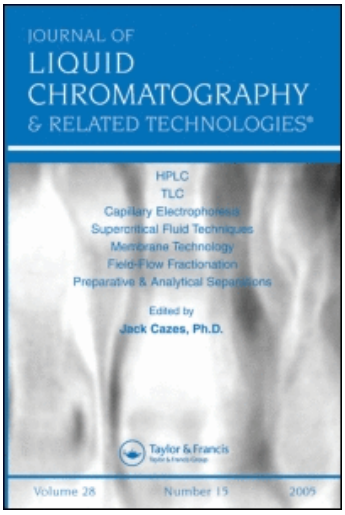

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Direct Liquid Chromatographic Resolution of Acidic Drugs Using a Chiral α_1-Acid Glycoprotein Column (Enantiopac®)

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DIRECT LIQUID CHROMATOGRAPHIC RESOLUTION OF ACIDIC DRUGS USING A CHIRAL α_1-ACID GLYCOPROTEIN COLUMN (ENANTIOPAC®)

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ABSTRACT

A chiral α_1-acid glycoprotein column (EnantioPac®) has been used for the separation of the enantiomers of some acidic (ibuprofen, ketoprofen, naproxen, 2-phenoxypropionic acid, bendroflumethiazide, ethotoin and hexobarbital) and basic drugs (disopyramide and RAC 109).

The column is prepared by immobilization of the human plasma protein α_1-acid glycoprotein on silica particles. The retention and the enantioselectivity of the solutes can easily be regulated by the addition of the tertiary amine N,N,-dimethyloctylamine (DMOA) to the mobile phase. DMOA decreases the retention and the enantioselectivity of the weaker acids, whereas the retention and the enantioselectivity of the stronger acids increase drastically with increasing DMOA concentration.

The influence of column temperatures between 25 and 80 ^{o}C on the separation factor, separation efficiency and the resolution was also evaluated. Stability studies indicate that the α_1-acid glycoprotein column (EnantioPac®) is very stable. It can be used at elevated temperatures, it tolerates pure 2-propanol and has been stored in a water-2-propanol mixture long periods (12 months) with <10% changes of the capacity and separation factors.

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INTRODUCTION

An enantiomer and its antipode have identical physical and chemical properties like reactivity and solubility in a symmetrical environment. However, if the enantiomers are introduced in a chiral environment, for exampel in the human body, the enantiomers are handled by receptors (1), enzymes (2) and proteins as two quite different molecules. This property has been utilized to resolve enantiomers chromatographically. In a series of papers we have described the use of the human plasma protein, α_1-acid glycoprotein, as the chiral stationary phase for the resolution of racemic drugs (3-7). Basic drugs of different character, as well as amides and esters have been resolved using the α_1-AGP column (now available as EnantioPac®). The column has also been used in bioanalysis for the separation and quantitation of the enantiomers of disopyramide in human plasma (8).

Many of the advantages with the EnantioPac® column are associated with the reversed phase character of the column, which gives many possibilities to regulate the retention and the enantioselectivity, as well as the possibility for direct injection of aqueous samples. Moreover, another important property of the EnantioPac® column is that it enables the resolution of many basic and acidic drug substances without the need for derivatization.

The above mentioned properties are well illustrated in the present study which describes the use of the EnantioPac® column for the direct resolution of racemic α-methylarylacetic acid anti inflammatory agents and some weaker acids, without derivatization. Some of the α-methylarylacetic acids have been separated using another chiral phase (9). However, the preparation of amide derivatives was a prerequisite for obtaining a resolution using that phase. Furthermore, 1.23 was the highest separation factor obtained and in most cases only partial resolution was achieved.

EXPERIMENTAL

Apparatus

The chromatographic system contained a LKB 2150 HPLC pump, a Waters model U6K injector and a Shimadzu SPD-2A UV detector with variable wavelength, operating at 215 nm. For pH measurements an Orion Research model 701, equipped with an Amagruss pHc-1016 electrode was used. A Klein thermostate K4 from Lauda thermostated the HPLC system.

Chemicals

The RAC 109 enantiomers were kindly supplied by Dr R Sandberg, Astra Läkemedel AB, Södertälje, Sweden. Racemic ketoprofen was a gift from AB Leo Rhodia, Helsingborg, Sweden. Racemic disopyramide, bupivacaine, naproxen, ethotion, hexobarbital and bendroflumethiazide were obtained from drug manufacturers. Mandelic acid, mandelic acid methyl ester and mandelic acid ethyl ester were from Sigma, Missouri, U.S.A. and 2-phenoxypropionic acid was from Schuchardt, München, Germany. The structures of the substances are presented in Table 1. N,N-dimethyloctylamine, DMOA, was obtained from ICN Pharmaceuticals, Inc, Plainview, N.Y., U.S.A. All other chemicals used were of analytical grade.

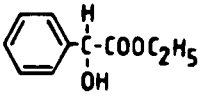
Chromatographic technique

The column used was either a chiral 100 x 4.0 mm (l x i.d) EnantioPac® column (particle size ~10 μm), developed by Hermansson (3, 4) and now available from LKB, S-161 25 Bromma, Sweden,

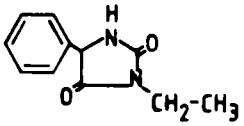
TABLE 1.

Chemical Structures

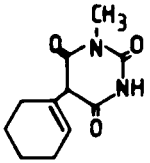
Mandelic acid ethyl ester



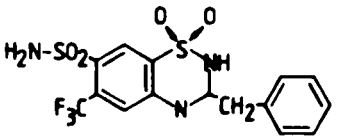
Ethotoin



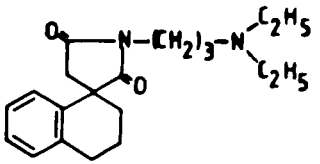
Hexobarbital

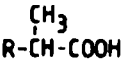


Bendroflumethiazide



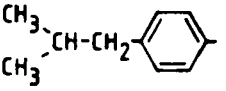
RAC 109



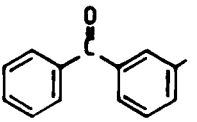


R

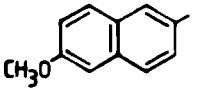
Ibuprofen



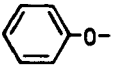
Ketoprofen



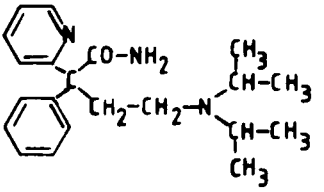
Naproxen



2-Phenoxypropionic acid



Disopyramid



or a 100 x 3.0 mm α_1-acid glycoprotein column (α_1-AGP) (particle size ~13 µm) prepared in our laboratory. The mobile phases were prepared by dissolving appropriate concentrations of amine in an aqueous solution of sodiumdihydrogen phosphate. After adjusting pH with sodiumhydroxid 2-propanol was added. The final concentration of phosphate and 2-propanol were 20 mM and 0.5% (v/v), respectively, unless otherwise stated in the figure or table text. The mobile phases were degassed in an ultrasonic bath before being used. When the temperature effect on the capacity factors and the separation factors was examined, the mobile phase reservoir was kept in a thermostated water bath. The column was thermostated by pumping water from the water bath through a glass-jacket mounted on the column. All the capillary tubes were insulated. In all other cases the chromatographic experiments were performed at room temperature. The retention volume for an unretarded compound, Vm, was determined by injecting destilled water or mobile phase with a slight difference in composition.

RESULTS AND DISCUSSION

It has been demonstrated in previous papers (3-7) that the α_1-AGP column (now available as EnantioPac®) is an effective tool for the direct resolution of amines and non-protolytic compounds. This paper demonstrates that this column also can be used for the resolution of racemic acids of different strength (pK_a-values 4-8.5).

Regulation of the retention of uncharged acidic compounds

The retention of the enantiomers of bendroflumethiazide and mandelic acid ethyl ester can be regulated by addition of N,N-

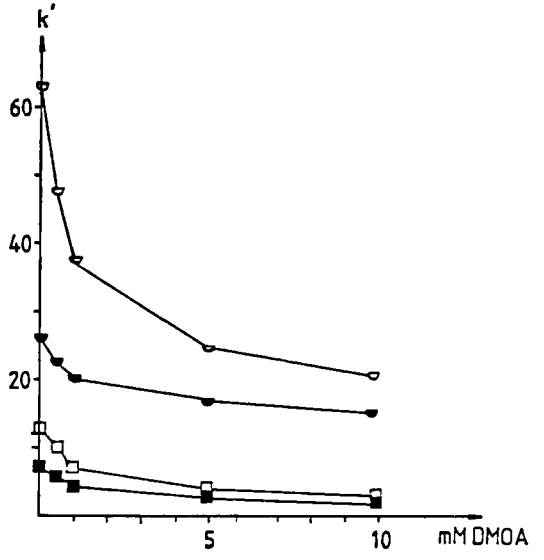


Fig. 1. Influence of the DMOA concentration on the capacity factors. Column: EnantioPac. Mobile phase: phosphate buffer pH 7.0 containing 0.5% (v/v) 2-propanol and different amount of DMOA. Flow-rate: 0.9 ml/min. Samples: ◡ bendroflumethiazide I, ◡ bendroflumethiazide II, ■ mandelic acid ethyl ester I and □ mandelic acid ethyl ester II.

dimethyloctylamine (DMOA) to the mobile phase which is demonstrated in Fig. 1. Bendroflumethiazide is a weak acid with pK_a= 8.53 (10), which means that this compound is mainly present in uncharged form at pH 7.

The retention of the enantiomers of bendroflumethiazide and mandelic acid ethyl ester decreases strongly with DMOA concentrations up to about 1 mM. DMOA concentrations >5 mM influences the capacity factors to a limited extent. Similar effects have been observed previously for phosphate ion-pairs of cationic compounds using either non-chiral reversed phase columns (11) or an α_1-AGP column (4).

This effect is assumed to be due to that the solutes are retained at two different sites on the stationary phase. One site, A_s, where DMOA (Q^+) competes with the solute (S) for

adsorption and another site, A_s^*, where the adsorption of DMOA (Q^+) can be neglected. If it is assumed that DMOA is adsorbed as phosphate ion-pair (QZ) the distribution of (QZ) to the stationary phase can be expressed by

$$Q^+_m + Z^-_m + A_s \rightleftarrows QZA_s$$

and the equilibrium constant for the process is given by

$$K_{QZA_s} = \frac{QZA_s}{[Q^+]_m \times [Z^-_m] \times [A]_s} \quad (1)$$

The equilibrium constant for the distribution of the solute to the stationary phase can be expressed in analogy with eq. 1.

The adsorption capacity of the sites, K_o and K^*_o (moles/g), is limited and can be expressed by

$$K_o = [A]_s + [SA]_s + [QZA]_s \quad (2)$$

and

$$K^*_o = [A^*]_s + [SA^*]_s \quad (3)$$

$[SA]_s$, $[SA^*]_s$ and $[QZA]_s$ are the concentrations (moles/g) of adsorbed uncharged solute and the phosphate ion-pair of DMOA, respectively. $[A]_s$ and $[A^*]_s$ are the number of available adsorption sites expressed in moles/g solid phase.

The capacity factor of the sample, k', depends on the phase ratio on the column, q (g/l) and the distribution ratio of the solute between the stationary and the mobile phase according to

$$k' = q \left(\frac{[SA]_s + [SA^*]_s}{[S]_m}\right) \quad (4)$$

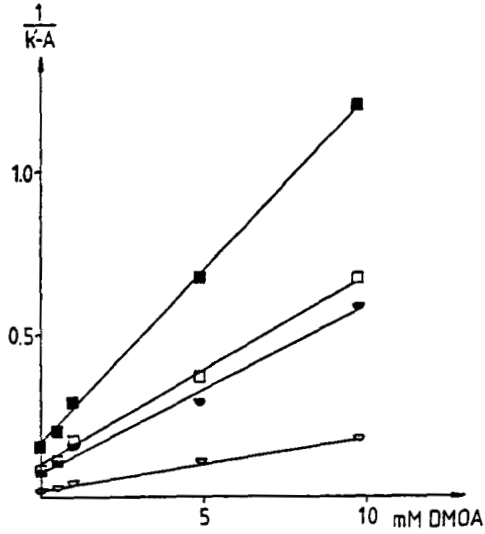


Fig. 2. Plots according to eq. 7. Conditions and samples as in Fig. 1.

A combination of eqs. 1 - 4 and the expressions for the distribution of the solute to the stationary phase gives the following expressions of k'

$$k' = \frac{q \times K_o \times K_{SA_s}}{1 + K_{SA_s} \times [S]_m + K_{QZA_s} \times [Q^+]_m \times [Z^-]_m} + \frac{q \times K^*_o \times K^*_{SA^*_s}}{1 + K^*_{SA^*_s} \times [S]_m} \quad (5)$$

As the peak symmetry was good, indicating that the sample concentration does not affect the capacity factor, eq. 5 can be reduced to

$$k' = \frac{q \times K_o \times K_{SA_s}}{1 + K_{QZA_s} \times [Q^+]_m \times [Z^-]_m} + q \times K^*_o \times K^*_{SA^*_s} \quad (6)$$

The experiments were performed at constant phosphate concentration. Substitution of $q \times K^*_o \times K^*_{SA^*_s}$ with A and rearrangement and linearization of eq. 6 by inversion gives

$$\frac{1}{k' - A} = \frac{1}{q \times K_o \times K_{SA_s}} + \frac{K_{QZA_s} \times [Q^+]_m \times [Z^-]_m}{q \times K_o \times K_{SA_s}} \quad (7)$$

The retention data of the uncharged enantiomeric pairs were plotted according to eq. 7 (See Fig. 2). Values of A giving a straight line relationship between 1/k'-A and the DMOA concentration, $[Q^+]_m$, were obtained by testing.

The linearity of the plots in combination with the agreement between the $K_{QZAs} \times [Z^-]_m$ constant, calculated from the slopes

TABLE 2.

Constants calculated from the plots in Fig. 2.

Sample	$q \times K_o \times K_{SA_s}$	$q \times K^*_o \times K^*_{SA_s}$	$K_{QZA_s} \times [Z^-]_m$
Mandelic acid ethyl ester I	5.89	0.800	624
Mandelic acid ethyl ester II	10.8	1.10	635
Bendroflumethiazide I	12.0	13.5	597
Bendroflumethiazide II	40.0	14.8	620

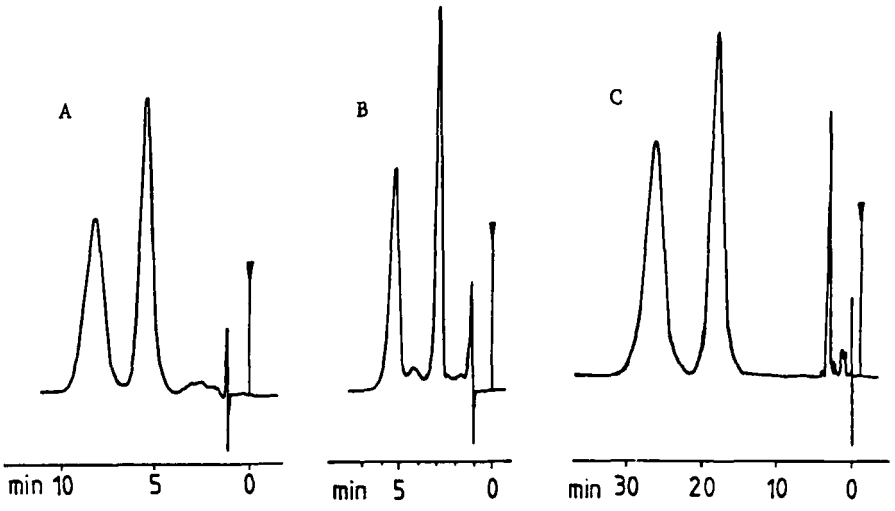


Fig. 3 A - C. Resolution of racemic weak acids on the chiral EnantioPac. Flow-rate: 0.9 ml/min. A. Hexobarbital. Mobile phase: as in Fig. 1. with 0.98 mM DMOA. B. Ethotoin. Mobile phase: phosphate buffer pH 7.15 (μ= 0.02) 1% 2-propanol. C. Bendroflumethiazide. Mobile phase: as in Fig. 1. with 4.9 mM DMOA.

and the intercepts from the four lines, indicates that eq. 7 is an acceptable approximation of the relation between the capacity factor for the uncharged solutes and the DMOA concentration. Constants estimated from the curves are summarized in Table 2. The separation of the enantiomers of bendroflumethiazide and two other weak acids, hexobarbital and ethotoin is demonstrated in Figs. 3 A-C.

Regulation of the retention and the enantioselectivity of charged acids

The retention of ibuprofen, naproxen, ketoprofen and 2-phenoxypropionic acid, with pK_a-values ~4.5 (12, 13) can also be regulated by the DMOA concentration in the mobile phase which is

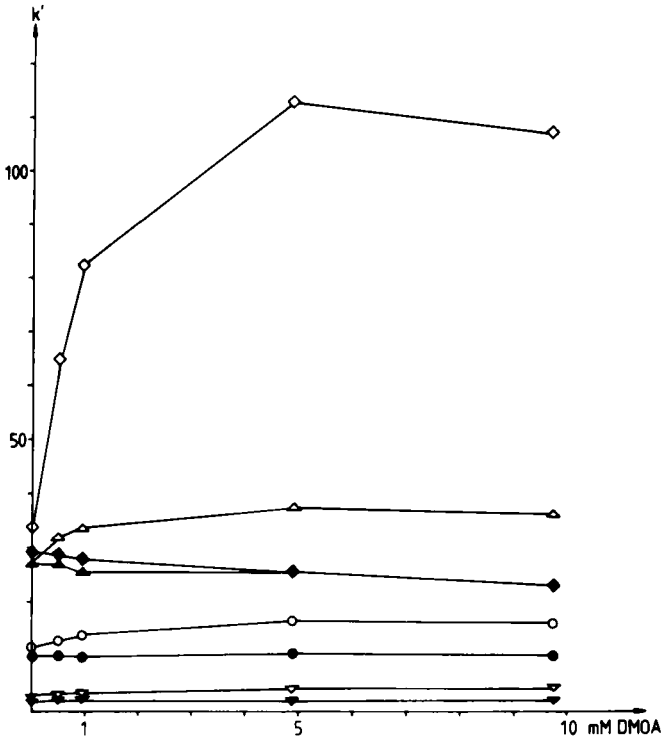


Fig. 4. Variation of the retention with the DMOA concentration in mobile phase. Conditions as in Fig. 1. Samples: ▼ 2-phenoxypropionic acid I, ▽ 2-phenoxypropionic acid II, ● ibuprofen I, ○ ibuprofen II, ▲ ketoprofen I, △ ketoprofen II, ◆ naproxen (-), ◇ naproxen (+).

demonstrated in Fig. 4. The capacity factors of one of the enantiomers in each of the four enantiomeric pairs increase with increasing DMOA concentration, whereas the capacity factors for their antipodes are almost unaffected or decrease slightly. It can also be noticed that the separation factors of the stronger acids are very low without DMOA in the mobile phase (See Fig. 5), i.e. with only sodium as counter ion. Addition of DMOA to the mobile phase drastically increases the separation factors (α) for

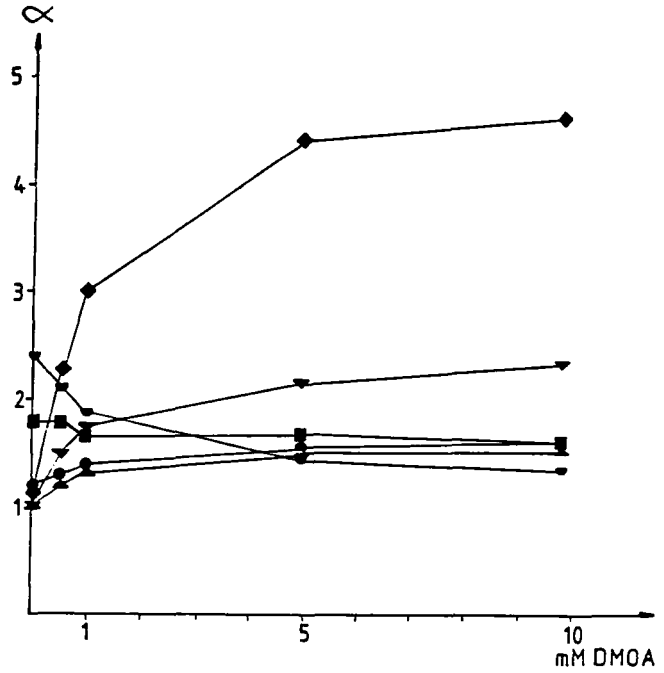


Fig. 5. Effect of the DMOA concentraion on the separatio-factors α. Condition as in Fig. 1. Samples: ▼ 2-phenoxypro-pionic acid, ▲ ketoprofen, ● ibuprofen, ■ mandelic acid ethyl ester, ◒ bendroflumethiazide, ⬤ naproxen

the stronger acids, whereas the separation factors for the uncharged solutes with no possibility to form DMOA ion-pairs, decrease with increasing DMOA concentration.

These observations, together with the fact that the retention of only one enantiomer in each pair can be regulated by the DMOA concentration may indicate that the solutes are distributed as ion-pairs with DMOA to the stationary phase. Furthermore, it indicates that the bulky DMOA ion-pair of one enantiomer in each pair fits better in the chiral active site of the protein due to sterical reasons.

The separation of the enantiomers of 2-phenoxypropionic acid and ibuprofen is demonstrated in Figs. 6 A-B.

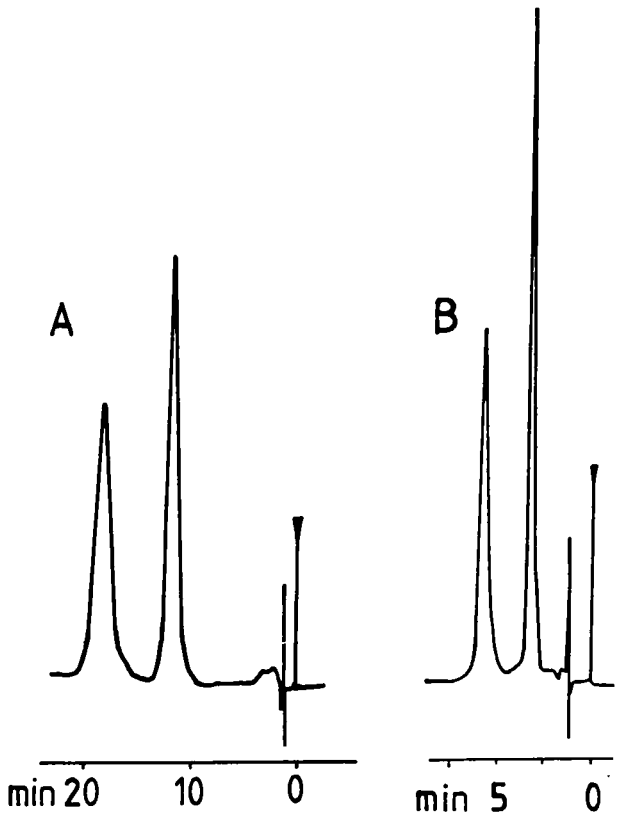


Fig. 6 A - B. Resolution of racemic acids. Column: EnantioPac. Flow-rate: 0.9 ml/min. A. Ibuprofen. Mobile phase: as in Fig. 1. with 9.8 mM DMOA. B. 2-phenoxypropionic acid. Mobile phase: as in Fig. 1. with 4.9 mM DMOA.

Regulation of the retention and the enantioselectivity with pH

A variation of the pH of the mobile phase between 3.9 and 7.0 influences the retention and the separation factors (α) for both uncharged solutes, i.e. mandelic acid esters, and acids with pk_a-values in the range 4 to 8.5 which is demonstrated in Table 3. As expected the retention of 2-phenoxypropionic acid, ibuprofen, ketoprofen and naproxen, increases with decreasing pH,

TABLE 3

pH influence on the capacity factors and the separation factors. Column: EnantioPac. Mobile phase: Phosphate buffer of different pH containing 1% 2-propanol. (The final concentration of phosphate was 6.6 - 10 mM).

Sample	pk_a (ref.)	pH= 3.92		pH= 4.95		pH= 6.02		pH= 7.02	
		k'**	α	k'**	α	k'**	α	k'**	α
Bendroflumethiazide	8.53 (10)	16.4	1.46	18.5	1.67	20.4	1.89	22.9	2.16
Hexobarbital	8.5 (14)	2.95	1.34	3.62	1.53	4.43	1.77	5.24	2.13
Ethotoin	- (15)	1.70	2.13	1.69	2.18	1.82	2.11	1.74	2.11
Mandelic acid	3.45 (16)	17.8	1.00	10.24	1.00	4.63	1.00	2.11	1.00
Mandelic acid methyl ester	-	1.36	1.21	1.71	1.32	1.84	1.34	2.05	1.33
Mandelic acid ethyl ester	-	2.76	1.51	3.70	1.64	3.99	1.68	4.53	1.72
Naproxen	4.2 (12)	-	-	-	-	80.2*	1.09	41.4*	1.05
Ibuprofen	4.4, 5.2 (12)	-	-	85.7	1.19	27.3	1.14	11.7	1.00
Ketoprofen	4.75 (13)	-	-	-	-	66.8	1.00	29.2	1.00
2-phenoxypropionic acid	-	37.9	1.08	17.9	1.00	6.34	1.00	0.945	1.00

* (-)-isomer

** capacity factor for the first eluted enantiomer

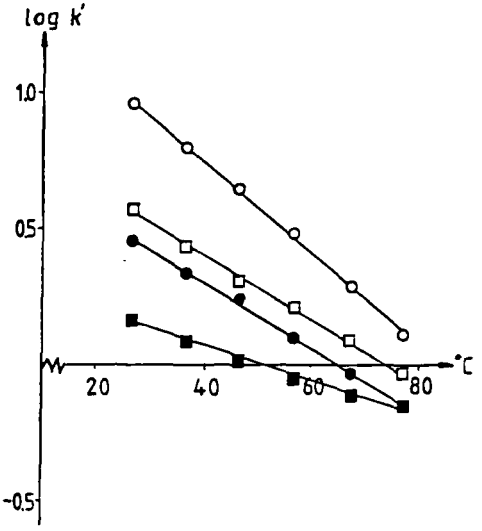


Fig. 7. Regulation of the retention with the temperature. Column: α_1-AGP column 100 x 3.0 mm I.D.) with 190 mg protein per gram solid phase. Mobile phase: phosphate buffer pH 7.13 μ= 0.02, containing 7% v/v 2-propanol. Flow-rate: 0.5 ml/min. Samples: ● disopyramide (R), ○ disopyramide (S), ■ RAC 109 I, □ RAC 109 II.

whereas the retention for the esters and the weaker acids (pk_a ~8.5), chromatographed in uncharged form, decreases with decreasing pH of the mobile phase. This indicates that the chiral phase, with many proteolytic functions (17), changes properties with a change of the pH.

The separation factor, α, is also affected by the pH of the mobile phase. The enantioselectivity of hexobarbital and bendroflumethiazide is improved with increasing pH, whereas the separation factors for ethotoin and the mandelic acid ethyl esters were almost unaffected by the pH.

Temperature variation

The influence of the temperature on the capacity factor and the resolution (R_s) was investigated using two racemic tertiary

amines, disopyramide and RAC 109 as model compounds. Fig. 7 demonstrates the relation between the logarithm of the capacity factor and the temperature for the enantiomers of the test compounds. The capacity factors as well as the separation factors increase with decreasing temperature. Furthermore, the separation efficiency is improved at higher temperatures. However, the resolution (R_s) decreases with increasing column temperature which is demonstrated in Fig. 8.

Separation efficiency

The relation between the separation efficiency expressed as the reduced plate height, h, and the linear velocity of the mobile phase was studied using racemic 2-phenoxypropionic acid (k'_1= 1.63, k'_2= 2.92) and racemic ibuprofen (k'_1= 8.47, k'_2= 11.8). The separation efficiency decreases with increasing flow velocity and h-values ≤10 was obtained at flow velocities ≤0.17 mm/sec (See Fig. 9).

Column stability

The stability of the α_1-AGP column has been discussed previously (4). In this paper the stability studies have been extended and were performed using an α_1-AGP column (110 mg α_1-AGP/g solid phase) prepared in our laboratory. The long term stability of the column was studied during storage in phosphate buffer pH 7.0 with 6% 2-propanol for 11.5 months at room temperature. Three test compounds i.e. racemic disopyramide, RAC 109 and bupivacaine, were chromatographed before and after storage and the change of the capacity factors and the separation factors was <10%. Moreover, the column was used at elevated temperatures, up to 76.5 ^{o}C without noticeable deterioration of the column. Furthermore, pure

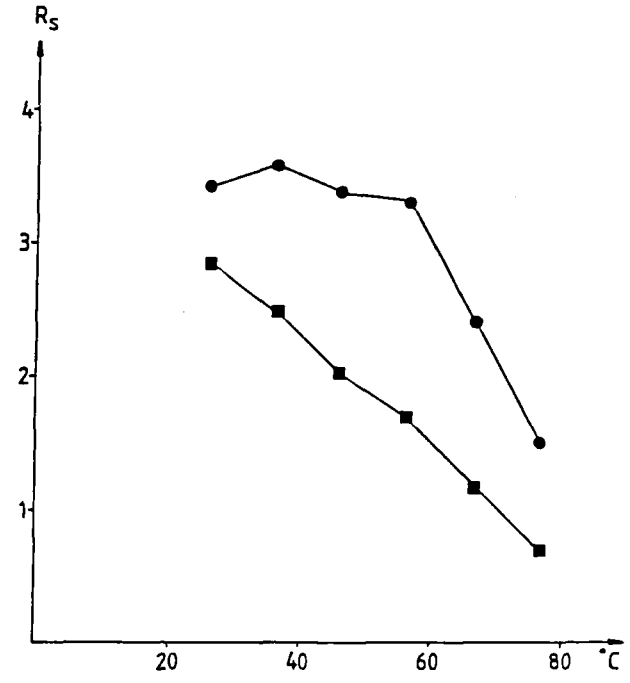


Fig. 8. Regulation of the resolution factors R_S with the temperature. Conditions as in Fig. 7. Samples: ■ RAC 109 ● disopyramide.

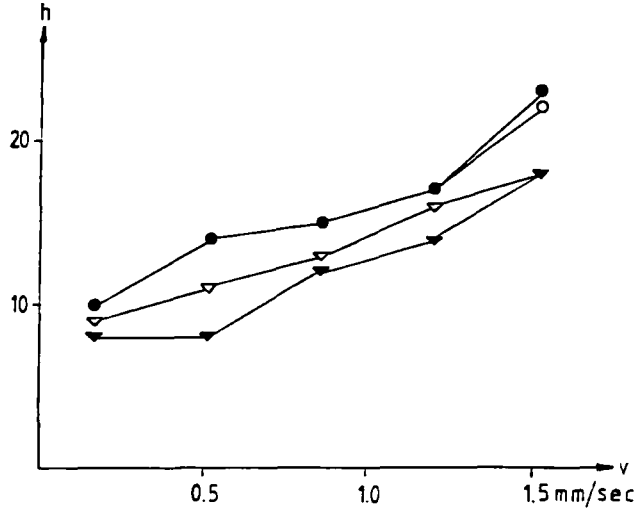


Fig. 9. Reduced plate hight (h) versus mobile phase speed (v). Column: EnantioPac, Mobile phase: as in Fig. 1. with 0.98 mM DMOA. Samples: ● ibuprofen I, ○ ibuprofen II, ▼ 2-phenoxypropionic acid I, ▽ 2-phenoxypropionic acid II.

2-propanol (180 ml) was also pumped through the column under a 14 h period without ruining the separation properties of the column.

It is well known that amine additives in combination with a high pH and a low content of organic modifier in the mobile phase decrease the lifetime of reversed phase columns. The same effect has been observed for the protein column.

However, the α_1-AGP column (EnantioPac®) shows very good stability. The column can, if necessary, operate with mobile phases with a high content of propanol or other organic modifiers (4), at elevated temperatures and at different mobile phase pH.

Moreover, the α_1-AGP column (EnantioPac®) has been used for the direct resolution of many racemic drugs from different compound classes, such as amines (4, 7, 8) and acids without the need for derivatization.

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