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Gradient, reversed-phase HPLC method for simultaneous determination of chemical and enantiomeric impurities of dexibuprofen in a single run

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ABSTRACT

In this work, a gradient reversed-phase HPLC method is presented for the simultaneous determination of four potential chemical impurities in dexibuprofen, as well as its chiral isomer, (*R*)-ibuprofen. Among nine polysaccharide-based chiral columns tested, enantioselectivity was observed only with the column containing cellulose tris(4-methylbenzoate) under polar organic mode conditions. To enhance enantioresolution, a reversed-phase mode was employed by adding water to the mobile phase. Examination of the water content's impact on retention time revealed a typical reversed-phase retention behavior in alcohol–water mixtures, whereas acetonitrile–water mixtures displayed HILIC-like retention characteristics. A full factorial design optimization strategy was applied to identify the ideal conditions for determining organic and enantiomeric impurities in dexibuprofen. Under the optimized conditions—Lux Cellulose-3 stationary phase using a methanol:water:acetic acid 70:30:0.1 (v/v/v) mobile phase, at a flow rate of 0.5 mL/min and temperature of 35 °C—all target compounds were effectively separated with clear baseline resolution. The initial analysis required 60 min, primarily due to the prolonged retention of highly lipophilic ester impurities. To reduce the analysis time, a gradient elution strategy was developed, successfully cutting the runtime by half. Furthermore, the stability and robustness of the gradient system were confirmed by consistent performance over one hundred consecutive injections. Following regulatory validation guidelines, the developed HPLC method was effectively applied for the quality assessment of commercially available pharmaceutical formulations.

1. Introduction

The development of polysaccharide-based chiral columns represents a significant advancement in the field of chiral separations. These chiral selectors have been demonstrated to be effective in the enantioseparation of a wide range of compounds in HPLC. Furthermore, their versatility extends to diverse separation modes, including normal phase, polar organic mode, and reversed-phase mode [1–7].

A number of significant advancements have recently been made in the field of chiral separation, including ultrafast enantioseparation within seconds [8], mass spectrometer compatibility [9,10], prediction of the success of chiral separation [11,12], and the simultaneous separation of multiple components, including both chiral and chemical impurities in a single run [13,14]. In the compendia (EP, USP), enantiomeric purity analysis focuses exclusively on the quantification of the distomer. However, it is important to acknowledge that chiral stationary phases exhibit not only enantioselectivity, but also chemoselectivity. By separating the chemical and chiral impurities in a single run, significant amounts of time, solvent, and money can be saved. Papp et al. provided an in-depth overview of single-column HPLC approaches that allow the co-separation of chiral and achiral impurities within one analytical run [13]. It has been demonstrated that a wide range of chiral columns can separate achiral and chiral substances; however, in the overwhelming majority of cases, polysaccharide-based stationary phases were employed [14–17].

Ibuprofen is a widely utilized nonsteroidal anti-inflammatory drug

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(NSAID) indicated for the management of pain, fever, and inflammatory conditions [18]. The substance is a racemic mixture containing equimolar amounts of (*S*)-ibuprofen and (*R*)–ibuprofen. Among these, (*S*)-ibuprofen is the pharmacologically active enantiomer responsible for cyclooxygenase inhibition, while (*R*)–ibuprofen lacks this activity [19]. Pharmacokinetic differences have been documented, particularly regarding metabolic processes [20]. In contrast to (*S*)-ibuprofen, (*R*)–ibuprofen forms a thioester intermediate with coenzyme A, resulting in the incorporation of hybrid triglycerides into lipid metabolism. As a result of this unusual metabolic transformation (*S*)-ibuprofen is metabolically "cleaner" than the racemic mixture. Furthermore, metabolic inversion converts a significant amount of R-ibuprofen (50–60 %) to the active S-enantiomer, though this process varies depending on individual metabolism and dosing conditions. Given these pharmacological and metabolic differences, the concept of chiral switching has gained increasing attention, leading to the development of dexibuprofen, the pure (*S*)-enantiomer of ibuprofen [19,21]. This approach aims to enhance drug efficacy while allowing for a lower therapeutic dose, thereby minimizing potential side effects and metabolic complications. Studies have shown that 200 mg of dexibuprofen provides equivalent or superior pain relief compared to 400 mg of racemic ibuprofen [22,23]. The introduction of dexibuprofen to the market also necessitates novel chiral analytical techniques. In addition to the analysis of chemical impurities, there is now a necessity to determine the enantiomeric impurity as well. A comprehensive review of the available literature reveals numerous enantioseparation methods for ibuprofen, including capillary electrophoresis [24,25], thin layer chromatography [26,27], and mainly HPLC [28–31]. These methods have also been summarized in review articles [21,32]. The main purpose of these methods is to investigate the pharmacokinetics of the different enantiomers, and/or to detect enantiomers in different biological fluids [33–36].

Interestingly, only a limited number of studies focus on pharmaceutical analysis in this context [30], and even fewer address the quantification of the enantiomeric purity of dexibuprofen specifically [37]. Camilo and Foley introduced a reversed-phase HPLC technique that achieves concurrent separation of chiral and achiral NSAIDs, such as naproxen, ibuprofen, ketoprofen, and flurbiprofen. Their approach employed a tandem-column setup, consisting of a biphenyl column coupled with a chiral column coated with silica gel and packed with amylose tris(3,5-dimethylphenylcarbamate) as the chiral selector [38]. However, to the best of our knowledge, no method has yet been reported in the literature that enables the simultaneous separation of both chemical and enantiomeric impurities of dexibuprofen.

The simultaneous separation of chiral and chemical impurities is not merely methodological refinement but represents a significant scientific advancement in analytical method development. Achieving this in a single chromatographic run results in reduced eluent consumption, lower energy usage, and shorter analysis time — all contributing to a more environmentally sustainable process. In the current landscape, the development of green analytical methods should be considered a fundamental requirement. Despite the widespread use of hexane-based normal-phase chromatography in chiral separations, alternative approaches such as polar organic mode or reversed-phase mode often provide comparable or superior performance with a significantly lower environmental burden. Furthermore, gradient elution — commonly employed in achiral analysis — offers an opportunity to reduce solvent consumption compared to long isocratic runs. However, its application in chiral separations remains limited, as most methods are designed solely for enantiomer resolution, for which isocratic conditions are typically sufficient. When the analytical goal extends beyond enantiomeric separation to include structurally related impurities, it becomes crucial to demonstrate that gradient elution is compatible with chiral stationary phases, even under industrially relevant conditions. To our knowledge, comprehensive studies addressing this aspect are still lacking in the literature.

The objective of this study was to establish a method for the simultaneous determination of dexibuprofen impurities, including (*R*)–ibuprofen, oxo-ibuprofen (Ibuprofen related compound J), 4′-Isobutylacetophenone (Ibuprofen related compound C), ibuprofen methyl ester, and ibuprofen ethyl ester (Fig. 1). Each impurity was required to remain below 0.1 %, using a green, reversed-phase method. An extensive examination of chromatographic factors affecting the separation process was also performed. Furthermore, the feasibility of applying a gradient method on a chiral column was also investigated. A comparative evaluation was also conducted to assess the environmental and operational performance of the conventional, non-simultaneous method alongside our newly developed isocratic and gradient methods, using established green analytical metrics.

2. Materials and methods

2.1. Materials

Reference standards used in this study included dexibuprofen ((2S)-2-(4-isobutylphenyl)propanoic acid; CAS No. 51,146–56–6), racemic ibuprofen (2-(4-isobutylphenyl)propanoic acid; CAS No. 15,687–27–1), ibuprofen-related compound C (4′-isobutylacetophenone; CAS No. 38,861–78–8), and compound J ((2RS)-2-(4-isobutyrylphenyl)propanoic acid; oxo-ibuprofen; CAS No. 65,813–55–0), all of which were obtained from Sigma-Aldrich (Budapest, Hungary). Ibuprofen methyl ester (Methyl 2-(4-isobutylphenyl)propanoate) and ethyl ester (Ethyl 2-(4-isobutylphenyl)propionate) were synthetized according to literature methods [39,40]. HPLC-grade acetonitrile (ACN) methanol (MeOH), and acetic acid (100 %, HPLC suitable) was also acquired from Sigma-Aldrich. A Milli-Q water purification system form Merck-Millipore (Burlington, MA, USA) was utilized to produce deionized water. Seractil® 400 mg dexibuprofen tablets were purchased at local pharmacy in Kazincbarcika, Hungary.

Nine polysaccharide-based chiral columns were utilized in the study, as listed in Table 1.

2.2. HPLC analysis

The same chromatographic system was employed as previously reported [17]. In the preliminary screening, the enantioseparation capabilities of nine polysaccharide-based HPLC columns, as listed in Table 1, were assessed under polar organic mode, utilizing ACN, MeOH, and EtOH as eluent, each modified with 0.1 % acetic acid. A fixed set of chromatographic parameters was used throughout the screening phase, with 1 µL injection volume, a column held at 25 °C, and a flow rate of 1.0 mL/min maintained in all runs. During the method development, methanolic stock solutions of dexibuprofen were prepared at a concentration of 400 µg/mL and subsequently spiked with all impurities examined at a level of 1 % (around 4 µg/mL). A full factorial design approach was employed to investigate the effects of three independent variables: flow rate, column temperature, flow rate, and methanol percentage in the mobile phase. The experimental matrix was constructed using Design-Expert software (v7.0.0, Stat-Ease Inc., Minneapolis, MN, USA). The final dexibuprofen test solution for method validation was prepared at a concentration of 4 mg/mL. Impurity levels were calculated relative to the sample concentration; for example, in a 4000 µg dexibuprofen sample, an impurity level of 0.1 % corresponds to 4 µg of impurity. The detection wavelengths were established at 220 nm and 254 nm.

2.3. Real sample analysis

Ten Seractil® film-coated tablets, each containing 400 mg of dexibuprofen, were ground in a porcelain mortar. A precisely measured amount of powdered tablet was subjected to extraction with 50.0 mL of MeOH in an ultrasonic bath for 30 min, yielding a dexibuprofen solution with the ultimate concentration of 8 mg/mL. The suspension was

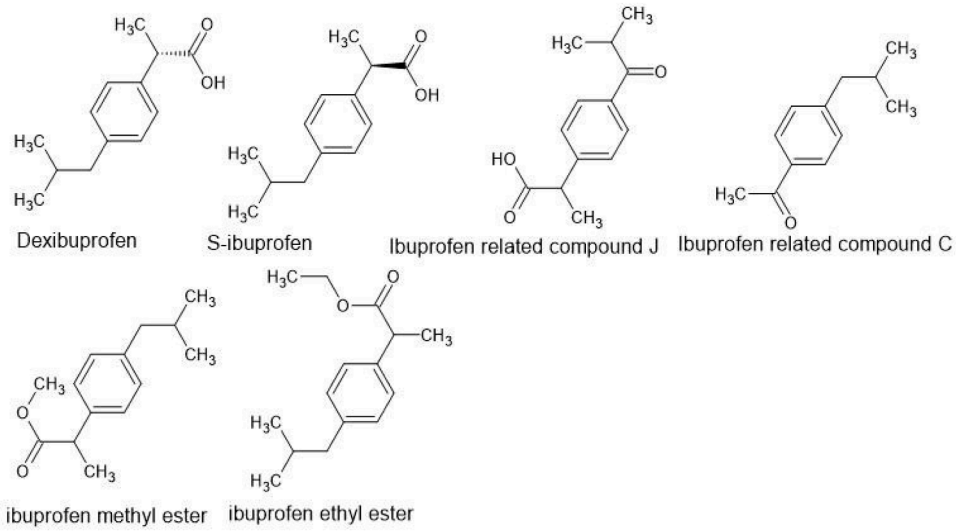


Fig. 1. Chemical structure of the investigated compounds.

Table 1
List of chiral columns used and their corresponding chemical selectors.

Column Name	Chiral Selector	Dimension	Supplier
Lux Cellulose-1	Cellulose tris(3,5-dimethylphenylcarbamate)	150 × 4.6 mm; particle size: 5 μm	Phenomenex
Lux Cellulose-2	Cellulose tris(3-chloro-4-methylphenylcarbamate)	150 × 4.6 mm; particle size: 5 μm	Phenomenex
Lux Cellulose-3	Cellulose tris(4-methylbenzoate)	150 × 4.6 mm; particle size: 5 μm	Phenomenex
Lux Cellulose-4	Cellulose tris(4-chloro-3-methylphenylcarbamate)	150 × 4.6 mm; particle size: 5 μm	Phenomenex
Lux Amylose-1	Amylose tris(3,5-dimethylphenylcarbamate)	150 × 4.6 mm; particle size: 5 μm	Phenomenex
Chiralcel OD	Cellulose tris(3,5-dimethylphenylcarbamate)	250 × 4.6 mm; particle size 10 μm	Daicel
Chiralcel OJ	Cellulose tris(4-methylbenzoate)	250 × 4.6 mm; particle size 10 μm	Daicel
Chiralpak AD	Amylose tris(3,5-dimethylphenylcarbamate)	250 × 4.6 mm; particle size 10 μm	Daicel
Chiralpak IA	Amylose tris(3,5-dimethylphenylcarbamate) (immobilized phase)	250 × 4.6 mm; particle size 10 μm	Daicel

centrifuged using a MiniSpin centrifuge (Eppendorf, Germany) at 4000 rpm for 5 min to facilitate phase separation, after which the supernatant was passed through a fine-mesh PTFE membrane filter (Millipore Corp., Cork, Ireland) for clarification. Subsequently, to achieve a final dexibuprofen concentration of 4 mg/mL, a 0.5 mL aliquot of the filtrate was diluted with 1.0 mL of MeOH.

3. Results and discussion

3.1. Screening phase

The initial stage of method development focused on the chiral resolution of ibuprofen enantiomers, which constitutes a pivotal challenge in the overall separation process. The enantioseparation performance of nine polysaccharide-based chiral stationary phases (CSPs) was investigated under polar organic mode conditions. Enantiorecognition was observed exclusively with a methanolic polar organic mobile phase on Chiralcel OJ and Lux Cellulose-3 columns, both containing the same chiral selector, cellulose tris(4-methylbenzoate). Since these columns share the same chiral selector, no significant difference was observed in their enantioseparation ability ($R_s = 1.34$ for Chiralcel OJ, and $R_s = 1.22$ for Lux Cellulose-3, respectively). The enantiomers eluted in the same order on both columns, with R-ibuprofen eluting first. Further method development was performed on the shorter, Lux Cellulose-3 column, as it displayed similar selectivity with faster runtime.

The mobile phase selection step involved identifying the mobile phase that maximizes the chiral recognition ability of the Lux Cellulose-3 column. Different eluents were evaluated, consisting of neat ACN, MeOH, and EtOH (containing 0.1 v/v % acetic acid) in polar organic mode or each organic eluent with water in varying compositions (from 0 to 50 % water in the corresponding organic eluent) in reversed-phase mode. The enantiomeric elution order did not change under any of the applied conditions. For ACN, separation of the enantiomers was only observed at 60:40:0.1 and 50:50:0.1 ACN:water:acetic acid compositions (v/v/v). Matarashvili et al. similarly investigated the effect of water for enantiodiscrimination of chiral weak acids in aqueous–organic mobile phases using polysaccharide-based chiral stationary phases. Like in our study, they observed enantioresolution in ACN:water mixtures; however, they reported enantiomer elution order reversal at higher water content. In our case, higher water content was avoided due to significantly increased retention times and the occurrence of an undesired enantiomer elution order [41].

Enantiomeric resolution was achieved with both acidified MeOH and the acidified MeOH:water mixture, regardless of the mobile phase composition. Increasing the water content in the mobile phase enhanced the resolution between the enantiomers, but this improvement was accompanied by greater peak shape distortion (Supplementary Figure 1). Similar trends were observed using EtOH–water mixtures; however, no enantioseparation occurred in polar organic mode when using acidified EtOH alone. The effect of eluent water content on retention times was examined. The MeOH–water and EtOH–water mixtures showed typical reversed-phase retention behavior, with retention time increasing progressively as the water content increased. (Fig. 2A and 2B). This phenomenon can be explained by the observation that water serves as a weaker eluent compared to MeOH and EtOH in reversed-phase mode. The ACN–water combination demonstrated characteristics of a mixed-mode elution profile, involving both reversed-phase and hydrophilic interaction liquid chromatography (HILIC) mechanisms. At low water contents, the retention factor decreased; however, starting from 10 % water, it began to increase, forming a U-

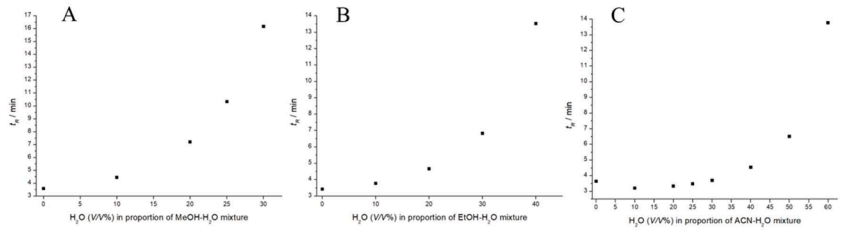


Fig. 2. Variation of retention time with water content in different solvents: (A) tR plotted against water percentage in MeOH, (B) t_R plotted against water percentage in EtOH, and (C) tR plotted against water percentage in ACN. Experimental conditions: Lux Cellulose-3 column, flow rate 0.7 mL/min, column temperature 25 °C, injection volume 1 μL.

shaped retention curve (Fig. 2C) [17,41,42].

Since the MeOH:water:acetic acid-containing mobile phases demonstrated the most promising results on the Lux Cellulose-3 column, all subsequent method development was performed using these conditions.

3.2. Method development

An initial analysis encompassing all impurities was undertaken to ascertain the best parameter ranges and identify the chromatographic parameters that most significantly affected the separation of enantiomeric and chemical impurities. The incorporation of acetic acid as an acidic modifier in the eluent proved crucial for attaining optimal peak shapes and enantioseparation. An optimal acetic acid concentration of 0.1 % was chosen, because higher concentrations, along with lower pH, could potentially reduce column lifetime. As discussed in Section 3.1, increasing the concentration of water in the mobile phase improved resolution; nevertheless, MeOH mixtures containing less than 20 % or more than 30 % water led to inadequate separation of organic impurities due to peak broadening, and prolonged retention times. The flow rate within the investigated range of 0.5–0.8 mL/min influenced retention times, with elevated flow rates decreasing analysis duration. In most instances, however, this was followed by a reduction in resolution. Flow rates above 0.8 mL/min were excluded due to increased backpressure. The investigation of column temperature effects was conducted within the range 25 – 40 °C and demonstrated a substantial impact on separation efficacy. Temperatures exceeding 40 °C were not utilized to maintain adherence to the column operating parameters.

Based on this a full factorial design was utilized for method optimization, incorporating flow rate (0.5 to 0.8 mL/min), mobile phase composition (70 to 80 % v/v MeOH in water), and column temperature (25 °C to 40 °C) as input variables. This method facilitated a systematic enhancement of the crucial parameters that influence the separation process. The selected response variables for optimization comprised resolution values (R_s) and the retention time of the final peak (analysis duration). Supplementary Table 1 outlines the experimental matrix alongside the corresponding results, including a parallel set of the original input data. The software randomly shuffled the data to enhance the robustness and reliability of the statistical analyses and outcomes. Baseline separation of all analytes was aimed for, with an $R_s \geq 1.5$ per peak pair and an analysis time of less than 60 min. It is important to note that additional peaks also appear after the elution of dexibuprofen. However, in this context, resolution values typically exceed five; therefore, the critical resolution is between the enantiomers. The parameters were optimized to maximize resolution and to reduce analysis time. All variables were assigned the same weight, but the critical resolution (R_{s4}, resolution between enantiomers) was given the highest importance, while all other dependent variables were assigned moderate importance. The quadratic models underwent analysis via the ANOVA test. All examined parameters (flow rate, temperature, and MeOH %) significantly influence all resolution values and analysis duration. The Derringer desirability function was utilized through the optimization capabilities of statistical software to attain the optimal chromatographic conditions for all responses. The resulting three-dimensional response surface plots, produced from the regression models, are displayed in Fig. 3.

Based on the optimization through the desirability function, the most favorable chromatographic conditions included the use of a Lux Cellulose-3 column at 35 °C temperature, with a mobile phase consisting of methanol/water/acetic acid (70:30:0.1, v/v/v) and a flow rate of 0.5 mL/min. As depicted in Fig. 4A–B, these conditions enabled full baseline separation of all analytes within 60 min. Ibuprofen related compound J is a racemic mixture of (*S*)-oxo-ibuprofen and (*R*)-oxo-ibuprofen, which are separated in our system. Oxo-ibuprofen has an absorbance maximum at higher wavelength, therefore it is determined at 254 nm, while other compounds are determined at 220 nm.

3.3. Gradient elution: an efficient approach to reduce analysis time

The developed method can be easily applied for the determination of both chemical and enantiomeric impurities in dexibuprofen samples. However, it should be noted that the retention time is long, due to the prolonged retention of the two ester derivatives. In addition, there is a 20-minute gap between the peaks of ibuprofen and ibuprofen methyl ester. Thus, gradient elution was employed to reduce retention times. While gradient elution is a common approach in reversed-phase achiral chromatography, its use in chiral chromatography is relatively rare [14, 43,44]. As part of the validation process, it should be demonstrated first that gradient elution can be used for chiral analysis, and retention times remain stable, even after multiple injections.

The following HPLC gradient program was employed utilized a mobile phase composed of A (MeOH:AcOH, 99.9:0.1 v/v %) and B (water:AcOH, 99.9:0.1 v/v %), with a flow rate of 0.5 mL/min: 0–20 min (70 % A, 30 % B), 20–30 min (90 % A, 10 % B), followed by re-equilibration to (70 % A, 30 % B) from 30 to 40 min as detailed in Table 2.

Although gradient elution is not commonly used in chiral separations, its application in this study significantly reduced the analysis time, nearly halving it, as the retention time of the last eluting impurity was less than 30 min. The experiment repeated over one hundred consecutive injections on two different HPLC systems, demonstrating high reproducibility and consistent retention times, as illustrated in Fig. 5.

The gradient method was subsequently validated in accordance with the Q2(R2) guidelines of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use quantifying available organic impurities, and enantiomeric impurity emphasizing essential parameters, including LOD, LOQ, accuracy, precision, and linearity [45]. The results are summarized in Table 3.

Limit of detection (LOD) and limit of quantification (LOQ) were determined based on signal-to-noise ratios of 3:1 and 10:1, respectively. Impurity validation was performed in the 0.05–0.30 % range relative to a dexibuprofen concentration of 4 mg/mL. Linearity was confirmed across seven concentration levels, with all impurities exhibiting consistent responses and correlation coefficients exceeding 0.9946.

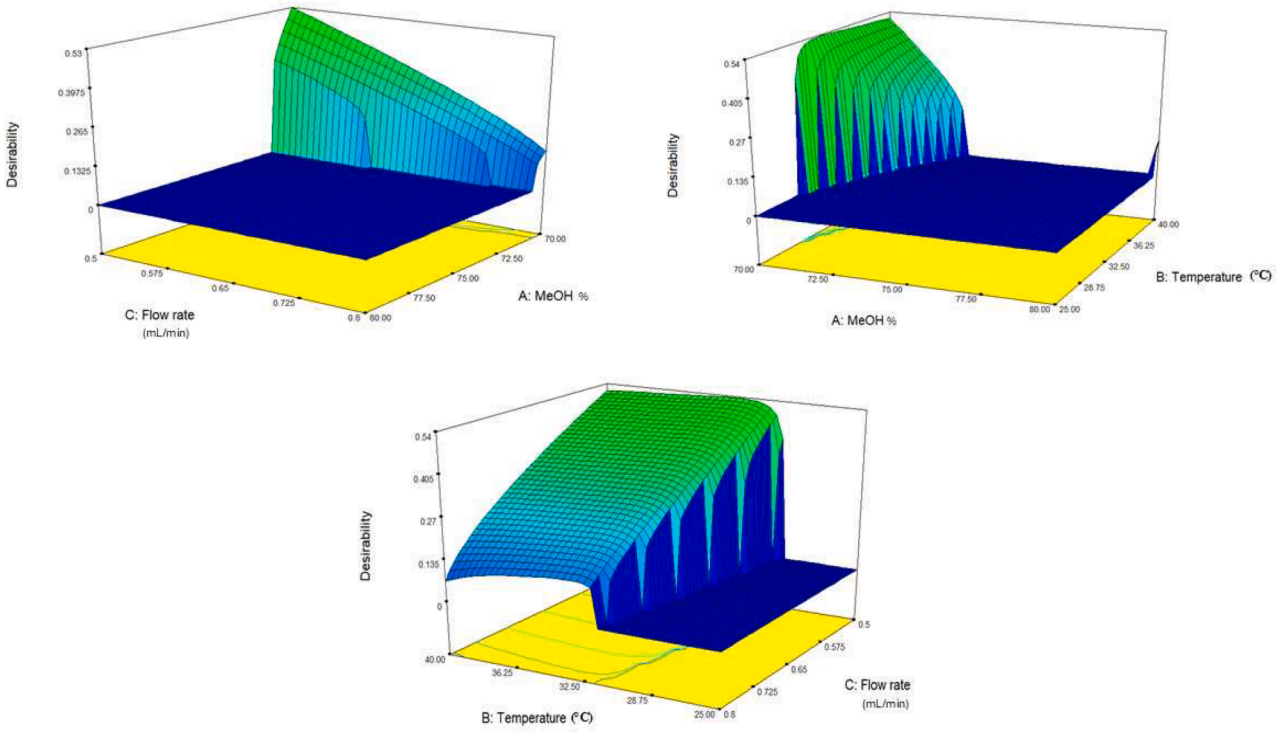


Fig. 3. Three-dimensional response surface plots obtained for desirability.

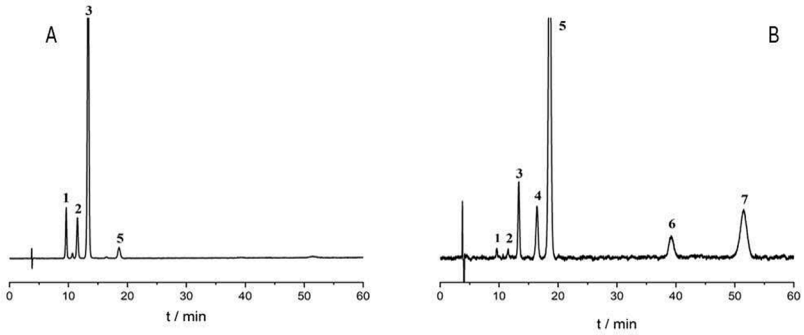


Fig. 4. Chromatograms exemplifying technique (A) and (B) display dexibuprofen samples spiked with 0.1 % impurities at wavelengths of 254 nm and 220 nm, respectively. Chromatographic parameters: Lux Cellulose-3 column using a methanol/water/acetic acid mobile phase (70:30:0.1, v/v/v), at 35 °C and a flow rate of 0.5 mL/min. (1 and 2— Enantiomers of ibuprofen related compound J, 3 – Ibuprofen related compound C, 4—(*R*)–ibuprofen, 5—Dexibuprofen, 6— ibuprofen methyl ester and 7— ibuprofen ethyl ester).

Table 2
Mobile phase gradient of the optimized method.

Time (min)	MeOH:AcOH 99.9:0.1 v/v (%)	water:AcOH 99.9:0.1 v/v (%)
0	70	30
20	90	10
30	90	10
31	70	30
40	70	30

Furthermore, the absence of systematic trends in residuals, along with 95 % confidence intervals for the y-intercepts covering zero, indicated a statistically sound model fit. The accuracy and precision of the method were evaluated through both interday and intraday experiments. For each impurity, five injections were made at three different concentrations within the established linear range to assess performance. Injections were made on the same day and then repeated over two additional days. Table 3 presents a summary of the validation data. The approach was similarly utilized on commercially available Seractil® film-coated tablets, each bearing a nominal dexibuprofen dosage of 400 mg (Supplementary Figure 2). The analyzed product was found to contain (*R*)-ibuprofen at a percentage of 0.27 ± 0.06 % compared to dexibuprofen, with no other impurities within the applied concentration range detected.

According to the International Council of Harmonization (ICH) Q3B (R2) guideline, the presence of the impurity cannot exceed 0.1 % of the active pharmaceutical ingredient when the maximum daily dose is 1 g and 0.05 % when the maximum daily dose is 1 g [46]. Irrespective of the maximum daily dose, the product exhibited an excessive percentage of (*R*)-ibuprofen impurity. As specific limit requirements for enantiomeric impurities have not been established yet, chiral switch formulations do not appear to comply with the general regulations on impurities. The following enantiomeric impurity limits for chiral switch compounds, as listed in various pharmacopeias, include escitalopram 3 % in USP, 2 % in

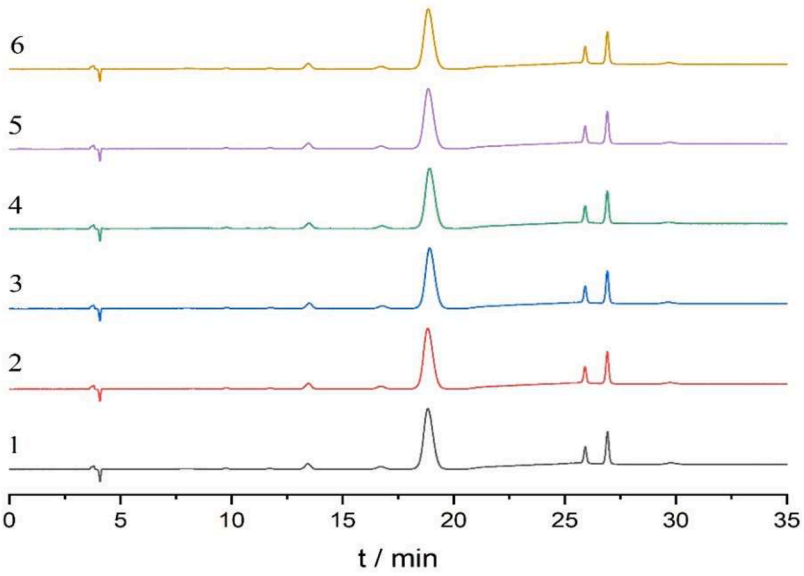


Fig. 5. Representative chromatograms of dexibuprofen enantiomeric separation using a gradient elution method. Chromatographic conditions: Lux Cellulose-3 column with a mobile phase of A (MeOH:AcOH, 99.9:0.1 v/v %) and B (water:AcOH, 99.9:0.1 v/v %), 0–20 min (70 % A, 30 % B), 20–30 min (90 % A, 10 % B), followed by re-equilibration to (70 % A, 30 % B) from 31 to 40 min, a flow rate of 0.5 mL/min, and a column temperature of 35 °C (1— First injection, 2— Fifth injection, 3— Sixteenth injection, 4— Twentieth injection, 5— Thirty-fifth injection and 6— one hundredth injection).

Table 3
Consolidated results from the method validation process for the dual determination of chemical and enantiomeric purity in a dexibuprofen sample (4 mg/mL).

Parameter	Level	Imp-J first peak	Imp-J second peak	4′-Isobutylacetophenone	(*R*)–ibuprofen	methyl ester	ethyl ester
Range (%)		0.05–0.30	0.05–0.30	0.05–0.30	0.05–0.30	0.05–0.30	0.05–0.30
Equation		2.565x+3.281	2.225x+15.083	21.887x+12.063	10.190x+8.432	41.883x−2.352	77.037x+21.467
r^2		0.9989	0.9992	0.9990	0.9946	0.9975	0.9994
LOD (ug/ml)		0.35	0.35	0.25	0.4	0.6	0.6
LOQ (ug/ml)		1.2	1.2	0.8	1.3	2	2
Accuracy	I (0.05 %)	98.3 %	99.7 %	98.1 %	96.05 %	100.9 %	101.8 %
	II (0.10 %)	97.4 %	101.5 %	101 %	97.65 %	102.05 %	99.6 %
	III (0.20 %)	101.05 %	100.3 %	101.9 %	97.97 %	98.8 %	99.9 %
Intraday precision (RSD %)	I (0.05 %)	1.06 %	1.22 %	1.05 %	1.12 %	0.75 %	1.56 %
	II (0.10 %)	1.64 %	1.12 %	1.37 %	1.01 %	1.01 %	0.86 %
	III (0.20 %)	1.81 %	1.36 %	1.01 %	1.26 %	0.89 %	1.08 %
Intermediate precision (RSD %)	I (0.05 %)	1.06 %	1.31 %	1.16 %	1.05 %	0.59 %	1.72 %
	II (0.10 %)	1.03 %	1.44 %	1.21 %	0.83 %	0.79 %	1.02 %
	III (0.20 %)	1.34 %	1.42 %	1.16 %	1.11 %	0.69 %	1.64 %

EP, esomeprazole 0.2 % in USP and 0.6 % in EP, levofloxacin 1.0 % in USP and 0.5 % in EP, levocetirizine 2 % in USP, and in EP. However, it is important to highlight that the presence of enantiomeric impurities in NSAIDs belonging to phenylpropionic acid derivatives is not considered detrimental, as demonstrated by various well-established racemic formulations in the market. Comparable results were obtained for dexketoprofen formulation [17]. Naproxen is the only 2-arylpropionic acid derivative that has been marketed exclusively as a single-enantiomer drug from the beginning, with the (*S*)-enantiomer serving as the eutomer. According to European Pharmacopoeia regulations, the (*R*)-naproxen content must not exceed 2.5 %, which is high compared to the impurity limits set for most enantiopure pharmaceuticals.

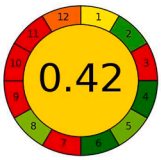
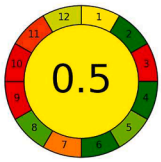
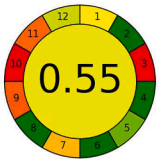
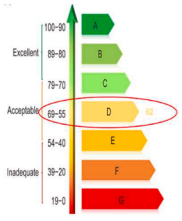
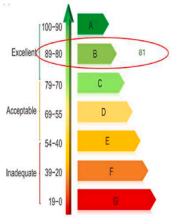
3.4. Comparative evaluation of analytical approaches

An in-depth comparative study was conducted to evaluate the environmental and operational performance of the combined non-simultaneous method, as well as our developed isocratic and gradient methods, using established green analytical metrics. These included the AGREE metric [47], the Analytical Eco-Scale [48,49] and a Modified Eco-Scale [50].

In the combined non-simultaneous approach, the method was based on the European Pharmacopoeia procedure for chemical impurities, complemented by a separate chiral method using a MeOH:water:acetic acid (90:10:0.1, v/v/v) mobile phase using Lux Cellulose-3 column. The result of comparison is summarized in Table 4.

Among the tested approaches, the gradient method demonstrated the most favorable environmental profile, achieving the highest AGREE score (0.55), an excellent Eco-Scale value of 81, and a "B" classification under the Modified Eco-Scale highlighting its superior sustainability and alignment with green chemistry principles. In addition to its environmental advantages, the gradient method offers enhanced operational efficiency. It utilizes a single chiral chromatographic column, in contrast to the combined, conventional, non-simultaneous method, which necessitates the use of both chiral and achiral columns. The separate method results in increased cost, instrument workload, and procedural complexity. Moreover, it involves substantial use of organic solvents and reagents, thereby elevating the environmental burden through hazardous waste generation and prolonged run times exceeding one hour. Although the isocratic method employs similar instrumentation to the gradient method, it requires longer analysis duration (~60 min), leading to greater solvent consumption, waste output, and energy usage. In

Table 4
Comparison between the greenness profiles of the Combined non-simultaneous, Gradient and Isocratic methods via Eco, modified eco-scale, and AGREE tools.

Greenness profile	Combined non-simultaneous method	Isocratic method	Gradient method
AGREE*			
Eco-Scale**	62 (Acceptable level)	74 (Acceptable level)	81(Excellent level)
Modified Eco-Scale			

* The detailed description of Complex AGREE method is illustrated in Supplementary Tables (2–4).
** The detailed description of Eco-Scale method is illustrated in Supplementary Tables (5–7).

contrast, the gradient method not only shortens analysis time but also improves separation efficiency and minimizes chemical waste. Based on this, a method that can separate chiral and chemical impurities has many advantages. Moreover, if it is necessary the analysis time should be reduced by gradient method, while ensuring stable and reproducible results on chiral columns.

4. Conclusion

One of the key challenges in chiral separations is the simultaneous separation of chemical and chiral impurities in a single run. The most cost-effective approach is to perform this on a single chiral column. In our work, we developed a unique gradient method for the separation of both chiral and chemical impurities of dexibuprofen. The method was rigorously validated across various parameters, including linearity, sensitivity, and reproducibility. Furthermore, we applied this method to pharmaceutical formulation, demonstrating its robustness and versatility. The ability to simultaneously analyze chiral and chemical impurities simplifies the workflow and reduces the time and cost associated with multiple analyses. This advancement holds significant promise for the quality control of chiral drugs, ensuring their safety and efficacy for patient use.

CRediT authorship contribution statement

Ali Mhammad: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Gergely Dombi:** Writing – original draft, Visualization, Validation, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Máté Dobó:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Data curation. **Balázs Simon:** Writing – review & editing, Validation, Supervision, Methodology. **Zoltán-István Szabó:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Gergő Tóth:** Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.466210.

Data availability

Data will be made available on request.

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