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Research Article

Chiral Separation of Three Anti-Inflammatory Drugs by Liquid Chromatography on Polysaccharide-Type Stationary Phases and Pirkle-Type Phase

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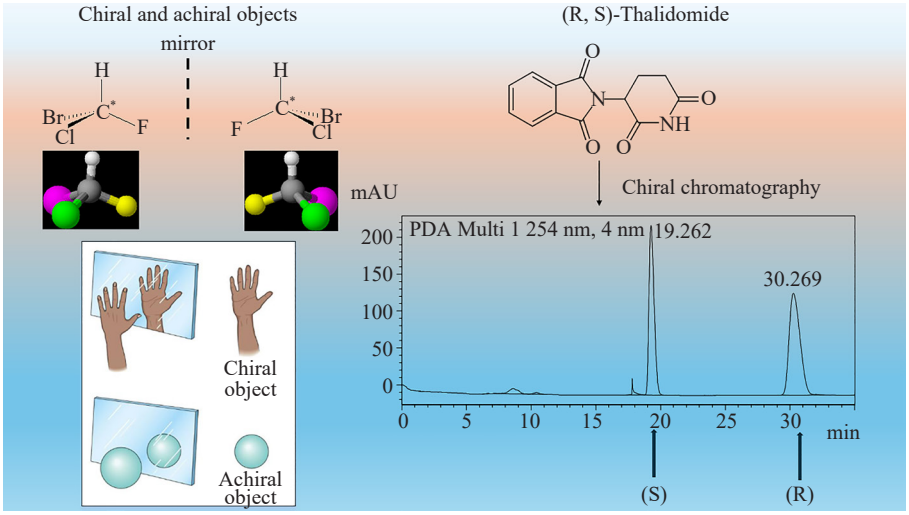
Graphical Abstract:



Abstract: In this work, the enantioseparation of three anti-inflammatory drugs (naproxen, ibuprofen, and thalidomide) was investigated on seven different polysaccharide-type stationary phases (ReproSil Chiral-MIG, ReproSil Chiral-MID, ReproSil Chiral-AM, ReproSil Chiral-JM, ReproSil Chiral-MIC, Lux i-Amylose-1, and Lux Amylose-2) and a Pirkle-type phase (ReproSil Chiral-NR) using Normal (NP) and Reverse (RP) phase systems. Along with the separation capacity of the applied systems, our study focuses on the resolution, the effect of mobile phase mixtures, retention times, selectivity, and the number of theoretical plates. The studies enabled the determination of optimal chromatographic conditions that provide the best separation results for the individual enantiomers of anti-inflammatory drugs. Thalidomide separated best on a ReproSil Chiral-MIG amylose column in a system of 99% acetonitrile and 1% water (v/v) with a resolution (R_s) of 9.410. Naproxen separated best on a column with a Pirkle-type phase in the same

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solvent system, 95 : 5 (v/v), with a resolution (R_s) of 1.186. Ibuprofen did not separate on columns with a Pirkle-type phase or cellulose packing. Partial separation was achieved on the ReproSil Chiral-MID amylose column in a system of 85% *n*-heptane and 15% 1-propanol (v/v), yielding a resolution (R_s) of 1.169. These studies enabled the verification of the separation of three drugs, for which only one of the enantiomers typically has a therapeutic effect. This finding is valuable from the perspective of human health. Our studies focus on a wide range of polysaccharide stationary phases, a brush column, and as many as ten solvent systems; furthermore, the elution order was determined wherever possible. Several variables enabled the drawing of both general and specific conclusions, considering the structure of the analytes and the column packings.

***Keywords*:** anti-inflammatory drugs, enantiomer, racemate, resolution, chiral chromatography

1. Introduction

A drug's chirality has a significant impact on its ability to bind to specific receptors, enzymes, or other biological targets in living organisms. Due to differences in the spatial orientation of enantiomer molecules, each isomer may exhibit different affinities for these targets. This, in turn, determines the specific interactions that drugs will have in the body, influencing their therapeutic effects. Chirality plays a key role in pharmaceutical chemistry.[1] Many chemical compounds with medicinal use exist as enantiomers, which can differ in their effects on the body. In the case of anti-inflammatory drugs, one of the enantiomers may be therapeutically active, while the other may not exhibit pharmacological activity or cause adverse effects.[2-5] Effective separation into enantiomers, therefore, becomes crucial to ensure the quality and safety of the drug.

One of the most effective methods for chiral separation is High-Performance Liquid Chromatography (HPLC).[6-8] The requirements for the purity of enantiomers in drugs are constantly increasing, and analytical processes must comply with strict standards, which places chiral chromatography at the forefront of methods used in the pharmaceutical industry. The development of new chromatographic materials and the optimization of HPLC parameters make this method increasingly efficient and accessible, which opens new possibilities in the field of pharmacology.

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are among the most used groups in pain pharmacology. They exhibit anti-inflammatory and antipyretic effects. They are used in both general and specialist medicine, such as rheumatology or orthopedics. Their popularity is primarily due to their ease of availability (most of these drugs are sold over the counter without a prescription), their relatively low price compared to other treatment methods, and the relief of varying intensities and origins of inflammation.[9-12] Despite their numerous advantages, they are the drugs for which adverse effects are most frequently reported. The therapeutic effect and safety profile may vary significantly depending on the chemical structure, particularly the chirality of individual substances.[13-15]

The primary mechanism of action of NSAIDs is the inhibition of the Cyclooxygenase (COX-1 and COX-2) enzyme. Cyclooxygenase is required to convert arachidonic acid into thromboxanes, prostaglandins, and prostacyclins.[16] The therapeutic effects of NSAIDs are attributed to the lack of these eicosanoids. Specifically, thromboxanes play a role in platelet adhesion, prostaglandins cause vasodilation, increase the temperature set point in the hypothalamus, and contribute to anti-nociception. There are two cyclooxygenase isoenzymes, COX-1 and COX-2. COX-1 is constitutively expressed in the body and plays a role in maintaining the gastrointestinal mucosa lining, kidney function, and platelet aggregation. COX-2 is not constitutively expressed in the body but is inducibly expressed during an inflammatory response. Most of the NSAIDs are nonselective and inhibit both COX-1 and COX-2. However, COX-2 selective NSAIDs (ex., celecoxib) only target COX-2 and therefore have a different side effect profile. Because COX-1 is the primary mediator of ensuring gastric mucosal integrity, and COX-2 is primarily involved in inflammation, COX-2-selective NSAIDs are expected to provide anti-inflammatory relief without compromising gastric mucosal integrity.[17]

A list of common NSAIDs includes ibuprofen, aspirin, and naproxen. Ibuprofen 2-(4-isobutylphenyl) propionic acid, as shown in Figure 1, is a propionic acid derivative. It was introduced in 1969 as a safer alternative to Aspirin. Its primary use is pain relief, fever reduction, and inflammation management.[18-19]

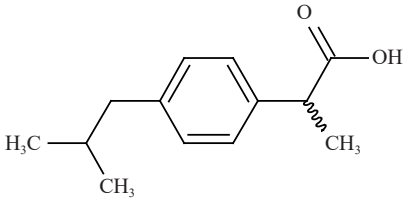


Figure 1. Chemical structure of ibuprofen

The pharmacological properties of ibuprofen are almost exclusively related to the (S) enantiomer; however, some of the (R) ibuprofen is metabolized to its pharmacologically active mirror form. Therefore, the racemic mixture (R, S) is mainly used clinically. Racemic ibuprofen and the (S) enantiomer are primarily used to treat mild to moderate pain associated with dysmenorrhea, headache, migraine, spondylitis, osteoarthritis, rheumatoid arthritis, and soft tissue disorders. It is also one of the most effective and widely used NSAIDs in the treatment of toothache.[20]

Naproxen, or 2-(6-methoxy-2-naphthyl)propionic acid (Figure 2), treats various painful and inflammatory conditions, including acute postoperative pain. It is often combined with sodium to improve its solubility for oral administration. The advantages of naproxen include its rapid absorption and long duration of action, which result from its long biological half-life (approximately 13 hours).[21] Naproxen has been used in clinical practice since 1976 as a single enantiomer (S)-naproxen. It has a higher affinity for Cyclooxygenase (COX), whereas (R)-naproxen is hepatotoxic, causing liver damage. According to the regulations of the European Pharmacopoeia, the content of (R)-naproxen should not exceed 2.5%, which can be considered a relatively high limit compared to most enantiomerically pure pharmaceuticals.[22,23]

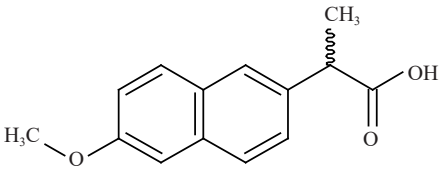


Figure 2. Chemical structure of naproxen

Besides, thalidomide (2-(2,6-dioxopiperidin-3-yl)isoindole-1,3-dione), as shown in Figure 3, also exhibits anti-inflammatory properties and was initially prescribed to pregnant women in the late 1950's as an antiemetic. It is known to cause severe congenital disabilities, such as phocomelia, in babies whose mothers took the drug during pregnancy.[24-26] Although thalidomide has a poor reputation, clinical interest in this drug is currently growing due to its immunomodulatory (enhancing immunity and supporting the body's repair functions) and antiangiogenic (inhibiting the formation of new blood vessels, thereby slowing tumor growth) properties. Modern studies have shown that thalidomide reduces the level of Tumor Necrosis Factor-alpha (TNF-α), which is responsible for inflammatory processes, and is also effective in combating Human Immunodeficiency Virus (HIV)-associated wasting syndrome, HIV-associated diarrhea, aphthous mouth ulcers, and oral ulcers.[27-31]

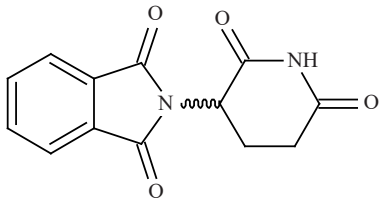


Figure 3. Chemical structure of thalidomide

The reason for thalidomide's notoriety and harmfulness was related to the fact that the drug was initially sold as a mixture of two enantiomers. Studies have shown that only the "R" form of thalidomide was therapeutically active and had a healing effect. On the other hand, the "S" form, which is a mirror image, was not only ineffective but also caused congenital disabilities in newborns. This discovery explained why, despite its effectiveness in treating some diseases, the drug became so controversial and dangerous to use. The history of the drug thalidomide illustrates why stereochemistry plays such a significant role in drug development.[31-35]

This research aimed to develop a method that obtains baseline separation of three drugs (naproxen, ibuprofen, and thalidomide) with a reasonably short analysis time using polysaccharide-type and Pirkle-type stationary phases. During the studies, the type of column, solvent volume ratio, and mobile phase flow rate were varied. The elution order was also determined to determine which enantiomer elutes first on a given column, as enantiomers have different therapeutic effects. Chiral recognition mechanisms for the individual types of stationary phases were also described. Finding optimal conditions for separating enantiomers of anti-inflammatory drug substances is significant from the perspective of patient health.[36] Despite numerous publications on this subject,[37-47] it is worth continually seeking optimal solutions, as the enantioseparation process is also influenced by factors such as the column manufacturer, solvent sources, or the drugs themselves. Furthermore, numerous variables are involved in chromatographic analysis, making it challenging to find identical conditions across publications due to variations in sample concentrations, detection wavelengths, column dimensions, solvent purity, measurement temperatures, mobile phase flow rates, mobile phase systems, and modifier additions, among others.

2. Experimental

2.1 *Materials and methods*

2.1.1 *Anti-inflammatory drugs*

The subject of the analysis was three anti-inflammatory drugs that differed in their chemical structure. The structure of thalidomide consists of a phthalimide ring connected to a glutarimide group. Due to the presence of amide groups, it is a reasonably polar compound. Ibuprofen is a derivative of propionic acid. Its structure consists of a benzene ring, which is substituted with two functional groups (isobutyl and carboxyl). Ibuprofen has low polarity due to the presence of a large, apolar alkyl group. Naproxen, like ibuprofen, is a propionic acid derivative, but its structure is more complex. Unlike ibuprofen, naproxen contains a naphthalene ring, which is substituted with a carboxyl and methoxy group. Due to these substituents, the naproxen molecule is more polar than ibuprofen but still less polar than thalidomide. Table 1 presents the acronyms of these substances.

Table 1. The acronyms of the studied racemates

Acronym	Name
NAP (R, S)	Naproxen
IBU (R, S)	Ibuprofen
THAL (R, S)	Thalidomide

The sample preparation process began with the preparation of weights. Approximately 2 mg of each of the drug substance mixtures was weighed into vials. The next step involved dissolving the weighed samples in the appropriate solvents. Acetonitrile and 2-propanol were used in a 2 mL volume to dissolve the samples. Of the three substances, only thalidomide did not show solubility in 2-propanol. Therefore, it was dissolved only in acetonitrile. Weights of non-equimolar mixtures were also prepared to determine the elution order. Since approximately 1 mg of the racemic mixture and approximately 1 mg of the S enantiomer were used to prepare such mixtures, the enantiomeric excess (e.e.) was 50%. The samples of non-equimolar mixtures were dissolved in 2 mL of acetonitrile.

Commercially available drugs were used for the studies: (S)-(+)-NAP (Tokyo Chemical Industry Co., Ltd. (TCI) Europe nv, purity > 99%), NAP (R, S) (Fluorochem, purity > 98%), (S)-(+)-IBU (Fluka, purity > 98%), IBU (R, S) (BLDpharm, purity 99.7%), (S)-(-)-THAL (Sigma Aldrich, purity > 98%), THAL (R, S) (TCI, purity > 98%).

2.1.2 *Chiral HPLC separation*

Enantioseparation of the three drugs using seven different polysaccharide-type Chiral Stationary Phases (CSPs), including five amylose-based columns, two cellulose-based columns, and one Pirkle-type column, has been performed. The columns had a particle size of 5 μm, dimensions of 250 mm × 4.6 mm i.d., and a pore size of 1,000 Å. The chemical structures and names of the eight chiral selectors are depicted in Table 2. Dr. Maisch (Germany) or Phenomenex (United States) manufactured the columns.[48,49] The ReproSil Chiral-AM and Lux i-Amylose-1 columns have the same packing material, but the first is coated, while the second is immobilized.[50-53] Pirkle's chiral stationary phases were presented by his research group in the eighties.[54-55] Their chemical structure is characterized by the presence of π-donor and π-acceptor groups, which form hydrogen bonds. Chiral molecules are distributed on an inert substrate, like bristles on a brush; hence, the English name of these phases is brush-type.[56] Such a distribution of molecules facilitates access of the analyte to the chiral selector.

The Shimadzu LC-20AP HPLC system (Kyoto, Japan), comprising a binary solvent delivery pump, an autosampler (SIL-10AP), a communications bus module (CBM-20A), a diode array detector (SPD-M20A), and a fraction collector (FRC-10A), was utilized for the separation and detection of analytes. Data acquisition was performed by Shimadzu software (LabSolutions, 2010-2017 Shimadzu Corporation). The injection volume of samples was 5-25 μL. The measurements were carried out at room temperature. The mobile phase flow rate was 0.3, 0.5, or 1 mL/min, and the detection wavelength was 254 nm.

Table 2. The structures and names of the chiral selectors (R = amylose or cellulose)

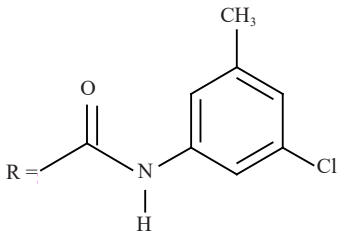
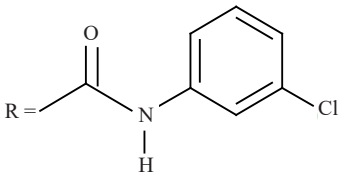
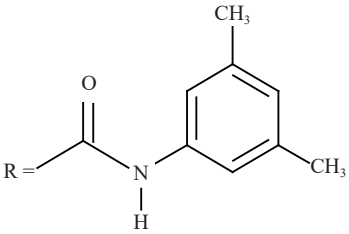
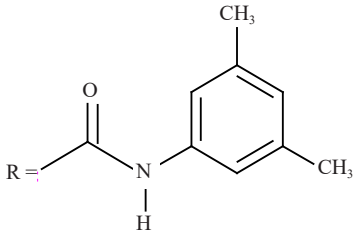
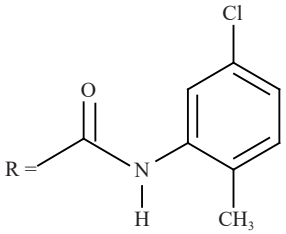
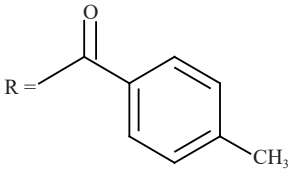
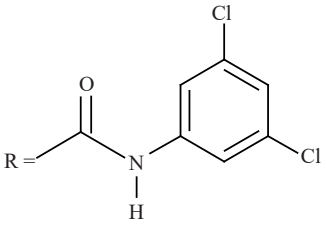
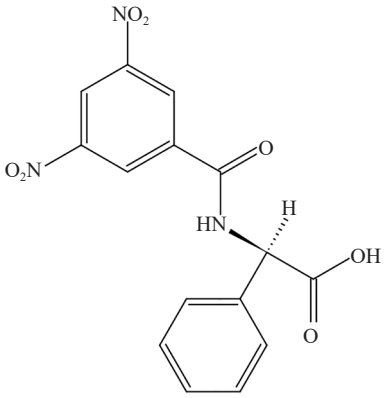
Name	Structure
ReproSil Chiral-MIG[1] Amylose tris(3-chloro-5-methylphenylcarbamate)	
ReproSil Chiral-MID[1] Amylose tris(3-chlorophenylcarbamate)	
ReproSil Chiral-AM[1] Amylose tris(3,5-dimethylphenylcarbamate)	

Table 2. (cont.)

Name	Structure
Lux i-Amylose-1[2] Amylose tris(3,5-dimethylphenylcarbamate)	
Lux Amylose-2[2] Amylose tris(5-chloro-2-methylphenylcarbamate)	
Reprosil Chiral-JM[1] Cellulose tris(4-methylbenzoate)	
ReproSil Chiral-MIC[1] Cellulose tris(3,5-chlorophenylcarbamate)	
ReproSil Chiral-NR[1] N-(3,5-dinitrobenzoyl)phenylglycine	

[1]Dr. Maisch columns; [2]Phenomenex columns

During the analysis, various solvent systems were used, as shown in Table 3. Both normal-phase and reversed-phase systems were used.[57-61] All solvents were used as purchased, and ultrapure water, after double distillation, was used. HPLC grade *n*-hexane, cyclohexane, 1-propanol, 1-butanol, and ethanol were purchased from Chempur, Poland. HPLC-grade *n*-heptane was purchased from Sigma-Aldrich, South Korea; acetonitrile was purchased from LabScan, Poland; 2-propanol was purchased from Honeywell, Germany; and methanol was purchased from Fisher Chemical, Belgium.

Table 3. Mobile phase systems used in the analyses

Solvent system	Acronym
n-Hexane/2-Propanol	HEX/IPA
n-Heptane/Ethanol	HEP/EtOH
Cyclohexane/2-Propanol	*c*-HEX/IPA
n-Heptane/2-Propanol	HEP/IPA
n-Heptane/1-Propanol	HEP/PrOH
n-Heptane/1-Butanol	HEP/BuOH
Acetonitrile/Water[1]	ACN/H_2O
Acetonitrile/Ethanol[1]	ACN/EtOH
Acetonitrile/Methanol[1]	ACN/MeOH
Acetonitrile/2-Propanol[1]	ACN/IPA

[1]Reversed-phase system

3. Results

3.1 *Amylose columns*

On the ReproSil Chiral-MIG column, analyses were performed using five different solvent systems, as listed in Table 4.

Table 4. Parameters of analyses on the ReproSil Chiral-MIG column

Mobile phase	Volume ratio	The volume of an injected sample [µl]	Flow rate [mL/min]
HEX/IPA	90 : 10 85 : 15 70 : 30	5	1
ACN/H_2O	99 : 1 95 : 5 90 : 10	10	0.5 or 1
ACN/MeOH	99 : 1	10	1
ACN/EtOH	99 : 1	10	1
ACN/IPA	99 : 1	10	1

Representative chromatograms showing enantiorecognition are presented in the Appendix (Figures S1-S3). The results obtained are summarized in Table 5. The baseline separation was achieved for thalidomide in all solvent systems (R_s > 1.5). The racemic mixtures, which contained a carboxyl group in their structure, namely naproxen and ibuprofen, were partially separated, and the best results were obtained using the ACN/MeOH system. None of the mixtures was separated in the normal-phase solvent system (HEX/IPA).

Table 5. Chromatographic data: resolution, Enantiomer Elution Order (EEO), retention times, selectivity, the number of theoretical plates, flow rate 1 mL/min (ReproSil Chiral-MIG column)

Drugs	Mobile phase 99 : 1 (v/v)	R_s	EEO	t_{r1}; t_{r2}	α	N
THAL (R, S)	ACN/H_2O	8.226	S > R	8.966; 14.695	2.226	4,400; 5,000
	ACN/MeOH	7.278	S > R	9.881; 15.760	1.921	3,900; 4,300
	ACN/EtOH	5.856	S > R	10.312; 15.916	1.823	2,900; 3,200
	ACN/IPA	6.912	S > R	9.842; 15.624	1.910	3,500; 4,000
NAP (R, S)	ACN/H_2O	0.483	R < S	4.414; 4.596	1.397	3,300; 1,700
	ACN/MeOH	0.554	R < S	4.697; 4.885	1.259	4,000; 2,600
	ACN/EtOH	0.419	R < S	4.719; 4.910	1.043	2,000; 1,600
	ACN/IPA	0.394	R < S	4.699; 4.850	1.148	1,700; 1,800
IBU (R, S)	ACN/H_2O	-	-	4.058	1.000	900
	ACN/MeOH	0.444	R < S	4.063; 4229	1.221	2,200; 1,800
	ACN/EtOH	0.348	R < S	4.050; 4.210	1.320	1,300; 1,305
	ACN/IPA	0.381	R < S	4.037; 4.202	1.343	1,600; 1,300

The flow rate of the mobile phase was also modified to improve separation. Decreasing the flow rate increased the resolution parameter value, but simultaneously, a twofold increase in retention times was observed. The obtained results are presented in Table 6.

Table 6. Chromatographic data: resolution, retention times, flow rate 0.5 mL/min (ReproSil Chiral-MIG column)

Drugs	Mobile phase 99 : 1 (v/v)	R_s	t_{r1}; t_{r2}
THAL (R, S)	ACN/H_2O	9.410	19.262; 30.269
NAP (R, S)	ACN/MeOH	0.497	9.374; 9.761
IBU (R, S)	ACN/MeOH	0.570	8.078; 8.423

Table 7. Summary of the analysis results in the ACN/H_2O 99 : 1 + 0.1% of CH_3COOH solvent system (ReproSil Chiral-MIG column)

Drugs	Mobile phase 99 : 1 : 0.1 (v/v/v)	R_s	α	N_R; N_S
NAP (R, S)	ACN/H_2O/CH_3COOH	0.087	1.022	1,200; 120
IBU (R, S)	ACN/H_2O/CH_3COOH	0.273	1.037	1,900; 700

In addition to standard solvent systems, an ACN/H_2O system was prepared with minimal organic acid addition to the aqueous phase. The organic acid acts as a pH stabilizer in the mobile phase, which can affect the interaction properties between the analytes and the stationary phase. Naproxen and ibuprofen were analysed in this system due to the carboxyl groups present in the structure of these analytes. The solvent system consisted of 99% acetonitrile, 1% H_2O, and 0.1% acetic acid (CH_3COOH) (v/v/v). Analyses were performed at a flow rate of 0.5 mL/min. In contrast to the system without added acid, ibuprofen separated slightly. The resolution of naproxen was worsened, as shown in Table 7.

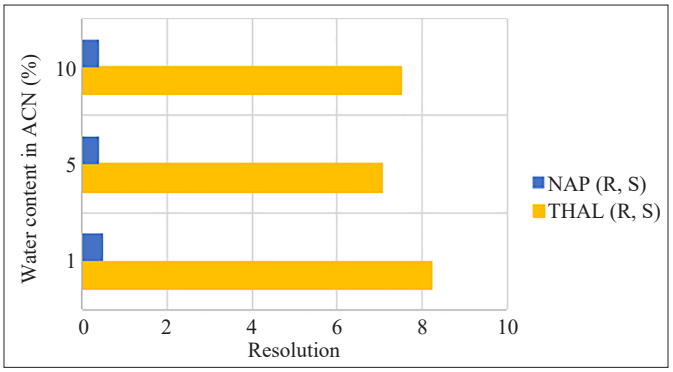


Figure 4. The effect of water content in ACN on the resolution of thalidomide and naproxen on the Reprosil Chiral-MIG column

As part of the studies conducted in the acetonitrile/water solvent system, chromatographic parameters were analysed for water content in ACN. 1%, 5%, and 10% of water were added to the system, respectively, and then the changes in resolution were assessed. The results are presented in Figure 4. Thalidomide and naproxen achieved the highest resolution with the lowest volume percentage of water in the system.

Drugs in four different solvent systems were analyzed on the ReproSil Chiral-MID column (see Table 8).

Table 8. Parameters of analyses on the ReproSil Chiral-MID column

Mobile phase	Volume ratio	The volume of an injected sample [µl]	Flow rate [mL/min]
HEP/IPA	85 : 15 80 : 20 75 : 25	10	1
ACN/H_2O	99 : 1 95 : 5 90 : 10	15	0.5 or 1
HEP/PrOH	90 : 10 85 : 15	10	1
HEP/BuOH	85 : 15	10	1

Of all the mixtures, only naproxen did not separate in any of the solvent systems. Thalidomide achieved baseline separation in the reversed-phase mode, while ibuprofen was partially separated in the normal-phase mode. The resolution of thalidomide, depending on the water content in the system, is presented in Figure 5. With the increase in water content in the system, an improvement in resolution was observed, suggesting that a higher water concentration favors better separation of the mixture components.

Chromatographic parameters for thalidomide are presented in Table 9.

The increased water content in the system improved resolution, suggesting that higher water concentration promoted better separation of the mixture components. The shortest retention time was obtained with the highest water content in the mobile phase. Higher water concentration promoted faster movement of the analyte through the column. Analysis of thalidomide selectivity as a function of water content revealed that the highest selectivity was achieved with 1% water, while the lowest selectivity was observed with 10% water. These results are opposite to the resolution results. Lower selectivity with higher water content suggests that although the components are better separated, the differences in their interactions with the stationary phase become less pronounced. As a result, the ability to distinguish between enantiomers may be worse. The highest number of theoretical plates was obtained with the highest water concentration in the mobile phase. The elution order of thalidomide was identical to that obtained on the ReproSil Chiral-MIG column, i.e., (S > R); see Figure S4 in the Appendix.

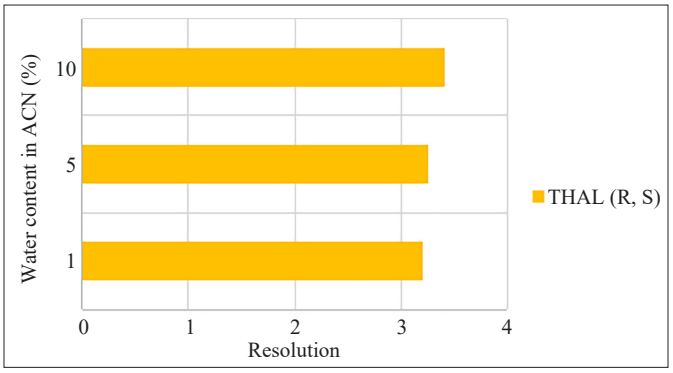


Figure 5. The effect of water content in ACN on the resolution of thalidomide on the ReproSil Chiral-MID column

Table 9. Chromatographic data: resolution, enantiomer elution order, retention times, selectivity, the number of theoretical plates, flow rate 1 mL/min (ReproSil Chiral-MID column)

Drug	Mobile phase (v/v)	R_s	EEO	t_{r1}; t_{r2}	α	N_S; N_R
THAL (R, S)	ACN/H_2O (99 : 1)	3.201	S > R	5.806; 8.429	2.332	2,400; 800
	ACN/H_2O (95 : 5)	3.261	S > R	5.220; 7.514	1.719	2,900; 800
	ACN/H_2O (90 : 10)	3.415	S > R	4.981; 7.308	1.568	3,000; 850

In the case of ibuprofen, the highest resolution (but not baseline) was obtained in the *n*-heptane/1-propanol system, which exhibited the highest separation efficiency, as shown in Figure 6.

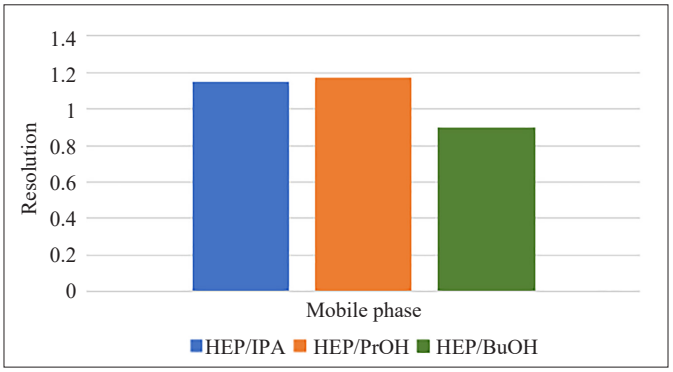


Figure 6. The resolution of ibuprofen in different solvent systems at a ratio of 85 : 15 (v/v) on the ReproSil Chiral-MID column, with a flow rate of 1 mL/min

Analyses were performed in three different solvent systems to optimize the separation process on the ReproSil Chiral-AM column (see Table 10).

Table 10. Parameters of analyses on the ReproSil Chiral-AM column

Mobile phase	Volume ratio	The volume of an injected sample [µl]	Flow rate [mL/min]
c-HEX/IPA	90 : 10 80 : 20	10	1
ACN/H_2O	99 : 1 95 : 5 90 : 10	10	0.3 or 0.5 or 1
ACN/MeOH	99 : 1	10	0.3 or 0.5 or 1

In the case of ibuprofen, no separation was obtained in any of the tested systems, which suggests that the stationary phase was not appropriately selected for the separation of this analyte.

The cyclohexane/2-propanol system proved to be unsuitable for the separation of the analyzed mixtures of substances on the ReproSil Chiral-AM column. Despite attempts to examine different solvent concentrations, no satisfactory results were obtained for any mixtures.

Thalidomide and naproxen were slightly separated in the reversed-phase mode. The results of the resolution analysis in the solvent volume ratio of 99 : 1 are presented in Table 11. The highest resolutions for thalidomide were obtained in methanol, and for naproxen in the water systems. The differences in retention times between these two solvent systems (ACN/H_2O and ACN/MeOH) were only a few milliseconds. The retention times are short, but the interactions between the analytes and the selector are insufficient to achieve baseline separation. For both naproxen and thalidomide, the (R) enantiomer eluted first. The chromatograms are shown in Figures S5 and S6 in the Appendix.

Table 11. Chromatographic data: resolution, enantiomer elution order, and retention times, flow rate 1 mL/min (ReproSil Chiral-AM column)

Drug	Mobile phase (99 : 1) (v/v)	R_s	EEO	t_{r1}; t_{r2}
THAL (R, S)	ACN/H_2O	0.778	R < S	5.874; 6.221
	ACN/MeOH	0.841	R < S	5.843; 6.232
NAP (R, S)	ACN/H_2O	0.804	R < S	3.653; 3.903
	ACN/MeOH	0.659	R < S	3.662; 3.898

Both solvent systems provide partial separation of the mixtures, but neither changing the solvent volume ratio nor modifying the flow rate (Table 12) significantly improved resolution.

Table 12. Resolution of thalidomide from the flow rate of the mobile phase in different systems at a ratio of 99 : 1 on the ReproSil Chiral-AM column

Drug	Mobile phase (99 : 1) (v/v)	Flow rate [mL/min]	R_s
THAL (R, S)	ACN/H_2O	0.3	0.976
		0.5	0.904
		1.0	0.778
	ACN/MeOH	0.3	0.841
		0.5	0.722
		1.0	0.788

For thalidomide, the resolution parameter was also tested depending on the water content in the system (Figure 7). The best result was obtained for the lowest water content, while in the system with 10% water, the resolution decreased to almost zero.

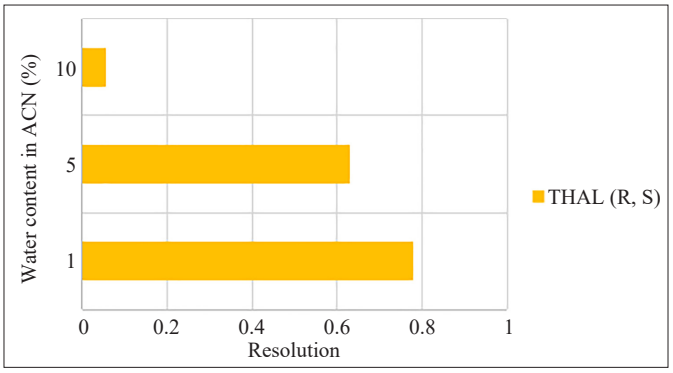


Figure 7. The effect of water content in ACN on the resolution of thalidomide on the ReproSil Chiral-AM column

Analysis of the results obtained on the Lux i-Amylose-1 column, which has the same packing as the ReproSil Chiral-AM column, showed significant differences in separation efficiency. Despite conducting analyses in normal and reversed phase modes, none of the studied mixtures were separated on the Lux i-Amylose-1 column, which has an immobilized packing. In contrast, on the ReproSil Chiral-AM column, with coated packing, two mixtures were partially separated. These differences may be due to the characteristics of the immobilized packing, which can limit interactions between the analytes and the stationary phase.

Table 13. Parameters of analyses on the Lux Amylose-2 column

Mobile phase	Volume ratio	The volume of an injected sample [µl]	Flow rate [mL/min]
HEX/IPA	85 : 15 80 : 20	25	1
ACN/H_2O	99 : 1 95 : 5	25	1
ACN/MeOH	99 : 1 95 : 5	25	1
ACN/EtOH	99 : 1 95 : 5	25	1

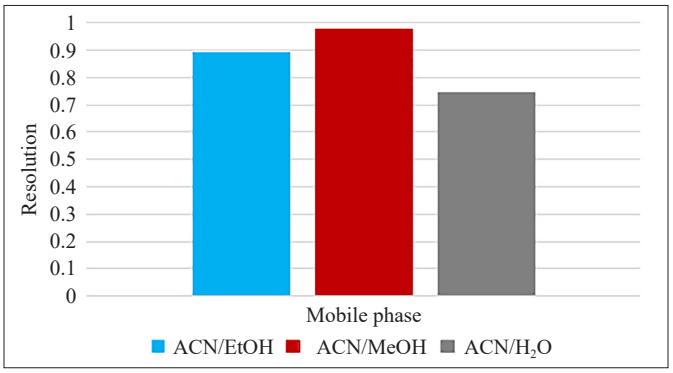


Figure 8. Thalidomide resolution in different solvent systems in the ratio of 99 : 1 on the Lux Amylose-2 column, flow rate 1 mL/min

Studies were conducted on the Lux Amylose-2 column using four different solvent systems (Table 13). None of the mixtures separated in the HEX/IPA system. In the reversed-phase systems, partial separation of some mixtures occurred. Naproxen and ibuprofen showed resolutions close to zero. Thalidomide obtained higher resolution values, but baseline

separation was not achieved. Figure 8 shows the dependence of thalidomide resolution on the solvent system used. The highest resolution result was obtained for the acetonitrile/methanol solvent system.

The shortest retention time was observed at the lowest resolution, i.e., for the ACN/H_2O system. The highest selectivity was obtained for the same system (see Table 14). The (R) enantiomer was eluted first; see Figure S7 in the Appendix.

Table 14. Chromatographic data: selectivity, enantiomer elution order, and retention times, flow rate 1 mL/min (Lux Amylose-2 column)

Drug	Mobile phase (99 : 1) (v/v)	α	EEO	t_{r1}; t_{r2}
THAL (R, S)	ACN/H_2O	1.341	R < S	4.106; 4.320
	ACN/MeOH	1,337	R < S	4.182; 4.461
	ACN/EtOH	1.067	R < S	4.191; 4.453

3.2 *Cellulose columns*

Racemic mixtures on the ReproSil Chiral-JM column were analyzed using ACN/H_2O, ACN/IPA, and ACN/EtOH. Despite changes in the mobile phase composition, the expected separation could not be obtained for any of the mixtures due to the lack of proper interactions between the analyte and the stationary phase.

Analyses on the ReproSil Chiral-MIC column were performed in four solvent systems (see Table 15).

Table 15. Parameters of analyses on the ReproSil Chiral-MIC column

Mobile phase	Volume ratio	The volume of an injected sample [µl]	Flow rate [mL/min]
HEX/IPA	85 : 15	5	0.5 or 1
ACN/H_2O	99 : 1 95 : 5 90 : 10	5	0.5 or 1
ACN/BuOH	99 : 1	5	0.5 or 1
ACN/EtOH	99 : 1 95 : 5	5	0.5 or 1

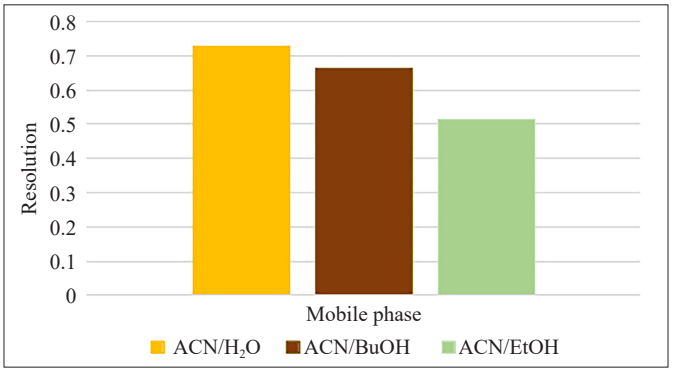


Figure 9. Thalidomide resolution in different solvent systems in the ratio of 99 : 1 on the ReproSil Chiral-MIC column, flow rate 1 mL/min

In the HEX/IPA system, none of the mixtures were separated. In the remaining systems, only thalidomide was partially separated. Figure 9 compares the thalidomide resolution parameter as a function of the solvent system used. The highest resolution result was obtained for the acetonitrile/water system.

Despite attempts at optimization by modifying the volume ratio of solvents and adjusting the flow rate of the mobile phase, no significant improvement in the resolution of thalidomide could be achieved, as shown in Figure 10. The longest retention time occurred in the ACN/H_2O solvent system.

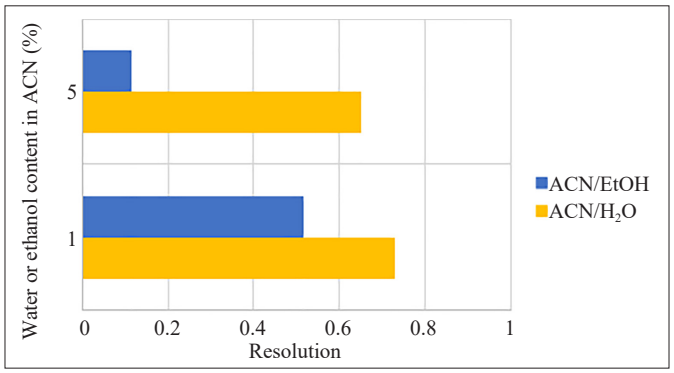


Figure 10. The resolution of thalidomide from the water/ethanol content in the acetonitrile system on the ReproSil Chiral-MIC column, with a flow rate of 1 mL/min

The highest selectivity value was obtained for the ACN/BuOH system, as shown in Table 16. Thalidomide, as an analyte with a polar amide group, may exhibit more complex interactions with butanol, which improves selectivity in the separation process. The (R) enantiomer was the first to elute, as shown in Figure S8 in the Appendix. In the ACN/H_2O solvent system, the number of theoretical plates was the highest, allowing for the narrow peaks to be obtained.

Table 16. Chromatographic data: resolution, enantiomer elution order, retention times, and selectivity (ReproSil Chiral-MIC column)

Drug	Mobile phase (99 : 1) (v/v)	R_s	EEO	t_{r1}; t_{r2}	α	N_R; N_S
THAL (R, S)	ACN/H_2O 1 mL/min 0.5 mL/min[1]	 0.729 0.419	R < S	 4.697: 4.899 -	 1.079 -	 5,000; 4,500 -
	ACN/BuOH 1 mL/min 0.5 mL/min[1]	 0.665 0.637	R < S	 4.475; 4.673 -	 1.466 -	 4,100; 3,800 -
	ACN/EtOH 1 mL/min 0.5 mL/min[1]	 0.515 0.617	R < S	 4.433; 4.612 -	 1.045 -	 3,200; 2,200 -

[1]Retention times, selectivity, and the number of theoretical plates for a flow rate of 0.5 mL/min were not provided because the resolution results varied, sometimes increasing and sometimes decreasing. It had no significance in further analyses

3.3 *Brush-type column*

Partial separation on the ReproSil Chiral-NR column was obtained only for naproxen. This substance strongly interacts with the Pirkle phase due to its aromatic, hydrophobic structure, leading to the separation of enantiomers. However, the peaks were broad and diffuse due to the low number of theoretical plates, contributing to the lower resolution (see Figure 11). The number of theoretical plates is given in Table 17.

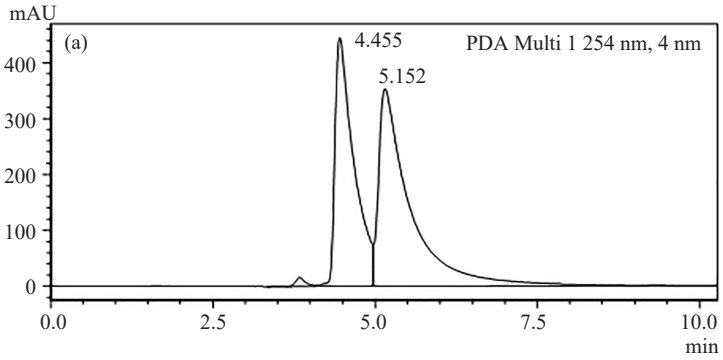


Figure 11. Chromatogram showing naproxen enantiomers in the ACN/H_2O solvent system (ratio 99 : 1) on the ReproSil Chiral-NR column, with a flow rate of 1 mL/min

Table 17. The number of theoretical plates for naproxen, flow rate 1 mL/min (ReproSil Chiral-NR column)

Drug	Mobile phase (v/v)	N_R; N_S
NAP (R, S)	ACN/H_2O (99 : 1)	1,200; 900
	ACN/H_2O (95 : 5)	1,300; 2,000
	ACN/H_2O (90 : 10)	200; 900

The optimization of the separation consisted of examining the resolution as a function of the water content in the system using ACN. The highest resolution was achieved for a 5% water solution. At 10% water content, the resolution dropped by a factor of two. The results are presented in Figure 12.

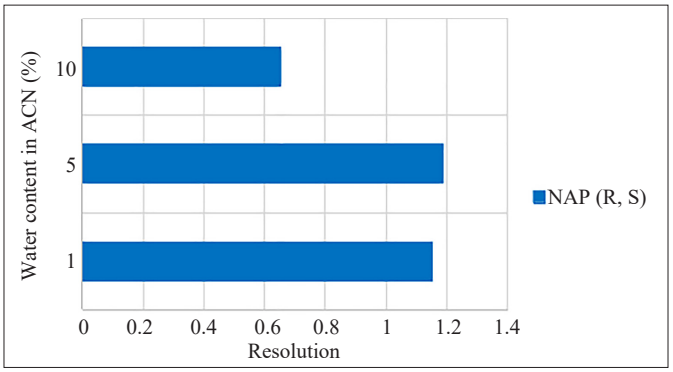


Figure 12. Resolution of naproxen from water content in the ACN/H_2O system on the ReproSil Chiral-NR column, with a flow rate of 1 mL/min

The order of elution of the naproxen enantiomers on the ReproSil Chiral-NR column is shown in the chromatograms below (Figure 13). The (R) enantiomer eluted first (R < S).

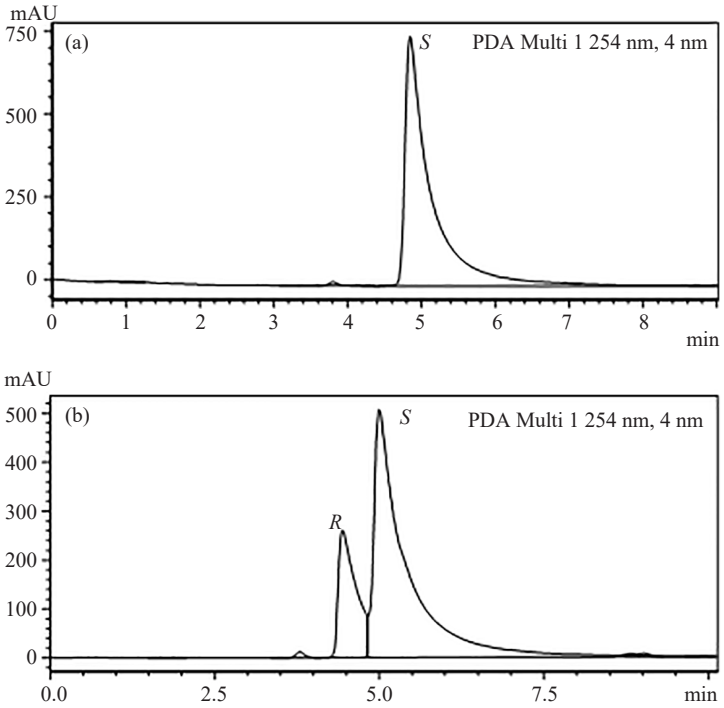


Figure 13. Chromatograms of (a) the NAP (S) enantiomer and (b) the NAP (R, S) + (S) mixture in the solvent system ACN/H_2O in the ratio 99 : 1 on the ReproSil Chiral-NR column, flow rate 1 mL/min

4. Discussion

The graphs (Figures 14-16) summarize the resolution results of the analyzed drugs on the columns used. Table 18 compares this chromatographic parameter for racemic mixtures of thalidomide, naproxen, and ibuprofen obtained on three chromatographic columns with different types of column packing.

Table 18. Comparison of the resolution results of thalidomide, naproxen, and ibuprofen on columns with different stationary phases in ACN/H_2O and ACN/EtOH systems in a ratio of 99 : 1 (v/v), with a flow rate of 1 mL/min

Drugs	ReproSil Chiral-MIG (amylose column)		ReproSil Chiral-MIC (cellulose column)		ReproSil Chiral-NR (Pirkle-type column)	
	ACN/H_2O	ACN/EtOH	ACN/H_2O	ACN/EtOH	ACN/H_2O	ACN/EtOH
THAL (R, S)	8.226	5.856	0.729	0.515	-	-
NAP (R, S)	0.483	0.419	-	-	1.152	0.555
IBU (R, S)	-	0.348	-	-	-	-

Thalidomide achieved the best separation in the acetonitrile/water solvent system on the ReproSil Chiral-MIG amylose column with the retention times below 15 minutes. The presence of a chlorine atom in the packing structure increases its polarity, which allows for a stronger interaction of the hydroxyl groups of amylose with the polar amide groups in thalidomide. Chlorine has a relatively large volume, which also introduces additional steric effects in interactions with analyte molecules. In the case of the cellulose column-ReproSil Chiral-MIC, the interactions between thalidomide and the stationary phase are weaker because the methyl substituents do not interact so strongly with the functional groups of thalidomide. The resolution in the system with water was better than in the system with ethanol due to the greater polarity of water, which affects the strength of electrostatic interactions.

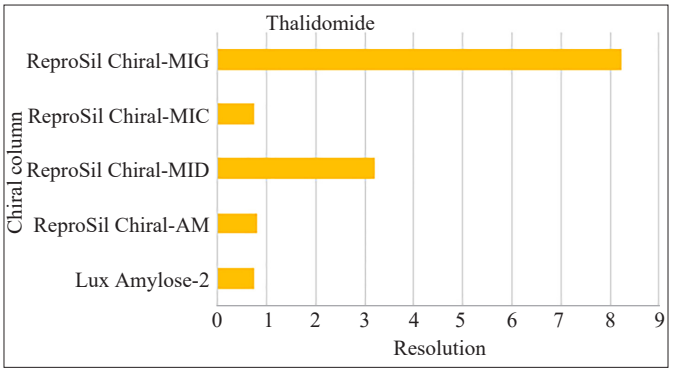


Figure 14. Comparison of thalidomide resolution results on different columns in the solvent system ACN/H_2O (99 : 1, v/v), with a flow rate of 1 mL/min

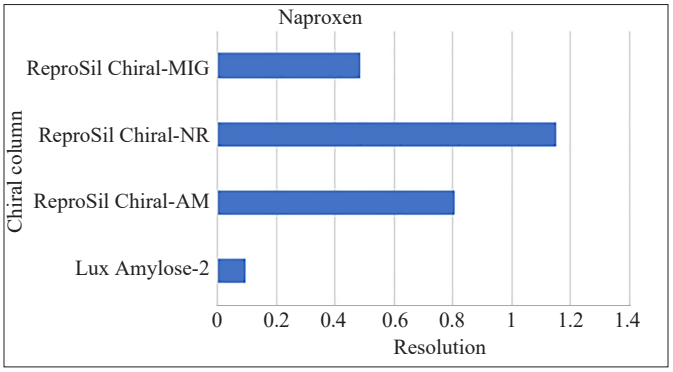


Figure 15. Comparison of naproxen resolution results on different columns in the ACN/H_2O solvent system at a 99 : 1 (v/v) ratio, with a flow rate of 1 mL/min

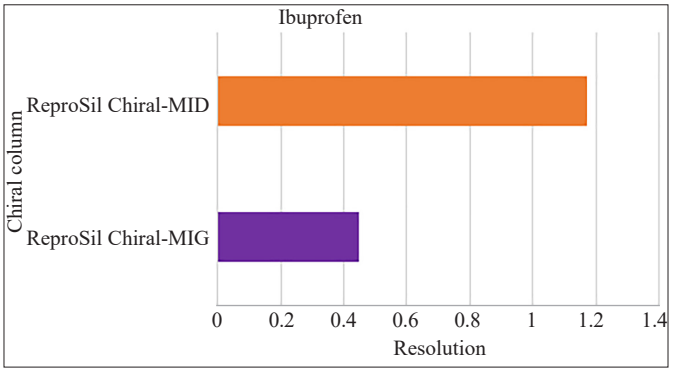


Figure 16. Comparison of ibuprofen resolution results on different columns in the ACN/MeOH solvent system in a ratio of 99 : 1 (v/v)-purple colour, and HEP/PrOH solvent system in a ratio of 85 : 15 (v/v)-orange colour at a flow rate of 1 mL/min

The racemic mixture of naproxen is best separated on the column with Pirkle-type packing due to specific donor-acceptor interactions between the analysed substance and the stationary phase. The nitro groups in the stationary phase are electronegative, which allows interaction with the carboxyl groups of naproxen. The hydroxyl group in phenylglycine can act as a proton donor and form hydrogen bonds with the carboxyl substituents in the tested mixture. Methyl groups in the stationary phase of the cellulose column promote hydrophobic interactions, which may be too weak for effective separation of naproxen enantiomers.

Ibuprofen, unlike naproxen, did not separate on columns with Pirkle or cellulose packing. However, it partially separated on the amylose columns in the RP system, using acetonitrile and different alcohols, and in the NP system,

using *n*-heptane and different alcohols. The isobutyl group in the ibuprofen structure introduces a significant steric barrier, which limits the molecule's ability to interact effectively with the stationary phase. Alcohols are polar solvents, but they are less polar than water. Thanks to this, ibuprofen, which has a partially hydrophobic character, interacts better with the stationary phase.

The method of attaching the column packing could also influence the separation process. Table 19 compares the resolution results on two columns: immobilized ReproSil Chiral-MIG and coated ReproSil Chiral-AM.

Table 19. Comparison of the resolution of racemic mixtures depending on the method of binding the stationary phase to the column, at a flow rate of 1 mL/min

Drugs	ReproSil Chiral-MIG (immobilized column)	ReproSil Chiral-AM (coated column)
	ACN/H_2O (99 : 1)	ACN/H_2O (99 : 1)
THAL (R, S)	8.226	0.778
NAP (R, S)	0.483	0.804
IBU (R, S)	-	-

Thalidomide, having amide groups, is more polar and forms stronger electrostatic and hydrogen interactions with the stationary phase. On columns with immobilized packing, these interactions are more controlled, which favors a better separation of the thalidomide enantiomers. Naproxen separates better on coated columns because the rigidity of the immobilized stationary phase limits its interactions. On immobilized columns, naproxen does not separate efficiently because the rigidity of the stationary phase limits its interactions with the analyte. In contrast, a more flexible stationary phase on coated columns enables better separation due to dynamic interactions with naproxen.

5. Conclusions

Successful enantioseparation of the inflammatory drugs studied was achieved only in the case of thalidomide. This drug was baseline separated from two amylose columns, which indicates that this type of stationary phase is best suited to its structure. Thalidomide separated best on a ReproSil Chiral-MIG amylose column in a system of 99% acetonitrile and 1% water (v/v). These conditions favored strong electrostatic interactions between the amide groups of thalidomide and the hydroxyl groups of amylose, reinforced by the presence of a chlorine atom in the stationary phase.

Naproxen separated best on the column with a Pirkle-type phase in the ACN/H_2O solvent system. This is due to specific donor-acceptor interactions between the carboxyl groups of naproxen and the nitro and hydroxyl groups in the stationary phase.

Ibuprofen did not separate on columns with a Pirkle-type phase or cellulose packing. Partial separation was achieved on amylose columns due to the steric barrier introduced by the isobutyl group, which limits interactions with the stationary phase. In contrast to thalidomide and naproxen, which achieved the highest resolution in the ACN/H_2O system, ibuprofen was best separated in the normal-phase system-HEP/PrOH.

Our studies describe two cases of EEO reversal concerning thalidomide and the ReproSil Chiral-MIG and ReproSil Chiral-MID columns, where the selector is an amylose derivative. Different backbones or substituents of the polysaccharide CSPs often lead to EEO reversal. The mobile phase did not influence the elution order.

We have demonstrated that amylose-based chiral selectors are particularly effective in separating anti-inflammatory drugs, such as thalidomide and ibuprofen. Increasing the resolution of ibuprofen and naproxen to the baseline could be achieved by increasing the temperature or using modified mobile phase additives, such as buffers or organic acids.

Our work may help enhance the separation of other drugs from the class of propionic acid derivatives[62-67] and α-N-phthalimidoglutarimide derivatives (for example, pomalidomide, lenalidomide, and apremilast).[68-70]

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Conflict of interest

The authors declare no conflict of interest.

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Appendix

Chiral separation of three anti-inflammatory drugs by liquid chromatography on polysaccharide-type stationary phases and pirkle-type phase

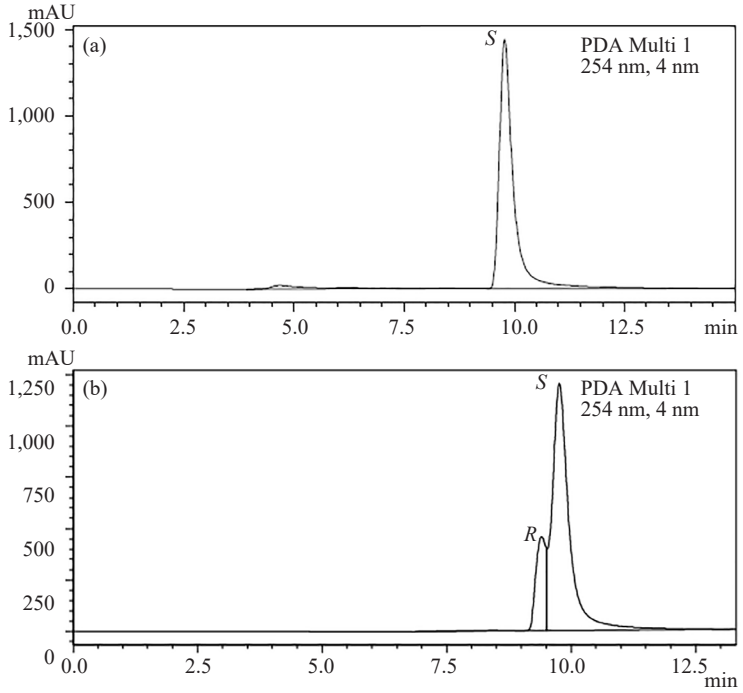

Figure S1. Chromatograms showing (a) NAP (S) enantiomer and (b) NAP (R, S) + (S) mixture in ACN/H_2O solvent system in ratio 99 : 1 on the ReproSil Chiral-MIG column, flow rate 1 mL/min

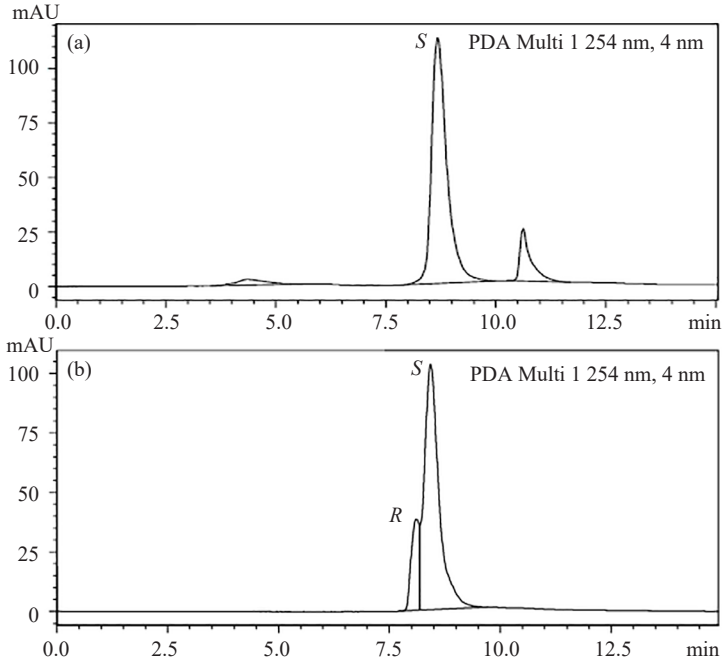

Figure S2. Chromatograms showing (a) IBU (S) enantiomer and (b) IBU (R, S) + (S) mixture in ACN/EtOH solvent system in ratio 99 : 1 on the ReproSil Chiral-MIG column, flow rate 1 mL/min

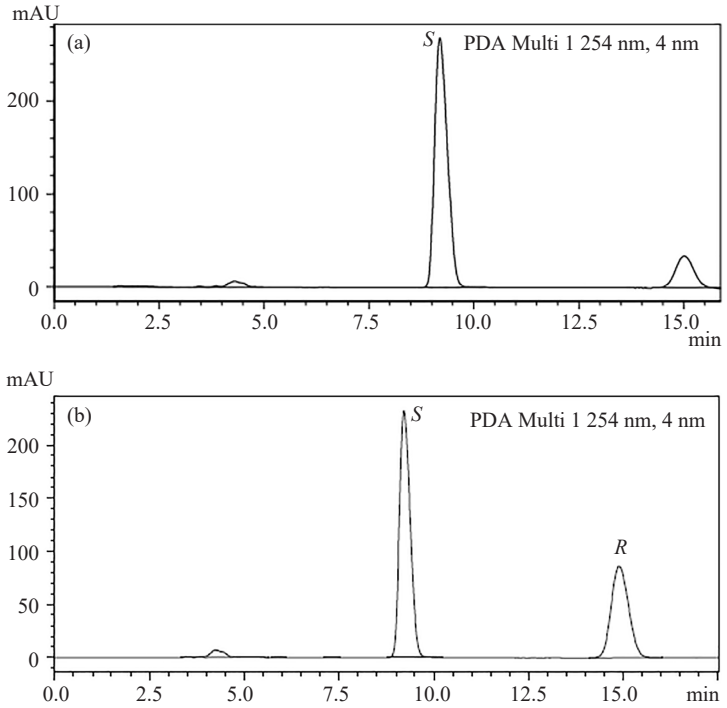

Figure S3. Chromatograms showing (a) TAL (S) enantiomer and (b) TAL (R, S) + (S) mixture in 99 : 1 ACN/H_2O system on ReproSil Chiral-MIG column, flow rate 1 mL/min

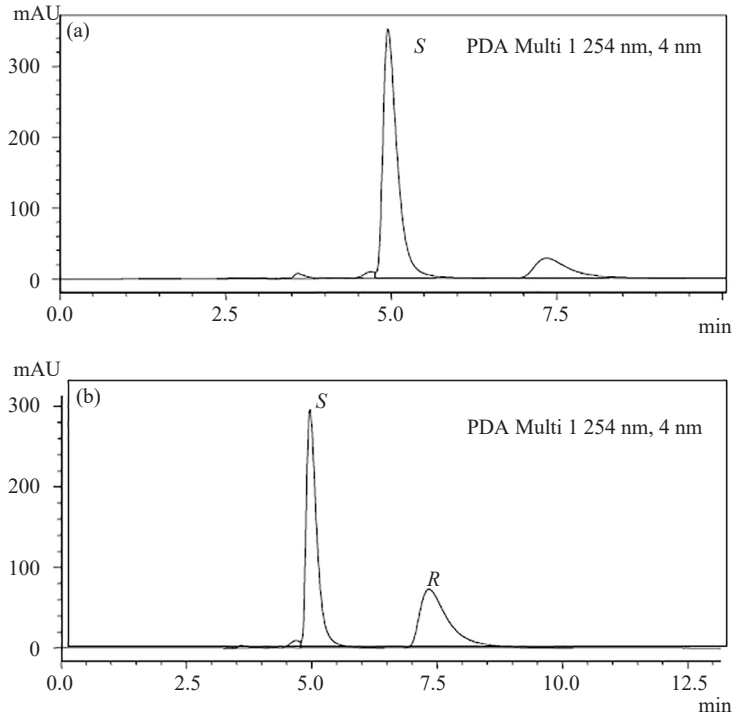

Figure S4. Chromatograms showing (a) TAL (S) enantiomer and (b) TAL (R, S) + (S) mixture in ACN/H_2O solvent system in ratio 90 : 10 on the ReproSil Chiral-MID column, flow rate 1 mL/min.

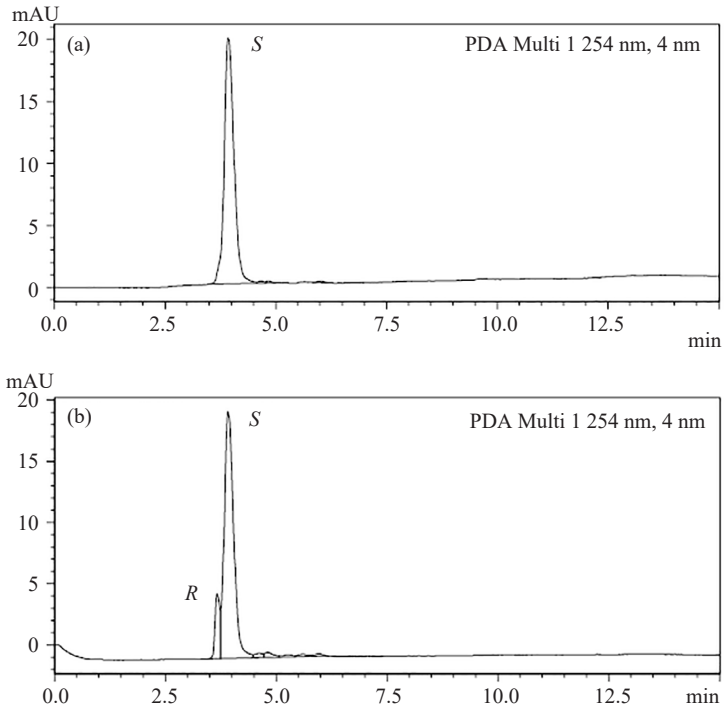

Figure S5. Chromatograms showing (a) NAP (S) enantiomer and (b) NAP (R, S) + (S) mixture in ACN/H_2O solvent system in ratio 99 : 1 on the ReproSil Chiral-AM column, flow rate 1 mL/min.

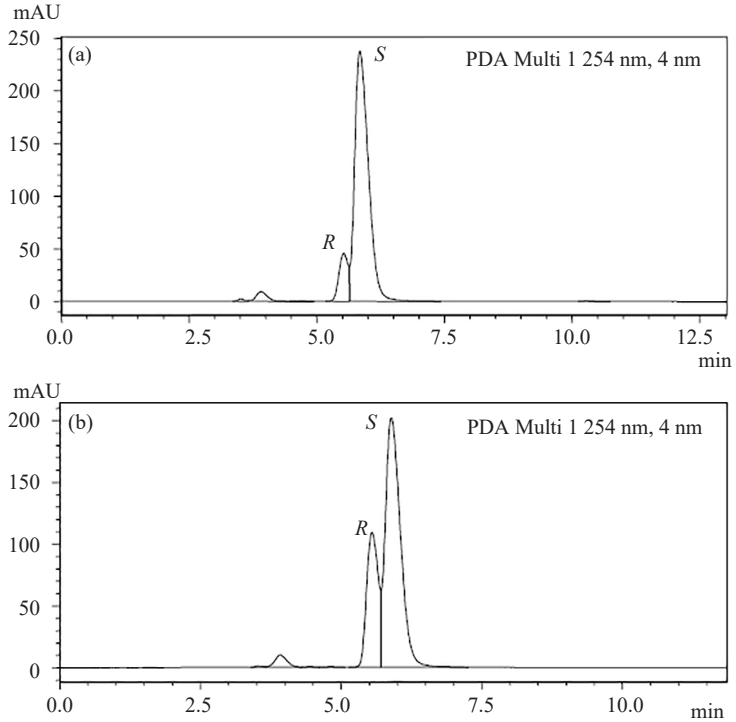

Figure S6. Chromatograms showing (a) the TAL (S) enantiomer and (b) the TAL (R, S) + (S) mixture in ACN/H_2O solvent system in ratio 99 : 1 on the ReproSil Chiral-AM column, flow rate 1 mL/min.

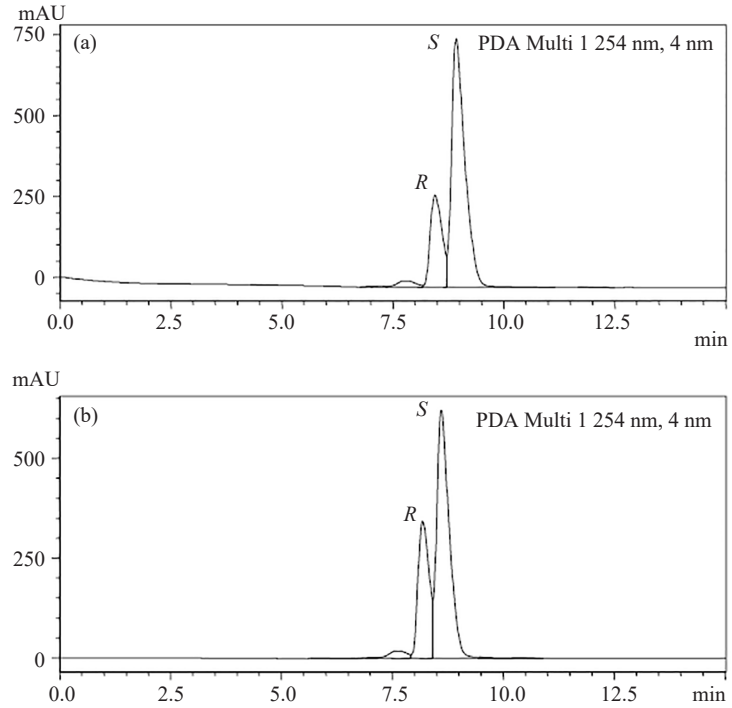

Figure S7. Chromatograms showing (a) TAL (S) enantiomer and (b) TAL (R, S) + (S) mixture in ACN/H_2O solvent system in ratio 99 : 1 on the Lux Amylose-2 column, flow rate 1 mL/min.

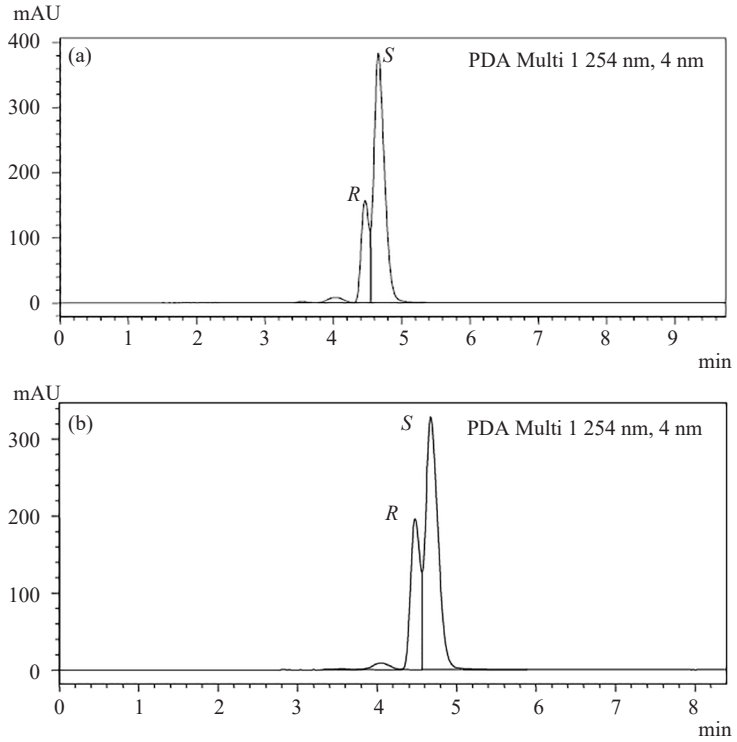

Figure S8. Chromatograms of (a) TAL (S) enantiomer and (b) TAL (R, S) + (S) mixture in ACN/H_2O solvent system in ratio 99 : 1 on the ReproSil Chiral-MIC column, flow rate 1 mL/min