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The Effect of the Stationary Phase on Resolution in the HPLC-Based Separation of Racemic Mixtures Using Vancomycin as a Chiral Selector: A Case Study with Profen Nonsteroidal Anti-Inflammatory Drugs

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Abstract: Chiral resolution is a technique of choice, making it possible to obtain asymmetric and enantiomerically pure compounds from a racemic mixture. This study investigated the behavior of vancomycin when used as a chiral additive in high-performance liquid chromatography (HPLC) to separate enantiomers of nonsteroidal anti-inflammatory drugs (NSAIDs), including ketoprofen, ibuprofen, flurbiprofen, and naproxen enantiomeric impurities. We compared two achiral stationary phases (C18 and NH_2) to assess the impact of mobile phase composition and stationary phase on the vancomycin retention time in the racemic resolution of drug enantiomers. Our results demonstrated the successful enantioseparation of all drugs using vancomycin in the mobile phase (phosphate buffer 0.05 M/2-propanol, 50/50) with an NH_2 column. This enhanced separation on the NH_2 column resulted from the chromatography system's efficiency and vancomycin dimers' stereoselective interaction on the NH_2 surface. This study underscores the importance of stationary phase selection in the chiral resolution of NSAIDs with vancomycin as a chiral additive. It offers valuable insights for future research and development of NSAID chiral separation methods, highlighting potential vancomycin applications in this context.

Keywords: achiral stationary phases; enantiomeric separations; profen drugs; vancomycin chiral mobile phase additive

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1. Introduction

Enantiomer separation holds significant importance, particularly within the pharmaceutical and environmental domains, due to the prevalence of chiral compounds such as drugs and pesticides [1]. In some cases, only one of the enantiomers has a therapeutic effect, while the other may exert opposed pharmacological activities or cause unwanted side effects [2]. Hence, there is considerable interest in developing novel enantioseparation techniques with enhanced efficiency and sensitivity. These methods are sought after to achieve the stereoselective synthesis of enantiomers and ensure enantiomeric purity control, thereby preventing therapeutic disasters similar to the thalidomide incident of the 1960s [3,4]. Chromatography stands as one of the most effective techniques for enantiomer separation, widely employed in both academic and industrial settings for analytical and preparative objectives [5].

Practically, direct enantiomeric resolution is only feasible in chromatographic systems that contain an appropriate chiral selector. This selector can be a chiral stationary phase or be covalently bonded to the surface of the column packing material (linkage) or coated onto the surface of the chromatographic support [6,7]. Enantioselective chromatography can be achieved on achiral chromatographic columns by incorporating a specific chiral selector such as a chiral mobile phase or a chiral mobile phase additive (CMPA) [8]. It is also possible to use combinations of chiral selectors in the mobile phase in conjunction with chiral stationary phases for this purpose [9,10].

In CMPA-based methods, the chiral selectors dissolved in the mobile phase interact with the chiral analytes, forming transient diastereomeric complexes [11]. The separation of enantiomers in this process is driven by differences in the formation kinetics or in the relative stability of these transient diastereomeric complexes, as well as by variations in their partitioning between the mobile phase and the stationary phase [12]. HPLC methods utilizing CMPAs offer several advantages, including flexibility, the availability of a wide range of available additives, and lower costs compared to those employing equivalent chiral stationary phases (CSPs) [13].

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a highly prevalent analytical technique, accounting for approximately 70–80% of all analytical separations. Due to its widespread use, considerable interest has been devoted to understanding fundamental aspects of this technique. One such area that has garnered significant attention is mobile-phase optimization [14]. The typical approach for predicting the optimal solvent composition for a specific separation problem involves chromatographing a set of solutes using various combinations of mobile phases and expressing the retention time through quantitative parameters and interactions [15,16]. Vancomycin has emerged as the most widely employed glycopeptide among the various chiral selectors used for chiral separation, whether as a stationary phase or as a chiral additive in the mobile phase [17,18]. Armstrong et al. were pioneers in introducing vancomycin for enantioseparation [19]. This powerful chiral selector has been successfully utilized to resolve over 100 racemates, including nonsteroidal anti-inflammatory drugs [20], antineoplastic drugs [1], pesticides [21], and numerous N-derivatized amino acids [22]. Its versatility and effectiveness make it a valuable tool in the field of enantioselective chromatography.

The unique structural features of vancomycin (Figure 1) make it an excellent chiral selector in enantioseparations [17]. Its basket-like structure provides a hydrophobic environment that can accommodate the analytes, and steric effects and hydrophobic–hydrophobic interactions can significantly contribute to the enantioselectivity of the separation [17,23]. The presence of the two sugar moieties also adds to the chiral recognition properties of vancomycin [20]. Moreover, the π-acidic nature of the aromatic rings with two chlorine substituents and ionic interactions involving the carboxyl and amino groups play a crucial role in chiral recognition. These structural features allow vancomycin to selectively interact with enantiomers, leading to the efficient separation of chiral compounds [17,21,23]. All these polar and ionizable groups have a structure proximate to the ring structure and can

establish strong hydrogen bonding and electrostatic interactions, respectively, with solute molecules [24–26].

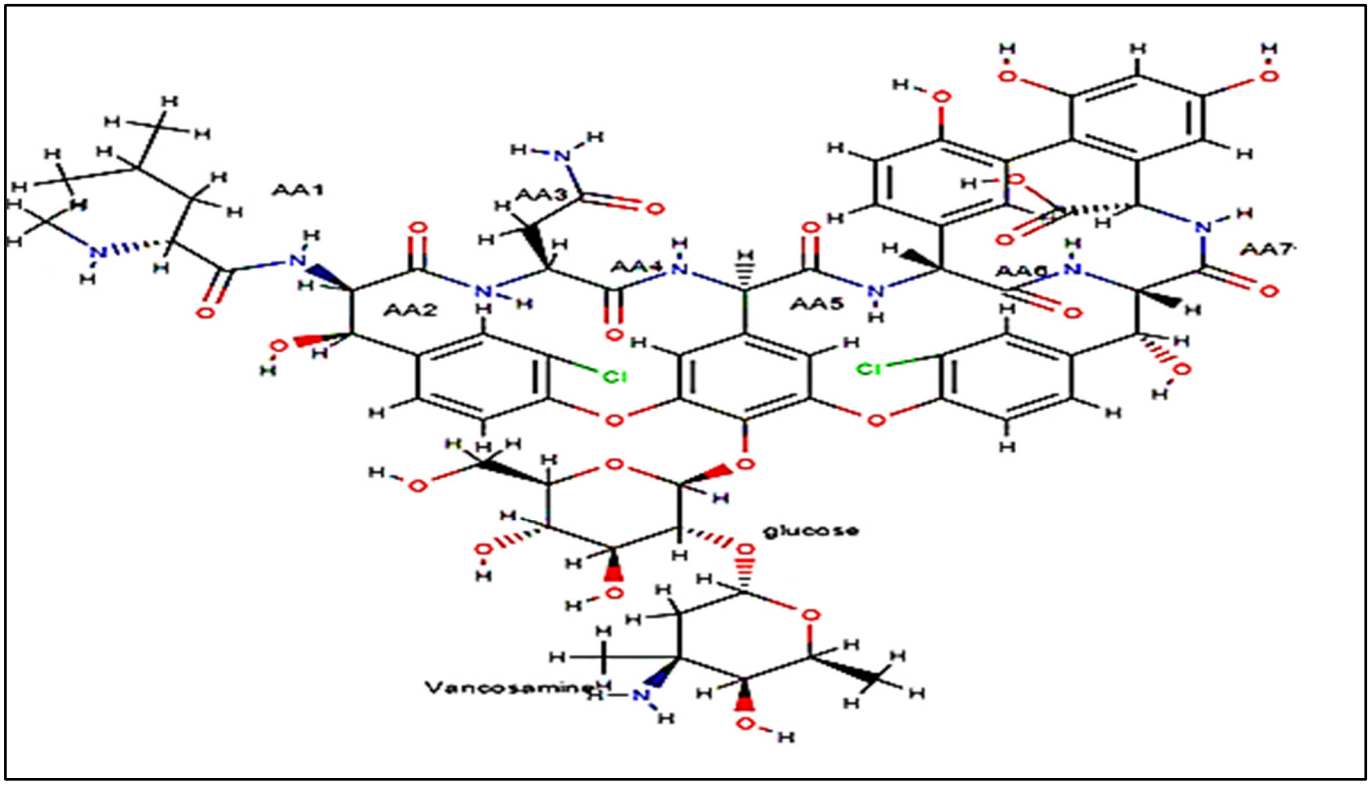

Figure 1. Vancomycin structure.

The use of chiral selectors combined with achiral columns is a widely accepted method for resolving chiral mixtures. The selectivity of the separation is dependent on the chromatographic conditions of both the stationary and the mobile phases. The effectiveness of the method can vary due to differences in the column or the mobile phase. As a result, it is crucial to determine the optimal chromatographic conditions for applying a chiral selector such as a CMPA.

This study focused on investigating the behavior of vancomycin +under different chromatographic conditions and determining the best conditions for its use as a chiral selector. The key objective was to identify the experimental settings that would result in the longest vancomycin retention time on the stationary phase, which would lead to the optimal chromatographic conditions. To achieve a high chiral resolution of profen NSAID drugs, the effect of buffer pH on vancomycin elution using achiral stationary phases such as C18 and NH_2 columns and the effect of organic solvent on vancomycin elution were investigated. The comparison between C18 and NH_2 columns has never been investigated and constitutes the originality of the present research. Finally, the selectivity obtained was evaluated by comparing the results with previous studies using the same samples and chiral selector. Overall, this study provides a comprehensive description of vancomycin behavior under different chromatographic conditions and identifies the optimal conditions for using it as a chiral selector to improve the chiral resolution of profen NSAID drugs. This study provides an alternative strategy to asymmetric synthesis, making it possible to take advantage of the asymmetric nature of vancomycin in order to resolve the symmetry present in racemic mixtures.

2. Materials and Methods

2.1. Materials

The chromatographic experiments were conducted using the Shimadzu HPLC System, manufactured in Tokyo, Japan. The system comprises various components, including an LC-10AT vp pump, an SCL-10A vp system controller, an SPD-10AV vp UV/VIS detector, an FCV-10AL low-pressure gradient unit, and a GT-104 degasser. To facilitate data collection and integration, a workstation equipped with Empower 2 software (Version 2.6.06) was employed.

The HPLC columns used in this study for reversed-phase RP-HPLC were a C18 column (LiChrosorb® RP-18, 250 mm × 4.6 mm, 5 µm particle size) and an NH_2 column (LiChrospher® 100 NH_2, 250 mm × 4.6 mm, 5 µm particle size), purchased from Merck KGaA (Darmstadt, Germany).

The vancomycin reference standard was procured from Sigma Aldrich, based in St. Louis, MO, USA. The solvents employed for HPLC, namely, acetonitrile (ACN), methanol (MeOH), and 2-propanol (2-Pro), were also purchased from Sigma Aldrich in St. Louis, MO, USA. Additionally, analytical reagent-grade potassium hydroxide (KOH), potassium dihydrogen phosphate (KH_2PO_4), and phosphoric acid (H_3PO_4) were acquired from the same supplier, Sigma Aldrich in St. Louis, MO, USA.

The water used for the preparation of all the mobile phases was purified with a Milli-Q water purification system (Millipore, Billerica, MA, USA).

All the studied racemic profen NSAIDs, namely, ketoprofen, ibuprofen, flurbiprofen, and naproxen enantiomeric impurities, were purchased from Sigma-Aldrich (St. Louis, MO, USA). All substances were injected as 1.0 mg/mL methanol solutions in a 10 µL injection volume. The chemical structures of the analytes are shown in Figure 2.

2.2. Liquid Chromatography Method

The mobile phases utilized in the experiment consisted of a combination of different compounds and solvents, including phosphate buffer solution (PBS) 0.05 M, acetonitrile (ACN), methanol (MeOH), 2-propanol (2-Pro), and the free vancomycin chiral selector. These mobile phases were prepared at different pH and with different concentrations of vancomycin, using a 50/50 (v/v) mixture of PBS and organic modifier. To prepare the mobile phases, vancomycin was dissolved in pure buffer, and the appropriate amount of solvent was then added. Before use, the eluents were sonicated for a minimum of 15 min.

The 0.05 M PBS solution was prepared by dissolving 6.80 g of potassium dihydrogen phosphate (KH_2PO_4) in 1000 mL of distilled water, and then the pH of the final solution (pH 4.5) was adjusted to pH 4.0 using phosphoric acid. Using phosphate buffer as the mobile phase (polar solvent), it was determined how the pH affected the elution of vancomycin by the evaluation of the retention times at different pH values of the buffer varying from 4.0 to 6.5 in steps of 0.5 units. The same method as for the PBS pH 4.0 solution was followed to prepare 0.05 M PBS solutions with pH values higher than 4.5 (5.0, 5.5, 6.0, and 6.5), except that the pH was adjusted using potassium hydroxide 0.1 M.

In the chromatographic analysis conducted after incorporating organic solvents (ACN, MeOH, and 2-Pro) into the PBS mobile phase, the impact of the organic solvents was examined. The mobile phase was filtered through a 0.45 µm Whatman filter (Sigma-Aldrich, St. Louis, MO, USA) and then sonicated for 1 min before being finally used at a flow rate of 0.8 mL/min.

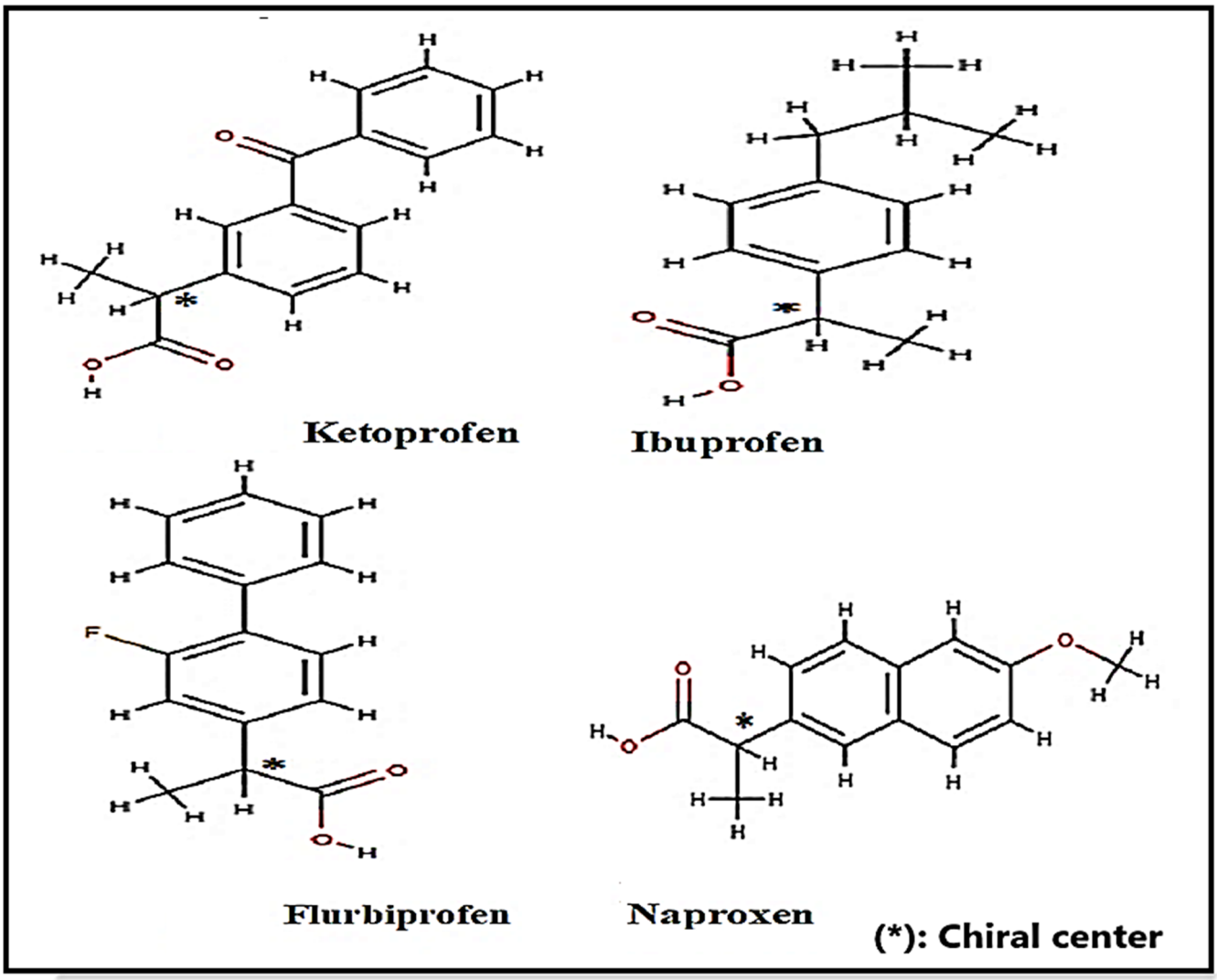

Figure 2. Chemical structures of the studied profen NSAIDs.

3. Results and Discussion

Vancomycin was chosen as the mobile phase additive, while two achiral columns, a LiChrosorb C18 RP-18 column and a LiChrospher NH_2 column, both with different chemically bonded chains, were evaluated for the enantioseparation of profen NSAIDs. Therefore, the impact of the stationary phase structure on the retention and enantiomeric separation of profen NSAIDs, as well as the impact of the buffer pH on vancomycin elution and retention time and the effect of the organic compound in the mobile phase on vancomycin elution were studied.

3.1. Effect of the Buffer pH on Vancomycin Elution

The elution behavior of vancomycin is significantly influenced by the pH of the mobile phase. The pH of the buffer used in the mobile phase plays a crucial role in determining the charge state of vancomycin, which, in turn, affects its elution time and peak shape.

Maintaining a precise pH control is particularly important when choosing chromatographic conditions for reversed-phase chromatography. This control governs the relative concentrations of protonated and unprotonated species [27]. The distribution between the non-polar stationary phase and the polar mobile phase directly impacts the retention times of compounds.

Phosphate buffers are widely preferred and extensively used due to their advantages, such as high purity, cost-effectiveness, and the ability to produce excellent chromatographic

results [28]. Additionally, these buffers can function optimally within useful pH ranges, which makes them a popular choice in analytical applications.

Vancomycin is an amphoteric (polybasic) molecule with charged amino, carboxyl, and phenol groups. For the ionization function of vancomycin, the estimated pKa constant values were calculated using Marvin Sketch software (version 20.11). The pKa values of the ionizable groups in vancomycin are reported in Figure 3. The tautomerization constants of various species and their concentration ratios can be calculated to determine the pKa values for various functional groups.

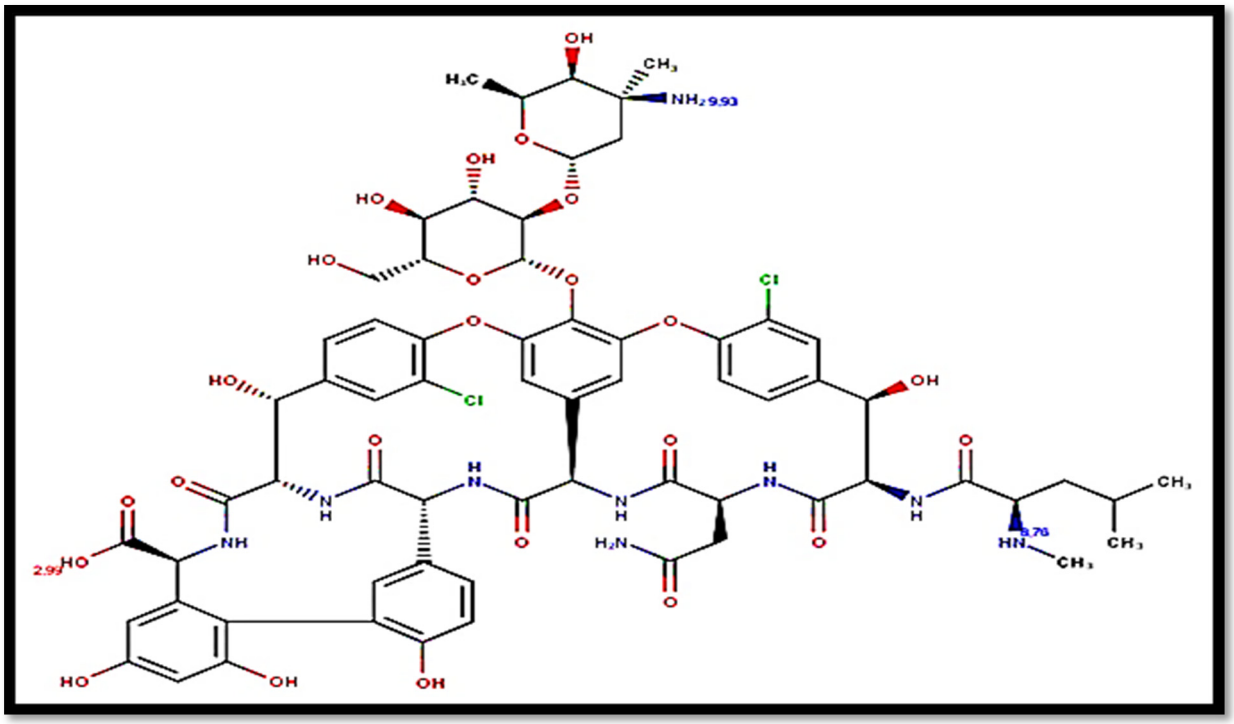

Figure 3. The different pKa values of the ionizable groups in vancomycin.

Vancomycin can change from a cation to an anion by altering the pH because of its macro-constant value. In the studied pH range (from pH 4.0 to pH 7.0), acid–base equilibrium can be observed, where vancomycin becomes charged as a result of the ionization of the vancosamine amino group, the side chain amino group, and the carboxyl group (Figure 4). At a low pH, the dissociation of the carboxyl group does not take place, and vancomycin ionization decreases (Figure 4a), while it increases, reaching its maximum, at pH 6.0 (Figure 4b). Furthermore, vancomycin acquires two positive charges and one negative charge, due to carboxylate, starting from pH 6.0, and phenolate groups appear in multiple overlapping stages (Figure 4c). In a strongly basic solution (pH 12.0), vancomycin bears four negative charges represented by three phenolate groups and one carboxylate group (Figure 4d), as opposed to a strong acid solution (pH 2.0), where vancomycin bears two positive charges represented by the vancosamine amino group and the side chain amino group of an amino acid residue [29].

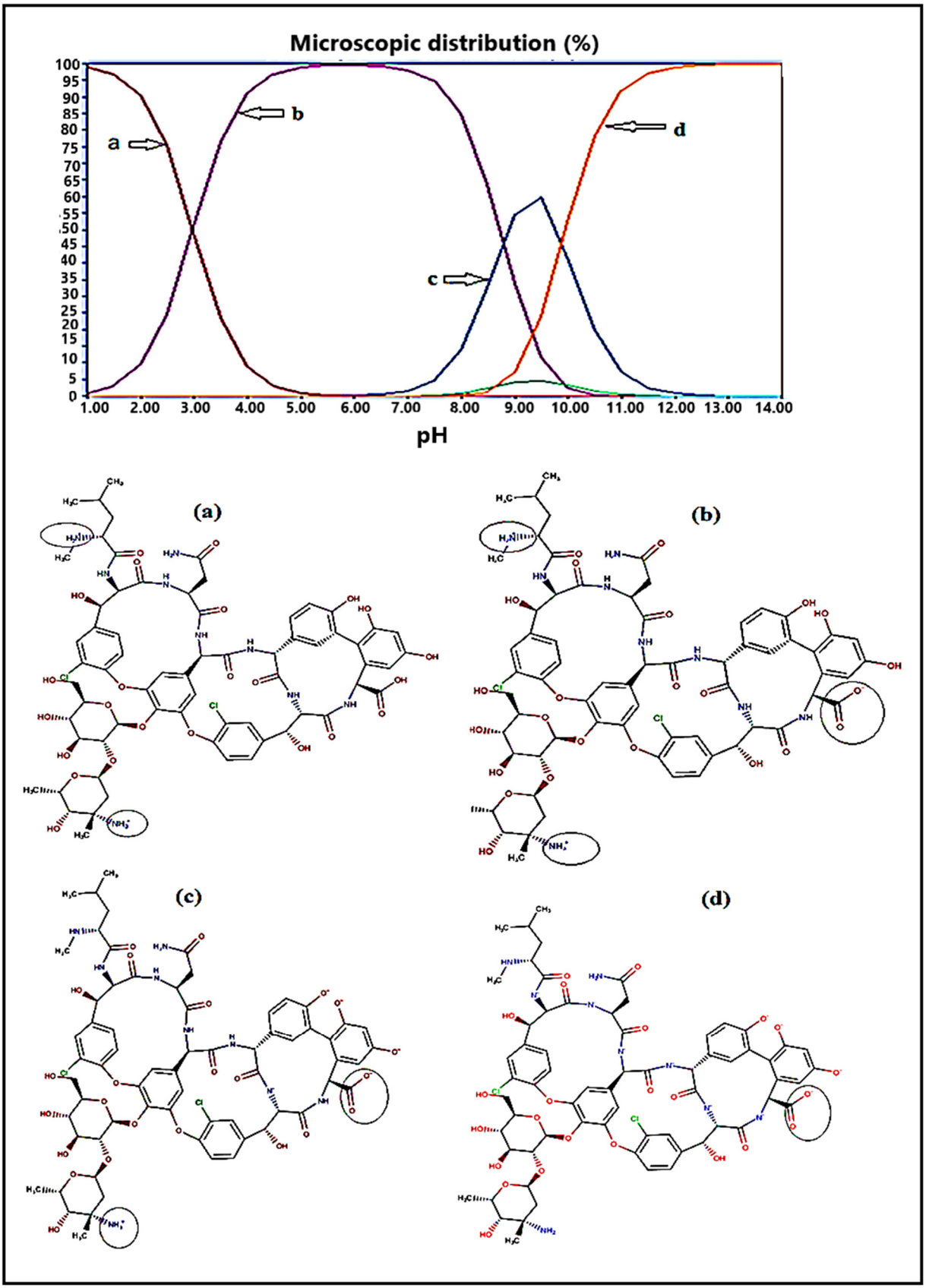

Figure 4. Distribution of vancomycin groups as a function of pH. Ionization of vancomycin groups (**a**) at pH = 2, (**b**) at pH = 6, (**c**) at pH = 9, (**d**) at pH = 12.

3.2. Effect of the pH on the Retention Time of Vancomycin

In high-performance liquid chromatography (HPLC), the retention time of vancomycin can be affected by the pH of the mobile phase. Vancomycin is classified as a polar compound and, at neutral pH, carries a net positive charge. Typically, when analyzing vancomycin, a reversed-phase mode is employed, where a nonpolar stationary phase is used, and a polar mobile phase is utilized to elute the compound [30]. This combination of stationary and mobile phases allows for the effective separation and elution of vancomycin during the chromatographic process.

The retention time of vancomycin is directly related to the strength of the interactions between the vancomycin molecule and the stationary phase, as well as the mobile phase. At low pH (acidic conditions), vancomycin is more protonated, which increases its overall polarity. This increased polarity leads to a stronger interaction with the polar stationary phase, and as a result, the retention time of vancomycin is longer. Conversely, at high pH (basic conditions), vancomycin is less protonated, which decreases its overall polarity [29]. This leads to weaker interactions with the stationary phase, and as a result, the retention time of vancomycin becomes shorter. Therefore, pH plays a critical role in controlling the retention time of vancomycin in HPLC. By adjusting the pH of the mobile phase, the retention time of vancomycin can be controlled and optimized for a particular analytical method [31,32].

By increasing vancomycin retention time on the stationary phase through appropriate chromatographic conditions, the activated sites can interact with the analyte and establish stronger interactions with the mobile phase [33].

According to different stationary phases, the vancomycin retention time varied with the buffer pH (Figure 5). It was found that as the buffer pH increased, the vancomycin retention time increased, reaching the max value at pH 5.5 with the octadecyl column and at pH 6.0 with the amino column. Above the buffer pH of 6.0, the vancomycin retention time decreased.

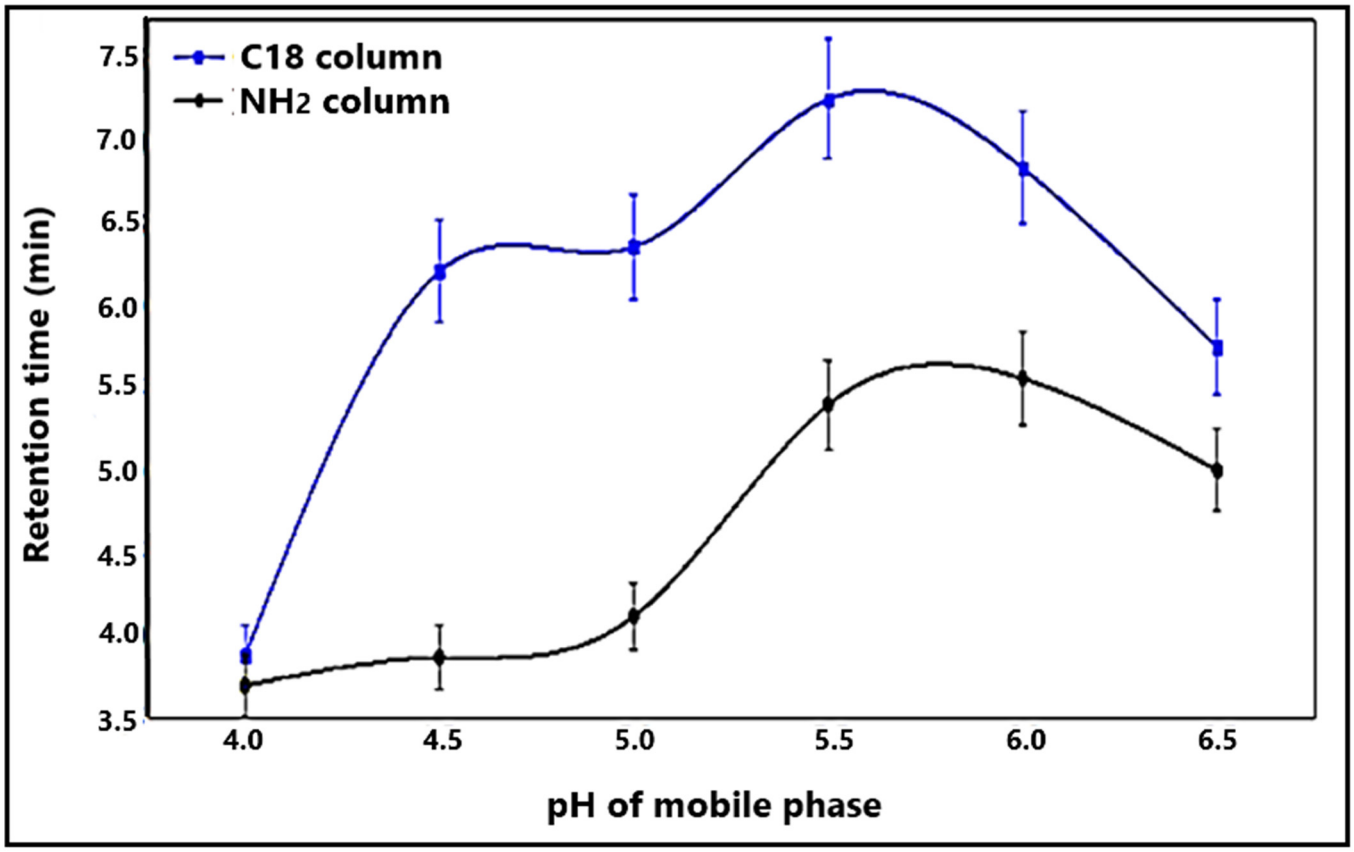

Figure 5. Effect of pH on the retention time of vancomycin using the buffer solution as the mobile phase.

The relative hydrophobicity of vancomycin species at a specific pH has an impact on retention overall. The outcome is the result of hydrogen bonding and van der Waals interactions with ionizable vancomycin, which produce the most ionized species between pH 5.5 and pH 6.0. Furthermore, the retention times on octadecyl columns are much longer than those on amino columns. Even though the surface of C18 is more hydrophobic than that of the amino column, vancomycin can bind to the amino column through hydrogen bonds and Van der Waals interactions [16].

3.3. Effect of the Mobile Phase on Vancomycin Elution

The impact of the concentration of the organic modifier is a significant aspect in reversed-phase liquid chromatography (RPLC), because optimizing the selectivity of this method often involves adjusting the mobile phase composition by adding organic solvents. Apart from the concentration, the choice of the organic modifier holds importance. It has been observed that using different organic modifiers can alter the retention mechanism. The organic modifiers weaken hydrogen bonding interactions and aid in the desorption of polypeptides from the column surface [34,35]. By carefully selecting and adjusting the organic modifier, scientists can fine-tune the chromatographic separation of the compounds of interest and achieve better resolution and elution.

After optimizing the pH for achieving maximum vancomycin retention, this research focused on examining the impact of the organic solvent added to the mobile phase on the retention time of vancomycin. In reversed-phase high-performance liquid chromatography (RP-HPLC), three organic solvents, namely, acetonitrile, methanol, and 2-propanol, are commonly used due to their excellent optical transparency at the detection wavelengths required for peptide and protein analysis [36].

Table 1 presents the results we obtained, showcasing the effect of the nature of the organic solvent on vancomycin retention time. This investigation helps in understanding how different organic solvents can influence the retention behavior of vancomycin in a chromatographic system, providing valuable insights for method optimization and for achieving the desired separation performance.

Table 1. Effect of the organic phase nature on vancomycin elution.

Mobile Phase Composition	Retention Time in Amino Column (min)	Retention Time in Octadecyl Column (min)
PBS 0.05 M pH 6.0	5.57	6.22
PBS 0.05 M pH 6.0/ACN (50/50)	5.60	3.06
PBS 0.05 M pH 6.0/MeOH (50/50)	7.95	4.05
PBS0.05 M pH 6.0/2-Pro (50/50)	10.70	3.82

According to the data, the influence of the organic solvent differed significantly depending on whether we employed the amino or the octadecyl column. Indeed, the retention time of vancomycin was longer on the C18 column when methanol was used than when acetonitrile was used. Because methanol is more polar than acetonitrile, the hydroxyl group of methanol can participate in hydrogen bonding.

The retention time of vancomycin on the C18 column was shorter when 2-propanol was used instead of methanol. This was because the solvents' polarities differ and because 2-propanol includes donor and acceptor hydrogen sites in its chemical structure.

On the NH_2 column, the organic modifier increased the vancomycin retention time because the organic solvent solubilized the NH_2 groups on the surface of the amino column and in the vancomycin side chain. The solubilization of these groups increased hydrogen bonding and van der Waals interactions [32]

Compared to methanol and acetonitrile, 2-propanol determined an increase in the vancomycin retention time. The higher viscosity and lower polarity of 2-propanol are

thought to be the cause of this variation. Additionally, the hydrophobicity of 2-propanol is higher than that of methanol, which explains the longer retention time for the NH_2 system containing 2-propanol, compared to that for the RP-18 system.

3.4. Effect of the Stationary Phase on the Vancomycin Retention Time

The effect of the stationary phase type on the vancomycin retention efficiency was examined using two different columns packed with C18 and NH_2 stationary phases. The obtained chromatograms are shown in Figure 6. In terms of peak separation efficiency, it is evident that, when using the ligand-exchange chromatography technique, the retention time of vancomycin on the NH_2 column (t = 10.81 min) was superior to that on the C18 column (t = 3.70 min). These findings suggest that enantioseparation depends on the kind of stationary phase used, although both stationary phases we used are achiral and present the same interaction characteristics when coming into contact with target species for elution [7]. Unfortunately, the non-selective retention of uncomplexed vancomycin on the C18 phase significantly contributed to the observed inefficient separation process [37].

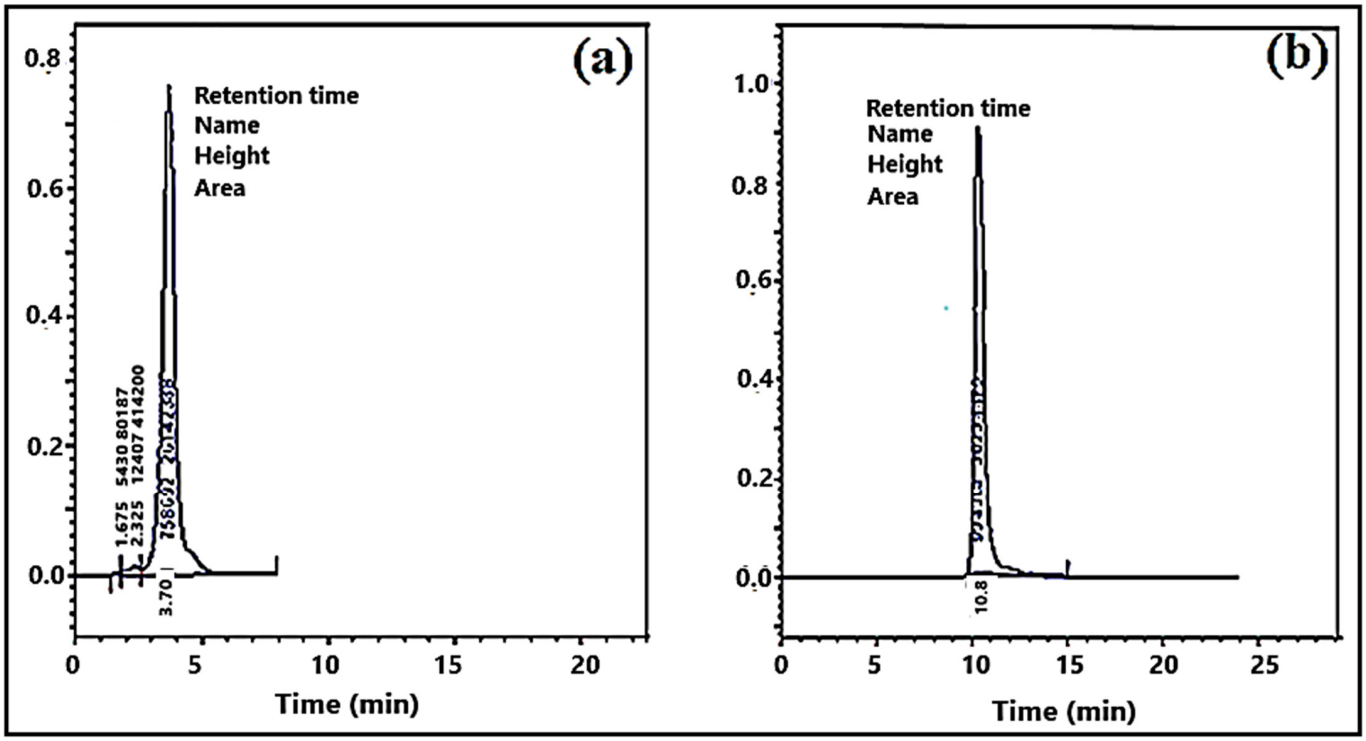

Figure 6. The chromatogram of vancomycin elution from RP-18 and NH_2 stationary phases. (**a**) C18 column (LiChrosorb® RP-18, 250 mm × 4.6 mm, 5 µm particle size), (**b**) NH_2 column (LiChrospher® 100 NH_2, 250 mm × 4.6 mm, 5 µm particle size); mobile-phase composition: 50:50 (*v*/*v*) pH 6.0 phosphate buffer 0.05 M/2-propanol.

The retention mechanism on the C18 surface column could involve van der Waals interactions between vancomycin and the C18 alkyl chain caused by London, Keesom, or Debye forces, as well as hydrogen bonding interactions between a vancomycin donor molecule and residual silanols.

On the NH_2 surface column, in addition to Van der Waals interactions and hydrogen bonds, electrostatic interactions were established between the functional groups present in vancomycin and the surface of the stationary phase when the carboxyl group COOH of the vancomycin side chain was deprotonated, leading to the formation of the negative $RCOO^-$ ion, and the amino group of the column was protonated, forming the positive NH_3^+ ion, under the considered experimental conditions.

Moreover, the retention time is related to the total interfacial surface of an RP packing and is based on a hydrophobic association between the solute and the hydrophobic absorbent on the surface. The vancomycin retention time on a reversed-phase (RP) packing is explained by Horvath's solvophobic theory of interactions [16,33–38], which are classified into three types.

Firstly, vancomycin molecules interact through Van der Waals forces, which encompass London, Keesom, or Debye forces, when they come into contact with each other. Additionally, within the vancomycin molecule, electrostatic intramolecular interactions occur between charged amino acids in the side chain, while intermolecular interactions take place between two charged vancomycin molecules. On the other hand, hydrogen bonding occurs between acceptor and donor groups within the glycopeptide molecule.

Secondly, two types of interactions can occur between vancomycin and the mobile phase buffer solution. The dissolution of vancomycin in the buffer solution results in a negative free energy change, involving Van der Waals and electrostatic interactions. The energy associated with Van der Waals interactions is roughly proportional to the molecular surface area of vancomycin, while the electrostatic forces depend on the dielectric constant of the phosphate buffer and the dipole moment of vancomycin [39]. Finally, vancomycin can interact with the stationary phase, while most of the molecules are exposed on the mobile phase and only one part of vancomycin (the side chain) is in contact with the reversed phase [37].

3.5. Enantioselective Separations of Profen NSAIDs

According to the obtained results in the first part of this study, the 2-propanol/buffer 0.05 M pH 6.0 (50:50 by volume) mixture was chosen as the ideal mobile phase for the addition of vancomycin, and the achiral NH_2 column was chosen as the stationary phase, since it showed higher retention. These findings suggest that polar or ionic interactions contribute to the retention mechanism, but hydrophobic interactions dominate in the reverse-phase separation mode.

The impact of varying the vancomycin concentration in the mobile phase, from 0 to 2 mM, on the retention of profen NSAIDs on the NH_2 column was investigated. The results obtained from these experiments are summarized and compared in Table 2.

Remarkable improvements in enantioselectivity were observed upon the addition of vancomycin. Elevating the vancomycin concentration resulted in increased retention, which proved highly beneficial for enantiomer discrimination. These results support those obtained in our previous research on ketoprofen where the concentration of vancomycin was set at 2 mM, which provided the reproducible values of the retention times t1 and t2 used in this present study [20].

Both selectivity and resolution of the tested molecules exhibited enhancement with the increasing vancomycin concentration. Figure 7 graphically illustrates the variations in enantiomer resolution and selectivity as vancomycin was added. Additionally, as indicated in Table 2, there was a simultaneous increase in selectivity and resolution with the rise in the CMPA concentration.

The best resolution in the separation of the ibuprofen enantiomers was obtained using 2 mM vancomycin in the mobile phase (Figure 8a). This result was due to the dimerization of vancomycin as a consequence of its high concentration, which promoted hydrophobic interactions in its basket-like structure [5,40,41].

Table 2. Influence of vancomycin concentration in the mobile phase on the retention time of profen drugs at a flow rate of 0.8 mL/min. The initial mobile phase consisted of pH 6.0 phosphate buffer (0.05 M)/2-propanol (50/50); t1 and t2 represent the retention times of the drug enantiomers.

Profen Drugs	Molarity of Vancomycin (mM)	Retention Time (min)
Ibuprofen	0	4.420
	0.5	t_1 = 4.530
		t_2 = 4.881
	1.0	t_1 = 4.325
		t_2 = 4.854
	1.5	t_1 = 4.216
		t_2 = 4.924
	2.0	t_1 = 4.152
		t_2 = 5.075
Flurbiprofen	0	3.854
	0.5	t_1 = 4.215
		t_2 = 4.882
	1.0	t_1 = 4.621
		t_2 = 4.958
	1.5	t_1 = 4.401
		t_2 = 5.274
	2.0	t_1 = 4.406
		t_2 = 5.281
Ketoprofen	0	4.605
	0.5	t_1 = 5.134
		t_2 = 5.477
	1.0	t_1 = 5.358
		t_2 = 6.874
	1.5	t_1 = 6.217
		t_2 = 10.584
	2.0	t_1 = 6.879
		t_2 = 14.130
Naproxen	0	3.080
	0.5	t_1 = 1.211
		t_2 = 3.154
	1.0	t_1 = 1.325
		t_2 = 3.885
	1.5	t_1 = 1.889
		t_2 = 4.251
	2.0	t_1 = 2.867
		t_2 = 5.308

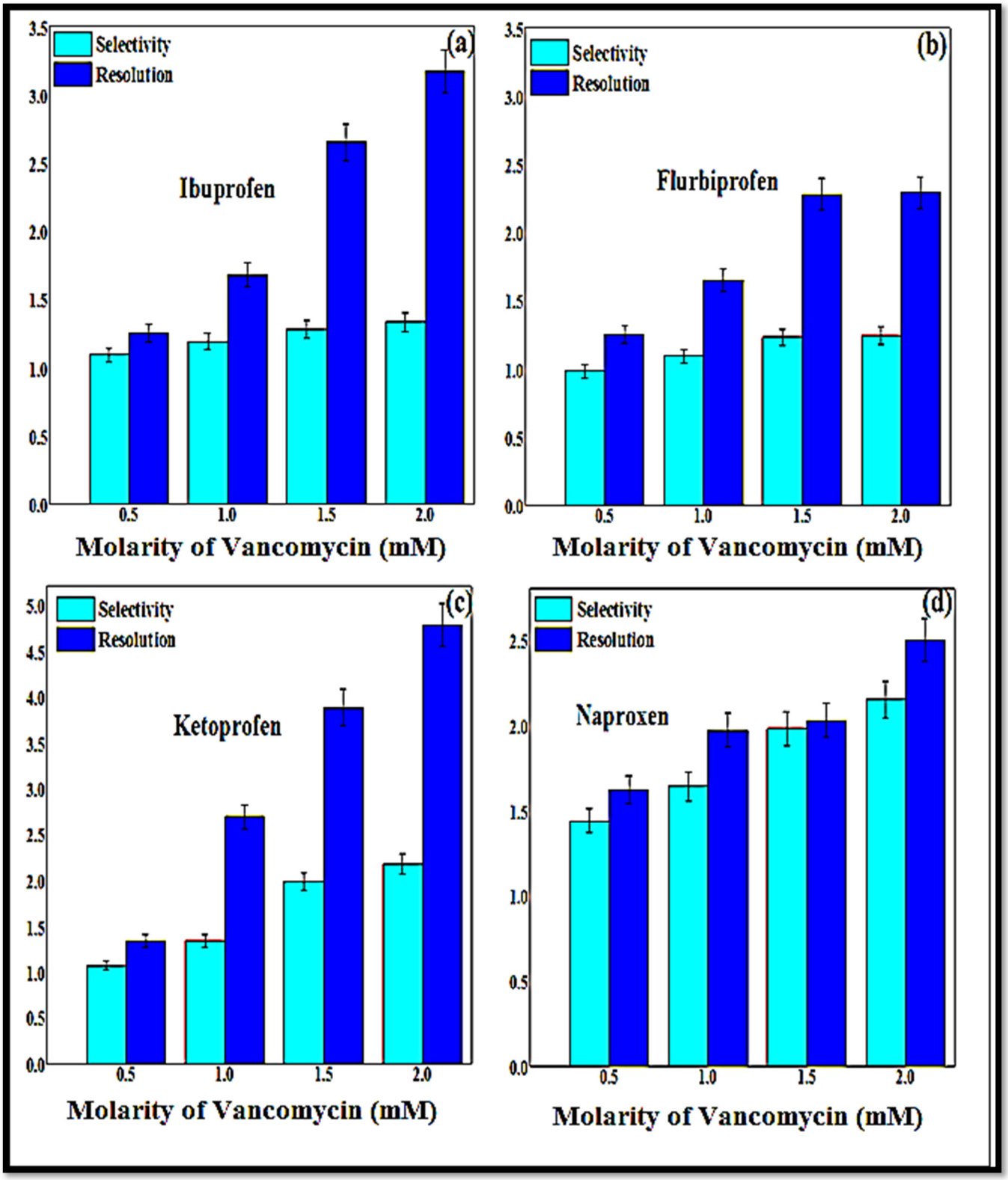

Figure 7. Variation in the resolution and selectivity of profen NSAIDs with mobile phases containing different vancomycin concentrations. The mobile phase was pH 6.0 phosphate buffer 0.05 M/2-propanol (50/50), at a flow rate of 0.8 mL/min. (**a**) Ibuprofen, (**b**) Flurbiprofen, (**c**) Ketoprofen, (**d**) Naproxen.

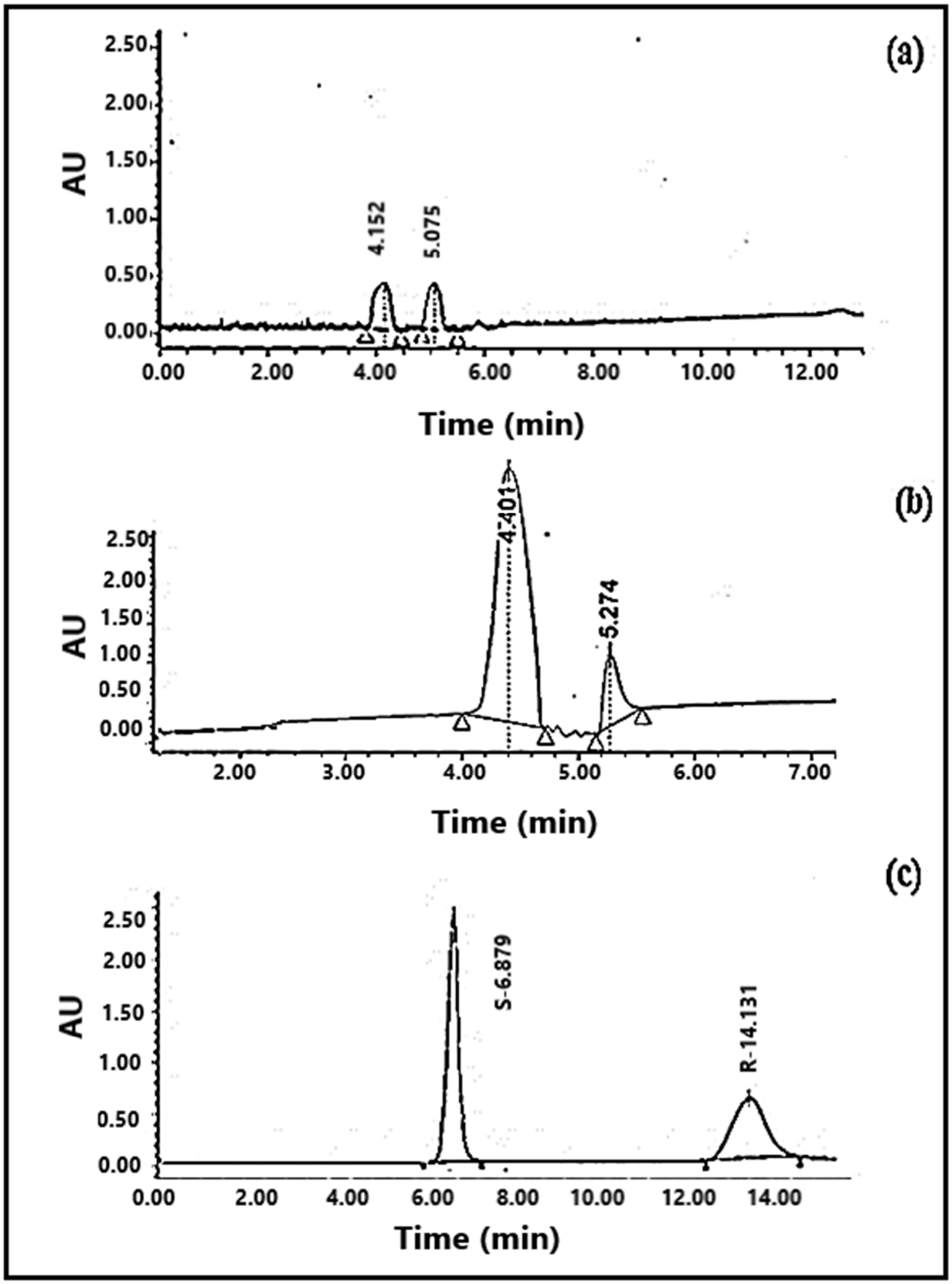

Figure 8. Chromatograms of NSAID enantioseparation using vancomycin as a CMPA, with pH 6.0 phosphate buffer 0.05 M/2-propanol. (**a**) Ibuprofen, 2.0 mM CMPA, (**b**) flurbiprofen, 1.5 mM CMPA, (**c**) ketoprofen, 2.0 mM CMPA.

The chromatograms resulting from the separation of racemic NSAIDs, including ketoprofen, ibuprofen, and flurbiprofen, are displayed in Figure 8. The use of low concentrations of vancomycin as a chiral selector was found to improve the enantiomeric resolution of these profen drugs. The best resolution for the separation of ibuprofen enantiomers was achieved using 2 mM vancomycin in the mobile phase, as shown in Figure 8a. The obtained results can be explained by vancomycin dimerization stimulated by high vancomycin concentrations, which intensified the hydrophobic interactions within the vancomycin molecule [5,40,41]. Previous studies obtained a selectivity of up to 1.74 for ibuprofen enantiomers when using vancomycin in chiral stationary phases, but with a low resolution, which was also observed when using vancomycin as an additive in the mobile phase [17,42,43]. However, in this study, a selectivity of 1.33 and a high resolution of 3.17 were achieved using 2 mM vancomycin as a CMPA in phosphate buffer 0.05 M/2-propanol (50/50) in the reversed-phase mode, with the achiral NH_2 column serving as the stationary phase.

To separate the flurbiprofen enantiomers, we successfully used 1.5 mM vancomycin in the mobile phase, which induced vancomycin dimerization (Figure 8b). When 2 mM vancomycin was added to the mobile phase, we were able to achieve a selectivity of 1.23 and a resolution of 2.29 for flurbiprofen. These results are comparable to those obtained by Tesarova et al., who used 3 mM vancomycin with a C8 achiral column [44], and are superior to those obtained when vancomycin was used as a chiral column [42]. The addition of the chiral selector increased the retention time of flurbiprofen compared to when the chiral selector was not added to the mobile phase [45]. The observed difference in retention times is attributable to the interactions between the vancomycin chiral selector, the stationary phase, and flurbiprofen [18,46,47].

As the concentration of vancomycin in the mobile phase increased, the chiral resolution and selectivity of the ketoprofen racemate also increased [20,48]. On an achiral NH_2 column, ketoprofen was separated with a good selectivity of 2.17 and a short retention time at a concentration of 2 mM vancomycin (Figure 8c). We started the resolution of ketoprofen using 0.5 mM vancomycin as a CMPA, which yielded a selectivity of 2.17 and a resolution of 4.78. This result is similar to the findings of Bouchair et al. [43], who used vancomycin as a chiral column and obtained a selectivity of 2.02 and a resolution of 3.62. In addition, this result is superior to those obtained by Guo et al., who used the same selector at a concentration of 3 mM as a CMPA and the C18 column and obtained a selectivity of 1.20 and resolution of 1.95 [49].

The use of an amino column as the stationary phase and of a mobile phase consisting of propanol/phosphate buffer (pH 6.0, 0.05 M) (50:50, v/v) with vancomycin as a chiral selector was found to be optimal for identifying the enantiomeric impurities of S-naproxen (Figure 9). This result was attributed to the establishment of various interactions between vancomycin and naproxen, such as hydrogen bonding and electrostatic and Van der Waals interactions [50,51]. The testing of the chiral purity of S-naproxen under these chromatographic conditions resulted in a good selectivity of 2.15 and a high resolution of 2.5. Similar results were obtained in the optical purity test of S-naproxen using vancomycin as a chiral column, while no previous studies using vancomycin as a CMPA are available [50–52].

The effect of vancomycin concentration on resolution and selectivity in the racemic separation of profen drugs depends on several factors, including the concentration of vancomycin, the properties of the profen drugs, and the experimental conditions.

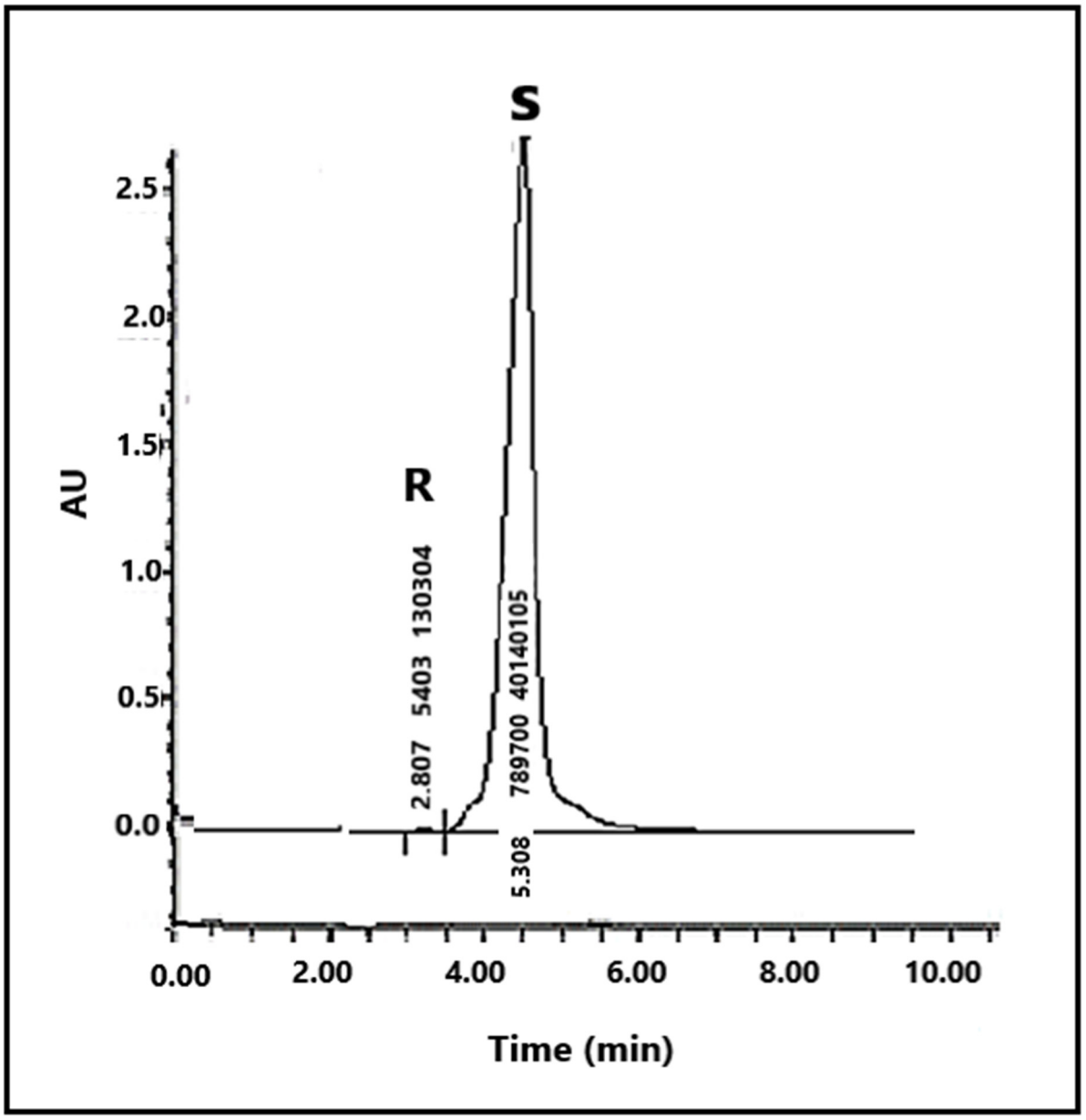

Figure 9. Chromatograms of naproxen enantiomeric purity using vancomycin as a CMPA (2.0 mM) and pH 6.0 phosphate buffer 0.05 M/2-propanol.

In Figure 10, it is observed that increasing the concentration of vancomycin in the mobile phase improved the resolution (Figure 10a) and selectivity (Figure 10b) of the racemic separation of the examined profen drugs. This was due to the increased interaction between the stationary phase and the enantiomers in the racemic mixtures, resulting in a better separation and higher resolution and selectivity. Therefore, adjusting the concentration of vancomycin as a chiral selector in the mobile phase can be an effective strategy for optimizing the chiral separation of profen drugs. Stronger interactions between the analyte–vancomycin complex and the immobilizing surface could be the cause of the increased retention seen at higher vancomycin concentrations [20,23].

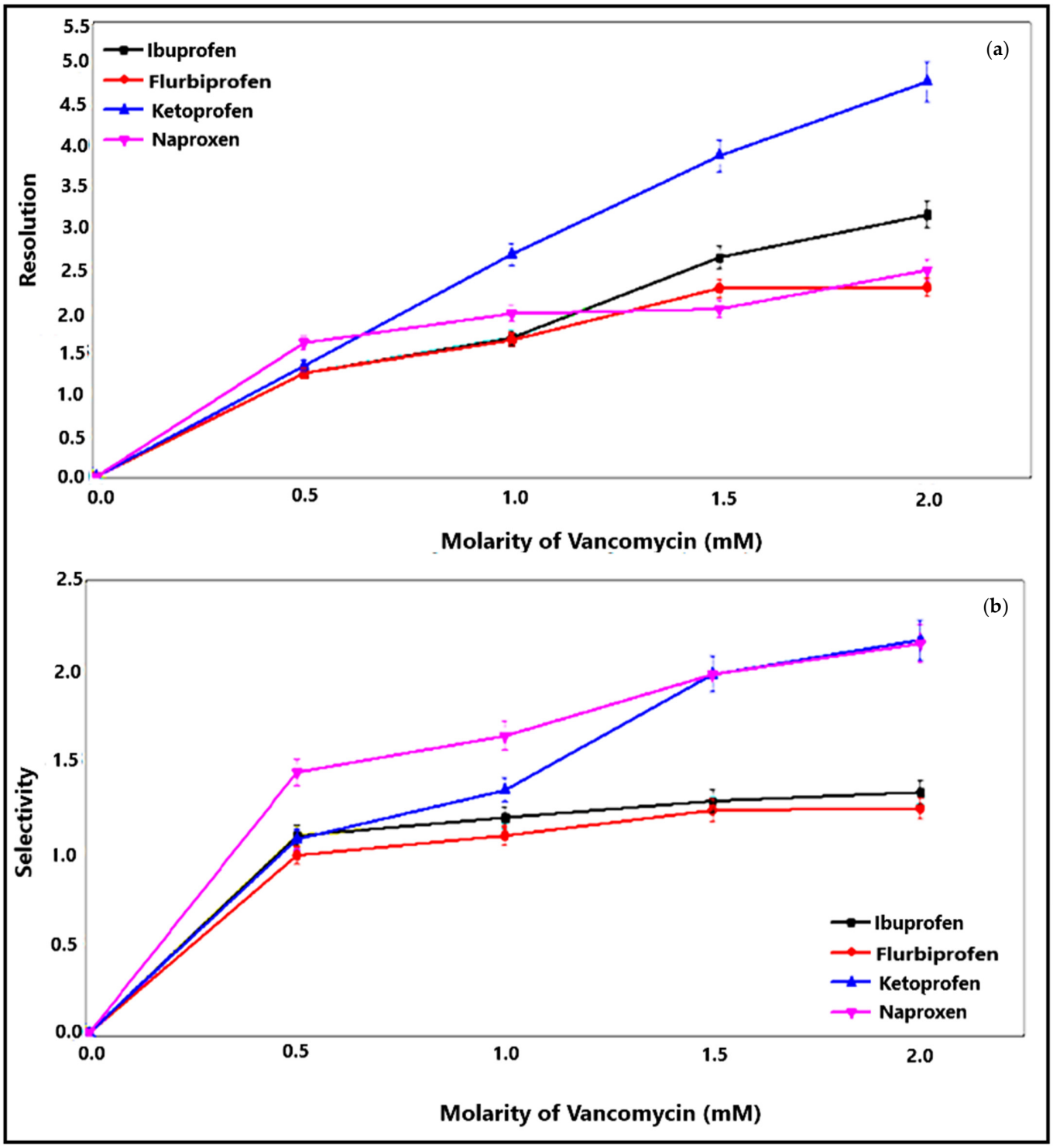

Figure 10. Effect of the vancomycin concentration in the mobile phase on resolution (**a**) and selectivity (**b**) of profen NSAIDs.

This phenomenon contributed to the overall improvement in resolution and selectivity of the chiral separation process. It is important to note that there is a limit to the effect of vancomycin concentration on resolution and selectivity in the racemic separation of profen drugs. Beyond a certain point, increasing the concentration of vancomycin may not lead to further improvements in separation and may even result in decreased resolution and selectivity. This is due to the overloading of the stationary phase and the formation of

vancomycin aggregates [23,53]. Therefore, it is crucial to determine the optimal vancomycin concentration for each analyte experimentally. In this study, the optimal concentration was found to be around 1.5 mM, which was high enough to provide good separation but not so high as to lead to decreased resolution and selectivity.

The vancomycin–analyte complex forms interactions within the mobile phase before engaging with the stationary phase [23,53]. However, it should be noted that vancomycin in the mobile phase may also adsorb onto the achiral stationary phase before vancomycin–analyte interactions take place [22]. As a result, the retention mechanism is typically a hybrid of these two possibilities. Both the structure of the stationary phase and the composition of the mobile phase influence the extent of adsorption of the vancomycin chiral selector on the surface of the stationary phase [22,54].

The presence of vancomycin dimers in solution, as reported by numerous authors [17,55], is an intriguing aspect that influences the mechanism of analyte interaction with vancomycin as a chiral mobile phase additive (CMPA). The dimerization of vancomycin seems to enhance its affinity for analytes containing carboxyl groups. The higher stability of these dimers likely leads to the proper conformation required for the chiral recognition of these analytes, which could explain the increased retention observed in experiments with higher vancomycin concentrations. Furthermore, utilizing vancomycin as a CMPA offers a more flexible separation approach compared to using it solely as a chiral stationary phase, providing greater control over the separation process and ultimately resulting in improved resolution and selectivity.

4. Conclusions

This study aimed to explore the potential of vancomycin as a chiral additive for the enantioseparation of various NSAIDs, including ketoprofen, ibuprofen, flurbiprofen, and chiral impurities of S-naproxen. Several factors, such as type of achiral column (C18 and NH_2), mobile phase pH, polarity, and vancomycin concentration, were investigated for their effects on the separation process.

Through reverse-phase chromatography, employing an achiral NH_2 column and a mobile phase consisting of phosphate buffer 0.05 M and 2-propanol (50/50) at pH 6, the effective enantiomeric separation of profen derivatives using vancomycin as a mobile phase additive was achieved. Notably, this study revealed that lower concentrations of vancomycin (ranging from 0.5 to 2 mM) led to improved enantiomeric resolutions.

This research provides valuable insights into employing vancomycin as a chiral selector in the chromatographic separation of NSAIDs, and the identified optimal conditions hold promise for future research in this domain. The enhanced enantioseparation on the NH_2 column was attributed to the high separation efficiency of the analytical chromatography system and the stereoselective interaction of vancomycin dimers with the surface of the NH_2 column. Overall, this study suggests that the spatial arrangement of the dissolved chiral selector facilitates its interaction with profen analytes, and the formation of vancomycin dimers enhances chiral discrimination.

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