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A green liquid chromatographic method using ethanol in mobile phase for the determination of nimesulide and naproxen in gel formulations

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Abstract

Determination of nonsteroidal anti-inflammatory drugs (NSAIDs) in pharmaceutical formulations helps to ensure that they are manufactured to the correct specifications. Consequently, simple, reliable, and environmentally friendly analytical methods are needed for the quality control of NSAID formulations. In this study, a novel and green liquid chromatographic method was developed for the determination of nimesulide (NIM) and naproxen (NAP) in gel formulations by employing ethanol as a green alternative to hazardous methanol and acetonitrile. The high viscosity of ethanol was compensated by employing a Chromolith High Resolution RP18e monolithic column (100 × 4.6 mm) with low flow resistance. Box-Behnken design with desirability function was employed for the optimization of selected significant parameters: pH (2.8 – 4), ethanol ratio in the mobile phase (35-55%), and flow rate (0.7–1.5 mL/min). Under optimum conditions, satisfactory separation of analytes was achieved within 5 min. Calibration curves for both analytes were linear between 1–50 µg/mL. Accuracies of intra- and inter-day experiments at low-, middle-, and high-quality control levels ranged from 99.0–101.5% with relative standard deviation values lower than 2.3%. The detection limits were 0.27 and 0.62 µg/mL for NIM and NAP, respectively. The applicability of the method was demonstrated by analyzing gel formulations. The results of this study indicated that monolithic columns in combination with ethanol as a mobile phase component could be considered a desirable and green alternative for the routine analysis of NSAIDs in quality control laboratories.

Keywords: Anti-inflammatory drugs, liquid chromatography, pharmaceutical analysis, design of experiments

1. Introduction

Nimesulide (NIM) and naproxen (NAP) are nonsteroidal anti-inflammatory drugs (NSAIDs) commonly utilized for their potent analgesic, anti-inflammatory, and antipyretic effects. These medications exhibit distinct pharmacological effects and have been widely prescribed for various inflammatory conditions and pain management [1].

NIM selectively inhibits cyclooxygenase-2 (COX-2) enzyme, which is primarily responsible for the production of prostaglandins involved in inflammation and pain. This selectivity minimizes the adverse gastrointestinal effects often associated with nonselective NSAIDs, such as gastric irritation and ulceration. Nimesulide has also shown anti-inflammatory and analgesic properties, making it a valuable option for managing acute and chronic pain and musculoskeletal disorders [2,3]. On the other hand, NAP is a nonselective NSAID that inhibits both COX-1 and COX-2 enzymes, leading to a more comprehensive suppression of prostaglandin synthesis. The broader spectrum of action of NAP is advantageous in treating several inflammatory conditions, particularly in rheumatoid arthritis and osteoarthritis [4,5].

Accurate quantification of drugs ensures that the formulations maintain their intended therapeutic potency. Additionally, quality control (QC) measures are essential to ensure drugs meet stringent regulatory standards. In this manner, there is a need for reliable analytical methodologies for the determination of NIM and NAP in pharmaceutical formulations. High-performance liquid chromatography (HPLC) with UV or diode array detector (DAD) detection has a leading position in the analysis of NSAIDs [6–15]. On the other hand, HPLC methods generally require the use of solvents such as methanol and acetonitrile, which present health and environmental issues and, therefore, are classified as "class 2" in the International Conference on Harmonization (ICH) guidelines [16].

Green analytical chemistry (GAC) is a field of science that focuses on minimizing the negative environmental

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impact of medical, environmental, food, and industrial analysis laboratories [17]. Today, there is a shift towards applying GAC concepts to replace conventional methods with eco-friendly ones displaying similar or better analytical performance [18]. In this manner, ethanol has been considered as a green mobile phase component in LC due to its wide availability, safety, and relatively low cost. It has an eluotropic strength and a UV cut-off value similar to acetonitrile and methanol, respectively [19]. Although ethanol has a high viscosity, which may lead to the formation of elevated column back pressures, this issue can be solved by using monolithic columns consisting of a single rod of high-purity polymeric silica gel, which creates significantly lower flow resistance compared to traditional particle-based columns due to bimodal pore structure of macro and mesopores [20,21]. For these reasons, there is a growing interest in the use of ethanolic mobile phases to separate various analytes, including natural compounds [22] and drugs [23–26].

In HPLC analysis, several chromatographic parameters influence separation efficiency, including mobile phase pH, organic solvent ratio, gradient slope, flow rate, temperature, etc. These parameters are generally optimized by employing a one-factor-at-a-time (OFAT) approach, which requires performing many experiments and is unable to detect factor interactions. In this manner, Design of Experiments (DOE) has recently attracted considerable interest in the analytical chemistry community as a reliable chemometric tool for the optimization of analytical procedures while conducting a minimal number of experiments [27]. Instead of the traditional OFAT approach, where factors are altered individually, DOE allows for the simultaneous exploration of the effects of multiple variations on one or more responses. While conventional optimization approaches are often empirical, DOE offers a systematic and multivariate experimental framework to comprehensively understand a process and assess the statistical significance of factors. By creating an experimental matrix, DOE enables faster visualization and the evaluation of multiple factors simultaneously. Furthermore, unlike OFAT, which assumes that factors act independently, DOE models can reveal potential interactions between these factors, enhancing the depth of process understanding. The optimization is conducted by employing response surface methodology with desirability function based on different designs such as Box-Behnken design (BBD) and central composite design (CCD) [28–30]. The BBD is a second-order response surface design proposed for optimizing processes and studying the relationship between multiple independent factors and three-level responses. The main advantage of the BBD over the CCD is its simplicity and efficiency. The BBD requires fewer experimental runs compared to CCD, which makes it less resource-intensive and quicker to implement. It achieves this by omitting extreme factor combinations and focusing on factor settings closer to the center of the design space [30,31]. Many studies have been reported on using BBD to optimize chromatographic conditions [27,29].

The aim of this study is to develop a green HPLC method for the simultaneous determination of NIM and NAP in gel formulations. To our knowledge, ethanol was used for the first time as an eco-friendly mobile phase component for the separation of selected NSAIDs. The high viscosity of ethanol was compensated by employing a monolithic column with high permeability as the stationary phase. Chromatographic conditions were optimized by BBD with multi-response optimization based on the desirability function. The greenness of the method was examined by a recently reported assessment tool, i.e., AGREE [32]. The developed method was successfully applied to the determination of NIM and NAP in pharmaceutical formulations.

2. Material and methods

2.1. Chemicals and materials

All chemicals used in the present study were of analytical grade. Methanol, formic acid, NaOH, NAP, and NIM were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol was obtained from Smyrna. Ultrapure water was produced by Arium pro UV (Sartorius Stedim, Göttingen, Germany). Mobile phase pH was adjusted by dropwise addition of 3 mol/L NaOH solution. Nimes gel containing 1% NIM and Naprosyn gel containing 10% NAP were purchased from a local pharmacy.

2.2. Standard solutions

Individual stock solutions of NAP and NIM were prepared in methanol at 1000 µg/mL. Standard solutions (1, 2.5, 5, 10, 20, 25, 30, 50 µg/mL) for optimization studies and method validation were obtained by mixing and diluting stock solutions with water. The solutions were kept at +4 °C and protected from daylight.

2.3. Instrumentation and apparatus

Chromatographic analyses were carried out by a Prominence-20 series HPLC system coupled with an SPD-20A DAD. LC solution 1.25 software (Shimadzu, Japan) was used for system control and data acquisition. An HI 2211 pH meter from Hanna Instruments, a magnetic stirrer, a vortex mixer, and an ultrasonic bath

from Isolab Laborgerte were used for the preparation of samples and solutions.

2.4. Chromatographic conditions

NIM and NAP were separated on a Chromolith High Resolution RP18e monolithic column (100 × 4.6 mm) with a mobile phase consisting of 0.1% formic acid in water (pH 4): ethanol (60:40, v/v). The mobile phase was pumped through the system at a 1.5 mL/min flow rate. Column oven temperature was kept at 25 °C. A total of 20 µL sample or standard solution was injected into the system. The DAD was operated at 272 nm with 0.24 s time constant and 3.125 Hz data sampling rate. Under optimum conditions, retention times of NIM and NAP were 3.0 and 3.7 min, respectively.

2.5. Preparation of samples

Two-hundred fifty milligrams of Nimes or Naprosyn gels corresponding to 2.5 mg NIM or 25 mg NAP, respectively, were accurately weighed and transferred to a 25 mL volumetric flask. 20 mL of ethanol was added, and the mixture was magnetically stirred for 15 min. Then, the flask was subjected to ultrasonication for 5 min. After cooling at room temperature, each flask was made up to volume with ethanol. Five milliliters of the extracts were centrifuged at 5000 rpm for 5 min. Clear supernatants obtained for Nimes and Naprosyn gels were diluted 4- and 40-fold with ultrapure water to obtain a final drug concentration of 25 µg/mL, respectively. Twenty microliter of diluted extract was injected into the system. It should be noted that centrifugation was found to be sufficient to obtain a clean and particle-free extract. Therefore, no filtration was performed during sample preparation, which both simplified the sample preparation procedure and reduced the analysis cost per sample.

2.6. Optimization strategy

The effect of three factors: pH, % ethanol in the mobile phase, and flow rate on analysis time, resolution (*Rs*), and capacity factor (*k*) of the early eluting analyte (NIM), using the BBD. The simultaneous optimization of multiple responses was performed by the desirability approach [33]. Experimental design, data analysis, and desirability function calculations were performed by Design Expert 11.1.2 (Stat-Ease, Minneapolis, MN, USA). Duplicate measurements were performed for each experimental run to ensure the accuracy of the results.

2.7. Method validation

Validation experiments were conducted in accordance with ICH guidelines and official pharmacopeias [34,35]. A system suitability test (SST) was performed before validation experiments. For this purpose, a 25 µg/mL mixed standard solution of NIM and NAP was injected six times. The chromatographic performance was evaluated with regard to *Rs*, *k*, number of theoretical plates, tailing factor, selectivity factor, and repeatability of both retention times and peak areas. The linearity of the method was evaluated by analyzing mixed standard solutions of analytes at six concentrations (1, 5, 10, 25, 40, 50 µg/mL), each injected in triplicate. Regression parameters, including slopes, intercepts, and correlation coefficients, were obtained by Microsoft Excel. Intra- and inter-day accuracy and repeatability were evaluated by analyzing QC samples prepared at 20, 25, and 30 µg/mL corresponding to 80%, 100%, and 120% of final analyte levels in the extract, respectively. The intra-day measurements were assessed by four analyses at each concentration level within the same day, while inter-day measurements were evaluated by ten analyses in three consecutive days. Results for repeatability and accuracy were given as relative standard deviation (RSD) and % accuracy, respectively. The limits of detection (LOD) values were statistically calculated from the standard deviation of the y-intercept of the regression line and the slope of the corresponding calibration curve [36]. The limit of quantification (LOQ) for each drug was accepted as the lowest concentration level of the corresponding calibration curve. The specificity of the method was assessed by examining signals of potential interfering substances in the blank, standard, and sample solutions to ensure that the analyte peaks were free from interference.

3. Results and discussion

3.1. Optimization of chromatographic conditions by BBD

Based on its ability to generate second-order equations that establish correlations between the examined parameters and the observed responses, BBD was chosen as the experimental design method for optimizing the chromatographic separation of NIM and NAP. BBD is considered an attractive alternative to the CCD due to its capacity to yield appropriate mathematical models while reducing the number of required experimental runs. Unlike CCD, BBD avoids the utilization of extreme experimental conditions, which can sometimes yield undesirable outcomes [37]. In this study, we applied BBD to fine-tune the HPLC conditions, aiming to determine the shortest possible run time that still enables the acceptable separation of the NIM and NAP as well as the adequate retention for the first peak (NIM). After performing preliminary experiments, ethanol ratio (%) in the mobile phase, pH, and flow rate were selected as the main parameters that can affect the separation of analytes. Temperature was

Table 1. Experimental matrix and experimental plan of the BBD

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Std order	Run order	A: pH	B: Ethanol ratio (%)	C: Flow rate (mL/min)	*Rs* of NAP	*k* of NIM	Retention time of NAP (min)
1	10	2.8	35	1.1	7.607	3.657	9.053
2	12	4	35	1.1	6.061	3.697	8.51
3	11	2.8	55	1.1	1.365	0.415	2.11
4	13	4	55	1.1	1.116	0.418	2.068
5	14	2.8	45	0.7	3.044	1.062	5.319
6	17	4	45	0.7	2.505	1.07	5.15
7	3	2.8	45	1.5	3.163	1.065	2.526
8	6	4	45	1.5	2.558	1.076	2.442
9	8	3.4	35	0.7	7.144	3.665	13.909
10	16	3.4	55	0.7	1.152	0.41	3.255
11	5	3.4	35	1.5	7.153	3.646	6.571
12	2	3.4	55	1.5	1.28	0.412	1.546
13	4	3.4	45	1.1	3.197	1.072	3.407
14	9	3.4	45	1.1	3.142	1.063	3.391
15	1	3.4	45	1.1	3.23	1.067	3.418
16	7	3.4	45	1.1	3.137	1.062	3.396
17	15	3.4	45	1.1	3.11	1.059	3.374

deliberately omitted from the experimental design since its influence can be reasonably anticipated through common sense and the understanding of chromatographic principles [38]. The *Rs* between NIM-NAP, *k* of NIM, and the retention time of NAP were selected as responses for the optimization by desirability function. The effect of selected factors was evaluated at low and high levels in the following ranges: pH (2.8-4), ethanol ratio (35-55%), and flow rate (0.7-1.5 mL/min). BBD matrix and experimental results are shown in Table 1.

The relationships between the factors and responses were modeled by the second-order polynomial equations after applying multiple regression analysis. The log10 transformation was applied to all variables as it improved the models' ability to explain data. The model equations for the three responses are as follows:

$$Log_{10}(Y1) = 0{,}500085 - 0{,}0453716A - 0{,}377591B + 0{,}00900645C + 0{,}00280017AB - 0{,}00189043AC + 0{,}0113027BC - 0{,}0300783A^2 - 0{,}00836831B^2 - 0{,}0224439C^2$$

$$Log_{10}(Y2) = 0{,}0271826 + 0{,}00194682A - 0{,}473745B + 0{,}00043870C - 0{,}000399AB + 0{,}00030085AC + 0{,}00109267BC + 0{,}00282086A^2 + 0{,}0625462B^2 - 0{,}00133591C^2$$

$$Log_{10}(Y3) = 0{,}531117 - 0{,}00803817A - 0{,}313257B - 0{,}162058C + 0{,}00453276AB - 0{,}000166231AC + 0{,}000580734BC - 0{,}00409804A^2 + 0{,}10462B^2 + 0{,}0299352C^2$$

where Y1, Y2, and Y3 are the responses of *Rs*, *k*, and retention time of NAP, respectively. A, B, and C represent the three chromatographic parameters: pH, ethanol ratio (%) in the mobile phase, and flow rate, respectively. Statistical evaluation of models was carried out by analysis of variance (ANOVA) with the results presented in Table S1-3. All three models were statistically significant according to ANOVA ($p < 0.0001$). The determination coefficient (R^2), adjusted R^2, and predicted R^2 values were higher than 0.99, demonstrating that the models adequately interpret the interactions between factors and responses. Lack-of-fit values were found to be insignificant ($p > 0.1$) compared to the pure error, implying that the model could accurately estimate the response of the evaluated factors.

As can be seen from the equations and response surfaces (Fig. 1), the most significant parameter in all models was the ethanol ratio in the mobile phase, which was inversely proportional to all responses, i.e., *Rs* of peaks, *k* of NIM, and retention time of NAP. The *k* of NIM increased with the increase in pH while it negatively affected the *Rs* of peaks and retention time of NAP. An increase in the flow rate enhanced the *Rs*, decreased the retention of NAP, and did not significantly affect the *k* of NIM.

The optimal condition was established by the optimization tool of Design-Expert software according to the desirability function of Derringer and Suich [33]. The aim of the optimization studies was to obtain an *Rs* higher than 2 for the NAP-NIM peak pair, achieve a *k* of at least 2 for NIM, and minimize the retention time of NAP. Optimum chromatographic conditions were estimated as follows: mobile phase pH: 4; ethanol ratio of mobile phase: 40 %; and flow rate: 1.5 mL/min. The optimum values for Y1, Y2, and Y3 were estimated as 3.98, 2, and 3.81 min, respectively. The chromatogram obtained under these conditions is presented in Fig. 2. For Y1, Y2, and Y3, there are 4.02%, 2.15%, and 2.36% differences between the predicted and experimentally obtained values, respectively, proving the validity of the

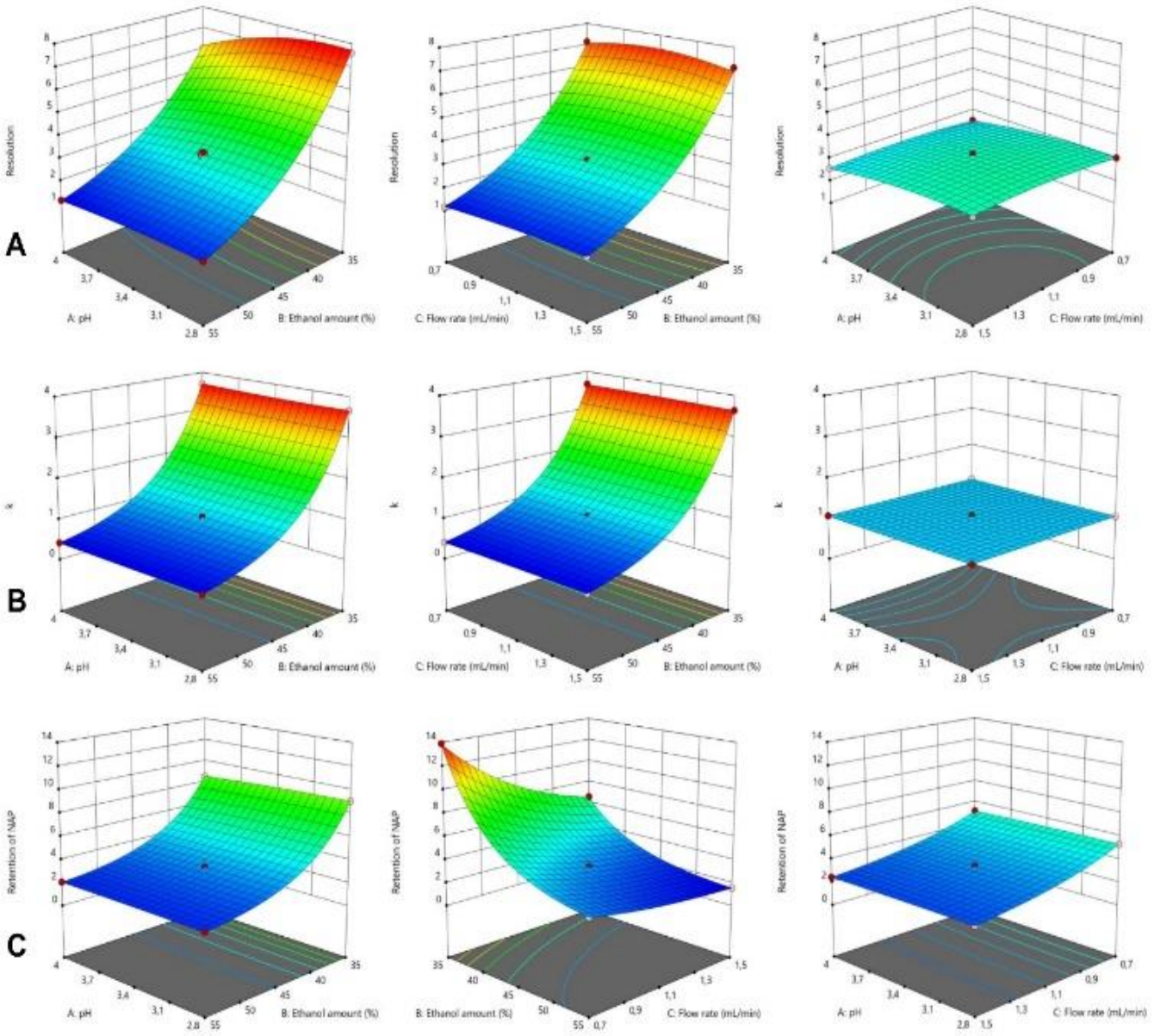

Figure 1. 3D-Response surface plots for (A) *Rs* of NAP, (B) *k* of NIM, and (C) Retention time of NAP

models used. Retention times of NIM and NAP were 3.0 and 3.7 min under optimum chromatographic conditions, respectively. The total run time was 5 min.

3.2. Method validation

In order to evaluate the appropriateness of the technique for the determination of NIM and NAP in pharmaceutical formulations, validation experiments were carried out as per the guidelines of ICH. An SST was performed as a control measure and an integral part of method validation. Results of SST studies obtained by injecting 10 µg/mL standard solution six times are presented in Table 2. Under optimum conditions, both analytes were adequately retained on the column. *Rs* value for NAP was 4.3. Tailing factors (<1.4) indicated that symmetric peaks were obtained. RSD values for both retention time and peak area are lower than 1%. All evaluated parameters were found to be in accordance with USP requirements.

The results of linearity and sensitivity studies are depicted in Table 3. All calibration curves had correlation coefficients >0.999, indicating that the developed method was linear. LOD values were 0.27 µg/mL and 0.62 µg/mL for NIM and NAP, respectively, while the lowest concentration of the calibration curve (1 µg/mL) was accepted as LOQ for both analytes.

Table 2. Results of system suitability tests for determination of NIM and NAP (n = 6)

	NIM	NAP	Recommended value
Retention time (min)	3.0	3.7	-
Tailing factor (*T*)	1.38	1.31	<2
Capacity factor (*k*)	2.0	2.7	>1
Resolution (*Rs*)	-	4.30	>2
Theoretical plates (N)	6012.9	6779.0	>2000
Selectivity factor (a)	-	1.36	>1.05
RSD% of retention time	0.85	0.92	<1
RSD% of peak area	0.52	0.48	<1

Table 3. Statistical evaluation of the calibration data of NIM and NAP

	NIM	NAP
Linear range (μg/mL)	1 – 50	1 – 50
Slope	15031	18146
Intercept	-1880.4	-2755.7
SE of slope	42.6	120.0
SE of intercept	1211.81	3412.2
Correlation coefficient (*r*)	0.9999	0.9996
LOD (μg/mL)	0.27	0.62
LOQ (μg/mL)	1.0	1.0

Table 4. Intra- and inter-day precision and accuracy for NSAIDs

Analyte	Concentration level (μg/mL)	Intra-day (n = 4) Accuracy (%)	Intra-day (n = 4) RSD (%)	Inter-day (n = 10) Accuracy (%)	Inter-day (n = 10) RSD (%)
NIM	20	99.2	2.05	99.0	2.29
	25	101.5	1.60	100.8	2.20
	30	99.1	1.54	99.4	1.33
NAP	20	100.3	2.03	99.8	1.80
	25	101.1	0.85	101.1	1.08
	30	101.0	1.23	100.9	1.01

Table 5. Assay data of NIM and NAP (n=3)

	Labeled amount (%, w/w)	Observed amount (%, w/w)	Recovery (%)	RSD (%)
NIM	1	0.989	98.95	0.88
NAP	10	9.921	99.22	0.20

QC solutions prepared at 20, 25, and 30 μg/mL concentration levels for all analytes were analyzed on the same day and three consecutive days to evaluate the intra- and inter-day accuracy and repeatability (Table 4). Intra-day accuracies were in the range of 99.1 – 101.5%, while inter-day accuracies ranged from 99.0% to 101.1%. RSD values of intra- and inter-day experiments at three QC levels were in the ranges of 0.85 – 2.05% and 1.01 – 2.29%, respectively. Results demonstrated that the developed method is precise and accurate for determining NIM and NAP.

3.3. Application on real samples

The feasibility of the developed method was demonstrated by analyzing gel formulations of NIM and NAP in the market. Nimes gel includes disodium EDTA, carbomer 940, polyethylene glycol 400, triethanolamine, dimethyl sulfoxide, and glycerin as excipients, while Naprosyn gel contains carbomer 940, ethyl alcohol, triethanolamine, sodium metabisulfite, rose essence. Gel formulations were prepared as explained in section "2.5. The preparation of samples" and obtained extracts were analyzed by the developed HPLC method. Triplicate measurements were performed for each sample. Chromatograms of Nimes and Naprosyn gels were presented in Fig. 3.

No interferences were observed in the retention windows of NIM and NAP.

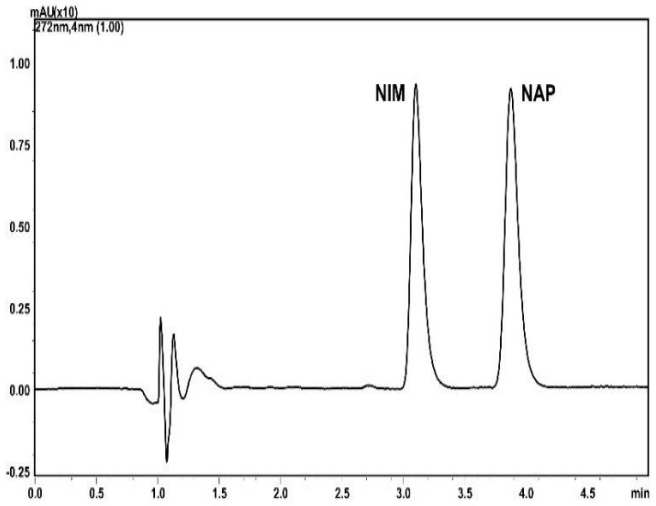

Figure 2. Chromatogram of a standard mixture (10 μg/mL) of NIM and NAP under optimized conditions at 272 nm

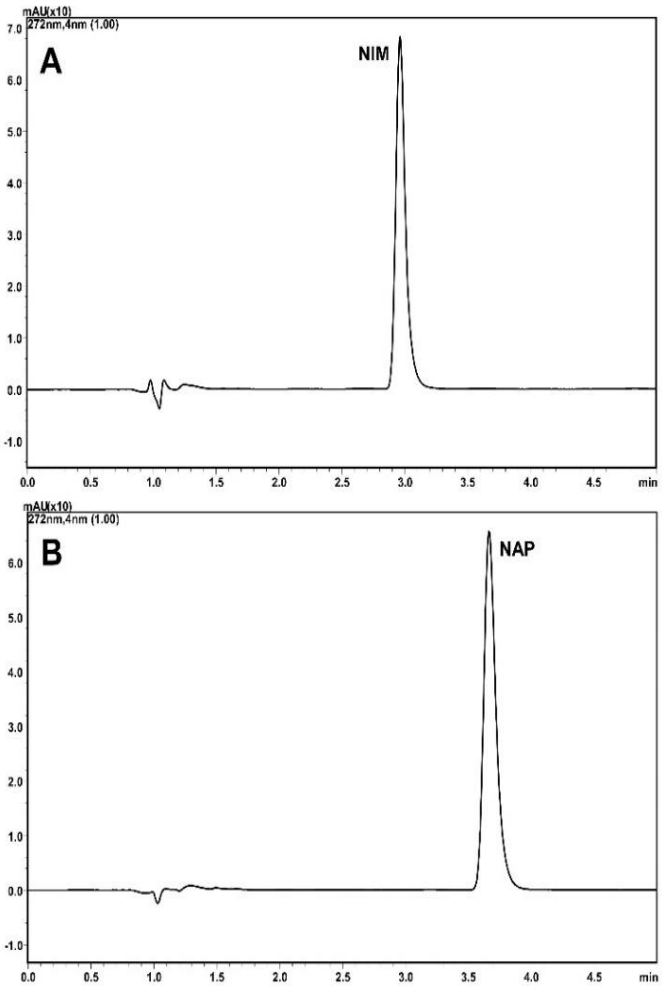

Figure 3. Chromatograms of Nimes (A) and Naprosyn (B) gel formulations at 272 nm (25 μg/mL)

Additionally, peak purity values obtained from real sample analyses were >0.9999 for both analytes, demonstrating the specificity of the developed green LC method towards NIM and NAP. Table 5 summarizes the results of the gel formulation analysis. The quantities obtained were consistent with the manufacturers' claims.

3.4. Greenness evaluation

The selection of analytical methodologies for use in routine analyses within pharmaceutical research and QC laboratories is determined by various factors.

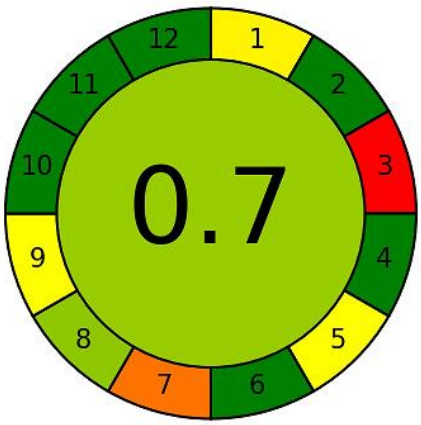

Figure 4. Greenness assessment of developed method using AGREE tool

Performance and throughput are paramount considerations. Cost efficiency is particularly critical for profit-seeking pharmaceutical entities. Recently, corporate social responsibility has become increasingly important, particularly with regard to the environmental impacts of business practices, including the regular implementation of analytical methods. Consequently, various metric tools have been introduced in order to accurately evaluate the "greenness" of analytical methods [39]. AGREE, introduced in 2020, stands as the latest comprehensive greenness assessment tool encompassing all 12 principles outlined by GAC [40]. As a result of the greenness assessment, the software generates a pictogram, which is divided into 12 adjustable sections, each marked by a distinct color scheme denoting its importance, from deep green (indicating a score of 1) to deep red (indicating a score of 0). The overall method greenness is depicted at the center of the circular pictogram as a final score ranging from 0 to 1. The closer the score is to one, the greener the method is considered [32].

The overall AGREE evaluation for the proposed HPLC-DAD method is shown in Fig. 4. An average score of 0.7 was achieved, demonstrating the environmental friendliness of the method. The lowest scores were obtained for the offline positioning of the analytical device (criteria 3) and waste production (criteria 7). It should be noted that offline sampling and sample transfer to the analysis laboratory are mandatory for QC laboratories to achieve separation of pharmaceutical production and QC sites [41]. Moreover, the generated waste during sample preparation and chromatographic separation mainly consists of ethanol, which is considered a safe and green solvent [42,43].

The method displayed prominent performance in terms of sections related to the use of safer solvents (criteria 2), high sample throughput (criteria 6), minimum energy consumption (criteria 8), and operator safety (criteria 10). The main advantages of the method are as follows: low sample amount (Criteria 2), reduced number of distinct steps (Criteria 4), the use of bio-based and non-toxic reagents (Criteria 10 and 11), and increased operator safety (Criteria 12).

4. Conclusion

A novel RP-HPLC-DAD method was developed for the simultaneous determination of NIM and NAP in pharmaceutical formulations. Chromatographic conditions were optimized to obtain adequate retention for both analytes with satisfactory *Rs* and run time by experimental design as a multivariate optimization tool that enables both the reduction of the total number of experiments and detection of the factor interactions. Satisfactory separation of analytes was achieved in a short time by using a monolithic column with a mobile phase containing ethanol, which was used as a strong organic component for the first time to separate NIM and NAP. Due to the porous structure of the monolithic column, acceptable column pressure was observed despite the relatively high viscosity of ethanol. The method was validated and successfully applied to determine NIM and NAP in commercial gels, demonstrating that satisfactory chromatographic performance and compliance with validation criteria can be achieved using green solvents. The calculated AGREE score of the method indicates the excellent greenness for pharmaceutical analysis as a result of using a greener solvent for sample and mobile phase preparation. The results of this study demonstrate that ethanol and word-based mobile phases can be used in pharmaceutical analysis, making it an important effort to reduce the toxicity levels of the HPLC process.

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Supplementary Informations

Table S1. Analysis of variance (ANOVA) of Box–Behnken design (BBD) for resolution (Y1)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.17	9	0.1295	1341.62	< 0.0001	**significant**
A-pH	0.0165	1	0.0165	170.68	< 0.0001	
B-Ethanol amount	1.14	1	1.14	11821.06	< 0.0001	
C-Flow rate	0.0006	1	0.0006	6.73	0.0358	
AB	0.0000	1	0.0000	0.3251	0.5864	
AC	0.0000	1	0.0000	0.1482	0.7117	
BC	0.0005	1	0.0005	5.30	0.0549	
A^2	0.0038	1	0.0038	39.48	0.0004	
B^2	0.0003	1	0.0003	3.06	0.1239	
C^2	0.0021	1	0.0021	21.98	0.0022	
Residual	0.0007	7	0.0001			
Lack of Fit	0.0005	3	0.0002	3.68	0.1201	**not significant**
Pure Error	0.0002	4	0.0000			
Cor Total	1.17	16				
Std. Dev.	0.0098		**R^2**	0.9994		
Mean	0.4714		**Adjusted R^2**	0.9987		
C.V. %	2.08		**Predicted R^2**	0.9930		
			Adeq Precision	112.2840		

Table S2. Analysis of variance (ANOVA) of Box-Behnken design (BBD) for capacity factor (Y2)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.81	9	0.2014	59152.35	< 0.0001	**significant**
A-pH	0.0000	1	0.0000	8.91	0.0204	
B-Ethanol amount	1.80	1	1.80	5.275E+05	< 0.0001	
C-Flow rate	1.540E-06	1	1.540E-06	0.4523	0.5228	
AB	6.370E-07	1	6.370E-07	0.1871	0.6783	
AC	3.620E-07	1	3.620E-07	0.1064	0.7539	
BC	4.776E-06	1	4.776E-06	1.40	0.2749	
A^2	0.0000	1	0.0000	9.84	0.0164	
B^2	0.0165	1	0.0165	4839.00	< 0.0001	
C^2	7.514E-06	1	7.514E-06	2.21	0.1809	
Residual	0.0000	7	3.404E-06			
Lack of Fit	7.021E-06	3	2.340E-06	0.5569	0.6709	**not significant**
Pure Error	0.0000	4	4.202E-06			
Cor Total	1.81	16				
Std. Dev.	0.0018		**R^2**	1.0000		
Mean	0.0573		**Adjusted R^2**	1.0000		
C.V. %	3.22		**Predicted R^2**	0.9999		
			Adeq Precision	675.2656		

Table S3. Analysis of variance (ANOVA) of Box–Behnken design (BBD) for retention time of NAP (Y3)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.05	9	0.1164	18867.05	< 0.0001	**significant**
A-pH	0.0005	1	0.0005	83.82	< 0.0001	
B-Ethanol amount	0.7850	1	0.7850	1.273E+05	< 0.0001	
C-Flow rate	0.2101	1	0.2101	34068.76	< 0.0001	
AB	0.0001	1	0.0001	13.33	0.0082	
AC	1.105E-07	1	1.105E-07	0.0179	0.8973	
BC	1.349E-06	1	1.349E-06	0.2187	0.6542	
A^2	0.0001	1	0.0001	11.47	0.0117	
B^2	0.0461	1	0.0461	7472.91	< 0.0001	
C^2	0.0038	1	0.0038	611.82	< 0.0001	
Residual	0.0000	7	6.167E-06			
Lack of Fit	0.0000	3	8.356E-06	1.85	0.2792	**not significant**
Pure Error	0.0000	4	4.526E-06			
Cor Total	1.05	16				
Std. Dev.	0.0025		**R^2**	1.0000		
Mean	0.5925		**Adjusted R^2**	0.9999		
C.V. %	0.4191		**Predicted R^2**	0.9996		
			Adeq Precision	499.1127		