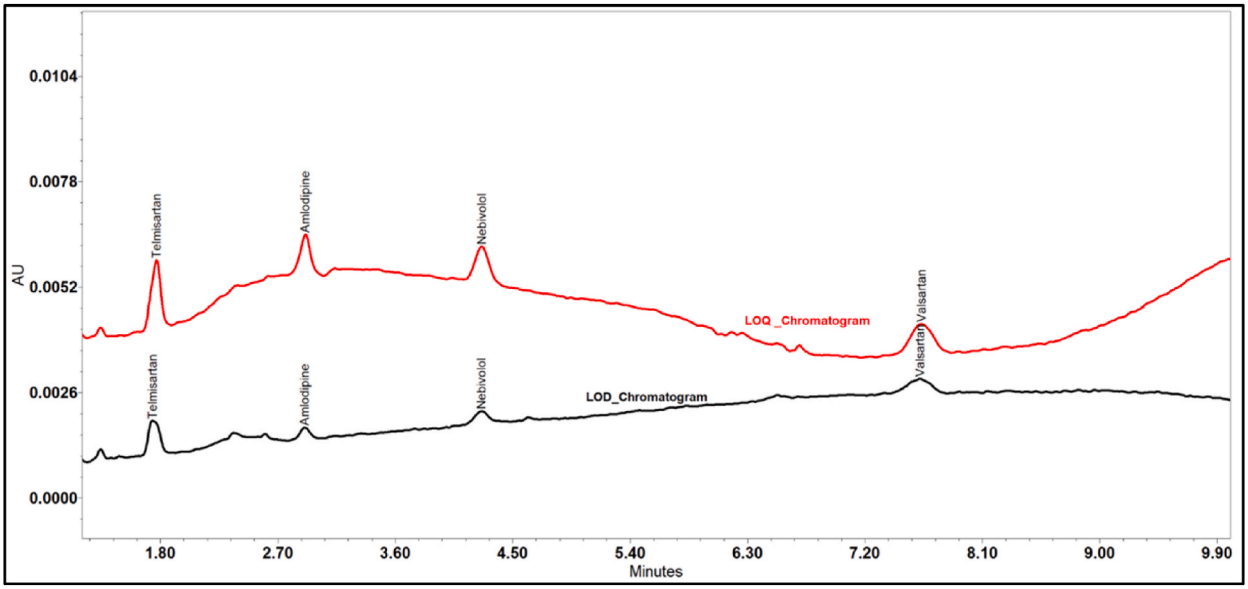

Fig. 4. Chromatograms from LOD and LOD standard solution.

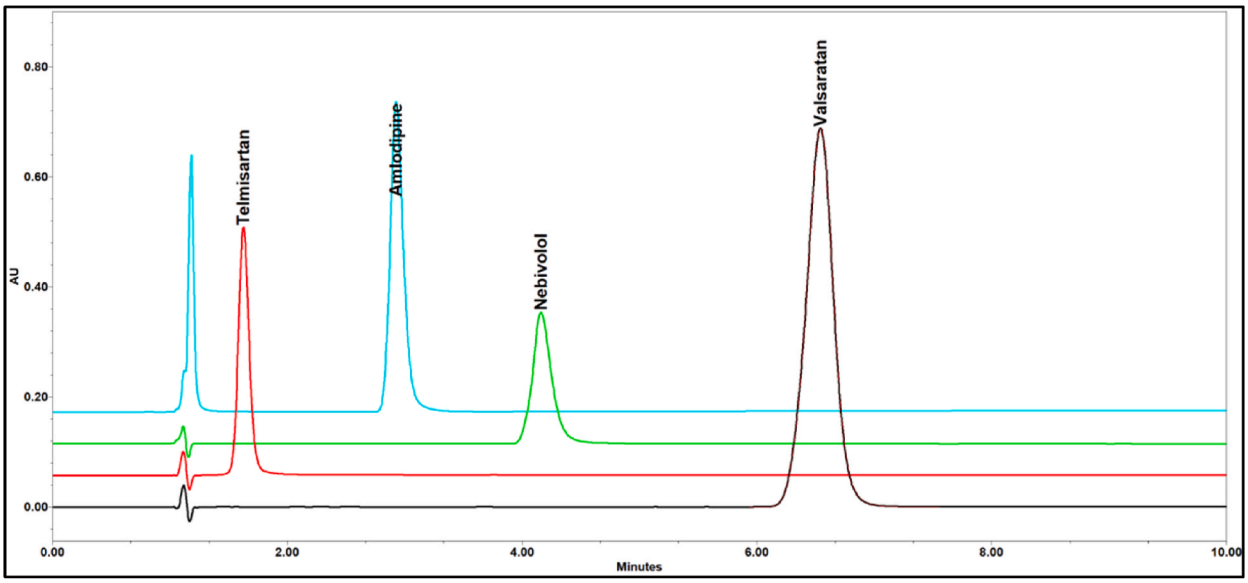

Fig. 5. Chromatograms from marketed formulation samples.

containing TEL concentrations ranging from 25 to 75 μg/mL and AML, NEB, and VAL concentrations ranging from 150 to 450 μg/mL. The obtained linear calibration plot demonstrated a high correlation coefficient exceeding 0.999, indicating a strong and reliable linear relationship between the analyte concentration and the method response. The results of the linearity study, including correlation coefficients, are presented in Table 3 The chromatogram of linearity, LOD and LOQ presented in Fig. 3 and 4.

4.2.6. Analysis on marketed formulation

We have conducted an analysis using commercially available tablets (specifically Amlip-5, Telma 40, Nebicard-5, and Valzaar-80) as real samples. we successfully determined the content of TEL, AML, NEB and VAL in these formulations. The results validate the practical applicability of our method, The chromatogram of individual formulations were presented in Fig. 5 and the Assay results were tabulated in Table 4.

Table 4
Summary of marketed formulations.

Parameter	TEL	AML	NEB	VAL
% Assay	100.16	99.73	99.15	99.98

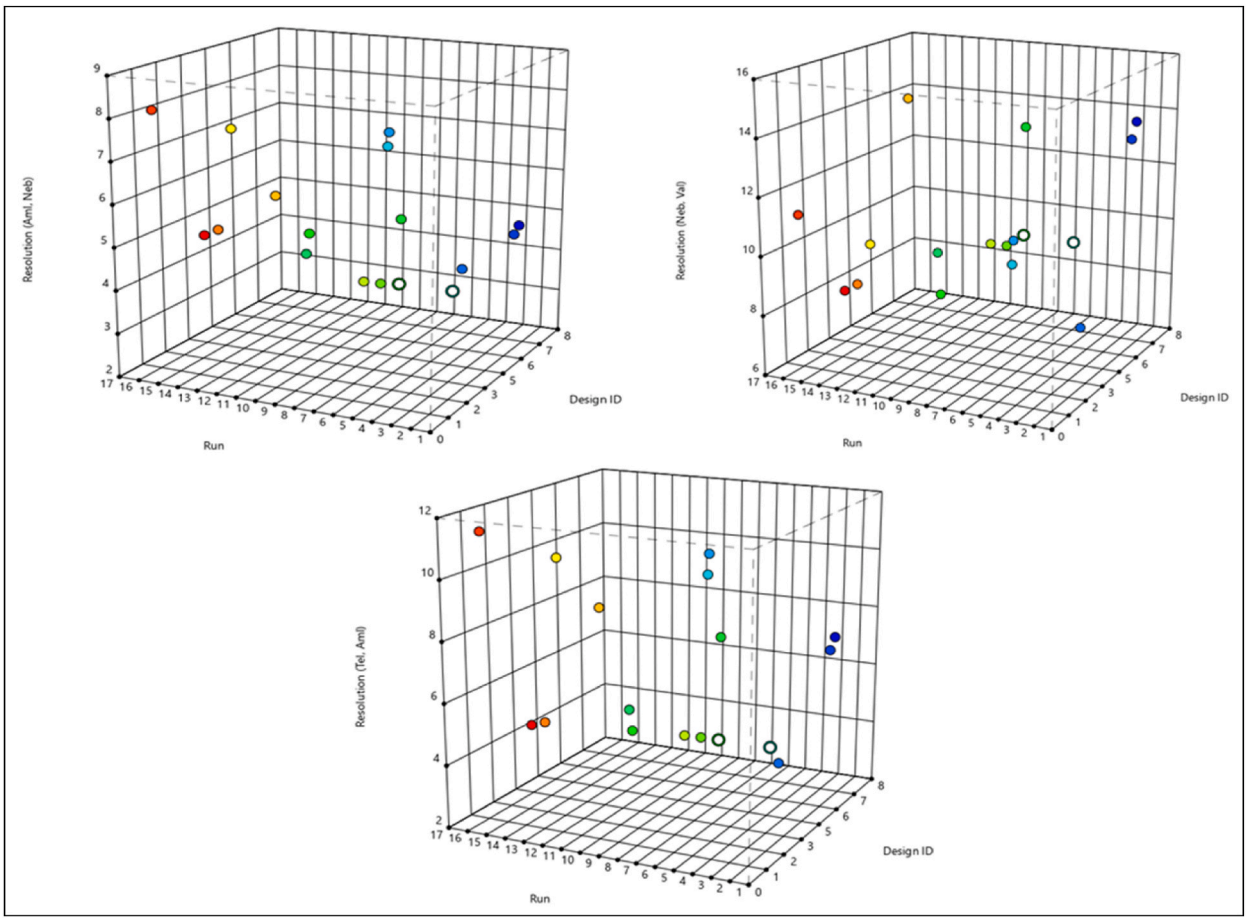

Fig. 6. Resolution obtained from robustness studies.

Table 5
ANOVA results for response (resolutions) obtained from experimental design.

	Model	A	B	C	AB	AC	BC	ABC	Prob > F
Resolution (TEL, AML)		−0.37375	−2.85375	−1.23625	0.0675	0.0375	0.47	0.02125	Significant
p-values	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	0.0107	<0.0001	0.0973	
F-value	11193.54	1090.26	63562.06	11928.30	35.56	10.98	1724.10	3.52	
Resolution (AML, NEB)	5.12	−0.24625	−1.385	−1.18875	0.00375	0.025	0.18375	0.0225	Significant
p-values		<0.0001	<0.0001	<0.0001	0.7821	0.0930	<0.0001	0.1245	
F-value	2848.33	351.81	11160.58	8221.83	0.0818	3.64	196.45	2.95	
Resolution (Neb, VAL)	10.3306	−0.479375	−2.03438	0.991875	0.038125	0.024375	−0.575625	−0.040625	Significant
p-values	<0.0001	<0.0001	<0.0001	<0.0001	0.0114	0.0703	<0.0001	0.0083	
F-value	5959.72	1685.64	30358.24	7216.53	10.66	4.36	2430.49	12.11	

5. Design of experiments for robustness study

To rigorously evaluate the robustness of our developed method, we employed the Design Expert software to construct a meticulous mathematical model. Utilizing a comprehensive 3 factorial experimental design, about 17 carefully planned experiments, we systematically varied the levels of three critical factors: flow rate, temperature, and mobile phase composition. Our primary focus was on

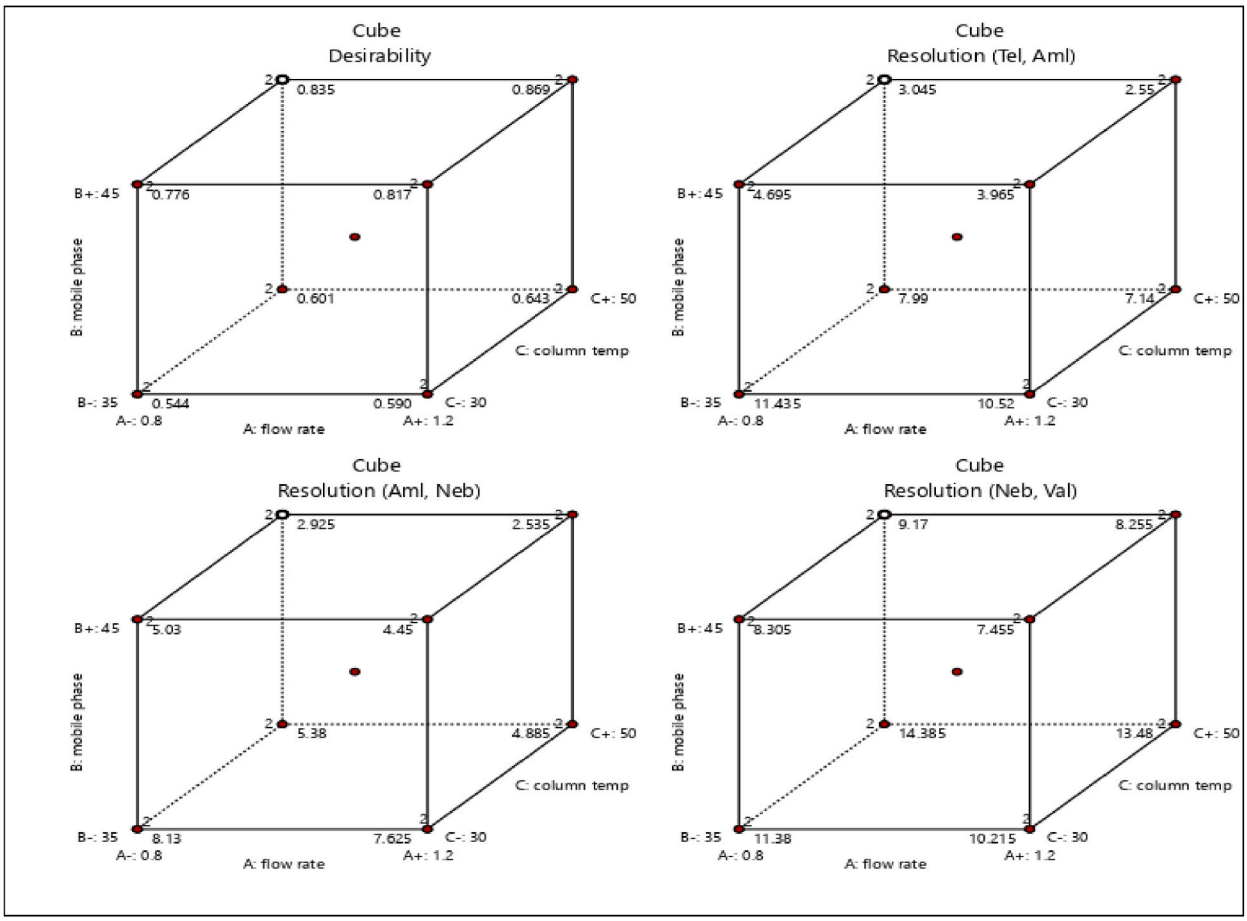

Fig. 7. Desirability and resolution between all 4 components as a response from the robustness studies.

achieving a resolution between TEL and AML, ensuring it remained below the predetermined threshold of 2.0. Throughout the experimental design, meticulous attention was giving to considering and controlling potential uncontrolled variables that could impact the experimental outcomes. The outcomes of our experiments were truly promising, as we observed resolutions exceeding the designated threshold of 2.0 in all instances. Even the lowest observed resolution reached an impressive value of 2.5, further validating the robustness of our method. Additionally, the consistently low tailing factor, remaining below 1.8 across all experiments, served as a testament to the reliability and stability of our method. To clearly present our findings, we portrayed the obtained results in an illustrative pictorial form, which is attractively depicted in Fig. 6. Furthermore, we subjected the data to rigorous statistical analysis employing the renowned Analysis of Variance (ANOVA) method. The results of this analysis unveiled the exceptional performance of our model, as evidenced by the attainment of a "P value $>$ F″ and a p-value below the critical threshold of 0.05. For complete insights into our findings, we request to refer the detailed summary available in Table 5. This tabulated representation serves as a valuable resource, encapsulating the key results and enabling a comprehensive understanding of the method's robustness. Furthermore, the contour plots provided invaluable information on critical parameters such as resolution, flow rate, temperature, and mobile phase composition, which are essential attributes of our method. By employing color-coded regions, we enabled a visual representation of the operational range, vividly illustrating higher resolution areas in striking red and poorer resolution areas in dull blue. Examining these contour plots, a distinct pattern emerged, presenting a remarkable increase in resolution as the working region transitioned from blue to red. To further confirm the robustness of our method, we turned to cube plots, which served as an additional verification to its reliability. These plots articulately demonstrated the independent effects of the three variables on resolution, setting our confidence in the method's stability. Notably, every single resolution response exceeded our initial predictions, reinforcing the notion of a highly robust method. To extend our understanding of the relationships between flow rate, temperature, and mobile phase composition, we thoroughly reviewed 3D-response surface data. This comprehensive dataset emphasized the substantial impact of these variables on

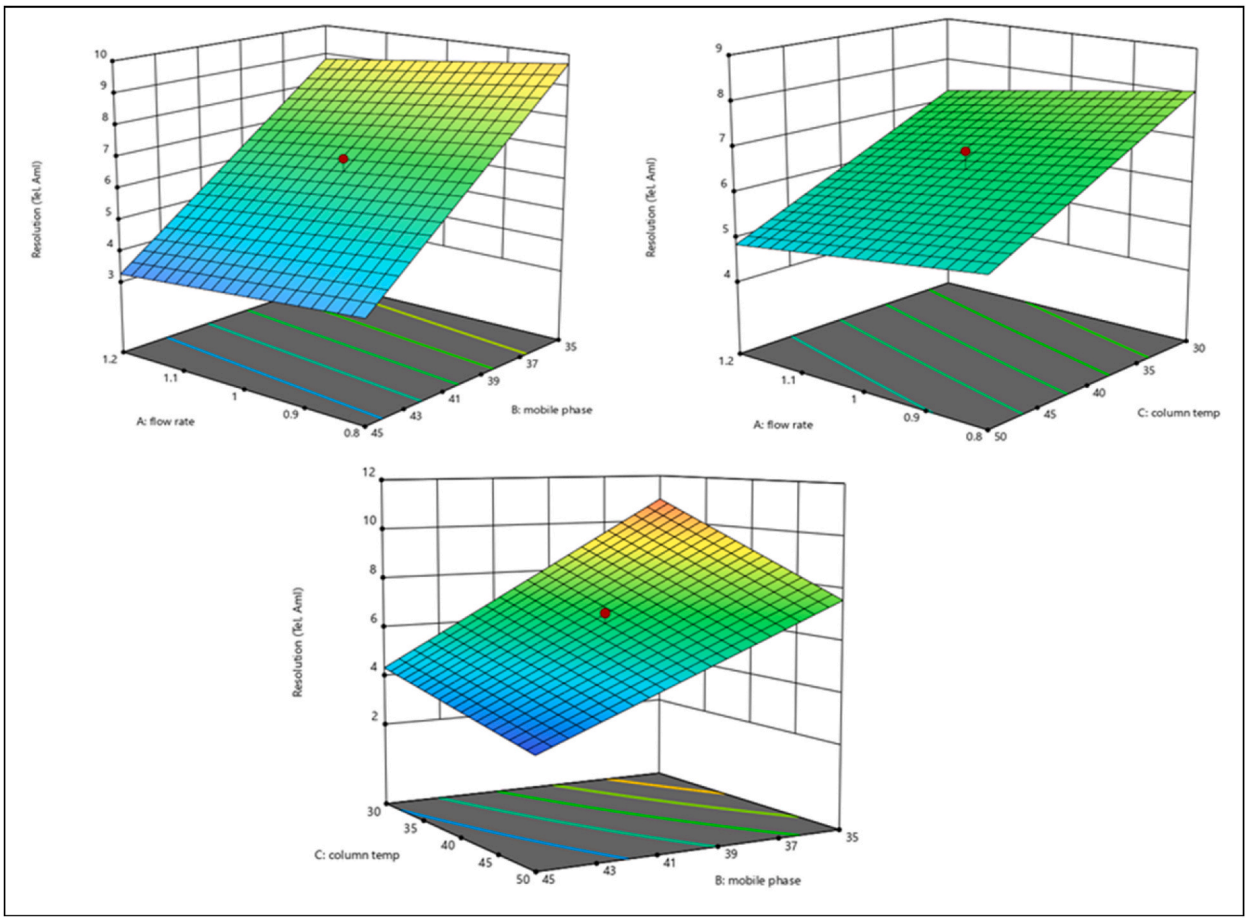

Fig. 8. Response surfaces obtained for resolution maintaining one variable at the central level.

resolution and provided valuable insights into the working region. For a visual representation of these insights refer to Figs. 7-9, which contains cube plots, 3D surface response and the contour plots, focusing on all resolutions while considering flow rate and temperature as critical method parameters. In summary, our comprehensive analysis of the contour plots, cube plots, and 3D-response surface data explicitly establishes the reliability and versatility of our method. Moreover, these visualization techniques played a pivotal role in calculating the method's desirability, a powerful metric that estimates its overall effectiveness. Notably, the obtained desirability value, which remains close to 1 Fig. 10, stands as a strong evidence to the exceptional robustness and efficacy of our method. The concept of "lack of fit" in regression modeling refers to the discrepancy between the model's predicted values and the actual observed data. It serves as an assessment of whether the model adequately captures the true underlying relationship between the predictor variables and the response variable. In the current study obtained lack of fit values are 1.17, 2.95, and 0.11. Since these values are greater than the significance level of 0.05, they are not statistically significant. Consequently, we can assume that the model is a good fit for the observed data.

6. Assessment of the environmental impact and sustainability features

In our research, we employed the innovative Analytical Greenness metric (AGREE) to meticulously assess the degree of greenness embodied by our advanced chromatographic method. To precisely quantify the method's eco-friendliness, we employed the Analytical Eco-Scale tool, which well assigned points to each distinct category of the method. Through a splendid visual representation, we revealed the eco-friendliness of our method using a vibrant pictogram with 12 essential elements. These elements gracefully encompassed critical process criteria, including sample preparation, solvent and reagent properties, hazardous materials usage, power

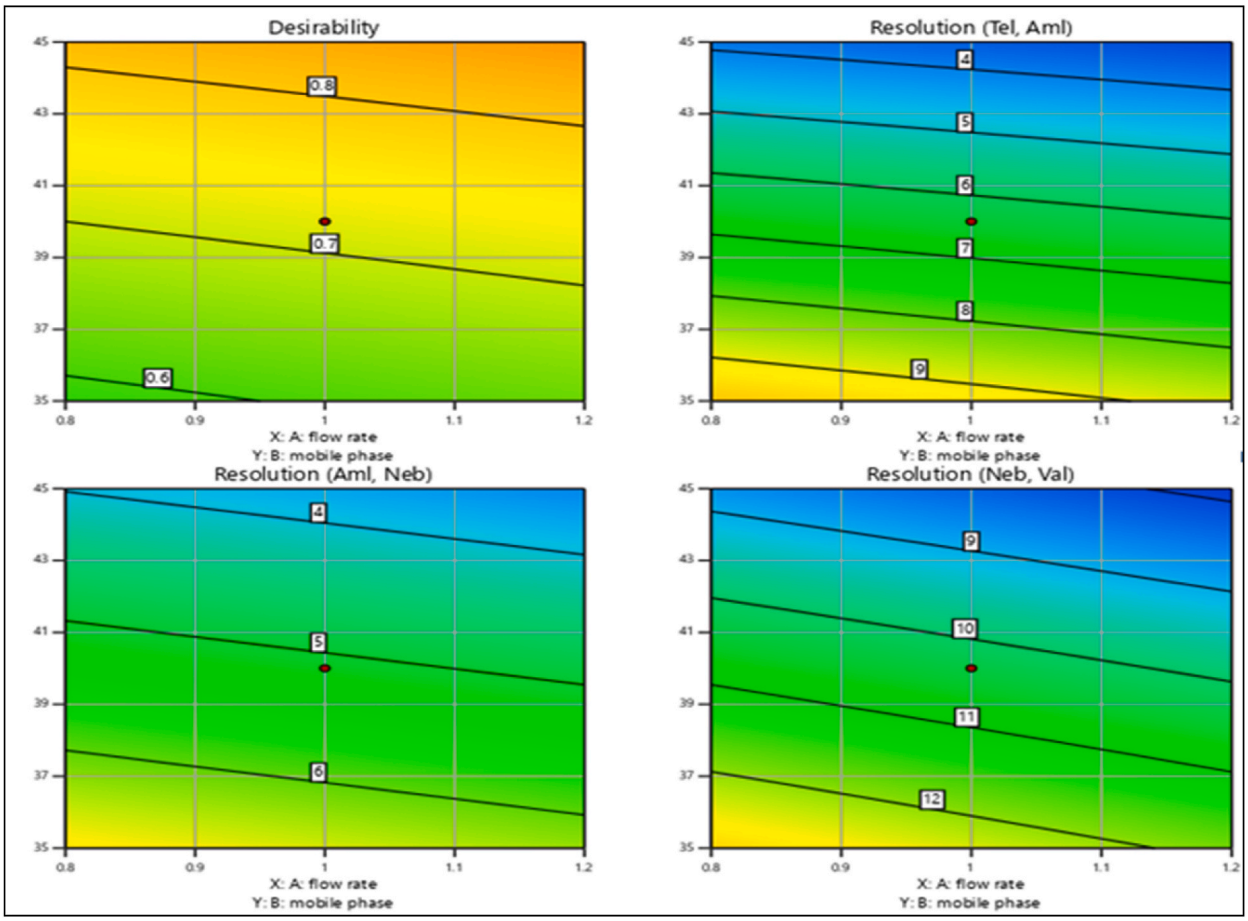

Fig. 9. Contour plots obtained for resolution maintaining one variable at the central level.

consumption, waste generation, and automation. We carefully highlighted non-green elements with a captivating red tint, while expertly categorizing each component as green, representing the essence of environmental friendliness, or yellow, signaling a slightly less green aspect. In the meticulous evaluation of our method, we observed the development of 8 brilliant green zones, accompanied by 1 yellow zone and 1 discreet red zone in Fig. 11, symbolizing its exceptional greenness. The obtained AGREE value of 0.81 showcases the method's commitment to sustainable and environmentally friendly practices in the field of analytical chemistry. This value not only demonstrates the method's greenness but also emphasizes its strengths in terms of eco-friendliness. Moreover, this extraordinary value not only exemplifies the method's remarkable greenness but also serves as a guiding light, explaining its inherent strengths in fostering an eco-friendly model. For assessing the environmental impact (greenness) of the methods, we employed the AMGS calculator from the American Chemical Society (ACS) website [10]. We input data related to flow rate, analysis run time, mobile phase composition, and the type and quantity of solvents used in sample preparation. The resulting AMGS score for the proposed method was 228.22 shown in Fig. 12., indicating its environmentally friendly and sustainable nature. "The assessment of greenness features using two distinct tools yielded consistent results, confirming the environmentally favorable attributes of the proposed method."

"While the AMGS calculator is robust, its limitations include handling a maximum of three mobile phase eluents and predefined solvent lists. In contrast, the AGREE tool stands out by accommodating diverse solvents, considering sample size, and providing a comprehensive toxicity assessment."

In the pharmaceutical industry, the demand for LC methods extends beyond environmental considerations to include analytical and economic attributes. We assessed the proposed method's whiteness using the RGB 12 algorithm Fig. 13. Analytical performance contributed to Red Score (79.4 %) Represents the analytical performance of the method, including precision, linearity, and accuracy. Green Score (82.8 %), Reflects the method's environmental impact, considering reagent consumption, waste generation, and occupational hazards. Blue Score (87.0 %), Indicates the practical aspects such as cost-efficiency, time efficiency, and sample consumption

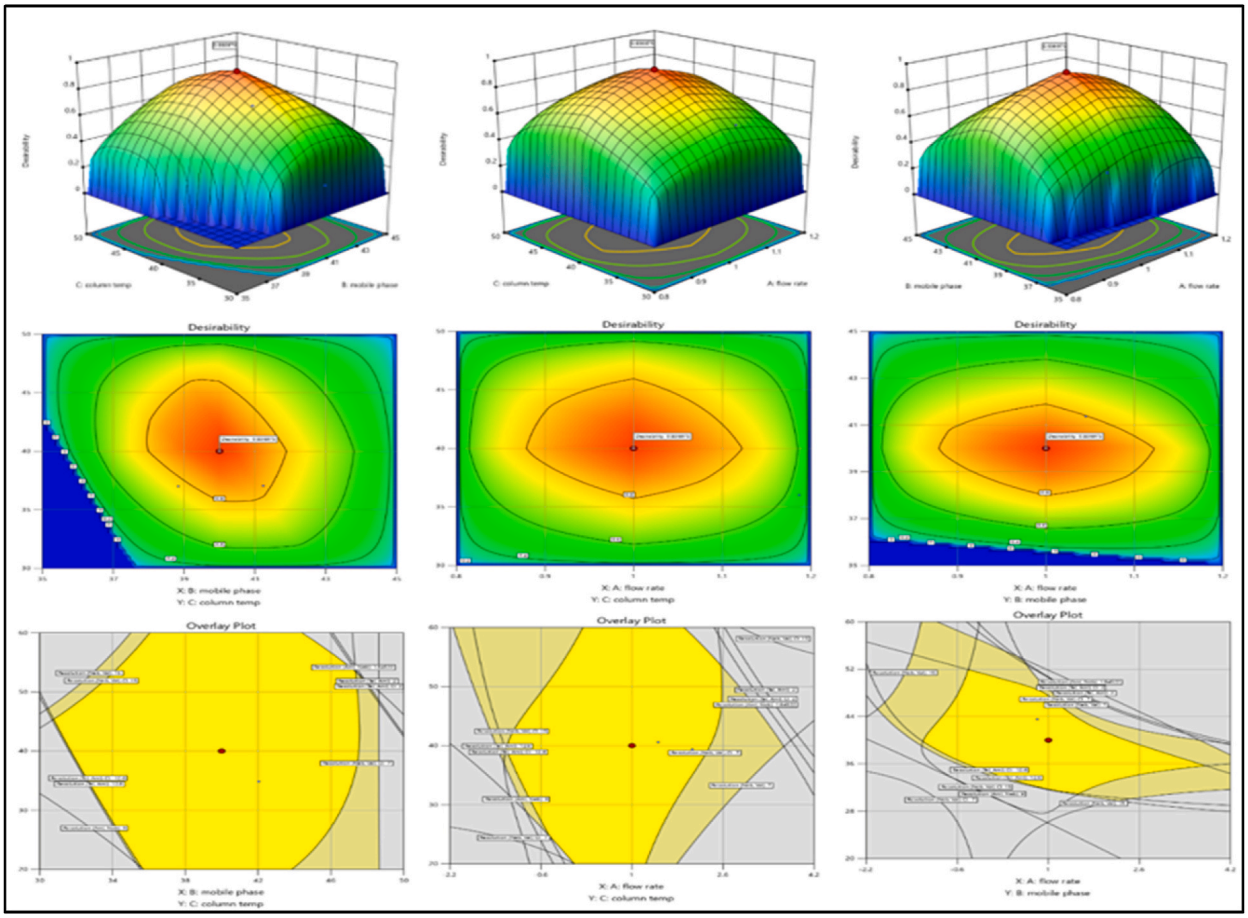

Fig. 10. 3D surface, counter plots and Overlay plots representing the design region.

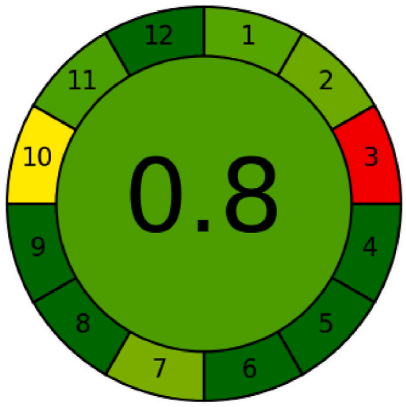

Fig. 11. AGREE green profile assessment of the proposed HPLC method. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and White Score (83.0 %), The overall score that combines the red, green, and blue scores, highlighting the method's balance of analytical quality, environmental safety, and cost-effectiveness. This approach ensures that the LC method is not only environmentally friendly but also maintains high analytical standards and is economically viable. It's a comprehensive way to evaluate the sustainability and efficiency of analytical methods in the pharmaceutical industry.

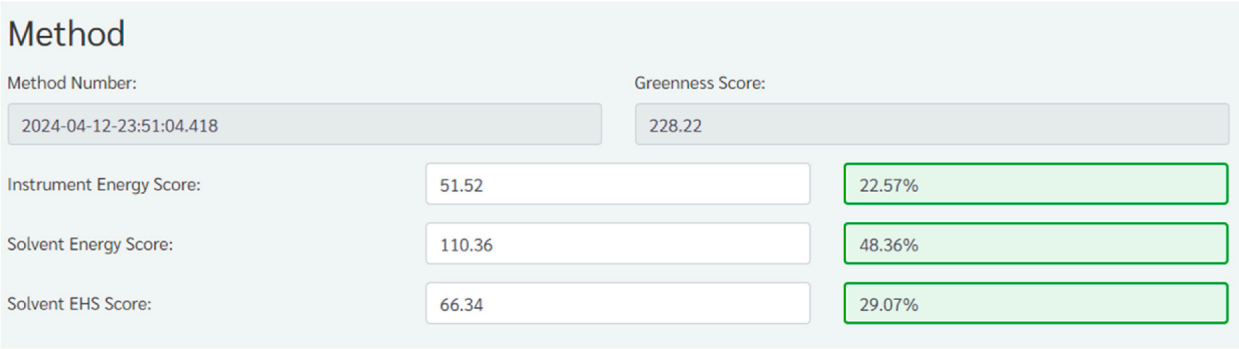

Fig. 12. AMGS score of the proposed HPLC method.

		w=3			w=3			w=3		
REDNESS (analytical performance)	W=3	Precision (RSD)			Accuracy (RE)			Linearity		
CS: 79.4%	LAV=33.3	2.0%			2.0%			0.999		
	LSV=66.6	0.5%			0.5%			1.0000		
	Result	0.40%			0.37%			0.9995		
	Score (0-100)	83	83	83	90	90	90	67	67	67
		w=3			w=3			w=3		
GREENNESS (safety and eco-friendliness)	W=3	Reagent consumption			Waste amount			Other occupational hazards		
CS: 82.8%	LAV=33.3	1200 mL			1500 mL			4 hazards		
	LSV=66.6	300 mL			400 mL			2 hazards		
	Result	250 mL			300 mL			0 hazards		
	Score (0-100)	80	80	80	89	89	89	80	80	80
		w=3			w=3			w=3		
BLUENESS (productivity / practical effectiveness)	W=3	Cost of analysis			Time of analysis			Sample consumption		
CS: 87.0%	LAV=33.3	250			600 min / 20 runs			acceptable		
	LSV=66.6	125			300 min / 20 runs			satisfactory		
	Result	83			200 min / 20 runs			satisfactory		
	Score (0-100)	100	100	100	99	99	99	66.6	66.6	66.6

FINAL COLOR:	REDNESS		GREENNESS		BLUENESS		BRILLIANCE (MB):	83.0%
WHITE	≥33.3%	≥66.6%	≥33.3%	≥66.6%	≥33.3%	≥66.6%		
	yes	yes	yes	yes	yes	yes		

Short annotation: 83.0white

Long annotation: 83.0white(79.4/3red-82.8/3green-87.0/3blue)

Fig. 13. White score for the proposed method.

7. Comparison of proposed method with previously reported methods

The literature survey revealed that most reported methods effectively quantify the content in drug products and bulk materials. A detailed summary of these methods is available in Table 6. However, these methods often use phosphate buffers and ion-pair reagents, such as hexane sulfonic acid, along with organic solvents like methanol or acetonitrile. These reagents and solvents may have significant environmental impacts and can be costly, whereas ethanol is a more environmentally friendly and less expensive alternative, reducing the overall cost of analysis. We compared the efficiency of the proposed method with the reported methods. In most cases, the proposed method exhibited higher efficiency by generating better LOD and LOQ results. Additionally, the proposed method can quantify four drug components simultaneously. While ethanol's high viscosity can lead to elevated back pressures, this limitation can be mitigated by using higher column temperatures.

In summary, the proposed approach is not only cost-effective but also environmentally friendly. By utilizing ethanol, we reduce the ecological footprint associated with drug content analysis while maintaining high efficiency and simplifying the process.

Table 6
Method coparision from literature search

Sr. No	Column Details and dimensions	Mobile phase	Samples evaluated	LOD and LOQ (µg/mL)	Reference
1	Hypersil BDS (250 × 4.6 mm 5 µm)	methanol, acetonitrile and 50 mM potassium dihydrogen phosphate (pH 6.0) in 10:45:45 v/v.	NEB, AML	AML 0.025 and 0.06 NEB 0.01 and 0.05	[47]
2	C-18 ODS (250 × 4.6 mm 5 µm)	potassium hydrogen phosphate buffer (pH 3.5): acetonitrile: methanol (15:50:35v/v/v)	NEB, VAL	TEL 0.78 and 2.12 NEB 0.08 and 0.26	[48]
3	Perfectsil target ODS3 (150 × 4.6 mm 5 µm)	Acetonitrile, 0.05M sodium dihydrogen phosphate buffer (60:40) pH 6.0	AML, TEL	TEL 2 and 4 AML 0.05 and 0.14	[49]
4	Lichrospher ODS RP-18 (250 × 4.0 mm 5 µm)	acetonitrile (ACN), phosphate buffer (pH 3.0), 40:60	AML,NEB	AML 0.062 and 0.188 NEB 0.01and 0.31	[50]
5	Phenomenex Gemini C18 (250 × 4.6 mm 5 µm)	methanol: acetonitrile: 0.02 M potassium dihydrogen phosphate (60:30:10, v/v/v; pH 4.0)	Neb	NEB 0.06 and 0.2	[51]
6	Inertsil ODS (250 × 4.6 mm 5 µm)	ACN: Buffer (pH 3.5 with dilute Ortho Phosphoric acid) in the fraction of 60:40 v/v	NEB, VAL	NEB- 0.05 and 0.15 VAL 0.81 and 2.44	[52]
7	RP C-18 Kromasil (250 × 4.6 mm 5 µm)	acetonitrile: potassium dihydrogen phosphate (0.02, pH 3.0) (56:44v/v)	VAL, AML	AML 0.03 to 0.089 VAL 0.018 to 0.054	[53]
8	C18 Hypersil BDS (250 × 4.6 mm 5 µm)	50 mM potassium dihydrogen phosphate buffer (add 2 mL triethylamine per liter of buffer and pH to 3.0 with orthophosphoric acid), acetonitrile (55:45, v/v)	NEB, VAL	NEB-0.03 to 0.09 VAL- 0.07 to 0.14	[54]
9	Eclipse Plus, (C18) 1.8 µm, 50 mm × 2.1 mm,	phosphate buffer pH 3.0 and acetonitrile in a ratio of 55:45	VAL, NEB, AML	NEB-0.99 to 0.299 VAL 0.009 to 0.027	[55]
10	YMC pack pro ODS (150 × 4.6 mm 5 µm)	methanol: acetonitrile: 0.05 M potassium dihydrogen phosphate buffer (pH 3.0 with 10 % ortho phosphoric acid after addition of 0.2 % triethylamine) (30:30:40, v/v/v)	NEB, VAL	NEB-0.38 to 1.15 VAL- 1.08 to 3.27	[56]
11	Nucleodur® C18 (250 × 4.6 mm 5 µm)	acetonitrile: phosphate buffer at pH 4.5 (60:40v/v)	TEL, AML	TEL 3.34 and 10.122 AML 0.02 and 0.06	[57]
12	Hypersil BDS C18 (100 × 4.6 mm, 5 µm)	Buffer orthoposhoric acid(pH 3.6 with triethylamine)and Acetonitrile in 60:40	TEL, AML	TEL 0.1 and 0.31 AML 0.19 and 0.58	[58]
13	Hypersil BDS C18 (100 × 4.6 mm, 5 µm)	Phosphatebuffer (pH 3.5): Acetonitrile taken in the ratio 57: 43	TEL, AML	TEL 0.62 and 1.88 AML 0.13 and 0.38	[59]
14	X-Bridge Shield RP18 (100 × 4.6 mm, 3.5 µm)	0.1 % formic acid in water (pH:2.5) and ethanol in the ratio 40:60	TEL, AML, NEB, VAL	TEL 0.01 and 0.04 AML 0.06 and 0.20 NEB 0.08 and 0.25 VAL 0.14 and 0.46	Present work

8. Conclusion

Our study has successfully developed and validated an HPLC method capable of simultaneously estimating 4 components, strictly adhering to established guidelines. To ensure the method's robustness, we employed well-designed experimental designs, meticulously verifying its reliability. The comprehensive summary of validation results unequivocally affirms the accuracy and robustness of our developed method. We evaluated the selected drugs according to the approved compendial methods and found that the assay values are consistent with the proposed method. The primary benefit of our developed method is its reduced environmental impact compared to traditional HPLC methods. It can determine all the selected molecules in a single run with a shorter runtime. Additionally, a positive

green score was achieved using various environmental assessment tools, such as AGREE and AMGS. The obtained white score using the RGB metric tool illustrates the method's whiteness towards the analytical, ecological and economical efficiency. Moreover, our study stands out for its commitment to environmental responsibility, as we have effectively incorporated green solvents, setting a commendable benchmark in sustainable analytical chemistry. The utilization of the Analytical Greenness metric and subsequent calculation of the AGREE score have provided a quantifiable and objective assessment of the method's eco-friendliness, reinforcing its alignment with sustainable practices in analytical chemistry. Our collective efforts, which encompass the adoption of green solvents, optimization of method parameters, and rigorous evaluation of greenness using the AGREE metric, make substantial contributions to the advancement of environmentally conscious analytical methods. This work serves as a solid foundation for future research endeavors and serves as an influential call to action, urging the wider adoption of sustainable approaches in analytical chemistry. The main merits of the proposed method are it is the single method for the estimation of drug components with shorter runtime. ethanol, the solvent used, is both renewable and biodegradable, making the developed method environmentally friendly. the cost of ethanol is comparatively low when compared with other organic solvents, so we can consider the method as cost effective. However, limitations include ethanol's high viscosity, which can lead to elevated back pressures. To mitigate this, higher column temperatures can be used, sometimes we may end up with sensitivity issues. Researchers are encouraged to use the methodologies and assessment tools mentioned in this study as a reference to develop more environmentally friendly methods that will have a positive effect on the environment.

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Research involving human participants and/or animals

The present work does not involve any human participants and/or animals.

Informed consent

Not applicable.

CRediT authorship contribution statement

Y.V.S. Veerendra: Writing – original draft, Validation, Formal analysis, Data curation. **Pradeep Kumar Brahman:** Supervision. **Sharad D. Mankumare:** Writing – review & editing, Supervision. **Jayaraju Ch:** Formal analysis, Data curation. **Vinod Kumar C:** Formal analysis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Dr. Pradeep Kumar Brahman reports was provided by KL Deemed to be University. Dr. Pradeep Kumar Brahman reports a relationship with KL Deemed to be University Department of Chemistry that includes: employment. Dr. Pradeep Kumar Brahman has patent no Patent pending to NOT APPLICABLE. No other relationship or activity with publisher If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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