Scientific Reports

https://doi.org/10.1038/s41598-026-36517-y

Article in Press

Development of a green chemistry based bioanalytical method using response surface methodology to analyze febuxostat and indomethacin in rabbit plasma

Received: 16 December 2024

Accepted: 13 January 2026

Published online: 17 January 2026

Cite this article as: Ansary A., Piyongsola , Paul B. *et al.* Development of a green chemistry based bioanalytical method using response surface methodology to analyze febuxostat and indomethacin in rabbit plasma. *Sci Rep* (2026). https://doi.org/10.1038/s41598-026-36517-y

Akramul Ansary, Piyongsola, Biprojit Paul, Koushik Nandan Dutta, Bhargab Jyoti Sahariah, Manoj Kumar Deka, Amit kumar Das & Manish Majumder

Research Article:

Development of a green chemistry based bioanalytical method using response surface methodology to analyze febuxostat and indomethacin in rabbit plasma

Akramul Ansary[a], Piyongsola[b], Biprojit Paul[a], Koushik Nandan Dutta[c], Bhargab Jyoti Sahariah[d], Manoj Kumar Deka[d], Amit kumar Das[d], Manish Majumder*[a,e]

a Department of Pharmaceutical Chemistry, NETES Institute of Pharmaceutical Science, Nemcare Group of Institution, Mirza, Kamrup - 781125, Assam, India

b Department of Pharmacology, NETES Institute of Pharmaceutical Science, Nemcare Group of Institution, Mirza, Kamrup - 781125, Assam, India.

c Department of Pharmacognosy, NETES Institute of Pharmaceutical Science, Nemcare Group of Institution, Mirza, Kamrup - 781125, Assam, India.

d Department of Pharmaceutics, NETES Institute of Pharmaceutical Science, Nemcare Group of Institution, Mirza, Kamrup - 781125, Assam, India.

e School of Pharmacy, Sainath University Ranchi, Jharkhand, India

*Corresponding Author Email: manishphar33@gmail.com, Tel: +91-9108451821

Abstract

This research established an environmentally friendly and sustainable approach to measure Febuxostat and Indomethacin levels in rabbit plasma samples, utilizing isocratic liquid chromatography guided by green analytical chemistry principles and Analytical Quality by Design (AQbD) methodology. Chromatographic separation was performed on an Eclipse Plus C18 column (25cm x 5cm,4.6µm), using a binary mobile phase of ethanol and 50 mM potassium dihydrogen orthophosphate (pH 4.5) in a 66:34 ratio, delivered at 0.8 mL/min for 15 minutes. Resolution and asymmetry factors were designated Critical Analytical Attributes (CAAs). Control Noise Experimentation (CNX) screening identified flow rate, mobile phase pH, and ethanol concentration as significant contributors to CAAs variability. Subsequent optimization utilizing Central Composite Design (CCD) refined the Critical Method Parameters (CMPs) to ensure optimal performance. Chromatographic analysis revealed Febuxostat and Indomethacin retention times of 4.41 and 7.35 minutes, respectively. The method's greenness and analytical quality were assessed using AGREE, ComplexGAPI, RGB, and AMGS tools. Validation studies confirmed linearity (R2: 0.9959 for Febuxostat, 0.9981 for Indomethacin) within 200-4600 ng/mL, alongside successful precision, accuracy, recovery, and stability evaluations at concentrations of 250, 750, 1500, and 3000 ng/mL.

Keywords: Febuxostat, Indomethacin, Analytical Quality by Design, Green Analytical Chemistry, Validation.

Abbreviation

FEB: Febuxostat. IND: indomethacin; HPLC: High Performance Liquid Chromatography; AQbD: Analytical Quality by Design; MODR: Method Operable Design Region; DoE: Design of Experiments; GAC: Green Analytical Chemistry; AGREE; Analytical Greenness Calculator; ComplexGAPI: Green Analytical Procedure Index; RGB: Red, Green, Blue; AMGS; Analytical Method Greenness Score; CCD: Central Composite Design; QC: Quality control; LLOQ: Lower limit of quantification; LQC: Low-quality control; MQC: Medium-quality control; HQC: High-quality control; IAEC: Institutional Animal Ethical Committee; LOD: limit of detection; LOQ: Limit of quantification; CS: Colour Score; C&S: Cause and Effect; MB: Method Brilliance;

LAV: Lowest Acceptable Value; LSV: Lowest Satisfactory Value; CNX: Control-Noise-Experimentation; Factor A: pH; Factor B: Ethanol concentration; Factor C: Flow rate; R1: Resolution (Rs); R2: Asymmetric factor (As); R1(FEB): Resolution of Febuxostat; R1(IND): Resolution of Indomethacin; R2(FEB): Asymmetric factor of Febuxostat; R2(IND): Asymmetric factor of Indomethacin; R: Redness(Analytical performance), R1: Scope of application, R2: LOD (R2.1)&LOQ (R2.2), R3: Precision, R4: Accuracy ; G: Greenness(Safety and eco-friendliness), G1: Toxicity of the reagents, G2: Amount of reagents and waste, G3: Consumption of energy and waste, G4: Direct impact (Occupational hazards and no of Genetically Modified Organism); B: Blueness (Productivity/ Practical effectiveness), B1: Cost-effectiveness, B2: Time efficiency, B3: Requirements: sample consumption (B3.1) & Advanced instruments (B3.2), B4: Operational simplicity: Mini-automatization (B4.1) & Portability (B4.2); SD, Standard Deviation;%RE Percentage of Relative Error; CV, coefficient of variation.

Introduction

Gout is a well-recognized and chronic metabolic disorder that continues to pose a significant and growing public health burden worldwide, particularly in developed countries, due to its increasing prevalence, lifestyle associations, and impact on quality of life.[1,2] Its rising incidence is linked to lifestyle changes, an ageing population, and dietary shifts, transforming gout from a "disease of affluence" to a widespread health concern. Recent studies as of 2020, gout affected an estimated 55.8 million people globally, reflecting its substantial burden. The global prevalence varies by region, with developed countries reporting rates between 1–4%, while the overall worldwide prevalence is estimated to be between 0.6% and 1.0% depending on population demographics and diagnostic criteria.[3–9]

Febuxostat (FEB) is a non-purine xanthine oxidase inhibitor that helps lower uric acid levels ($C_{16}H_{16}N_2O_3S$). In contrast, indomethacin (IND), a non-steroidal anti-inflammatory drug (NSAID) (C19H16ClNO4) alleviates pain and inflammation during acute gout flare-ups by inhibiting pro-inflammatory prostaglandins through the COX-1 and COX-2 enzymes[10]

Despite the availability of urate-lowering therapies such as febuxostat and allopurinol, gout treatment continues to face several challenges. Drug resistance and suboptimal response are observed in a subset of patients, often necessitating combination therapy or individualized dosing strategies. Genetic variability also plays a significant role in treatment outcomes—polymorphisms in genes such as *URAT1 (SLC22A12)*, *ABCG2*, and *HLA-B*5801* affect drug metabolism, efficacy, and risk of adverse reactions. Additionally, considerable inter-individual differences in uric acid metabolism, renal function, and comorbidities complicate standard treatment protocols. Patient non-compliance with long-term therapy is another critical issue, often driven by asymptomatic periods, medication side effects, or lack of disease awareness. These factors underscore the need for tailored treatment approaches and highlight the importance of developing robust, patient-adapted analytical methods to support therapeutic monitoring[11–13].

Effective gout treatment often involves using both FEB and IND, as these medications target uric acid levels and inflammation. Understanding these medications' actions, benefits, and potential side effects is crucial for successful gout management. The co-administration of febuxostat and indomethacin is often encountered in clinical practice, particularly during the initiation phase of urate-lowering therapy, where indomethacin or other NSAIDs are prescribed to prevent or manage acute gout flares. International guidelines such as those from the EULAR and ACR recommend concurrent use of anti-inflammatory agents during urate-lowering initiation to improve patient outcomes. Therefore, a robust, simultaneous analytical method for FEB and IND

is valuable for therapeutic monitoring, pharmacokinetic studies, and drug interaction assessments.[14] Additionally, reliable bioanalytical methods are needed to simultaneously monitor their levels in blood plasma, as current literature indicates a need for more techniques for this purpose.[15,16]

The AQbD strategy was not limited to theoretical considerations but was applied to address key challenges specific to the simultaneous estimation of febuxostat and indomethacin in plasma. Risk assessment identified matrix interferences, co-elution risks, and analyte degradation as critical concerns. Using a central composite design (CCD), we optimized mobile phase composition, pH, and flow rate to achieve robust separation and stability. Matrix complexity was mitigated through optimized extraction and specificity studies, while analyte stability was ensured through validated storage and handling protocols.[17] A central concept of AQbD is the establishment of a Method Operable Design Region (MODR), which identifies and optimizes critical method parameters like buffer pH, Ethanol concentration and flow rate which effects the responses like Resolution and asymmetric factor to maintain performance within desired limits. This approach integrates tools such as Design of Experiments (DoE), risk analysis, and control strategies to enhance method robustness and adaptability, ensuring compliance with regulatory and performance standards across various conditions.[18–20]

Moreover, Green Analytical Chemistry (GAC) principles are increasingly incorporated into bioanalytical method development to promote sustainable and environmentally friendly practices. GAC focuses on eco-friendly analytical practices, aiming to decrease the environmental footprint through reduced consumption of hazardous reagents and solvents, and improved energy efficiency.[21] For the development of the reverse-phase high-performance liquid chromatography (RP-HPLC) method, ethanol was selected as the green solvent of choice, providing a more sustainable alternative to methanol and acetonitrile with reduced environmental implications and the drugs are not soluble with other green solvents like methanol, acetonitrile , ethyl lactate or propylene carbonate so for better results in bioanalytical method ethanol is selected . By adopting GAC principles, bioanalytical methods can be developed to balance analytical performance with environmental responsibility. Ethanol led to slightly increased backpressure compared to acetonitrile, these trade-offs were considered acceptable in light of ethanol's lower toxicity, biodegradability, and renewable origin.[22] Tools such as Analytical Greenness Calculator (AGREE), Green Analytical Procedure Index (ComplexGAPI), Red, Green, Blue (RGB) evaluation, and Analytical Method Greenness Score (AMGS) calculator assist in quantifying and visualizing the sustainability of these methods, ensuring they meet quality and regulatory standards while aligning with global sustainability objectives.[23–25]

Combining AQbD and GAC approaches facilitates the formulation of efficient strategies that consider environmental impact throughout their lifecycle, promoting sustainable bioanalysis practices.[26]

Despite the frequent co-prescription of febuxostat and indomethacin during gout management, no validated method currently exists for their simultaneous estimation in plasma that also adheres to green analytical principles. This study addresses this gap by hypothesizing that a robust, ethanol-based RP-HPLC method can be developed using an Analytical Quality by Design (AQbD) framework to achieve both high analytical performance and environmental sustainability. The integration of GAC assessment tools such as AGREE and ComplexGAPI further enhances the relevance and impact of the method in advancing eco-conscious pharmaceutical bioanalysis.

Result

Risk Assessment

CNX was conducted to determine critical factors potentially impacting method characteristics through Cause and Effect (C&E) risk evaluation matrix for FEB and IND (Table 1).[27,28]

Table 1 Control-Noise-Experimentation (CNX) approach-for propose Analytical Method of Febuxostat and Indomethacin

Parent Parameter	Critical Method Parameter	Critical Method Attributes		Initial Risk assessment Scores	C,N,X	Experimental Strategy
		Resolution (Rs)	Asymmetric Factor (As)			
Pump	**Isocratic Quaternary Parameter**	2	2	40	C	**Calibrated**
	Flow Rate	10	10	200	X	**DOE**
Column	**Stationary Phase**	5	5	100	C	**New Column**
	Particle size	2	2	40	C	**Optimum**
	Dimension	2	2	40	C	**Standard**
	Column Temp	5	5	100	N	**Ambient**
Mobile Phase	**Buffer pH**	10	10	200	X	**DOE**
	% organic Modifier	10	10	200	X	**DOE**
	Solvent Grade	5	5	100	N	**HPLC grade**
Injection	**Injection Vol**	2	2	40	C	**20µL**
Flow cell	**Flow Cell temp**	5	5	100	C	**40^0 C**
Detector	**DAD**	5	5	100	N	**Standard**

Note: C-Control, N-Noise & X-Experiment Score Low Risk-2, Medium Risk-5 & High Risk-10.**Total Score** = (Risk level of First CMA×10) + (Risk level of Second CMA × 10)
DOE: Design of Experiment, DAD:Diode Array Detector

Design of Experiment

To optimize chromatographic separation, CCD was utilized, analyzing the impact of key settings on separation quality through Design Expert version 13.0.5 (Table 2)the investigated factor ranges were defined as: pH (3.5–5.5), ethanol concentration (61–71%), and flow rate (0.5–1.1 mL/min), all the factors based on preliminary risk assessment and their known influence on chromatographic performance. Using a Central Composite Design (CCD), a total of 20 experimental runs were conducted. The resulting responses such as resolution and asymmetry factor were recorded for each run Under these conditions, FEB and IND and showed an asymmetry factor of 1.012 and 1.013,

well within the acceptable ICH and USP range of 0.9–1.2, thereby demonstrating that the DoE strategy effectively enhanced peak shape and overall chromatographic performance All experiments under the CCD framework were randomized to mitigate the impact of uncontrolled variables. The design facilitated comprehensive evaluation of main, interaction, and quadratic effects. ANOVA assessed relationships between factors and responses, including p-values for FEB and IND (Asymmetric Factor and Resolution) (see Table 3).[29]

Table 2 Experimental Design: Central Composite design: 3 Factors with 2 Responses

	Factor 1	Factor 2	Factor 3	Response 1(Resolution): R1		Response 2(Asymmetric factor): R2	
Run	A:pH	B:Ethanol concentration	C:Flow rate	R1(FEB)	R1(IND)	R2(FEB)	R2(IND)
	pH	%	ml/min	Rs	Rs	As	As
1	3.5	61	0.5	11.525	11.689	1.125	1.127
2	4.5	66	0.8	5.434	8.037	1.075	1.012
3	4.5	66	0.8	5.482	8.045	1.087	1.013
4	6.181	66	0.8	4.314	4.426	1.001	1.093
5	4.5	57.591	0.8	9.385	11.787	0.908	1.065
6	4.5	66	0.295	4.627	9.876	1.066	1.209
7	4.5	66	0.8	5.536	8.025	1.087	1.013
8	4.5	66	0.8	5.541	8.124	1.098	1.021
9	5.5	61	0.5	11.525	11.689	1.125	1.127
10	4.5	66	0.8	5.554	8.303	1.078	1.013
11	3.5	71	1.1	5.581	6.818	1.131	1.186
12	5.5	71	1.1	3.549	3.756	1.053	1.141
13	4.5	66	0.8	5.421	8.021	1.084	1.011
14	5.5	61	1.1	7.199	6.565	0.902	1.059
15	3.5	71	0.5	5.813	8.163	1.274	1.233
16	5.5	71	0.5	3.715	4.108	1.044	1.147
17	2.818	66	0.8	13.099	5.191	0.990	1.096
18	4.5	74.409	0.8	2.910	4.977	1.175	1.182
19	3.5	61	1.1	10.464	9.925	0.959	1.089
20	4.5	66	1.30454	5.800	6.804	0.882	1.059

Factor A: pH; Factor B: Ethanol concentration; Factor C: Flow rate; R1: Resolution (Rs); R2: Asymmetric factor (As); R1(FEB): Resolution of Febuxostat; R1(IND): Resolution of Indomethacin; R2(FEB): Asymmetric factor of Febuxostat; R2(IND): Asymmetric factor of Indomethacin.

Table 3 ANOVA coefficients with p-values for Response 1 (Resolution) and Response 2 (Asymmetric factor)

	R1(FEB)	p-Value	R1(IND)	p-Value	R2(FEB)	p-Value	R2(IND)	p-Value

Intercept	+5.47		+8.07		+1.08		+1.01	
A-pH	-1.62	0.0013	-0.8614	0.0053	-0.0253	0.1524	-0.0122	0.0634
B-Ethanol concentration	-2.41	< 0.0001	-2.09	< 0.0001	+0.0616	0.0036	+0.0368	< 0.0001
C-Flow rate	-0.2790	0.4640	-1.01	0.0020	-0.0610	0.0039	-0.0302	0.0004
AB	-0.1081	0.8258	-0.4696	0.1702	-0.0314	0.1721	-0.0127	0.1273
AC	-0.3999	0.4230	-0.2958	0.3737	+0.0120	0.5875	+0.0014	0.8561
BC	+0.6236	0.2219	+0.6491	0.0683	+0.0317	0.1685	+0.0065	0.4125
A^2	+1.27	0.0051	-1.01	0.0016	-0.0151	0.3646	+0.0316	0.0002
B^2	+0.3700	0.3239	+0.2539	0.3087	+0.0011	0.9461	+0.0419	< 0.0001
C^2	+0.0398	0.9133	+0.2389	0.3366	-0.0229	0.1807	+0.0456	< 0.0001

Factor A: pH; Factor B: Ethanol concentration; C:Flow rate ; R1: Response1: Resolution (Rs), R2: Response2: Asymmetric factor (As); R1(FEB): Resolution of Febuxostat; R1(IND): Resolution of Indomethacin; R2(FEB): Asymmetric factor of Febuxostat;R2(IND): Asymmetric factor of Indomethacin.

Green evaluation Result
ComplexGAPI

The environmental impact of the developed RP-HPLC method was evaluated using the ComplexGAPI tool, which considers both sample preparation and analytical processes. Key factors assessed during the sample preparation stage included sample collection, preservation, transportation, storage, extraction scale, and associated health and safety hazards. For the analysis phase, instrumentation energy consumption, waste generation, waste treatment, pre-analysis yield, temperature, analysis time, and purity were evaluated. An E-Factor below 1.0 indicates a low-waste analytical process, aligning with green chemistry goals. The use of ethanol, minimal sample volume, and short analysis time all contributed to this reduced environmental impact. The method's E-Factor was calculated to be 0.4, indicating low waste production relative to sample output.

Each parameter was assessed and represented using a color-coded system: green for low environmental impact, yellow for moderate, and red for high. The ComplexGAPI pictogram generated for this method (Figure 1) shows that most segments were green, reflecting the method's minimal sample volume, use of ethanol as a green solvent, short run time, and simple sample preparation. A single red zone was noted due to the use of animal-derived plasma, which carries inherent environmental and ethical concerns. Overall, the ComplexGAPI evaluation confirms the eco-friendly nature of the proposed method across multiple operational stages.

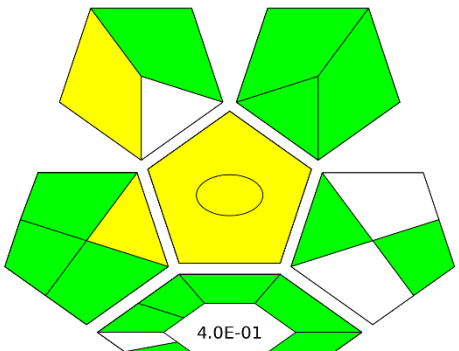

Figure1 Data on the greenness score of analytical methods for ComplexGAPI

AGREE

Analytical GREEnness (AGREE) is a sophisticated evaluative tool crafted to evaluate environmental sustainability or 'greenness' of analytical methodology in chemistry, facilitating the development and selection of sustainable practices. AGREE operates through a radar chart framework that employs twelve distinct criteria, each grounded in the 'Twelve Principles of Green Analytical Chemistry' (GAC), to systematically score and visualize the environmental implications of an analytical method. Each criterion is evaluated on a 0-1 scale, where a higher score signifies greater alignment with green chemistry principles. The comprehensive nature of AGREE allows for an in-depth comparison of analytical methodologies, highlighting areas for improvement and advancing the field toward environmentally sustainable, efficient, and safer practices. The RP-HPLC method demonstrated exceptional environmental sustainability, achieving a green score of 0.91, close to 1, indicating the method's greenness and environmental sustainability. For this RP-HPLC method, the AGREE graph was given in Figure 2.

Figure 2 Data on the greenness score of analytical methods, as assessed by AGREE

RGB

The RGB data is represented using two key metrics: Color Score (CS) and Method Brilliance (MB). These metrics offer a structured way to evaluate and visualize the effectiveness of analytical methods. The CS ranges from 0% to 100%, serving as a performance measure for specific criteria. A 66.6% or higher CS indicates a "satisfaction range," suggesting that the

method effectively meets expectations for that particular attribute, thereby earning an associated primary colour. On the other hand, if the CS falls between 33.3% and 66.6%, it is categorized as a "tolerance range." In this case, the method is considered neutral (colourless) concerning that specific attribute and unclear regarding two primary aspects. If one attribute is rated as tolerable while the others are satisfactory, the method is represented by a cyan, magenta, and yellow composite colour. Conversely, if one CS value is in the satisfaction range while two are in the tolerance range, the method is identified with a secondary colour, such as green (Safety and eco-friendliness), red (Analytical performance), or blue (Productivity/ Practical effectiveness). If all three CS values remain within the tolerance range but do not reach the satisfaction threshold, the method is deemed colourless and marked in grey. However, should any CS value drop below 33.3%, the process is classified as black, indicating a lack of transparency and unsatisfactory performance for any attribute. For each criterion, two reference values are predefined: the "Lowest Acceptable Value" (LAV), which corresponds to a score of 33.3, and the "Lowest Satisfactory Value" (LSV), aligned with a score of 66.6. In the case of the RP-HPLC method, the CS was recorded as 92.919%, 90.569%, and 90.189% for red, green, and blue, respectively. Additionally, the MB for the RP-HPLC process was found to be 91.218%, indicating that the method is environmentally sustainable and appears white regarding whiteness, as summarized in Table 4.

Table 4 Greenness assessment by RGB

Method	R1	R2		R3	R4	G1	G2	G3	G4		B1	B2	B3		B4		MB
HPLC Method	100	80.004	96.671	92.657	96.557	100	76.182	79.992	100	100	81.325	91.667	80	100	94.435	95.563	91.218
	CS: 92.919					CS: 90.569					CS: 90.189						

R: Redness(Analytical performance), R1: Scope of application, R2: LOD (R2.1)&LOQ (R2.2), R3: Precision, R4: Accuracy ; G: Greenness(Safety and eco- friendliness), G1: Toxicity of the reagents, G2: Amount of reagents and waste, G3: Consumption of energy and waste, G4: Direct impact (Occupational hazards and no of Genetically Modified Organism); B: Blueness (Productivity/ Practical effectiveness), B1: Cost-effectiveness, B2: Time efficiency, B3: Requirements: sample consumption (B3.1) & Advanced instruments (B3.2), B4: Operational simplicity: Mini-automatization (B4.1) & Portability (B4.2); CS: Color Score; MB: Method brilliance

AMGS

The AMGS score for the developed method was 1054.63 (Figure 3). Although not in the 'ideal' green range, it indicates a significant reduction in environmental impact compared to conventional RP-HPLC methods (typically >1300), especially those using hazardous solvents like acetonitrile. The score reflects the moderate-to-good greenness of the method, supported by ethanol use and simplified sample handling.

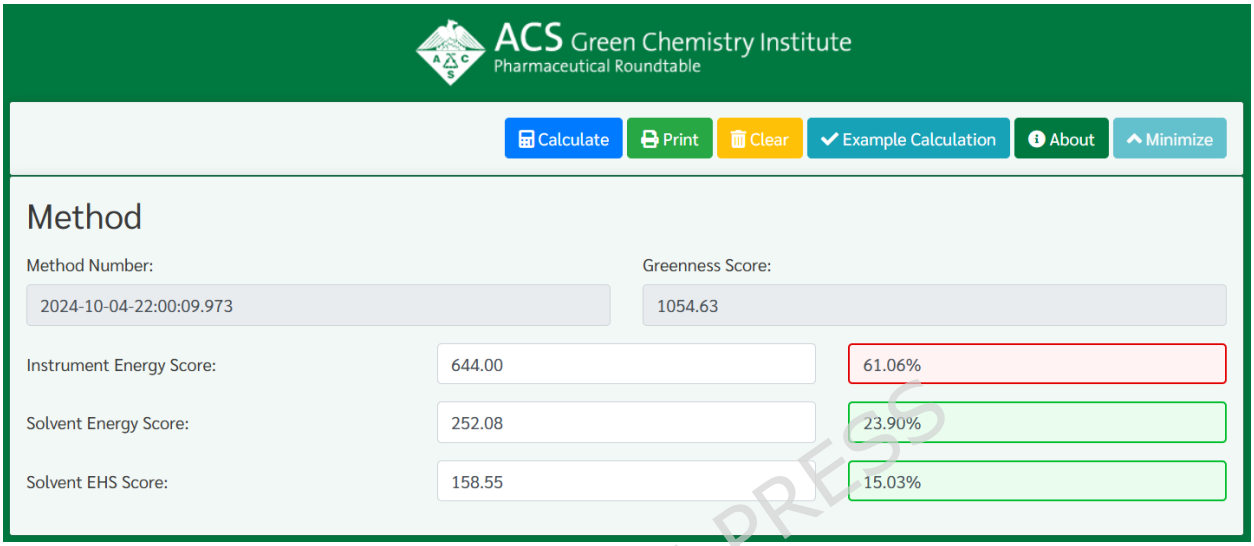

Figure 3 Data on the greenness score of analytical methods for AGMS.

Validation of the method

Specificity Study

The gradual resolution and separation of plasma and interference peaks from those of FEB and IND, as illustrated in Figure 4, confirm the specificity of the method. The blank chromatogram represents the injection of ethanol alone as the mobile phase component, serving as a system suitability control. This was compared with chromatograms obtained from standard drug solutions in ethanol and from spiked rabbit plasma samples. No significant shift in retention time was observed across these comparisons, indicating the method's consistency and the absence of major matrix interferences. The small shift on the chromatogram due to biological matrices such as plasma contain endogenous substances (e.g., proteins, lipids, salts, and phospholipids) that can influence chromatographic behavior, including retention time, peak shape, or signal intensity. These effects are commonly encountered in LC-based bioanalytical methods and are well-documented in regulatory guidelines. Therefore, this technique makes it possible to identify both medications in rabbit plasma precisely.[30]

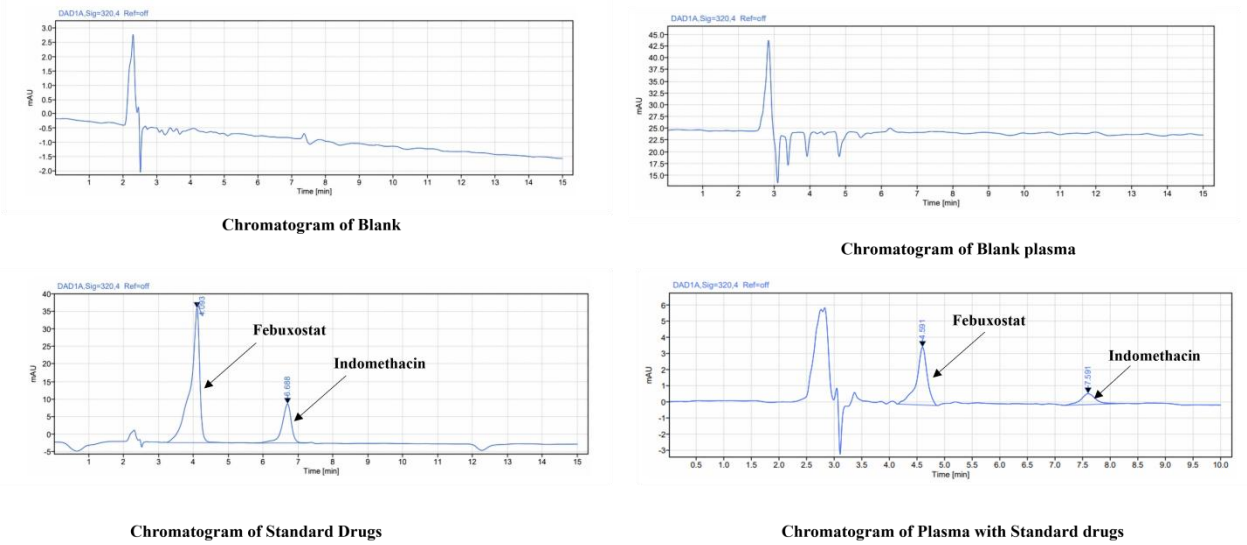

Figure 4 A specificity study of Febuxostat and Indomethacin in rabbit plasma.

Accuracy and precision

Tables 5 and 6 provide an overview of the accuracy and the intra- and inter-day precision, which are quantified as relative standard deviation (RSD). The method established herein demonstrates suitability for the precise and accurate quantification of individual pharmaceuticals in rabbit plasma. The results are further supported by LLOQ, LQC, MQC, and HQC triplicate values, which fall within established acceptance criteria (RSD ≤ 15% , %RE ±20% and %CV ≤ 2).[31–33]

Table 5 Accuracy data of FEB/IND (n=6).

QC Levels	Nominal conc(ng/ml)	FEB(ngml^{-1})			IND(ngml^{-1})		
		Mean conc±SD	%CV	%RE	Mean conc±SD	%CV	%RE
LLOQ	250	245.730±3.970	1.521	-3.105	225.941± 0.373	1.654	-9.623
LQC	750	749.608±2.938	0.392	-0.052	739.507± 2.795	0.377	-1.399
MQC	1500	1396.479±3.656	0.763	-6.901	1284.727±2.134	0.166	-14.351
HQC	3000	2972.859±4.938	0.166	-0.904	2983.565±0.322	0.108	-0.547

QC Level, Quality Control Level; CV, coefficient of variation; SD, Standard Deviation; LLOQ, Lower Limit of Quantification; LQC, Low Quality Control; MQC, Medium Quality Control; HQC, Quality Control; SD, Standard Deviation; %RE Percentage of Relative Error; CV, coefficient of variation**.**

Table 6 Intra and inter- day precision of FEB/IND (n=6)

Analyte	QC Levels	Nominal conc(ng ml^{-1})	Intraday precision			Interday precision		
			Measured Concentration (Mean ± SD)	%CV	%RE	Measured Concentration (Mean ± SD)	%CV	%RE
FEB(ng ml^{-1}	LLQC	250	244.283±2.102	1.871	-2.286	244.624 ± 2.120	1.960	-2.150

)	LQC	750	749.835±2.892	0.385	-0.021	748.022 ± 0.977	0.130	-0.263
	MQC	1500	1396.819±2.884	0.206	-6.878	1400.727±0.499	0.351	-6.618
	HQC	3000	2971.613±0.602	0.020	-0.946	2970.679±1.544	0.051	-0.977
IND(ng ml^{-1})	LLQC	250	227.233±2.909	1.280	-9.106	226.717±3.488	1.538	-9.313
	LQC	750	743.383±2.795	0.375	-0.082	743.771±1.689	0.227	-0.830
	MQC	1500	1284.221±1.747	0.136	-14.368	1285.890±1.863	0.142	-4.274
	HQC	3000	2993.771±3.214	0.775	-0.207	2985.890±0.806	0.027	-0.470

QC Level: Quality Control Level; CV: coefficient of variation; SD: Standard Deviation; LLOQ: Lower limit of quantification; LQC: Low Quality control; MQC: Medium Quality control; HQC: High Quality control; SD: Standard Deviation; %RE: Percentage of Relative Error; CV: coefficient of variation.

Linearity Study

A total injection volume of 20 µL was utilized under optimal chromatographic conditions, analyzing the entire set of calibration standards across six replicates (200-4600ng/ml). The peak areas were plotted against their corresponding concentrations to establish calibration curves. Both weighted and unweighted linear regression analyses were conducted to evaluate the calibration data Weighted regression was applied to address heteroscedasticity observed in the calibration data, where variance increased with concentration. We compared weighted and unweighted models and selected the weighted regression for validation due to its superior fit and more uniform residual distribution, as supported by improved correlation coefficients and residual analysis. Table 7 shows that the optimal weighting factors were determined based on R^2 values, and the percentage relative error (% ΣRE) is also presented in the table. The model showed statistically significant outcome for FEB (F=4.23, p=0.0172) and IND (F=14.74, p=0.001), The linearity of the calibration curve was evaluated using regression analysis, with the F-test applied to assess the significance of the linear relationship between concentration and response. An F-value of 4.23 for FEB indicates that the regression model significantly explains the variability in the data ($p < 0.05$), supporting the suitability of the linear model within the tested range. meeting the 95% confidence threshold.[34]

Table 7 Linearity data of FEB/IND (n=6).

Drug	**Weight Factor(w)**	**Weighting least square linear regression**			
		X	**1/X**	**1/√x**	**1/X^2**
FEB	**R^2**	0.9959	0.7747	0.5739	0.9013
	%RE	-14.958	565.399	27290.448	-30.245
IND	**R^2**	0.9981	0.6281	0.4222	0.7833
	%RE	30.691	-836.678	40202.94	7474.645

R2: Correlation Coefficient; %RE: Percentage of Relative Error; QC Level: Quality Control Level; CV: coefficient of variation; SD: Standard Deviation; LLOQ: Lower limit of quantification; LQC: Low Quality control; MQC: Medium Quality control; HQC: High Quality control; %RE: Percentage of Relative Error; CV: coefficient of variation SD: Standard Deviation**.**

Recovery Study

The absolute percentage recoveries for the LLQC, LQC, MQ, and HQC samples are presented in Table 8. Using ethanol as the extraction solvent significantly improved these recoveries The mean recovery ranged from 86.924% to 99.872% across all levels, meeting the acceptance criteria of 85–115% as recommended by regulatory guidelines for bioanalytical methods. The recovery percentages for each substance were sufficient to ensure precise and accurate quantification meeting the specified detection range. %CV ≤ 2 defines the method precision and lower matrix effect.[35–37]

Table 8 Percentage recovery studies of FEB/IND (n=3)

Analyte	QC Levels	Nominal conc(ng ml^{-1})	% Recovery			
			Mean ± SD	%CV	%RE	Recovery (%)
FEB	LLQC	250	239.583±2.173	1.740	-4.1667	95.833
	LQC	750	749.042±3.630	0.484	-0.127	99.872
	MQC	1500	1396.280±1.768	0.126	-6.914	93.085
	HQC	3000	2947.399±3.658	0.124	-1.753	98.246
IND	LLQC	250	228.267±2.926	1.281	-8.693	91.306
	LQC	750	692.996±0.834	1.203	-7.600	92.399
	MQC	1500	1259.133±1.794	1.418	-14.057	86.924
	HQC	3000	2947.389±1.567	0.531	-1.753	98.246

QC Level: Quality Control Level; CV: coefficient of variation; SD: Standard Deviation; LLOQ: Lower limit of quantification; LQC: Low Quality control; MQC: Medium Quality control; HQC: High Quality control; CV: coefficient of variation; SD: Standard Deviation; %RE: Percentage of Relative Error.

Stability Study

Both investigational candidates underwent stability tests in a variety of settings (RSD ≤ 15% , %RE ±20% and %CV ≤ 2) ; the findings are compiled in Table 9.[38–40]

Table 9 Stability of the analytes in rabbit plasma in four QC level (n=6).

Stability	Measured Concentration (Mean ± SD)			%CV		%RE	
	Nominal conc(ng ml^{-1})	FEB	IND	FEB	IND	FEB	IND
0h	250	186.935±0.932	231.755±3.378	0.498	1.457	-2.522	-7.97
	750	711.262±4.488	738.086±1.911	0.631	0.259	-5.165	-1.588
	1500	1396.054±0.960	1290.283±2.420	0.694	0.187	-6.929	-13.981
	3000	2944.227±3.204	2912.505±5.831	0.108	0.201	-1.859	-2.916

Bench Top (24 h) Rt	250	187.161±0.770	232.272±1.613	0.411	0.694	-2.513	-7.090
	750	713.698±1.669	739.378±2.271	0.233	0.307	-4.840	-1.416
	1500	1398.404±2.330	1290.025±2.134	0.166	0.165	-6.773	-13.998
	3000	2943.661±4.337	2910.309±3.977	0.147	0.136	-1.877	-2.989
Freeze and Thaw (-80^{0}C for 3 three cycles)	250	190.333±0.273	230.722±2.368	0.143	1.026	-2.386	-7.711
	750	713.244±0.429	739.378±2.271	0.259	0.307	-4.901	-1.416
	1500	1394.411±1.254	1292.609±2.541	0.297	0.196	-7.299	-13.826
	3000	2943.831±4.022	2912.376±5.467	0.136	0.187	-1.872	-2.920
Long term (-80^0 45 days)	250	185.745±0.6433	231.239±2.491	0.346	1.077	-2.570	-7.504
	750	711.460±4.292	748.422±1.720	0.603	2.299	-5.138	-0.210
	1500	1394.411±1.757	1292.867±4.624	0.543	0.357	-7.039	-13.808
	3000	2945.077±2.346	2935.373±3.294	0.079	0.112	-1.830	-2.154

QC level: Quality Control level; CV: coefficient of variation; SD: Standard Deviation; LLOQ: Lower limit of quantification; LQC: Low Quality control; MQC: Medium Quality control; HQC: High Quality control; CV: coefficient of variation SD: Standard Deviation; %RE: Percentage of Relative Error.

Discussion

We have developed a systematic RP-HPLC method to establish suitable chromatographic conditions and streamline the sample preparation process for the simultaneous estimation of FEB and IND in rabbit plasma. We intentionally optimized various liquid chromatographic parameters, such as flow rate, stationary phase, injection volume, mobile phase composition, and other system suitability factors, to achieve optimal resolution (Rs) and asymmetry factor (As). The LogD curve generated with the demo version of the ChemAxon Log-D predictor was instrumental in guiding the column selection and identifying the optimal pH range for the mobile phase buffer. Within a pH range of 2 to 5, the Log-D plot for FEB showed a nearly flat slope, indicating consistent retention times for this compound across the specified range. In contrast, the Log-D plot for IND displayed a curved profile, suggesting that its retention values were sensitive to even slight pH changes. The determined pKa values were 3.08 (weakly acidic) and 0.39 (strongly basic) for FEB, and 3.79 (weakly acidic) & -2.9 (strongly basic) for IND. Considering the physicochemical properties of both active pharmaceutical ingredients (APIs), we chose a pH range of 3 to 5 to optimize the buffer and column during method development and changes in the pH of the buffer will lead to lead to changes in the retention time, If the pH increased then both the drugs will ionized which will lead to shorter retention time because it will have less interaction with non-polar stationary phase.

We aimed to create a green analytical QbD hybrid fractionation liquid chromatography (HFLC) method to estimate both FEB and IND simultaneously. Given their moderate polarity, FEB and IND were more suitable for reverse-phase chromatography than normal-phase analysis. We optimized the RP-HPLC conditions to ensure adequate separation of the eluted compounds. Chromatographic separation took place using an Eclipse Plus C-18 column (250 × 4.6 mm, 5µm) at 25°C. Preliminary experiments were conducted at 20°C, 25°C, and 35°C to assess the impact of temperature on retention time, resolution (Rs), and peak symmetry. At 25°C, we observed optimal chromatographic performance, including consistent retention times, acceptable

resolution between critical pairs (Rs > 2.0), and improved peak symmetry (tailing factor < 1.5), with an injection volume of 20 µl and a total run time of 25 minutes for all solutions. The mobile phase and flow rate selection was based on peak characteristics: height, capacity, theoretical plates, tailing factor, and resolution. Initially, we used a mobile phase composed of ethanol and acetate buffer; we selected ethanol as it effectively dissolved both drugs and is recognized as a green solvent. We tested various proportions of ethanol and acetate buffer with a 1 ml/min flow rate. UV analysis identified 320 nm as the ideal wavelength for simultaneous detection of FEB and IND, providing a robust response. We maintained a constant column temperature of 25°C throughout the analysis. However, an acetate buffer (pH 4.5) was tested; however, it resulted in poor resolution between the analyte and its closely eluting impurity/metabolite, with a resolution (Rs) of <1.0. Upon switching to a phosphate buffer at pH 4.5, resolution improved significantly to an Rs value of >2.0, which meets the acceptance criteria for chromatographic separation. As a result, we adopted a mixture of ethanol and potassium dihydrogen orthophosphate (50 mM) as the mobile phase. We adjusted the pH to 4.5 using orthophosphoric acid, significantly impacting the method's selectivity due to the compounds' differing pKa values. The isocratic mode effectively separated the peaks of both drugs, yielding appropriate resolution and retention times at a flow rate of 0.8 ml/min. Given that IND is less hydrophobic than FEB, we utilized a method for protein precipitation with green solvent ethanol to extract both APIs from rabbit plasma. Ethanol was selected as a protein precipitating agent due to its favorable environmental profile, including low toxicity and renewable sourcing While ethanol is generally considered a green solvent. It is not being compared with ACN and methanol as both drugs do not completely soluble which will lead to problems in chromatographic method. As no significant matrix interferences were observed, confirming adequate selectivity. These results demonstrate that ethanol can serve as an effective and greener alternative, provided that extraction efficiency and selectivity are rigorously validated, as projected by the ChemAxon demo version. This approach resulted in clear chromatograms for blank plasma samples, demonstrating the method's effectiveness in providing reliable analytical results.

Noise Control Experimentation

To determine critical method parameters (CMPs) for optimal separation and elution, a Control Noise Experimentation (CNX) approach was integrated with C&E risk assessment matrix. The CMPs identified as pivotal to method performance included pH of the mobile phase, flow rate & ethanol concentration. The corresponding Critical Method Attributes (CMAs), defined as the resolution and asymmetry factor, were used to evaluate the quality and robustness of the method. These three CMPs and the associated CMAs were instrumental in generating the design space, ensuring an optimized and reliable analytical method for robust performance.[41]

Design of Experiment

Table 3 presents The Design of Experiments (DoE) analysis revealed that, for FEB, the most significant factors influencing resolution were A, B, and the quadratic term A^2, each with highly significant p-values (all below 0.01), while other factor combinations such as C, AB, AC, BC, B^2, and C^2 had no meaningful impact (all p-values above 0.2). For IND, resolution was significantly affected by A, B, C, and A^2, with interactions like AB, AC, BC, B^2, and C^2 remaining non-significant. Regarding the asymmetry factor, FEB was mainly influenced by B and C (p-values 0.036 and 0.039), whereas IND's asymmetry was significantly affected by B, C, A^2, B^2, and C^2. The high Model F-values (ranging from 26.80 to 64.53) confirm the statistical strength and reliability of the models, indicating that the main effects and quadratic terms of the

studied factors play a crucial role in determining both resolution and asymmetry for FEB and IND, while most interactions do not contribute significantly.

In this study, the coefficient of determination (R^2), adjusted R^2 values and predicted R^2 for resolution were 0.942,0.885 and 0.875 for FEB and 0.9299, 0.866 and 0.853 for IND, respectively. For the asymmetry factor, the R^2, adjusted R^2 and predicted R^2 values were 0.941, 0.854 and 0.845 for FEB and 0.952, 0.909 and 0.892 for IND, indicating excellent agreement between experimental data and model predictions as adjusted R^2 values ≥ 0.80, Value between adjusted and predicted R^2 value < 0.2 are indicative of good model fit and normal plot of residuals also added in the fig 5.

Adequate precision values were 9.761 for FEB, 13.868 for IND in terms of resolution, 10.484 for FEB and 13.511 for IND for the asymmetry factor, demonstrating an adequate signal for the model's suitability for the separation. The coefficients of variation (C.V.), which indicate model reproducibility and are considered reasonably reproducible when <10%, were found to be 9.4 for FEB and 8.65 for IND in terms of resolution, and 5.97 for FEB and 1.97 for IND for the asymmetry factor, underscoring the method's robustness and reproducibility.

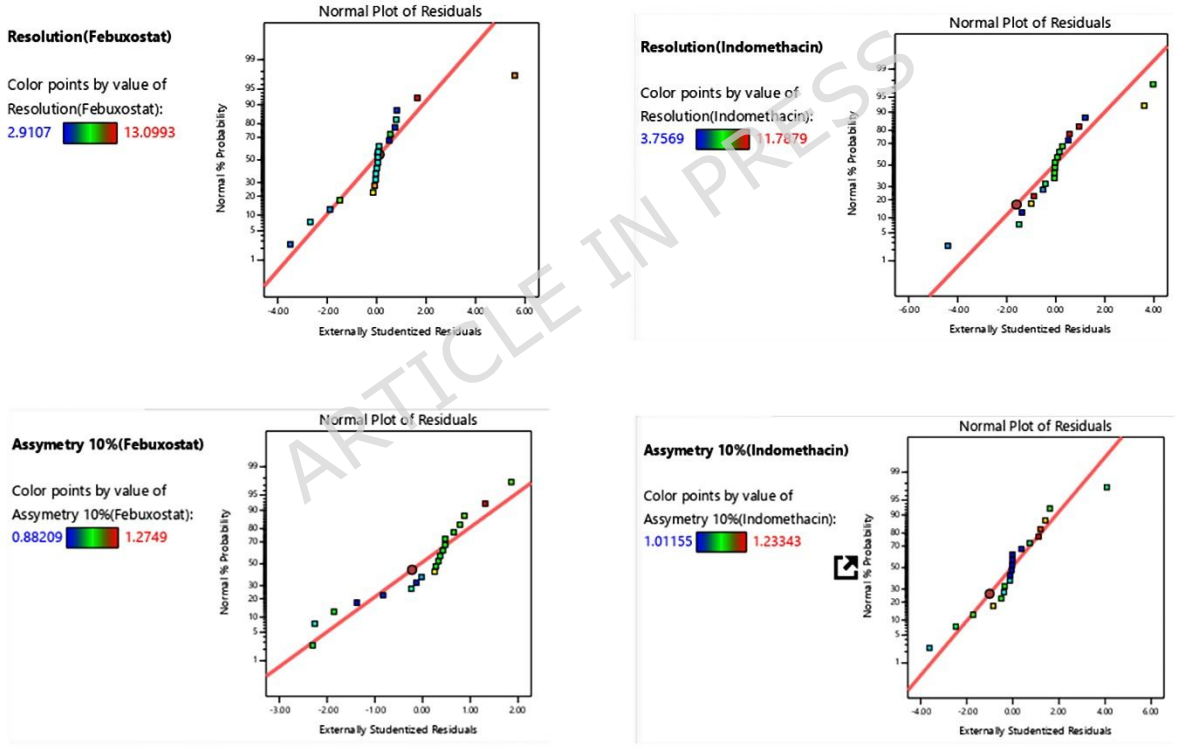

Fig 5 : Normal plot of residuals for the Febuxostat and Indomethacin for the response of Resolution and Asymmetric factor

Interaction Study

Response Surface Methodology (RSM) was used to examine and assess the effect of every individual variable and the interactive effects of the independent variables on each response.[42] (Figure 6) The correlation between the variables and the selected responses is illustrated through 3D contour plots for both FEB and IND. The 3D response surface plots for FEB reveal the following insights: (i) resolution declines linearly with a decrease in ethanol concentration and mobile phase pH, reaching a minimum at the lower value flow rates and pH values; and (ii) the asymmetry factor increases at the low pH, low flow rate, and maximum ethanol concentration. In the case of IND, the 3D response surface plots indicate that (iii) resolution achieves a maximum value if pH, ethanol concentration, and flow rate are minimized; & (iv) the asymmetry factor increases at lower pH and flow rate values, accompanied by a higher ethanol concentration.

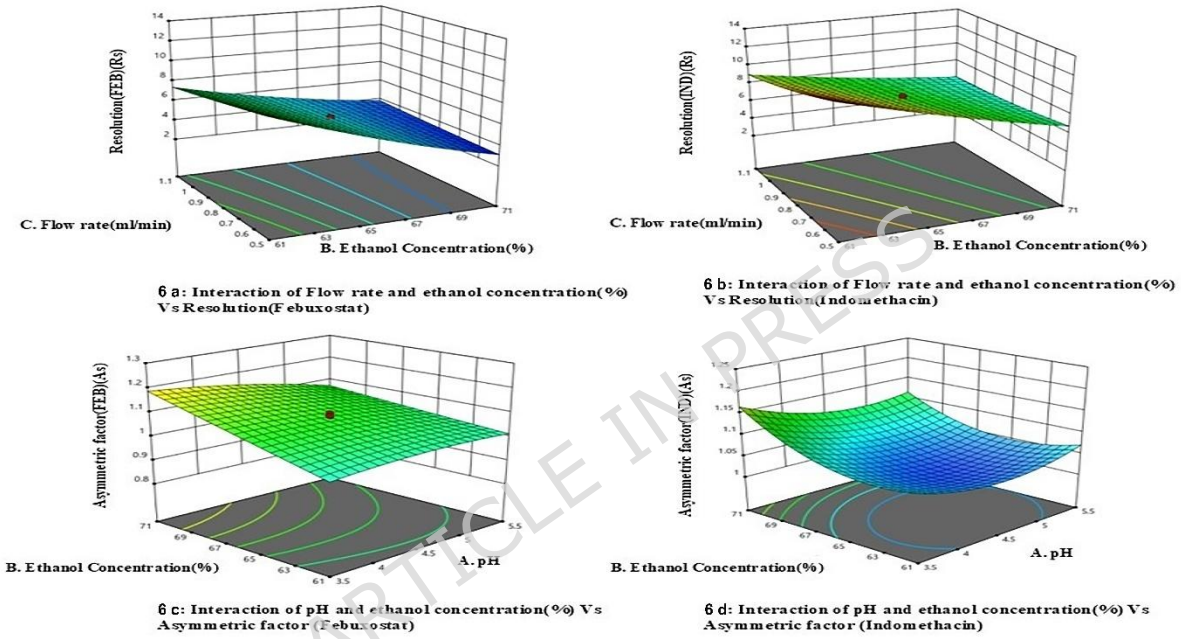

Figure 6 3D response surface plot illustrating the interaction study of flow rate, mobile phase pH, and ethanol concentration Vs resolution and Asymmetric factor.

Design Space and Desirability Optimization Using Derringer's Function

A two-dimensional design space (DS) was established by considering three key factors: flow rate, pH, and ethanol concentration, alongside two response variables, resolution and asymmetry factor. This design space is depicted in Figure 6, where the shaded red area within the 2D contour plots highlights the region that satisfies the criteria for both resolution and asymmetry factor, marking the robust region of the method. Our main goal was to reduce the asymmetry factor while enhancing the resolution of the asymmetrical peaks. We utilized the desirability function (D) to optimize multiple responses with varying targets. Figure 7 presents the response surface plot for the maximum desirability function (D = 1), illustrating the effectiveness of our mathematical model. The optimal parameters were a flow rate of 0.8 mL/min, a mobile phase

potassium dihydrogen orthophosphate buffer adjusted to a pH of 4.5, and an ethanol concentration of 34:66% (V/V). Under these conditions, we achieved ideal retention times of 4.539 minutes for FEB and 7.567 minutes for IND, respectively.

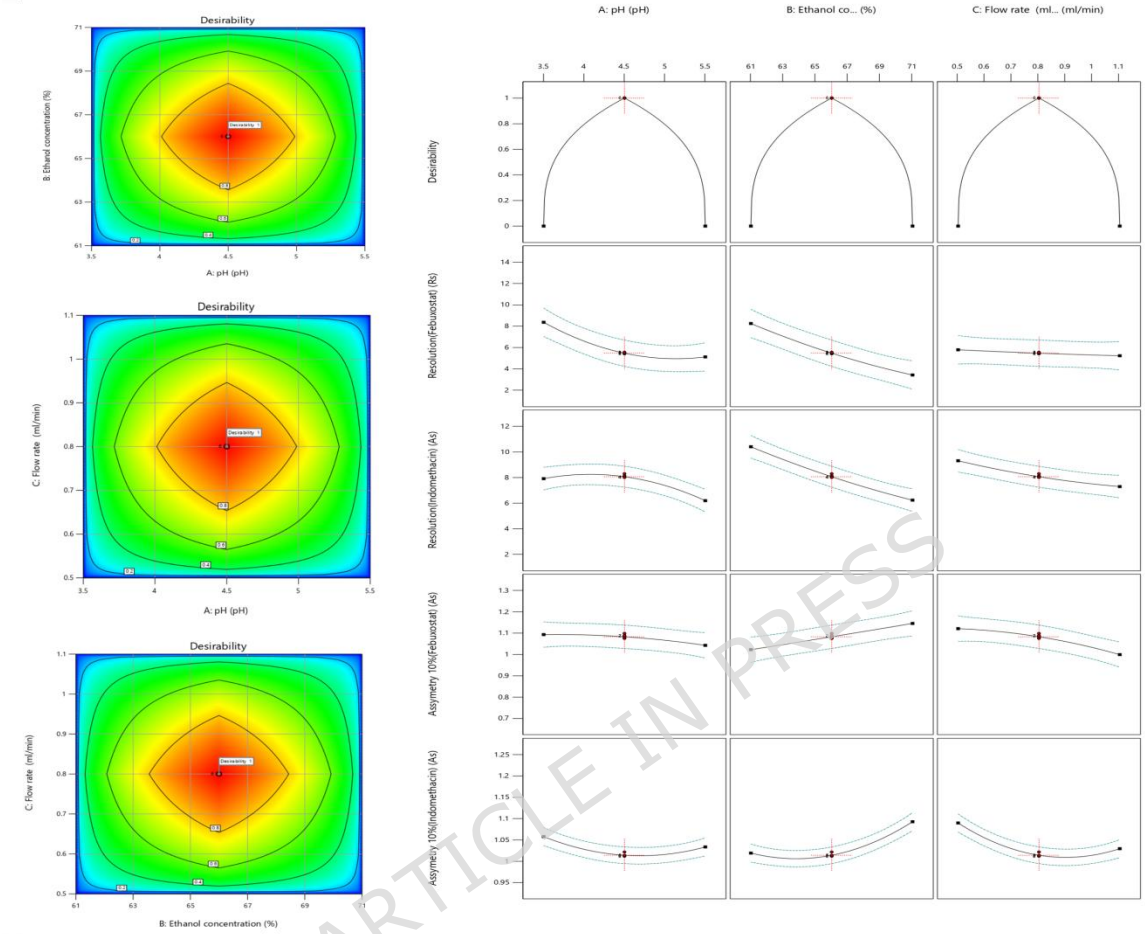

Figure 7 Design space and Derringer's desirability function

Outcome of Greenness evaluation

The tools ComplexGAPI E-Factor value ,AGREE score , RGB score, and AMGS score serve as essential instruments in defining the principles of Greenness and environmental sustainability, specifically within the framework of the RP-HPLC method.

At the forefront, the ComplexGAPI emerges as a measurable assessment tool that meticulously evaluates the ecological impact of various analytical methods, often called their "greenness." This innovative method enhances the foundational Green Analytical Procedure Index (GAPI) by incorporating additional criteria and deploying an extensive scoring system. It enables comprehensive evaluation of environmental sustainability across key analytical stages sample preparation, analysis, and waste disposal.

In the assessment of the RP-HPLC method, a notable E-factor value of 0.4 was recorded, signifying that this technique is greener and aligns with sustainable practices. The concept of Analytical Greenness is built on twelve distinct green analytical principles; each meticulously

assigned a score on a scale from 0 to 1. Here, a score that approaches 1 reflects a markedly greener methodology. For the RP-HPLC method, an impressive AGREE score of 0.91 was established, further affirming its credentials in terms of Greenness and sustainability.
Delving into the RGB methodology, the analysis yielded significant findings through the critical CS (Color Score) and MB (Matrix Block) parameters. Specifically, the RP-HPLC method produced CS values of 92.919% for red, 90.569% for green, and 90.189% for blue. In addition, the MB value was recorded at 91.218%. These metrics collectively suggest that the RP-HPLC method can be categorized as "white," thus highlighting its potential as a robust candidate for various applications.
Lastly, regarding the AMGS (Analytical Method Greenness Score Calculator) assessment, the data obtained culminated in a final score of 1054.63, as calculated by the AES Green Chemistry Institute's worksheet. This score is a testament to the RP-HPLC method's positive ecological impact, underscoring its commitment to sustainability in analytical procedures.

Conclusion

Our study combined Quality by Design (QbD) principles with green chemistry to develop an efficient, eco-friendly analytical method for the simultaneous determination of FEB and IND in rabbit plasma using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). We employed tools such as Complex GAPI, AGREE, RGB, and AMGS to develop a robust method with no interactions among them that utilizes ethanol as a sustainable solvent, thereby reducing its environmental impact. The QbD approach began with evaluating the physicochemical properties of FEB and IND to select input variables for the Design of Experiments (DoE) through a Central Composite Design (CCD). Rather than concentration, we focused on pH, flow rate, and ethanol concentration as independent variables, and on resolution and the asymmetry factor as dependent variables. We systematically examined the Analytical Quality by Design (AQbD) process to delineate the Design Space; using Response Surface plots to show how pH, flow rate, and ethanol concentration affected separation. Our method demonstrated accuracy, precision, linearity, cost-effectiveness, and environmental sustainability through validation. The AQbD framework supported the method development process and confirmed optimal conditions, reducing uncertainty. This approach is recognized as a safe, green, and reliable alternative to traditional methods, effectively enhancing analytical performance.

Several limitations are acknowledged in this study. While the method effectively combined QbD and green chemistry, optimization was limited to key variables, leaving other factors that may influence chromatographic performance unexamined. Additionally, the study was restricted to rabbit plasma in a single laboratory, indicating that applicability to other biological samples, long-term stability, and reproducibility across laboratories require further investigation.

Material and method

Reagent

FEB and IND were obtained as complimentary samples from Signova Pharma Pvt. Ltd. in Assam, India. Ethanol (HPLC grade) was sourced from CSI in China. Potassium dihydrogen orthophosphate, used for phosphate buffering, and orthophosphoric acid and potassium hydroxide—both of Analytical Reagent (AR) grade—were acquired from Loba Chemie, located in Mumbai, India.

Instrument

Chromatographic analysis was carried out using an Agilent Prominence HPLC system, with data integration and analysis facilitated by LC Open Lab software (1260 Infinity II, Serial Number: DEAC626165). The following equipment was used in the chromatographic method development: a weighing balance (WENSER, Serial Number: 109527 IND/09/08/559 HPB201), a digital pH meter (EUTECH INSTRUMENTS, Serial Number: 2606106), a water bath sonicator (ACZET PVT LTD, Serial Number: 1324050113042), and a cooling centrifuge (NEUATION, IFUGE UC02R, Serial Number: SD084957).

Greenness software

Analytical method greenness was evaluated using AGREE[43], ComplexGAPI[44], RGB[45], and AMGS tools[46].

Analytical Quality by Design Software

Data generation and testing were performed using Central Composite Design (CCD), implemented through Design Expert software (version 13.0.5, trial version).

Experimental Animal

The study utilized New Zealand White rabbits, weighing 1.5 to 2.5 kg, housed in the central animal facility at the NETES Institute of Pharmaceutical Science in Mirza. The facility maintained standard laboratory conditions, including a 22 ± 2 °C temperature and a 12-hour light-dark cycle, with a standard pellet diet and unrestricted access to water. The Institutional Animal Ethics Committee at the NETES Institute of Pharmaceutical Science (IAEC-NIPS/AH/24/015) approved the study involving these rabbits. The blood samples were extracted from the rabbits' ear veins without anaesthesia and then centrifuged to isolate drug-free plasma. Notably, the animals were neither euthanized nor sacrificed at any point during the study; only a single blood sample was obtained from each rabbit for plasma isolation. All procedures were carried out by the CPCSEA guidelines for laboratory animal facilities, and the study adhered to the ARRIVE guidelines.

Stock Solution

Stock solutions of FEB and IND (10,000 ng/mL) were prepared in ethanol and subsequently diluted to create working standards. The stock and working solutions were refrigerated (2-8°C) to maintain stability. Then, blank plasma was spiked with the working solutions to generate calibration standards and quality control (QC) samples.[47]

Calibrators and QC solution

We combined 300 µL of plasma with aliquots of 30, 60, 120, 240, 480, and 690 µL from a working standard solution with a 10,000 ng/mL concentration. This mixture produced final concentrations of 200, 400, 800, 1600, 3200, and 4600 ng/mL for both FEB and IND. The solution was brought to 1500 µL with ethanol. The exact working standard solution was also used to prepare QC samples for FEB and IND. The QC samples corresponded to the lower limit of quantification (LLOQ) at 250 ng/mL, low-quality control (LQC) at 750 ng/mL, medium-quality control (MQC) at 1500 ng/mL, and high-quality control (HQC) at 3000 ng/mL. The solutions were vigorously mixed (10 minutes) and centrifuged (4000 rpm, 10 minutes, 4°C).[48] The supernatant was collected, filtered (0.22 µm syringe filters), and sonicated (five minutes).

Samples Preparation using rabbit plasma

For sample preparation, Protein precipitation method is used where 0.3 mL of plasma was mixed with FEB and IND solutions in 2 mL Eppendorf tubes. Ethanol was added to adjust 1.5 mL, and the mixture was vigorously shaken (10 minutes) and centrifuged (4000 rpm, 10 minutes, 4°C). The supernatant was collected, filtered (0.22 µm), and analyzed.[49] All experimental procedures were conducted with New Zealand Albino white rabbit where blood was collected from marginal ear veins, with approval from the Institutional Animal Ethical Committee (IAEC), which issued an ethical clearance number NIPS/AH/24/015.

Initial Chromatographic condition

An Eclipse Plus C18 column (25 cm x 5 cm, 4.6 µm) was employed for chromatographic separation at 0.8 mL/min. A tailored mobile phase, comprising 50 mM potassium dihydrogen orthophosphate buffer (pH 4.5) and ethanol (34:66 ratio), ensured optimal peak symmetry and purity (peak purity index: 0.995). An isocratic method was employed, with both analytes being detected at a wavelength of 320 nm during a 15-minute run at ambient temperature.

Green evaluation methodology

ComplexGAPI

This tool involves a systematic analysis process, utilizing 15 parameters grouped into three environmental impact levels: green for low impact, yellow for moderate, and red for high impact. It assesses two critical factors related to sample preparation and the pre-analysis phase. Additionally, the Complex GAPI method incorporates extra criteria to assess the environmental footprint of an analytical method throughout its development and application stages. It looks into aspects such as sample preparation, detection techniques, waste management, and renewable materials, providing a comprehensive perspective on the method's sustainability.[49–53]

AGREE

The AGREE tool was applied to assess the greenness of the developed RP-HPLC method based on the twelve principles of green analytical chemistry. Rather than focusing solely on generic definitions, our analysis highlighted that key aspects such as the use of ethanol as a green solvent, minimal sample and reagent volumes, and a short analysis time contributed positively to the overall AGREE score. The visual radar plot confirmed strong alignment with multiple GAC principles, particularly in waste minimization, safety, and energy efficiency. Minor deductions were associated with the use of animal-derived plasma, an unavoidable aspect of bioanalytical studies. Overall, the AGREE score reflected a well-balanced method with a favorable environmental profile.[54–56]

RGB

This approach utilizes a three-color system - red, green, and blue - with red indicating analytical performance, assessed through factors like precision, accuracy, limit of detection (LOD), and Limit of quantification (LOQ). Green color signifies safety and eco-friendliness, focusing on the toxicity of reagents, the quantity of reagent usage, and waste energy consumption data. Meanwhile, the blue color embodies productivity and practical effectiveness, examining cost efficiency, time savings, sample consumption, and operational simplicity.[57] In this context, the color indication is represented through a Colour Score (CS) and Method Brilliance, quantifying how well a method aligns with red, green, or blue attributes. The CS is measured on a scale from 0% to 100%, with a score of 66.6% or higher indicating that the method meets one of these primary color standards—this is referred to as the "satisfaction range." Scores between 33.3% and 66.6% fall into the "tolerance range," which reflects partial conformity. Conversely, methods scoring below 33% are deemed unsuitable and are labeled "black," leading to their rejection.[58–60]

AMGS

This is a valuable tool designed for assessing the environmental sustainability of analytical methods. It systematically evaluates and compares these methodologies based on their sustainability or "greenness." The calculator assigns a numerical score by considering several important factors, such as chemical safety, energy use, waste generation, and the choice of solvents. A lower score indicates a greener and more sustainable method, which helps reduce ecological impact and diminish the health hazards linked to harmful chemicals. The AMGS's key elements include assessing reagent toxicity, minimizing energy consumption, cutting waste production, and promoting safer, environmentally friendly solvents. By offering a structured, quantitative framework for choosing analytical methods, the AMGS aligns with green chemistry principles, fostering more sustainable practices in laboratory settings.[61–63]

Bio analytical validation

The suggested approach has been assessed following US FDA Bioanalytical Technique Validation guidelines.[64,65]

Declaration of competing interest

The authors state that they have no financial interests or personal relationships that could affect the work presented in this paper.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

References

1. Coleshill, M. J. *et al.* Rebranding Gout: Could a Name Change for Gout Improve Adherence to Urate-Lowering Therapy? *Ther Innov Regul Sci* **55**, 138–141 (2021).

2. Azevedo, V. F. *et al.* Critical revision of the medical treatment of gout in Brazil. *Rev Bras Reumatol* **57**, 346–355 (2017).

3. Global, regional, and national burden of gout, 1990-2020, and projections to 2050: a systematic analysis of the Global Burden of Disease Study 2021. *Lancet Rheumatol* **6**, e507–e517 (2024).

4. Xia, Y. *et al.* Global, regional and national burden of gout, 1990-2017: a systematic analysis of the Global Burden of Disease Study. *Rheumatology (Oxford)* **59**, 1529–1538 (2020).

5. Rai, S. K. *et al.* The rising prevalence and incidence of gout in British Columbia, Canada: Population-based trends from 2000 to 2012. *Semin Arthritis Rheum* **46**, 451–456 (2017).

6. Mattiuzzi, C. & Lippi, G. Recent updates on worldwide gout epidemiology. *Clin Rheumatol* **39**, 1061–1063 (2020).

7. Kiadaliri, A. A., Uhlig, T. & Englund, M. Burden of gout in the Nordic region, 1990-2015: findings from the Global Burden of Disease Study 2015. *Scand J Rheumatol* **47**, 410–417 (2018).

8. Zhu, B. *et al.* Trend dynamics of gout prevalence among the Chinese population, 1990-2019: A joinpoint and age-period-cohort analysis. *Front Public Health* **10**, 1008598 (2022).

9. Roman, Y. M. Moving the Needle in Gout Management: The Role of Culture, Diet, Genetics, and Personalized Patient Care Practices. *Nutrients* **14**, (2022).

10. Rashad, A. Y. *et al.* Towards the Development of Dual Hypouricemic and Anti-inflammatory Candidates: Design, Synthesis, Stability Studies and Biological Evaluation of Some Mutual Ester Prodrugs of Febuxostat-NSAIDs. *Bioorg Chem* **135**, 106502 (2023).

11. Stamp, L. K. & Farquhar, H. Treatment advances in gout. *Best Pract Res Clin Rheumatol* **35**, (2021).

12. Dehlin, M., Jacobsson, L. & Roddy, E. Global epidemiology of gout: prevalence, incidence, treatment patterns and risk factors. *Nat Rev Rheumatol* **16**, 380–390 (2020).

13. Zhu, Y., Pandya, B. J. & Choi, H. K. Prevalence of gout and hyperuricemia in the US general population: The National Health and Nutrition Examination Survey 2007-2008. *Arthritis Rheum* **63**, 3136–3141 (2011).

14. Parisa, N., Kamaluddin, M. T., Saleh, M. I. & Sinaga, E. The inflammation process of gout arthritis and its treatment. *J Adv Pharm Technol Res* **14**, 166–170 (2023).

15. Gullick, D. R., Mott, K. B. & Bartlett, M. G. Chromatographic methods for the bioanalysis of pyrethroid pesticides. *Biomed Chromatogr* **30**, 772–789 (2016).

16. Shen, M. *et al.* Febuxostat in the treatment of gout patients with low serum uric acid level: 1-year finding of efficacy and safety study. *Clin Rheumatol* **37**, 3107–3113 (2018).

17. Deidda, R., Orlandini, S., Hubert, P. & Hubert, C. Risk-based approach for method development in pharmaceutical quality control context: A critical review. *J Pharm Biomed Anal* **161**, 110–121 (2018).

18. Moineau, I. *et al.* Using Analytical Quality by Design to improve analytical method development in vaccines quality control: Application to an optimized quantitative high-performance anion-exchange chromatographic method. *J Chromatogr B Analyt Technol Biomed Life Sci* **1233**, 123946 (2024).

19. Prajapati, P. B., Patel, P. R. & Shah, S. A. Chemometric and DoE-Based Analytical Quality Risk Management to HPTLC Method for Simultaneous Estimation of Metronidazole and Norfloxacin. *J Chromatogr Sci* **61**, 428–439 (2023).

20. Prajapati, P., Tamboli, J. & Mishra, A. Risk and DoE-Based Analytical Failure Mode Effect Analysis (AFMEA) to Simultaneous Estimation of Montelukast Sodium and Bilastine by HPTLC Method Using Enhanced AQbD Approach. *J Chromatogr Sci* **60**, 595–605 (2022).

21. Makhija, R., Barik, P., Mehta, A., Ganti, S. S. & Asati, V. Sustainable approaches to analyzing phenolic compounds: a green chemistry perspective. *Anal Sci* **40**, 1947–1968 (2024).

22. Prajapati, P. *et al.* Application of Principal Component Analysis and DoE-Driven Green Analytical Chemistry Concept to Liquid Chromatographic Method for Estimation of Co-formulated Anti-Hypertensive Drugs. *J AOAC Int* **106**, 1087–1097 (2023).

23. Lahkar, C. *et al.* A technique based on infrared spectroscopy for determining sulfanilamide levels sustainably: Progress and comparisons of greenness and whiteness using ComplexGAPI, AGREE, and RGB. *Spectrochim Acta A Mol Biomol Spectrosc* **318**, 124467 (2024).

24. Semysim, F. A., Hussain, B. K., Hussien, M. A., Azooz, E. A. & Snigur, D. Assessing the Greenness and Environmental Friendliness of Analytical Methods: Modern Approaches and Recent Computational Programs. *Crit Rev Anal Chem* 1–14 (2024) doi:10.1080/10408347.2024.2304552.

25. Katamesh, N. S., Abbas, A. E. F. & Mahmoud, S. A. Four chemometric models enhanced by Latin hypercube sampling design for quantification of anti-COVID drugs: sustainability profiling through multiple greenness, carbon footprint, blueness, and whiteness metrics. *BMC Chem* **18**, 54 (2024).

26. Patel, R. & Kotadiya, R. Stability-indicating green HPLC method for fixed-dose tablets containing remogliflozin etabonate and teneligliptin: an AQbD approach. *Drug Dev Ind Pharm* 1–13 (2024) doi:10.1080/03639045.2024.2400199.

27. Gurumukhi, V. C. *et al.* Quality-by-design based fabrication of febuxostat-loaded nanoemulsion: Statistical optimization, characterizations, permeability, and bioavailability studies. *Heliyon* **9**, e15404 (2023).

28. K M, P. *et al.* Development of a gradient method for sulfamethoxazole, trimethoprim, isoniazid, and pyridoxine hydrochloride in rabbit plasma through QbD-driven investigation. *Sci Rep* **14**, 25806 (2024).

29. Patel, R. B., Patel, N. M., Patel, M. R. & Solanki, A. B. Optimization of Robust HPLC Method for Quantitation of Ambroxol Hydrochloride and Roxithromycin Using a DoE Approach. *J Chromatogr Sci* **55**, 275–283 (2017).

30. Wu, B., Li, Q., Wang, L., Chen, F. & Jiang, J. Development and validation of bioanalytical methods to support clinical study of disitamab vedotin. *Bioanalysis* **16**, 385–400 (2024).

31. Zeng, W. & Bateman, K. P. Quantitative LC-MS/MS. 1. Impact of Points across a Peak on the Accuracy and Precision of Peak Area Measurements. *J Am Soc Mass Spectrom* **34**, 1136–1144 (2023).

32. Iwamoto, N., Shimada, T., Terakado, H. & Hamada, A. Validated LC-MS/MS analysis of immune checkpoint inhibitor Nivolumab in human plasma using a Fab peptide-selective quantitation method: nano-surface and molecular-orientation limited (nSMOL) proteolysis. *J Chromatogr B Analyt Technol Biomed Life Sci* **1023–1024**, 9–16 (2016).

33. Iwamoto, N. *et al.* Fully validated LCMS bioanalysis of Bevacizumab in human plasma using nano-surface and molecular-orientation limited (nSMOL) proteolysis. *Drug Metab Pharmacokinet* **31**, 46–50 (2016).

34. Barone, R. *et al.* Development and validation of a fast UPLC-MS/MS screening method for the detection of 68 psychoactive drugs and metabolites in whole blood and application to post-mortem cases. *J Pharm Biomed Anal* **228**, 115315 (2023).

35. Hailat, M., Al-Ani, I., Hamad, M., Zakareia, Z. & Abu Dayyih, W. Development and Validation of a Method for Quantification of Favipiravir as COVID-19 Management in Spiked Human Plasma. *Molecules* **26**, (2021).

36. Prajapati, P. *et al.* Whiteness, redness, blueness and greenness profile assessment and design of experiments approach to microwave-aided sensitive and green spectrofluorophotometric determination of baclofen. *Luminescence* **39**, e4911 (2024).

37. Prajapati, P., Pulusu, V. S. & Shah, S. White analytical chemistry-driven stability-indicating concomitant chromatographic estimation of thiocolchicoside and aceclofenac using response surface analysis and red, green, and blue model. *J Sep Sci* **46**, 2300139 (2023).

38. Han, D.-G. *et al.* Pharmacokinetic Evaluation of Metabolic Drug Interactions between Repaglinide and Celecoxib by a Bioanalytical HPLC Method for Their Simultaneous Determination with Fluorescence Detection. *Pharmaceutics* **11**, (2019).

39. Prajapati, P. *et al.* Green LC-MS/MS method for in-vivo pharmacokinetics of mirabegron-encapsulated nanostructured lipid carriers in rat plasma: Integrating white analytical chemistry and analytical quality by design approach. *Sustain Chem Pharm* **39**, 101523 (2024).

40. Prajapati, P., Prajapati, B., Pulusu, V. S. & Shah, S. Multivariate Analysis and Response Surface Modeling to Green Analytical Chemistry–Based RP-HPLC-PDA Method for Chromatographic Analysis of Vildagliptin and Remogliflozin Etabonate. *J AOAC Int* **106**, 601–612 (2023).

41. Shao, J., Cao, W., Qu, H., Pan, J. & Gong, X. A novel quality by design approach for developing an HPLC method to analyze herbal extracts: A case study of sugar content analysis. *PLoS One* **13**, e0198515 (2018).

42. Liu, Q.-D., Qin, K.-M., Shen, B.-J., Cai, H. & Cai, B.-C. Optimization of the processing technology of Fructus Arctii by response surface methodology. *Chin J Nat Med* **13**, 222–231 (2015).

43. Pena-Pereira, F., Wojnowski, W. & Tobiszewski, M. AGREE - Analytical GREEnness Metric Approach and Software. *Anal Chem* **92**, 10076–10082 (2020).

44. Płotka-Wasylka, J. & Wojnowski, W. Complementary green analytical procedure index (ComplexGAPI) and software. *Green Chemistry* **23**, 8657–8665 (2021).

45. Nowak, P. M. & Kościelniak, P. What color is your method? adaptation of the rgb additive color model to analytical method evaluation. *Anal Chem* **91**, 10343–10352 (2019).

46. Hicks, M. B. *et al.* Making the move towards modernized greener separations: introduction of the analytical method greenness score (AMGS) calculator. *Green Chemistry* **21**, 1816–1826 (2019).

47. Kang, Y.-J., Jeong, H.-C., Kim, T.-E. & Shin, K.-H. Bioanalytical Method Using Ultra-High-Performance Liquid Chromatography Coupled with High-Resolution Mass Spectrometry (UHPL-CHRMS) for the Detection of Metformin in Human Plasma. *Molecules* **25**, (2020).

48. Dong, H. *et al.* Liquid Chromatography-Tandem Mass Spectrometry Simultaneous Determination and Pharmacokinetic Study of Fourteen Alkaloid Components in Dog Plasma after Oral Administration of Corydalis bungeana Turcz Extract. *Molecules* **23**, (2018).

49. Heo, H.-M., Jo, H.-W., Chang, H.-R. & Moon, J.-K. Development of Simultaneous Analytical Method for Imidazolinone Herbicides from Livestock Products by UHPLC-MSMS. *Foods* **11**, (2022).

50. Al-Wasidi, A. S., Ahmed, H. A., Alshammari, M. F. A., Nafee, S. S. & Mohamed, M. A. Cutting-edge HPLC and MCR techniques for synchronically quantifying anticholinergic drugs in the presence of C12 and C14 homologs: Robust application to green and white chemistry. *Arch Pharm (Weinheim)* **357**, e2400256 (2024).

51. Płotka-Wasylka, J. A new tool for the evaluation of the analytical procedure: Green Analytical Procedure Index. *Talanta* **181**, 204–209 (2018).

52. Mostafa, A. Insights into the sustainability of liquid chromatographic methods for favipiravir bioanalysis: a comparative study. *RSC Adv* **14**, 19658–19679 (2024).

53. Sinzervinch, A., Torres, M. S. I. & Kogawa, C. A. Tools to Evaluate the Eco-efficiency of Analytical Methods in the Context of Green and White Analytical Chemistry: A Review. *Current Pharmaceutical Design* vol. 29 2442–2449 Preprint at https://doi.org/http://dx.doi.org/10.2174/0113816128266396231017072043 (2023).

54. Boutkhoum, O., Hanine, M., Boukhriss, H., Agouti, T. & Tikniouine, A. Multi-criteria decision support framework for sustainable implementation of effective green supply chain management practices. *Springerplus* **5**, 664 (2016).

55. Veerendra, Y. V. S., Brahman, P. K., Mankumare, S. D., Ch, J. & C, V. K. 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. *Heliyon* **10**, e35376 (2024).

56. Ražić, S., Arsenijević, J., Đogo Mračević, S., Mušović, J. & Trtić-Petrović, T. Greener chemistry in analytical sciences: from green solvents to applications in complex matrices. Current challenges and future perspectives: a critical review. *Analyst* **148**, 3130–3152 (2023).

57. Guo, L., Shi, Y., Li, K.-W., Yan, J. & Xu, R.-K. Using an inexpensive RGB color sensor for field quantitative assessment of soil accessible Cu(Ⅱ). *Environ Pollut* **344**, 123348 (2024).

58. Deka, M. K. *et al.* Development of three UV-spectroscopic methods for simultaneous estimation of raloxifene and aspirin in pharmaceutical dosage form: Whiteness and greenness assessment with application of ComplexGAPI, AGREE, and RGB. *Green Analytical Chemistry* **8**, 100088 (2024).

59. Nowak, P. M. & Kościelniak, P. What Color Is Your Method? Adaptation of the RGB Additive Color Model to Analytical Method Evaluation. *Anal Chem* **91**, 10343–10352 (2019).

60. Pujol-Cano, N. *et al.* Near-infrared fluorescence cholangiography at a very low dose of indocyanine green: quantification of fluorescence intensity using a colour analysis software based on the RGB color model. *Langenbecks Arch Surg* **407**, 3513–3524 (2022).

61. Jankech, T. *et al.* Current green capillary electrophoresis and liquid chromatography methods for analysis of pharmaceutical and biomedical samples (2019-2023) - A review. *Anal Chim Acta* **1323**, 342889 (2024).

62. Mohamed, H. M. & Lamie, N. T. Analytical Eco-Scale for Assessing the Greenness of a Developed RP-HPLC Method Used for Simultaneous Analysis of Combined Antihypertensive Medications. *J AOAC Int* **99**, 1260–1265 (2016).

63. Pena-Pereira, F., Wojnowski, W. & Tobiszewski, M. AGREE-Analytical GREEnness Metric Approach and Software. *Anal Chem* **92**, 10076–10082 (2020).

64. Starlin, Z., Harahap, Y. & S Sitepu, E. Method Validation of Acrylamide in Dried Blood Spot by Liquid Chromatography-tandem Mass Spectrometry. *Pak J Biol Sci* **23**, 1321–1331 (2020).

65. Abdelhamid, N. S., Magdy, M. A., Anwar, B. H. & Farid, N. F. US FDA-validated TLC method with four greenness assessment evaluations for simultaneous determination of prednisolone and esomeprazole in spiked human plasma. *Biomed Chromatogr* **36**, e5343 (2022).

CRediT authorship contribution statement

Akramul Ansary: Data curation, Methodology Visualization, Investigation; **Piyongsola, Biprojit Paul**: Data curation, Visualization, Methodology, Investigation; **Amit kumar Das**: Writing- Original draft preparation, Investigation, Software; **Koushik Nandan Dutta**: Original draft preparation, Investigation, Software; **Bhargab Jyoti Sahariah, Manoj Kumar Deka**: Supervision, Writing- Reviewing and Editing; **Manish Majumder**: Conceptualization, Supervision, writing, Reviewing and Editing.

Acknowledgement
The authors are thankful to NETES Institute of Pharmaceutical Science, Mirza, for providing all the research facilities to carry out the research work.