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Dynamic modification of the chiral bonding properties of a CHIRAL-AGP column by organic and inorganic additives

Separation of enantiomers of anti-inflammatory drugs

Jörgen Hermansson* and Inger Hermansson

ChromTech AB, Box 6056, 12906 Hägersten (Sweden)

ABSTRACT

Eight non-steroidal anti-inflammatory agents (NSAIDs) were used as model compounds for studies of organic and inorganic modifier-induced effects on enantioselectivity and retention. Addition of uncharged modifiers such as methanol, ethanol, 1-propanol, 2-propanol and acetonitrile decreases the retention. For some NSAIDs higher enantioselectivity was obtained with than without a modifier in the mobile phase. Tiaprofen could not be resolved using a phosphate buffer without a modifier as the mobile phase. Addition of 1- and 2-propanol to the mobile phase gave separation factors of 1.8 and 1.5, respectively. Modifiers with different hydrogen-bonding properties and hydrophobicity affected the enantioselectivity differently. Three different hydrophobic tertiary amines, N,N-dimethylheptylamine, N,N-dimethyloctylamine and N,N-dimethylnonylamine, were used as charged modifiers. Charged organic modifiers have more drastic effects on the enantioselectivity and retention than the uncharged modifiers. It was observed that the NSAIDs could be divided into two groups with respect to their behaviour in the presence of the tertiary amines; carprofen, flurbifrofen, naproxen and tiaprofen belong to group 1 and fenoprofen, ibuprofen, indoprofen and ketoprofen to group 2. The typical behaviour of a group 1 compound is that the retention of the last-eluted enantiomer increases drastically on addition of amine to the mobile phase, whereas the least-retained enantiomer is only affected to a limited extent. The group 2 compounds behave in a different way concerning retention. At amine concentrations $\geq$2.5 m*M* the retention was lower than that obtained without an amine in the mobile phase. Dramatic improvements in the enantioselectivity for both group 1 and 2 compounds could be obtained by increasing the amine concentration in the mobile phase. Separation factors up to 13 were obtained. It was also possible to affect both the enantioselectivity and the retention by varying the concentration of inorganic cations such as sodium and ammonium ions. The retention of both enantiomers of the NSAIDs increases with increasing concentration of the inorganic ions. A strong improvement of the enantioselectivity was also observed. For example, the enantioselectivity increased from 1.75 to 2.58 for naproxen on increasing the sodium concentration from 0.0148 to 0.16 *M*. The results obtained with both inorganic and organic cationic additives indicate that ion-pair distribution can be involved in the retention of the anionic solutes.

INTRODUCTION

A protein molecule is a biopolymer with a unique folded structure in solution. The conformation of certain proteins in water solution could be reversibly affected by decreasing the polarity of the solution by adding organic solvents such as acetonitrile and propanol [1] or by surfactants such as sodium dodecyl sulphate [2]. These types of additive could induce the formation of an α-helix in certain protein molecules [3]. Addition of sodium dodecyl sulphate to an α_1-acid glycoprotein (AGP) solution has been demonstrated to transform parts of the peptide chain with an unordered structure or β-con-

* Corresponding author.

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formation into an α-helix [3]. It is a very important finding that the protein conformation could be reversibly affected, as this could be utilized chromatographically to induce chiral selectivity of the solutes by addition of non-chiral additives to the mobile phase. Enquist and Hermansson [4] have demonstrated that both acetonitrile and propanol are adsorbed to a large extent on the AGP phase. It was also demonstrated that the enantioselectivity is greatly affected by both the type and the concentration of an organic modifier [4]. Small differences in the nature of the modifiers, such as between 1- and 2-propanol, could give differences in enantioselectivity for certain solutes. The very broad applicability of the AGP column can partly be ascribed to the unique property that the chiral selectivity could be induced by addition of non-chiral additives to the mobile phase, which reversibly affects the chiral bonding properties of the AGP molecule. A very large number of chiral drugs of different types have been resolved on a CHIRAL-AGP column [5–7]. This column has been used for purity determination of enantiomers of drugs and for the determination of drug enantiomers in biological materials [8–16].

In this work we studied the influence of inorganic and organic modifiers on the enantioselectivity and retention of a series of non-steroidal antiinflammatory drugs (NSAIDs). The aim was to obtain a deeper understanding of the mechanisms behind modifier-induced changes of the enantioselectivity and retention and to evaluate optimum separation conditions for NSAIDs on the CHIRAL-AGP column.

EXPERIMENTAL

Columns

CHIRAL-AGP columns (ChromTech, Norsborg, Sweden) with dimensions 100×4.0 mm I.D. and 50×4.0 mm I.D., packed with 5-μm particles, were used together with CHIRAL-AGP guard columns (10×3.0 mm I.D.).

Apparatus

The HPLC pump was an LKB 2150 (Pharmacia–LKB Biotechnology, Uppsala, Sweden). Two types of injectors were used; either a manual Model 7125 injector (Rheodyne, Cotati, CA, USA), or a Kontron Model 360 autosampler (Tegimenta, Rotkreutz, Switzerland). The detector was a Spectra 100 (Spectra-Physics, San Jose, CA, USA). All experimental data were collected and analysed on a Model 450 MT2 data system (Kontron, Eching/Münich, Germany).

Chemicals

Naproxen, tiaprofenic acid, flurbiprofen, ketoprofen, ibuprofen and fenoprofen were obtained as gifts from the manufacturers. Carprofen and indoprofen were purchased from Sigma (St. Louis, MO, USA).

Methanol, 1-propanol, 2-propanol and acetonitrile were of HPLC grade (Lab-Scan, Dublin, Ireland) and ethanol was obtained from Kemetyl (Stockholm, Sweden). Sodium phosphate and tris(hydroxymethyl)aminomethane (Tris) were purchased from Merck (Darmstadt, Germany), ammonium acetate from Janssen Chimica (Geel, Belgium), dimethyloctylamine from Lancaster Synthesis, dimethylheptylamine from Ames Labs. and dimethylnonylamine from Pfaltz & Bauer.

Chromatographic conditions

Mobile phases containing uncharged modifiers were prepared by adding appropriate concentrations of the organic solvent to a buffer solution with a certain pH, followed by dilution with distilled water to a known volume.

Mobile phases containing different concentrations of buffer at pH 7.0 were prepared as follows: an appropriate amount of sodium dihydrogenphosphate was placed in a beaker, water was added and pH was adjusted to 7 using sodium hydroxide solution of known concentration. Mobile phases containing different concentrations of buffer at pH 2.1 were prepared as follows: an appropriate amount of phosphoric acid was placed in a beaker, water was added and pH was adjusted to 2.1 using sodium hydroxide solution of known concentration. The final volume was adjusted with distilled water.

Mobile phases containing amines were prepared as follows: phosphoric acid and amine were weighed into a beaker, water was added and the pH was adjusted to 7.0 using sodium

hydroxide solution. The final volume was adjusted with distilled water.

Columns that have been used with mobile phases containing amines should not be used for chromatography with mobile phases without amine additives. It has been found that it can be difficult to remove completely all traces of amine from the stationary phase, which may affect the results obtained later using mobile phases without amine.

RESULTS AND DISCUSSION

Retention principles

Adsorption isotherm studies of the secondary amine terodiline indicated that this compound is adsorbed on at least two different sites on the AGP column, one a high-affinity site and the other a site to which the solutes are bound with lower affinity [17]. By using terodiline as a mobile phase additive and chromatography of solutes of different character, it has also been demonstrated that acidic, basic and non-protolytic solutes are bound to and compete for the same sites [17]. The binding sites are most likely areas or hydrophobic pockets on the protein molecule where an enrichment of hydrophobic amino acid residues such as tryptophan, phenylalanine, tyrosine and leucine could be found. The binding sites contain, in addition to the hydrophobic groups, also many charged groups, both anionic and cationic, and hydrogen-bonding groups of different kinds. The solutes can be bound to the protein phase by, in principle, two types of interactions, ionic binding and binding to uncharged groups of different types, such as hydrophobic and hydrogen-bonding groups.

AGP is a very acidic protein with an isoelectric point of 2.7 in phoshate buffer. This means that the protein in most instances in chromatographic experiments has a net negative charge. Increasing the pH increases the negative charge of the protein, which increases the ion-exchanging capacity for cations and decreases the capacity for anions. The retention of anions, according to this mechanism, is dependent on the OH^- concentration and the type and concentration of other anions in the mobile phase. The retention of cations, caused by ionic bonding, is affected by the H^+ concentration and the type and concentration of other cations in the mobile phase, such as the buffer ions.

The uncharged groups involved in the binding of the solutes can give different types of interactions with the solutes, such as hydrophobic interactions and hydrogen bonding. Both anionic and cationic solutes can be bound to the uncharged groups in uncharged form, or as uncharged ion pairs with a counter ion. Addition of an additive with the same charge as the solute affects both the ion-pairing process and the ion-exchange process in the same way. However, an additive with a charge opposite to that of the solute cannot affect the ion-exchange process. A hydrophobic charged additive with a charge opposite to that of the solute can, however, compete with the solute for binding to hydrophobic groups in the binding sites. The retention of uncharged solutes could be affected by both uncharged and charged modifiers.

Influence of uncharged modifiers on retention and enantioselectivity

It has been demonstrated that uncharged modifiers such as 1-propanol and acetonitrile are strongly adsorbed on the AGP phase [4]. It was observed that the more hydrophobic uncharged modifier 1-propanol was bound with higher affinity than acetonitrile. Adsorption isotherm studies indicated that a monolayer was formed at 10% and 15% (v/v) for 1-propanol and acetonitrile, respectively [4]. Further, the results suggest the formation of multilayers of solvent molecules on the protein surface at higher concentration [4]. The adsorption of the uncharged modifiers on the protein affects the enantioselectivity and the retention. Normally, both the enantioselectivity and the retention increase on decreasing the modifier concentration in the mobile phase. However, for certain solutes it is possible to improve the chiral selectivity by increasing the modifier concentration.

Five different modifiers, methanol, ethanol, 1-propanol, 2-propanol and acetonitrile, with different hydrophobicities and different hydrogen bonding properties were used as modifiers for the separation of some NSAIDs. The capacity factors and the separation factors obtained for the solutes are summarized in Table I. For

TABLE I

INFLUENCE OF THE NATURE OF AN UNCHARGED MODIFIER ON RETENTION AND STEREOSELECTIVITY

Mobile phase: 0.01 *M* phosphate buffer (pH 6.5) with addition of uncharged modifier.

Additive[a]	Tiaprofen		Fenoprofen		Flurbiprofen		Ibuprofen		Ketoprofen		Naproxen	
	k'	α	*k'*	α	*k'*	α	*k'*	α	*k'*	α	*k'*	α
–	8.4	1.0	23.7	1.4	21.9	1.4	5.5	1.5	12.7	1.2	6.2	2.0
0.75% ACN	5.3	1.0	14.3	1.2	15.3	1.1	3.5	1.6	6.3	1.1	3.6	1.8
0.5% MeOH	5.7	1.0	20.8	1.4	15.8	1.4	4.9	1.6	8.9	1.2	5.2	2.3
0.75% EtOH	5.2	1.0	15.1	1.3	11.9	1.3	3.1	1.4	5.0	1.2	3.5	1.8
1% 1-PrOH	4.2	1.8	5.7	1.2	8.6	1.2	1.4	1.3	4.4	1.0	2.3	1.3
1% 2-PrOH	3.8	1.5	6.9	1.3	8.2	1.3	1.7	1.4	4.1	1.2	2.6	1.5

[a]ACN = Acetonitrile; MeOH = methanol; EtOH = ethanol; PrOH = propanol.

comparison, the corresponding data obtained with 0.01 *M* phosphate buffer (pH 6.5) without modifier are included. It is interesting that tiaprofen does not show any separation using a pure buffer or buffer with addition of the most hydrophilic modifiers, acetonitrile and methanol. However, using 1- and 2-propanol separation factors of 1.75 and 1.47 were obtained.

The influence of 2-propanol concentration on the separation factor is demonstrated in Fig. 1. This study was performed using a mobile phase of 0.01 *M* phosphate buffer (pH 6.0) with 2-propanol. It is also possible to induce chiral selectivity for tiaprofen with ethanol as modifier in 0.01 *M* phosphate buffer (pH 6.0). The effect of ethanol is smaller than that of 1- and 2-propanol. From Table I can be seen that separation factors higher than 1.14 were obtained for ketoprofen with all modifiers except 1-propanol, where no separation was obtained. The highest separation factor for naproxen was 2.29 and it was obtained with a mobile phase containing 0.5% (v/v) of methanol. This separation factor was higher than that obtained without modifier. It has been observed previously for some hydantoin derivatives and barbituric acid derivatives that the enantioselectivity could be induced or improved by adding an uncharged modifier to the mobile phase [4]. For example, methylphenobarbital was strongly retained and no enantioselectivity was obtained using a mobile phase of phosphate buffer (pH 7) without modifier. However, addition of only 2% of 2-propanol resulted in a much lower retention and a complete baseline resolution [6]. So far such observations have never been made for basic compounds.

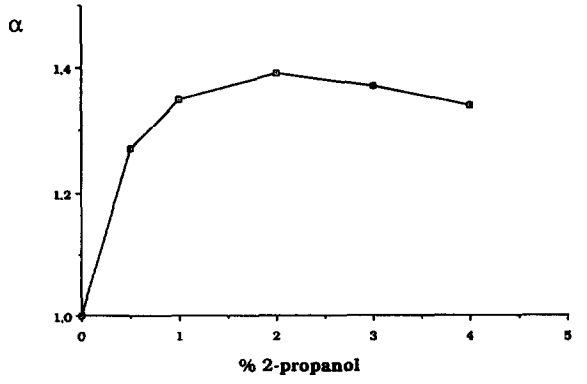

Fig. 1. Influence of 2-propanol concentration on enantioselectivity. Column, CHIRAL-AGP (100 × 4.0 mm I.D.); mobile phase, 10 m*M* sodium phosphate buffer (pH 6.5) containing different concentrations of 2-propanol; flow-rate, 0.9 ml/min; detection, UV at 225 nm; sample, tiaprofen (20 μg/ml).

The described observations cannot be explained by simple chromatographic theory. However, one explanation for the uncharged modifier-induced changes of the enantioselectivity could be that addition of organic solvents to the mobile phase can reversibly affect the secondary structure of the AGP molecule. It is known that the secondary structure can be affected by decreasing the polarity in aqueous solution by adding on organic solvent such as propanol [1].

The secondary structure of AGP has been studied by Aubert and Loucheux-Lefebvre [18], using the Lim [19] and Chou and Fasman method [20]. They found about 20% α-helix, about 20% β-conformation and a high degree of unordered structure. In another study, Jirgensons [3] concluded that no α-helix could be observed in AGP using circular dichroism (CD). The effects on the enantioselectivity could be due to transformation of parts of the peptide chain with β-conformation or an unordered structure into α-helix. This means that the enantiomers, after such a change, interact in a different way with the protein molecule and therefore the enantioselectivity could be affected. Enquist and Hermansson [4] utilized CD for studying the secondary structure of AGP in a solution containing 2-propanol and found no clear indication of the formation of α-helix. A reinvestigation of the old data together with new results might indicate the formation of α-helix when 2-propanol is added at high concentration, *i.e.* 40%, to a solution of AGP [21]. At a propanol concentration of 9.5% it was not possible to detect any formation of α-helix. However, it is reasonable to assume that only very small local changes of the secondary structure are required in order to observe effects on the enantioselectivity. Most likely transformation of such small parts of the peptide chain cannot be detected by CD. Another explanation of the modifier-induced changes of the enantioselectivity is that the modifiers compete with the solute enantiomers for hydrogen bonding. Thus, modifiers with different hydrogen-bonding properties affect the enantioselectivity in different ways. The modifiers can also be bound to the solutes by hydrogen bonding and in that way affect the stereoselective binding to the protein.

Influence of charged organic modifiers on enantioselectivity and retention

Charged organic modifiers often give more drastic effects than the uncharged modifiers on both the enantioselectivity and the retention. It has been demonstrated earlier for naproxen and ibuprofen that the enantioselectivity could be drastically improved by adding the tertiary amine N,N-dimethyloctylamine (DMOA) to the mobile phase [22]. The effect was caused by a selective increase in the retention of the last-eluted enantiomer, whereas the retention for the first-eluted enantiomer was almost unaffected. In order to obtain a deeper insight into the mechanisms behind these findings, a series of NSAIDs were studied using, in addition to DMOA, also two other analogues, N,N-dimethylheptylamine (DMHA) and N,N-dimethylnonylamine (DMNA), as mobile phase additives. Eight NSAIDs were studied, the structures of which are given in Fig. 2. The chromatographic data are summarized in Tables II and III. It was observed that the NSAIDs could be divided in two groups, naproxen, carprofen, tiaprofen and flurbiprofen belonging to group 1 and ketoprofen, ibuprofen, indoprofen and fenoprofen to group 2. The typical behaviour of a group 1 compound is that the retention of the last-eluted enantiomer increases drastically on addition of amine to the mobile phase, whereas the first enantiomer gives only a very limited increase in retention compared with the more retained enantiomer at the lowest amine concentration. Amine concentrations higher than 1 m*M* give a lower retention of the first-eluted enantiomer than is obtained without amine additive. Using DMNA as the mobile phase additive at the lowest concentration, 1 m*M*, the retention of the first-eluted enantiomer of all group 1 compounds increases. By using the least hydrophobic amine, DMHA, at the same concentration, both enantiomers of three of the compounds were more retained.

The group 1 compounds demonstrate an interesting behaviour with respect to the retention of both enantiomers. In order to test if they were bound to different binding sites, (+)-naproxen was added to the mobile phase at increasing concentration and racemic naproxen was injected on to the column. Fig. 3 demonstrates the results. The capacity factors for both enantiomers of naproxen were affected to the same extent and the separation factor was almost unaffected. From this experiment it can be concluded that the naproxen enantiomers are bound to the same binding sites.

The increase in the retention of the group 1 compounds obtained on adding the hydrophobic tertiary amines to the mobile phase might indi-

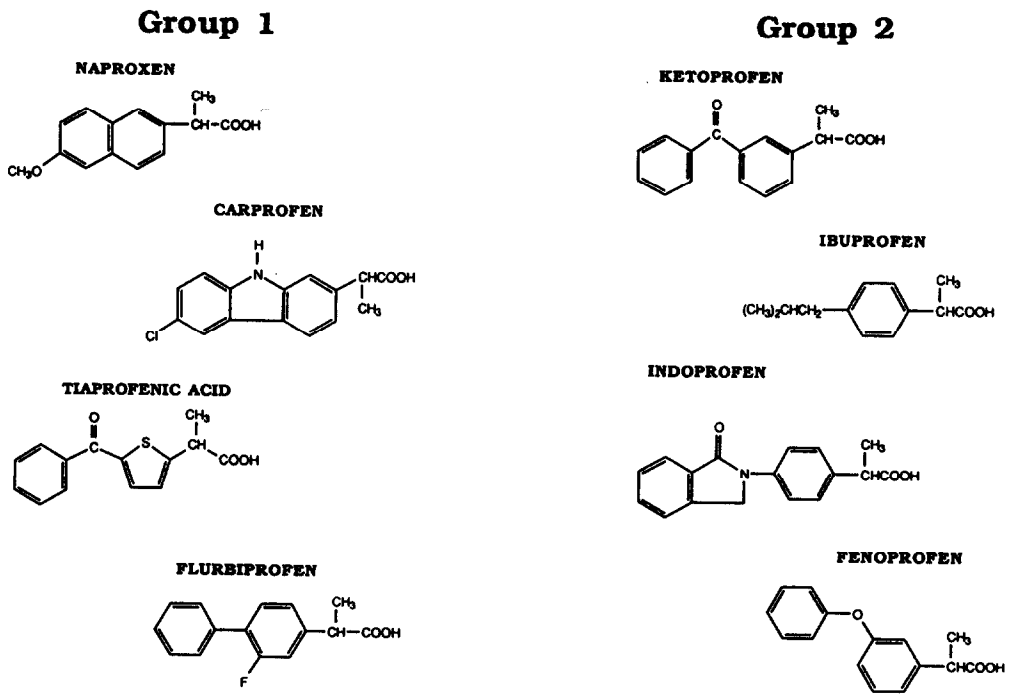

Fig. 2. Structures of NSAIDs.

cate that they are distributed as an ion pair. However, for some solutes the retention of the first-eluted enantiomer decreases with increasing amine concentration and this behaviour cannot be explained by ion-pair distribution. Chromatography of naproxen in a mobile phase containing DMOA in phosphate buffer at pH 2.2, where naproxen is uncharged and unable to form

TABLE II

INFLUENCE OF THE NATURE AND THE CONCENTRATION OF CATIONIC MODIFIERS ON THE RETENTION AND ENANTIOSELECTIVITY OF GROUP 1 NSAIDs

Mobile phase: 0.01 *M* sodium phosphate buffer (pH 7.0) containing different types of additives.

Additive	Naproxen			Carprofen			Tiaprofen			Flurbiprofen		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
None	5.23	9.14	1.75	35.8	35.8	1.00	10.1	10.1	1.00	15.8	2.12	1.34
1.0 m*M* DMHA	6.97	63.4	9.10	47.2	87.3	1.85	8.13	29.3	3.61	23.3	n.d.[a]	
2.5 m*M* DMHA	3.62	38.8	10.7	40.5	81.1	2.00	8.34	35.2	4.22	18.3	n.d.[a]	
5.0 m*M* DMHA	3.72	41.8	11.2	35.6	70.3	1.97	6.10	36.0	5.90	13.5	116	8.60
1.0 m*M* DMOA	4.95	61.0	12.3	–	–	–	10.0	54.0	5.41	25.9	n.d.[a]	
2.5 m*M* DMOA	4.08	43.8	11.0	–	–	–	8.79	52.9	6.02	24.1	112	4.65
5.0 m*M* DMOA	3.42	44.4	13.0	–	–	–	7.93	45.5	5.74	18.8	–	–
1.0 m*M* DMNA	5.64	37.1	6.58	44.4	72.9	1.64	10.3	41.0	3.97	16.7	109	6.54
2.5 m*M* DMNA	3.66	25.1	6.84	–	–	–	8.0	35.6	4.43	12.8	92.5	7.23
5.0 m*M* DMNA	3.18	19.3	6.06	32.3	49.3	1.52	5.8	24.7	4.29	10.5	78.9	7.54

n.d. = Not determined.

TABLE III

INFLUENCE OF THE NATURE AND THE CONCENTRATION OF CATIONIC MODIFIERS ON THE RETENTION AND ENANTIOSELECTIVITY OF GROUP 2 NSAIDs

Mobile phase: 0.01 *M* sodium phosphate buffer (pH 7.0) containing different types of additives.

Additive	Ketoprofen			Ibuprofen			Indoprofen			Fenoprofen		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
None	10.3	11.6	1.12	6.52	8.38	1.29	7.69	7.69	1.00	20.0	25.1	1.25
1.0 m*M* DMHA	8.85	13.9	1.57	4.39	7.93	1.80	9.27	11.2	1.20	15.4	26.2	1.69
2.5 m*M* DMHA	6.95	11.7	1.68	3.82	7.52	1.97	5.88	7.00	1.19	9.08	18.4	2.02
5.0 m*M* DMHA	5.56	10.8	1.93	3.29	6.85	2.08	–	–	–	7.67	14.2	1.85
1.0 m*M* DMOA	6.21	12.0	1.93	3.11	6.06	1.95	5.80	6.71	1.16	8.89	17.4	1.96
2.5 m*M* DMOA	5.26	10.9	2.08	2.54	5.37	2.10	4.88	5.61	1.15	6.67	14.1	2.10
5.0 m*M* DMOA	4.83	10.4	2.15	2.45	5.04	2.06	4.49	4.99	1.11	5.92	12.8	2.17
1.0 m*M* DMNA	6.23	9.84	1.58	3.82	5.28	1.38	6.48	7.40	1.14	7.37	11.9	1.60
2.5 m*M* DMNA	4.71	7.88	1.67	2.75	3.97	1.44	4.87	5.79	1.19	5.41	9.24	1.71
5.0 m*M* DMNA	3.65	5.93	1.63	1.96	3.09	1.58	3.76	4.46	1.19	4.32	7.66	1.77

an ion pair with DMOA, results in an almost unaffected retention when increasing the DMOA concentration. This finding supports an ion-pair distribution at pH 7. It has been demonstrated previously at pH 7 for uncharged solutes that an increasing concentration of DMOA in the mobile phase decreases the retention [22]. A reasonable explanation of the small effect of DMOA at pH 2.2 may be the lower degree of negative charge of the protein at this pH compared with pH 7, which reduces the binding capacity for cationic compounds, such as DMOA. This means that the retention of uncharged solutes is reduced more effectively at higher pH.

The group 2 compounds behave in a different way concerning the retention. At amine concentrations $\geq$2.5 m*M* the retentions for both enantiomers were lower than those obtained without amine in the mobile phase. However, a slight increase in retention was observed for some group 2 compounds using the most hydrophilic amine, DMHA, at the lowest concentration, 1 m*M*. Obviously ion-pair distribution influences the retention of some group 2 compounds using DMHA as mobile phase additive. However, when using the more hydrophobic amines, DMOA and DMNA, ion-pair distribution is not the dominant retention mechanism.

Adsorption isotherm studies demonstrated that the secondary amine terodiline, containing two aromatic rings, is adsorbed on the AGP column with high affinity. With increasing terodiline concentration the enantioselectivity for basic compounds disappears at a terodiline concentration of 15–20 μM in the mobile phase, *i.e.*, when the high affinity site is saturated with terodiline. However, high enantioselectivity was observed for the acidic and non-protolytic compounds even at the highest terodiline concentration, 80 μM. The retentions of both enantiomers of ibuprofen (group 2) and the non-protolytic compounds decreased with increasing terodiline concentration in the mobile phase [17]. Obviously, ion-pair distribution of ibuprofen with terodiline as the counter ion has no significant influence on the retention. The results indicate that terodiline and ibuprofen compete for binding to hydrophobic and hydrogen-bonding groups in the binding sites. As can be seen from Table III, ibuprofen behaves in the same way as with terodiline in the mobile phase concerning the retention and enantioselectivity on using hydrophobic aliphatic tertiary amines as mobile phase additives.

Dramatic improvements in the enantioselectivity for both group 1 and 2 compounds could be obtained by increasing the concentration of

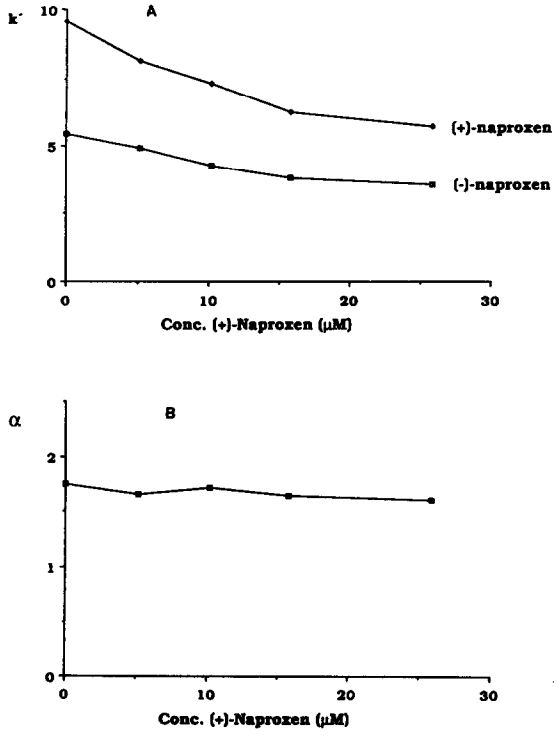

Fig. 3. Chromatography of naproxen with (+)-naproxen in the mobile phase. Column, CHIRAL-AGP (100 × 4.0 mm I.D.); mobile phase, 10 m*M* sodium phosphate buffer (pH 7.0) containing different concentrations of (+)-naproxen; flow-rate, 0.9 ml/min; detection, UV at 225 nm; sample, racemic naproxen (20 μg/ml). (A) Influence on retention; (B) influence on enantioselectivity.

the tertiary amine in the mobile phase. For example, a separation factor of 13.0 was obtained for naproxen with 5 m*M* DMOA in the mobile phase. The highest enantioselectivity was observed for the group 1 compounds. This is the result of the selective increase in the retention of the last-eluted enantiomer. The high separation factors and the high retentions obtained for certain compounds, using amine additives, could be reduced by using concentrations of the tertiary amines lower than 1 m*M* or by addition of an uncharged modifier to the mobile phase. It should be noted that no enantioselectivity was observed for tiaprofen without an amine in the mobile phase.

Fig. 4 demonstrates the separation of tiaprofen and ibuprofen enantiomers with tertiary amines in the mobile phase. It is a general finding for both group 1 and 2 compounds that the separation factor is lower without an amine in the mobile phase. For two of the group 1 compounds, carprofen and tiaprofen, a separation factor of 1.0 was obtained using a mobile phase of 0.01 *M* phosphate buffer (pH 7) without an amine. However, all amines tested induced high enantioselectivity for carprofen and tiaprofen, as can be seen from Table II. It cannot be excluded that some of the effects on the enantioselectivity and retention on addition of the tertiary amines to the mobile phase are caused by reversible changes of the protein conformation.

Effects of buffer concentration on retention and enantioselectivity

The influence of the buffer concentration was studied using two group 1 compounds, flurbiprofen and naproxen, and two group 2 compounds, fenoprofen and ketoprofen. The experiment was performed using mobile phases of 1% (v/v) 2-propanol in phosphate buffer (pH 7.0). The sodium concentration was varied in the range 0.01–0.4 *M*. At pH 7 these acids are fully ionized and can be retained as an ion pair with sodium.

From Figs. 5 and 6 it can be seen that the retention of both the first- and the last-eluted enantiomers of the four compounds increase with increasing concentration of sodium in the mobile phase. This indicates that the dominant retention mechanism of the NSAIDs might be ion-pair distribution with sodium as counter ion.

Chromatography of naproxen in uncharged form at pH 2.1 and increasing the sodium concentration from 0.006 to 0.062 *M* resulted in decreasing retention as is demonstrated in Fig. 7. These results support the assumption that the dominant retention mechanism at pH 7 may be ion-pair distribution. The mechanism behind the decrease in the retention obtained on increasing the sodium concentration at pH 2.1 is not yet understood. It is interesting to note the large difference in the behaviour of the NSAIDs observed with hydrophilic and hydrophobic cationic mobile phase additives at pH 7. Hydrophilic counter ions, such as sodium, gave increasing retentions of both enantiomers of the eight NSAIDs tested. The tertiary amine additives

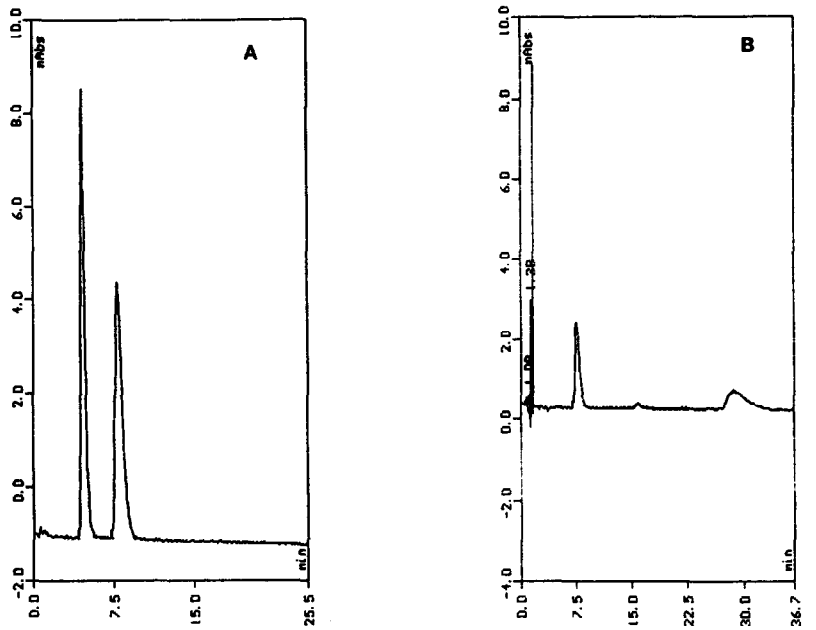

Fig. 4. Chromatography of ibuprofen and tiaprofen with tertiary amine in the mobile phase. Column, CHIRAL-AGP (100 × 4.0 mm I.D.); flow-rate, 0.9 ml/min; detection, UV at 225 nm. (A) Mobile phase, 10 m*M* sodium phosphate buffer (pH 7.0) containing 1 m*M* DMOA; sample, ibuprofen 20 (μg/ml). (B) Mobile phase, 10 m*M* sodium phosphate (pH 7.0) buffer containing 5 m*M* DMNA; sample, tiaprofen (60 μg/ml).

affect the retention of the group 1 and 2 compounds differently and in most instances the retention of the group 2 compounds decreases with increasing concentration of the tertiary amine. The retention of the last-eluted enantiomer of the group 1 compounds demonstrated a strong increase in retention. However, using the least hydrophobic tertiary amine, DMHA, at the lowest concentration resulted in a small increase in the retention of the first-eluted enantiomer for three of the four group 1 compounds. Some of the group 2 compounds also demonstrated a

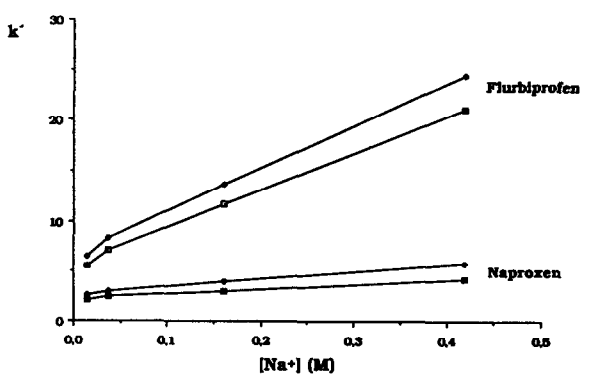

Fig. 5. Influence of buffer concentration on retention of group 1 NSAIDs. Column, CHIRAL-AGP (100 × 4.0 mm I.D.); mobile phase, sodium phosphate buffer (pH 7.0) at different concentrations containing 1% 2-propanol; flow-rate, 0.9 ml/min; detection, UV at 225 nm; samples, flurbiprofen (20 μg/ml) and naproxen (20 μg/ml).

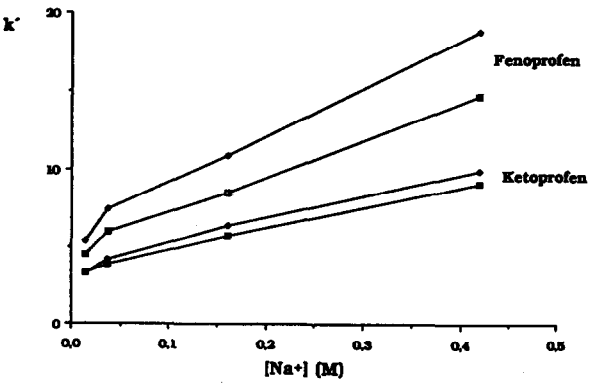

Fig. 6. Influence of buffer concentration on retention of group 2 NSAIDs. Column, CHIRAL-AGP (100 × 4.0 mm I.D.); mobile phase, sodium phosphate buffer (pH 7.0) at different concentrations containing 1% 2-propanol; flow-rate, 0.9 ml/min; detection, UV at 225 nm; samples, fenoprofen (20 μg/ml) and ketoprofen (20 μg/ml).

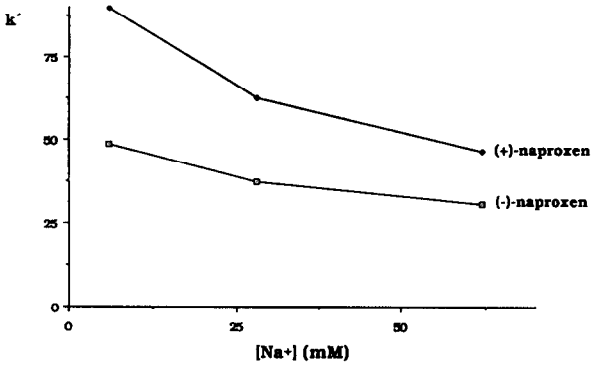

Fig. 7. Effect of the sodium concentration on retention of naproxen enantiomers at low pH. Column, CHIRAL-AGP (50 × 4.0 mm I.D.); mobile phase, sodium phosphate buffer (pH 2.1) at different concentrations; flow-rate, 0.9 ml/min; detection, UV at 225 nm; sample, naproxen (4 μg/ml).

small increase in retention using DMHA at the lowest concentration, 1 m*M*. By decreasing the hydrophobicity of the counter ion, from DMNA, DMOA and DMHA to sodium, the hydrophobic competing effect of the cation is reduced, which means that the ion-pair mechanism increases in importance. When using the tertiary amine additives, containing long aliphatic alkyl chains, in addition to the positively charged nitrogen, they can compete effectively with the solute for binding to hydrophobic groups in the binding sites, resulting in a decrease in retention. The tertiary amines are bound by ionic binding to negatively charged groups in the binding sites, in combination with hydrophobic interaction. The protein has a strong negative charge at pH 7. The fact that the retentions of the NSAIDs are affected by the nature and the concentration of the tertiary amines indicates that they are bound to the same domain (binding sites) of the protein. It also indicates that the negatively charged groups are located close to the uncharged hydrophobic groups interacting with the ion pairs of the NSAIDs. Hydrophilic cations such as sodium are also bound to the negatively charged groups in the binding sites of the protein. However, they lack the possibility of competing with the solutes for hydrophobic interaction and therefore ion-pair distribution might be the dominant retention mechanism for both enantiomers of the NSAIDs using such hydrophilic counter ions.

TABLE IV

INFLUENCE OF SODIUM CONCENTRATION ON ENANTIOSELECTIVITY

Mobile phase: sodium phosphate buffer (pH 7.0).

$[Na^+]$ (*M*)	α	
	Naproxen	Ibuprofen
0.0148	1.75	1.29
0.0372	1.91	1.33
0.1600	2.58	1.42

As demonstrated in Table IV, the enantioselectivity for naproxen is also greatly affected by the sodium concentration. However, the effects are small compared with those obtained using organic cations as reported above.

It was also observed that the separation efficiency was affected by the concentration of buffer in the mobile phase. Increasing the buffer concentration results in an increase in separation efficiency. This is demonstrated in Fig. 8 for the enantiomers of fenoprofen. Fig. 9 shows a comparison between different buffers concerning the retention. Ammonium acetate, sodium phosphate and Tris buffers were studied using ibuprofen as a model compound. The retentions of both enantiomers increase with increasing buffer concentration. The highest retention was ob-

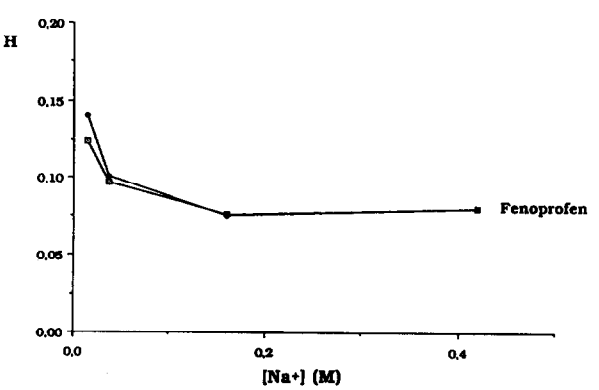

Fig. 8. Effect of buffer concentration on efficiency. Column, CHIRAL-AGP (100 × 4.0 mm I.D.); mobile phase, sodium phosphate buffer (pH 7.0) at different concentrations containing 1% 2-propanol; flow-rate, 0.9 ml/min; detection, UV at 225 nm; sample, fenoprofen (20 μg/m).

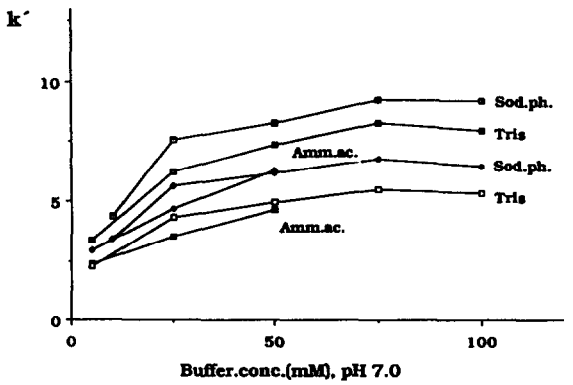

Fig. 9. Influence of buffer type on retention. Column, CHIRAL-AGP (100 × 4.0 mm I.D.); mobile phase, different buffers of 10–100 m*M* (pH 7.0); flow-rate, 0.9 ml/min; detection, UV at 225 nm; sample, ibuprofen (20 μg/ml).

tained using sodium phosphate, followed by Tris and ammonium acetate. The enantioselectivity for ibuprofen increases slightly in the order sodium phosphate, ammonium acetate and Tris.

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