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"Pioneering Eco-Friendly HPLC Analysis for Febuxostat and Aceclofenac Using Design of Experiments"

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KEYWORDS

High performance liquid chromatography, Febuxostat, Aceclofenac, Green Chemistry, AGREE, GAPI, BAGI, Analytical Eco-scale

ABSTRACT:

A novel "HPLC method was designed and validated" for the "simultaneous quantification of Aceclofenac and Febuxostat," a newer pharmaceutical combination. The "method" incorporates "green analytical chemistry" principles and utilizes "Design of Experiments" for optimization. "Chromatographic separation" was implemented on a Unison "C18 column, with Central Composite Design" optimizing the "mobile phase" using two factors: methanol volume and "flow rate". The optimal "mobile phase" consisted of "methanol, 0.01M KH_2PO_4 buffer (pH 5.0), and acetonitrile in a 30:50:20 ratios", with detection at "290 nm". "Retention times" were 11.415 and 9.82 minutes for Aceclofenac and Febuxostat, in that order. "Calibration plots were linear" over the "concentration" ranges of 9–27 "µg/ml" for Aceclofenac and 6–18 "µg/ml" for Febuxostat. The "method" demonstrated excellent precision, sensitivity, and durability, with percentage recoveries close to 100% and minimal variation. "The greenness of the method" was evaluated using four tools: "Analytical Eco-scale, Blue Applicability Grade Index, Complementary Green Analytical Procedure Index, and Analytical Greenness Metric Approach". The developed HPLC method is efficient, precise, and adheres to green analytical chemistry principles. It provides a reliable and environmentally friendly approach for the simultaneous quantification of Aceclofenac and Febuxostat.

1. Introduction

Green Analytical Chemistry (GAC) goals to develop "analytical" methods that decrease or completely eliminate the harm that "chemical analysis" causes to the surroundings and human health. It is consistent with the tenets of "green chemistry", which emphasize sustainability in the lab through energy conservation, waste reduction, and the use of less hazardous chemicals. Therefore, the primary goals are to decrease the "production of hazardous materials and waste, use safer reagents, materials, and solvents, increase the analytical process's energy efficiency, minimize sample size and the number of chemical reactions or processes that are required," and ensure that procedures are safe for the surroundings and human health. Researchers can use a variety of greenness evaluation tools to analyze and improve the effectiveness of analytical approach. By evaluating how well processes follow GAC principles, these tools help to improve or modify them to make them more sustainable. Therefore, the current study is to develop and optimize the HPLC methods for the

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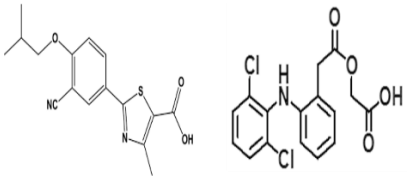
estimation of these medicines utilizing the concepts of green analytical chemistry.

Gout is brought on by urate crystals depositing in the tissues. Urate crystals are developed by increase in blood uric acid levels greater than a certain threshold. Crystals of uric acid can form in all tissues, but they are most common in and around joints, where they form tophi [1,2]. The "American College of Rheumatology" states that "analgesics, NSAIDs, corticosteroids, colchicine, xanthine oxidase inhibitors, and uricosurics" are all used in the treatment of gout [3-5]. A "xanthine oxidase inhibitor" (XOI) inhibits xanthine oxidase enzyme which is involved in purine metabolism. Because xanthine oxidase inhibition lowers "uric acid production in humans," it is recommended for the "treatment of hyperuricemia" and associated diseases, such as gout [1,5]. "Febuxostat (FEB) (2-[3-cyano-4-(2-methylpropoxy) phenyl]-4-methylthiazole-5-carboxylic acid" (fig. 1a) is a XOI that is more efficient than "allopurinol" in decreasing and maintaining blood "uric acid" levels. However, FEB causes inflammatory condition on initiation of therapy. Therefore, Aceclofenac (ACE) [(2, 6, Dichlorophenyl) amino] phenyl acetyl oxyacetic acid (fig. 1b), a potent analgesic and anti-inflammatory molecule is prescribed along with FEB to provide inflammatory action [6,7].



"Figure 1: (A) Chemical structure of Febuxostat (B) Chemical structure of Aceclofenac"

According to a review of the literature, HPTLC and HPLC techniques for FEB [8-12] and ACE [13-20] are reported either by themselves or in conjunction with other medications. This study uses a "Design of Experiments (DoE)" approach to synchronously quantify FEB and ACE in bulk and developed formulation for the first time. Therefore, the study designed to develop a green, robust and precise "HPLC method" for FEB and ACE loaded ufasomal gel.

2. Materials & Methodology

2.1 Materials

ACE and FEB was received as a gift sample from "J.B. Chemicals and Pharma Ltd., Ankleshwar" and Alembic Pharmaceuticals Ltd., Vadodara respectively. Milli Q water was purchased from SICART, Anand. "Methanol HPLC and HPLC grade Acetonitrile" (ACN) were obtained from Dutt Enterprise, Anand. Potassium dihydrogen ortho phosphate (KH_2PO_4) was purchased from Chemdyes Corporation, Rajkot.

2.2 Instrumentation

Analysis was performed by HPLC system, PC based LC solution 1.25 Shimadzu LC-2010_{HT} HPLC with SPD-M20A diode array detector. Unison, C_{18} (internal diameter 5µm, 250× 4.6mm) was used as packed column. Data collection and record was done by LC 2010 software. Analytical balance was used for weighing (Shimadzu Corporation ELB 300).

2.3 Standard stock solution preparation

FEB and ACE standard stock solutions were made independently by mixing 100 mg drug in 100 ml volumetric flasks, with HPLC grade Methanol, sonicating for "5 min", and then adding HPLC grade Methanol to make up the volume. The drug's concentration in the resultant solutions is 1 mg/mL. The concentrations of FEB and ACE were "10 µg/ml and 15 µg/ml", respectively, after diluting the solutions.

2.4 Mobile phase (MP) preparation

"KH_2PO_4 (1.36 g)" was solubilized in "1000 ml of Milli Q water". The "pH" of resulting buffer was 5.5, followed by degassing. Mix 300 ml of methanol, 500 ml of 0.01M phosphate buffer and 200 ml of ACN completely. Using a batch sonicator, sonication was performed for ten minutes to eliminate dissolved gas.

2.5 Chromatographic development

KH_2PO_4 with a variety of solvents in various ratios, including ACN and Methanol, were explored. At normal temperature (25±2°C), separation was accomplished on Unison "C18 (250 × 4.6 mm, packed with 5 µm)" using a tailored MP that included 30% methanol, 50% 0.01 M KH_2PO_4, and 20% ACN. The volume injected was 20

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µL, and the MP "flow was 1 ml/min". Using the MP, the quantification was carried out at 290 nm. Vials containing standard solutions in varying concentrations were stored in an auto sampler. The MP was prepared daily "degassed by ultrasonicator" and filtered by "0.22-mm membrane filter".

2.6 Software aided method optimization

When developing the method, a number of factors were taken into account, such as the "flow rate and the volume of organic solvents" in the MP. Therefore, using Design Expert 11.0.1, "Central composite design" (CCD) was used to check the impact of 2 distinct chromatographic settings on the 3 identified important dependent variables. The design, which included 19 experimental runs, assisted in screening components by assessing their primary impact in order to obtain study results. Two factors and three levels were chosen for a CCD. The "factors were (X_1)" the "% of organic solvent" (methanol) and "(X_2)" the flow rate. The 3 levels were -1, 0 and +1. Dependent factors were the retention time (R_T) of FEB (Y_1), ACE (Y_2), and resolution (Y_3). An appropriate technique for exploring and examining response behavior around optimal factor values and achieving optimal system performance was the response surface quadratic method. The model's relevance was investigated using the Analysis of Variance. Conditions for optimum approach were chosen from this and their performance was verified.

2.7 "Method Validation"

The aim of "method validation" is to show whether or not the approach is suitable for its intended use as per with "ICH guidelines" [21]. Several "validation" factors, including "linearity, precision, accuracy, specificity, limit of detection (LOD), limit of quantification (LOQ), and robustness," are tested for the developed method. Before validation, a "system suitability test" was conducted.

2.7.1 System suitability study

By measuring the "system suitability" characteristics, the system's performance was confirmed. In order to evaluate system, fit factors such as "resolution, theoretical plates, and tailing factor" of the 6 preparations was calculated. 6 replicates of preparations comprising the medication and HPLC grade Methanol were used to measure the system precision. Using LC 2010 solution software, the tailing factor and theoretical plate count were ascertained, and the % RSD was computed. For the five successive injections of the analyte's peak, the percentage RSD should always be less than 2.0% [22].

2.7.2 Linearity & Range

"Ability of an analytical technique to yield test results that are precisely proportionate to the analyte amount in a sample within a given range is called linearity." "The linearity of analytical procedures" is the range between the higher and "lower levels of analyte," encompassing levels that have been illustrated to be checked with "precision and accuracy" using the method. "The correlation coefficient of linear regression analysis" is used to express "linearity" [23]. A sequence of solutions in range from 6–18 "µg/ml" and 9–27 "µg/ml" of FEB and ACE, respectively, were injected to assess linearity. Six injections of each prepared concentration were made. Linearity between the injected concentration and its corresponding peak area was found by regression analysis.

LOQ and LOD

"LOD and LOQ" can be calculated from "signal to noise ratio". The "LOD and LOQ" were then computed using the response's "standard deviation (SD) and the calibration curve's slope (S)" at levels that approximated the "LOD and LOQ". The values of "LOD and LOQ" were determined as follows: [24,25]

"

$$LOD = 3.3\, x \frac{\sigma}{S}$$

$$LOQ = 10\, x \frac{\sigma}{S}$$

"

2.7.3 Precision

"3 distinct concentrations" were injected at the low, mid, and high ends of the "calibration curve to assess the precision" of the suggested HPLC method. Sets of 6 duplicates of the aforementioned 3 concentrations were examined for "intra-day variation, and on separate days for inter-day variation". "% RSD" of the all assays is also reported [26].

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2.7.4 Accuracy

"Closeness of test results to the true value (100%) is termed as accuracy." It was ascertained by applying analytical procedures to recovery studies, in which "pre-analyzed sample" solutions are "spiked with a known quantity of standard" [23]. 3 different amount 50 percent, 100 percent and 150 percent were selected. Due to unavailability of the commercial product in the local market, the mixture of Zerodol^{R} and Febuthal^{40} tablets were prepared and analyzed. The solutions were examined by the technique previously discussed. "Each experiment was performed in triplicate and % RSD was calculated" [25].

2.7.5 Specificity

"The ability to predict the analyte response in the presence of extraneous elements such contaminants, degradation products, and matrix is known as specificity." Using the "UV spectra" that the "UV detector in the HPLC system" had captured, the chromatographic peaks of FEB and ACE were assessed for spectral purity. The "peak purity" of FEB and ACE were checked by "comparing the R_T of standard and sample" of FEB and ACE respectively [27].

2.7.6 "Robustness"

"The robustness of an analytical procedure refers to its capability to remain unaffected by small but deliberate variations in the analytical method, as per the ICH guideline" [21]. The conditions studied were volume of MP 30 ± 1 ml, flow rate of MP "1 ± 0.1 ml/min" and "wavelength 290 ± 1 nm".

2.7.7 Analysis of developed formulation

Due to the unavailability of the commercial product in the local market, the developed formulation was made and evaluated by the proposed HPLC method. Accurately weigh equivalent to 100 mg FEB and 150 mg ACE in "100 ml volumetric flask" and dilute with "methanol". The resultant solution was "1 mg/ml". The "stock solution" was diluted to get the "concentration of 10 µg/ml of FEB and 15 µg/ml of ACE". The active ingredients FEB and ACE eluted at their specific R_T values. "No interfering peaks" were observed from any of the "inactive ingredients".

2.8 Predicting Greenness of HPLC method

A range of green assessment tools have been used to evaluate the approaches' sustainability and greenness. "The Analytical Eco-Scale, a semi-quantitative tool, assigns a score based on factors like the amount of reagents used, energy consumption, waste production, and safety." Up to a maximum score of 100, ineffective or dangerous practices are deducted. A greener approach is indicated by a higher score. With its numerical score and graphical representation of the analytical method's compliance with key evaluation criteria, such as the "least amount of hazardous chemicals used, the use of solvents, sample size, energy consumption, and waste production," AGREE is a tool that provides a thorough evaluation. When the AGREE "score is closer to 1," the method is deemed more environmentally friendly. "The five elements of the analytical process—sample preparation, reagents used, instrumentation, energy consumption, waste production, and disposal"—are all thoroughly assessed by GAPI, a visual assessment tool in the form of a pictogram. A symbol with a color code is used to evaluate each component. The Balance Analytical Green Index, or BAGI, is another tool that focuses on striking a balance between a number of "environmental impact, safety, and efficiency" factors. "The BAGI" tool assigns numerical scores to a number of parameters, such as energy consumption, waste output, and chemical hazards, in order to provide a clear and balanced assessment of the environmental and performance issues. Factors like the use of somewhat hazardous chemicals combined with minimal waste production are balanced by the BAGI score. A higher score indicates a more sustainable and eco-friendly analytical methodology. The final result, which typically ranges from 0 to 100, is then calculated by adding these scores together [27-30].

3. Results

3.1 DoE aided method optimization

The aim of this study was to generate a straightforward, reliable, and reasonably priced "HPLC process" for estimating FEB and ACE simultaneously. Preliminary experiments and a review of the literature were used to determine the key chromatographic conditions. In order

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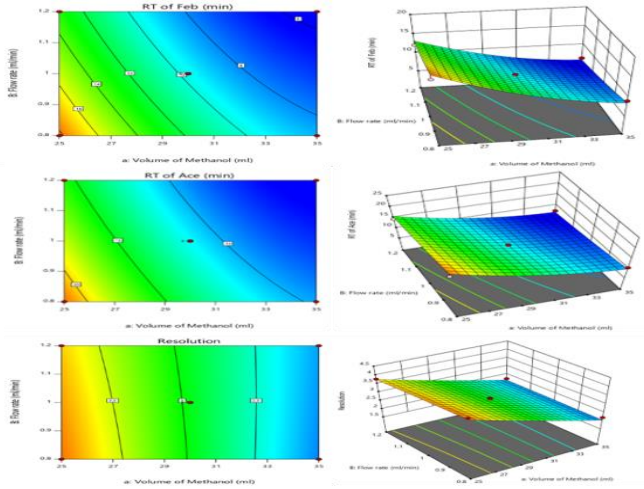
to separate FEB and ACE in a short amount of time, MP conditions needed to be modified, according to these examinations to choose the factor levels for "screening and optimization" studies. A modification in the volume of methanol led to a significant change in R_T, and it was discovered that the MP composition of "methanol, buffer, and ACN" was more appropriate for the synchronous estimation of both pharmaceuticals. As a result, it is one of the most dominant factors in method development. It was shown that "flow rate" had the most important effect on system suitability.

The current analytical technique optimization study made use of the CCD design. 2 factors—the "volume of the organic phase and the flow rate"—were shown in this effective and thorough experimental design, which is based on a methodical search for essential elements for the RPHPLC method's optimization. The multivariate technique DoE with CCD was used for the "RP-HPLC method" in order to optimize the mobile phase composition by studying the synchronous fluctuations of the parameters on examined responses, such as R_T of FEB (Y1), R_T of ACE (Y2), and resolution (Y3). Table 1 describes the circumstances and observed responses used to design 19 experiments. Based on the "effects of 2 factors" on replies and the examination of these results, it was possible to employ "intricate mathematical models" that had been marked to ascertain the relationship between "the factors and the responses" of interest. We found that the response surface quadratic "model" was the most well-fitting model for CCD. Using "Design Expert software," an ANOVA was also used to validate the model. There was less than a "0.2" discrepancy between the "adjusted R^2 values and the predicted R^2 values" of R_T of FEB (Y_1) and ACE (Y_2) and resolution (Y_3). The design space can be traversed using this quadratic model. Responses for the R_T of ACE (Y2), R_T of FEB (Y1), and resolution (Y3) had model F-values of 72.14, 79.35, and 201.66, respectively, indicating that the "model is significant." As a result, the "p-value" for significant responses was <0.05, indicating that the model terms are important. The high adjusted R^2 value and low standard deviation indicate a strong correlation between the fitted models and the experimental data. "2D and 3D contour plots" showing the "effect of mobile phase ratio (X1) and flow rate (X2) on retention time of FEB (Y1), ACE (Y2) and Resolution (Y3)" is shown in fig. 2.



"Figure 2: 2D and 3D contour plots showing the effect of mobile phase ratio (X1) and flow rate (X2) on retention time of FEB (Y1), ACE (Y2) and Resolution (Y3)"

"Table 1: Coded values for factor level and observed responses in CCD for 19 trials

Experiment (Run)	Volume of Methanol (ml) X_1	Flow rate (ml/min) X_2	Retention time of FEB (min) Y_1	Retention time of FEB (min) Y_2	Resolution Y_3
1	25	0.8	17.147	20.506	4.084
2	25	1.2	12.121	14.414	3.781
3	35	0.8	9.084	10.402	2.207
4	35	1.2	6.184	7.106	2.219
5	30	1	9.824	11.415	3.02
6	30	1	9.724	11.302	2.988
7	30	1	9.671	11.241	2.964
8	23	1	19.329	23.194	4.278
9	23	1	19.564	23.489	4.274
10	37	1	6.306	7.178	1.729

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11	37	1	6.284	7.161	1.73
12	30	0.72	13.185	15.3	2.854
13	30	1.28	7.651	8.896	2.84
14	30	1	9.665	11.232	2.952
15	30	1	9.647	11.21	2.938
16	30	1	9.638	11.2	2.931
17	30	1	9.63	11.19	2.918
18	30	1	9.623	11.184	2.896
19	30	1	9.632	11.193	2.907

”

For certain levels of each element, the reaction can be predicted using the equations in terms of coded factors. By comparing the factor coefficients, this equation helps determine the relative importance of the elements. Resultant equations are:

FEB: $Y1= +9.68-4.29X_1-1.98X_2+0.53X_1X_2+1.55X_1^2+0.217X_2^2$;

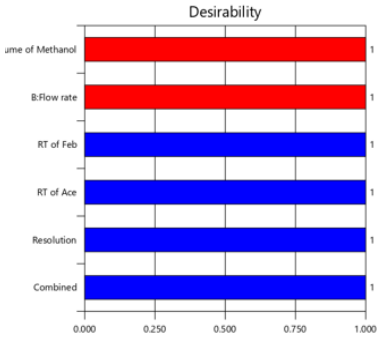
ACE: $Y2= +11.25-5.3X_1-2.32X_2+0.699X_1X_2+1.96X_1^2+0.254X_2^2$;

Resolution: $Y3= +2.94-0.89X_1-0.04X_2+0.08X_1X_2+0.05X_1^2-0.006X_2^2$

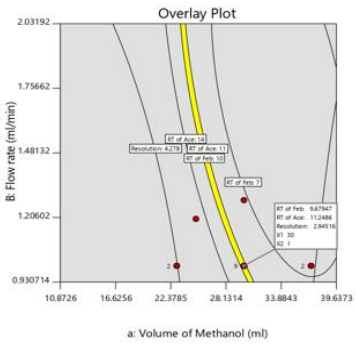
The R_T of FEB and ACE, Y_1 and Y_2, as well as the resolution, Y_3, were negatively impacted by parameters such MP composition (X_1), according to the coefficient values and signs from the aforementioned equations. The R_T of FEB and ACE, Y_1 and Y_2, as well as the resolution, Y_3, were adversely affected by flow rate (X_2). Y_1, Y_2, and Y_3 were all positively impacted by interactions between X_1 and X_2. All chromatographic responses were positively impacted by the squares of factors X_1^2 and X_2^2, with the exception of X_2^2 on resolution Y_3.

To observe how the factors and their interactions affected the “responses, response surface and contour plots” were examined. The curvature of the contour plots indicated a nonlinear relationship between the factors and the answers.

To determine the ideal set of circumstances based on the objectives and constraints for every answer, a composite desirability is used. The entire experimental region was investigated for the composition, where the “constraints set were met to the maximum, i.e., 1”, as illustrated in fig. 3, and the “desirability function "R," equal to unity,” demonstrated the achievement of desired goals in the constraints established. The MP (X1) ratio of methanol: buffer: ACN 30:50:20 and “flow rate” of “1 ml/min” were determined to be the ideal chromatographic conditions for RP-HPLC (fig. 4). As illustrated in fig. 5, these results produced R_T of FEB (Y1) 9.82, ACE (Y2) 11.415, and resolution (Y3) 3.02, respectively.



“Figure 3: Desirability function representation basis unity=1”



“Figure 4: Optimized chromatographic conditions”

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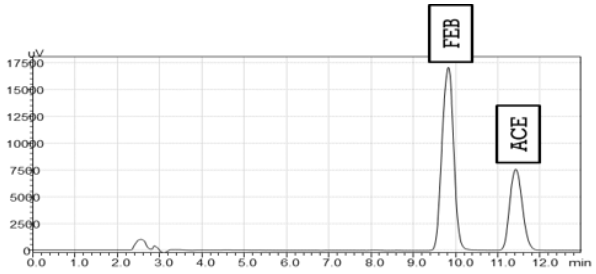

"Figure 5: Optimized RP-HPLC chromatogram for FEB and ACE at 290 nm"

3.2 "Method Validation"

As per "ICH guidelines (2005)", "system suitability tests" were performed in "liquid chromatographic technique." According to several theoretical plates, the column efficiency for both medications was above 2000, the resolution was greater than 2, and the tailing was "<2". It has demonstrated good injection repeatability because the "% RSD" was less than 2%. Plotting the "linearity curve" over concentrations ranging from "6 to 18 µg/ml for FEB and 9 to 27 µg/mL for ACE" verified the developed method's linearity. The "correlation coefficients (r2=0.9904 and 0.9999) for FEB and ACE", respectively, are displayed in Table 2. Different sample concentrations "(50, 100, and 150%)" of standard concentrations for both medicines were created for the recovery investigation, and the results showed recovery rates of 98.918–100.44 percent for FEB and 98.157–100.35 percent for ACE. Data from the intermediate accuracy and repeatability tests are shown in Table 2. Both medications had precision levels below 2%, demonstrating the method's accuracy. The results showed that the "LOD and LOQ" for ACE were "0.028 and 0.085 µg/ml", respectively, and for FEB they were "0.062 and 0.187 µg/ml". The durability of the "RP-HPLC" technology to such small changes demonstrated its insensitivity to small changes un the optimum experimental adjustments. The flow velocity and composition of the MP have a major impact on the R_T of FEB and ACE, and resolution.

"Table 2: Validation Parameters

Parameters		**FEB**	**ACE**
System suitability parameters			
No. of theoretical plates	Mean±SD	3613.62±15.61	3965.08±16.35
	%RSD	0.432	0.412
Resolution	Mean±SD	13.05±0.16	2.34±0.04
	%RSD	1.206	1.686
Tailing factor	Mean±SD	1.00±0.01	1.30±0.003
	%RSD	0.863	0.268
Linearity			
Range (µg/ml)		6-18	9-27
Slope		99.62	25.74
Intercept		982.82	280.59
Correlation coefficient		0.990	0.999
Accuracy			
Recovery studies		98.91-100.44	98.16-100.35
%RSD		0.308-0.759	0.054-1.38
Precision			
Repeatability	%RSD	0.062	0.040
Intermediate precision	%RSD	0.037-0.120	0.013-0.115
LOD (µg/ml)		0.062	0.028
LOQ (µg/ml)		0.187	0.085

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3.3 Predicting Greenness of HPLC method

An ideal green analytical method receives a starting score of 100 points from the Eco-Scale. Depending on how serious the environmental impact is, deductions from this score based on a number of hazardous and unfavorable environmental aspects related to the method can range from minor to major penalties. The score gets closer to 100 the greener the technique. The suggested methods provide a score of 86 (Table 3). Due to the requirement for huge sample quantities, sample preparation has a score of -5. Similarly, for energy use, -1 marks for necessitating a procedure that uses a lot of energy. Hazardous waste production has a 5-point penalty for waste generation. The waste handling has penalty of -3 point. Therefore, even if the proposed method is considered environmentally friendly, there is undoubtedly room for improvement in terms of reducing the use of hazardous solvents and waste generation.

"Table 3: Analytical Eco-scale

Items of the method	**Value**	**Penalty points**
Reagents		
Acetonitrile		2
Phosphate buffer	<10 ml	0
Methanol		3
Instrument		
Energy	<1.5 Kwh/sample	1
Occupational Hazard	Process Hermetization	0
Waste	>10ml	5
Waste treatment	None	3
Total penalty points		14
Analytical eco scale score (HPLC)		**86**

"

The AGREE tool provides a visual representation, typically in the form of a radar chart, along with a numerical score (ranging from 0 to 1), making it easy to comprehend the method's overall environmental impact. The final AGREE score is determined by averaging the scores of the 12 GAC principles; the more environmentally friendly a method is, the closer its score is to 1. With a score of 0.7, the recommended approach is green but not perfect. This implies that while the process complies with other green guidelines, some aspects still need to be improved (e.g., by switching to greener solvents or lowering the energy used in the process).

The "Green Analytical Procedure Index" (GAPI) is a tool that displays the results in a color-coded pictogram. According to the color scheme, green is the most environmentally friendly color, yellow has a moderate effect, and red has a more negative effect. The pictogram shows largely green and yellow signs and fewer red signs for sample collection and preparation and solvents, green for instrumentation, red for waste generation, and mainly green signs and fewer yellow sign for pre-analysis process after the GAPI tool has been applied.

A high BAGI score indicates that the process is highly ecologically friendly and strikes a balance between environmental and safety considerations. Although there is potential for improvement, particularly in the areas of energy consumption and reagent toxicity, as indicated by the scores of 80, the method is regarded as being somewhat green. It suggests using fewer hazardous chemicals, producing less waste, using less energy, and posing less of a risk to the environment and public health. This implies that although the instruments used for evaluation demonstrate that both approaches are ecologically friendly, they could be improved by identifying safer chemical alternatives and further reducing waste generation [31,32]. The pictograms are shown in fig. 6.

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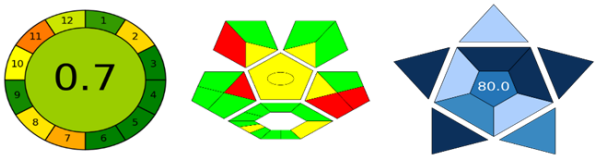

Figure 6: AGREE, GAPI and BAGI tools for HPLC

4. Discussion

A straightforward, quick, "accurate", and economical "RP-HPLC technique" for the synchronous measurement of FEB and ACE is being developed in this study using a systematic DoE approach. The composition of the MP and the "flow rate" are two important components that are studied in the experimental design. The modeling software made it easier to comprehend the variables affecting the method's optimization and the separation of ACE and FEB. CCD was used to maximize the resolution as a quick response between FEB and ACE. Methanol, buffer, and ACN at a "30:50:20 v/v/v" ratio demonstrate the suitability for estimating FEB and ACE in the optimized model. "1 ml/min" was the ideal MP flow rate. By verifying that the approach was "accurate, linear, precise, and robust," the validation study helped to validate the choice of the ideal conditions. Consequently, the application of the response surface technique offers improved insight for robustness testing and "method development." Under regulatory flexibility, this created procedure is appropriate for regulatory submission and satisfies the design space idea. The greenness assessment tools like the "Eco-Scale, GAPI, AGREE, and BAGI" were used to evaluate the greenness of the various aspects indicating that the method is greener and environmentally benign resulting in reduced environmental and health impacts.

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