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Journal of Chromatography A, 866 (2000) 15–23

JOURNAL OF CHROMATOGRAPHY A

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Enantiomeric separation of amino alcohols on protein phases using statistical experimental design A comparative study

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Received 23 July 1999; received in revised form 27 September 1999; accepted 28 September 1999

Abstract

Two LC supports often used for giving enantioselective retention were tested and compared in the reversed-phase mode using statistical experimental design. The two supports contain two different proteins, α_1-acid glycoprotein or cellulase immobilised to silica particles, as the chiral selectors. The two chromatographic columns are commercially available as Chiral-AGP and Chiral-CBH. Twelve closely structurally related amino alcohols were used as the testing solutes. For each column three important mobile phase descriptors, that improve the chiral recognition, were chosen as variables and retention and separation factors were used as responses. All the tested solutes were separated using the two protein based supports. However, the highest enantioselectivities, i.e., separation factors higher than 10 were obtained using the Chiral-CBH column. The solute structure, e.g., distance between the nitrogen atom and the chiral carbon atom, and position as well as type of substituent in the aromatic ring highly influence the enantioselectivity on both columns. For one of the solutes the choice of mobile phase composition could be used to control the retention order of the two enantiomers.

Keywords: Enantiomer separation; Amino alcohols; Protein phases

1. Introduction

Liquid chromatography (LC) is well known as an excellent method for separating and analysing mixtures of stereoisomers and normal-phase [1] as well as reversed-phase [2] LC is commonly used for this purpose.

Enantiomers have identical chemical and physical properties in an isotropic environment, however, they often exhibit significant differences in interactions with other chiral species.

In LC the enantiomers can be directly separated by using a chiral mobile phase additive (CMPA) [3] or by using a chiral stationary phase (CSP) [4]. Enantiomers are separated due to differences in adsorption properties to the chiral selector when using CSPs. A number of proteins have been used for this purpose e.g., α_1-acid glycoprotein [5], human serum albumin [6], α-chymotrypsin [7], ovomucoid [8] and cellulase [9]. In these publications the influence on enantioselective retention of several mobile phase parameters, e.g., type and concentration of mobile

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)01040-7

phase additives, mobile phase buffer pH, column temperature and ionic strength were studied.

In the present work a set of structurally closely related amino alcohols were studied regarding enantioselective retention on two protein based supports, i.e., Chiral-AGP and Chiral-CBH. A screening of mobile phase parameters for the two columns resulted in three descriptor variables to study for each chiral support. The influence of mobile phase buffer pH and column temperature were studied using both the chiral supports. These variables and the ionic strength using the Chiral-AGP column and concentration of 2-propanol for the Chiral-CBH column were examined using statistical experimental design. Screening as well optimisation experiments regarding chromatography and by using chemometrics have previously been presented in the literature [10,11].

Differences in enantioselective retention were observed between the two tested chiral stationary phases. Interestingly, reversal of the retention order was obtained when altering the concentration of 2-propanol in the mobile phase and also by changing the column temperature for the Chiral-CBH stationary phase.

2. Experimental

2.1. Instrumentation

The chromatographic system consisted of a Binary LC pump 250 (Perkin-Elmer, Norwalk, CT, USA), an AS-3000 autosampler (Spectra-Physics Analytical, San Jose, CA, USA), and a LC detector Chrompack UV–Vis (Chrompack, The Netherlands). The Chiral-AGP column (150×4.0 mm, 5 μm), consisting of α_1-acid glycoprotein as the immobilized protein and the Chiral-CBH column (150×4.0 mm, 5 μm), consisting of cellulase as the immobilised protein, were purchased from ChromTech (Stockholm, Sweden). The temperature of the column and solvent reservoir was maintained by a waterbath (Grant LTD 6; Cambridge, UK). The mobile phase flow-rate was kept constant at 1.0 ml min^{-1}. The analyte solutions, injected twice, and the mobile phases were all freshly prepared. The solutes were detected at 272 nm unless otherwise stated. The injection volume was 20 μl and the sample concentration was around 0.1 m*M* for all the solutes.

2.2. Chemicals

Acetonitrile, 2-propanol, sodium acetate, acetic acid, sodium dihydrogenphosphate and disodium hydrogenphosphate (all analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). All the solutes, structures shown in Fig. 1, were synthesised at the department of Medicinal Chemistry at AstraZeneca R&D (Mölndal, Sweden).

2.3. LC methods

The retention factor, k was defined as $k=t_r/t_0-1$ where t_0 was the transport time from injection to the detector cell by a non-retained component. t_0 was calculated from the first disturbance of the baseline obtained after injection (0.7 min). The separation factor α was calculated by the k for the later eluting enantiomer over k for the faster eluting enantiomer.

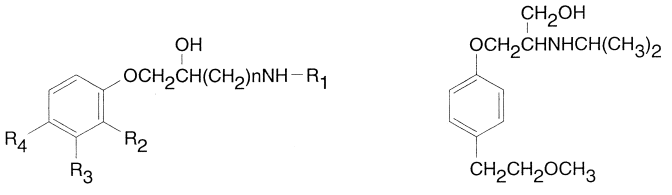
2.4. Statistical methods

A 2^3 full factorial design was used to examine the influence of the descriptor variables on the chromatographic responses [12]. All designs were produced by the Modde software (version 3.0, Umetri, Umeå, Sweden) and center-point experiments in triplicate were included in order to estimate the precision of the used method. The data were evaluated by multivariate analyses using partial least-squares (PLS) [13,14] by the Modde software. The statistical models were validated by cross validation [15].

3. Results and discussion

3.1. Solute structure and enantioselective retention using Chiral-AGP or Chiral-CHB as the stationary phase

Enantioselective retention was studied on two protein phases for metoprolol and closely related compounds, solute structures in Fig. 1. The influence of three mobile phase parameters were evaluated



Solute 12

Solute. No	n	R_1	R_2	R_3	R_4
1	1	$CH(CH_3)_2$	H	H	H
2	1	$CH(CH_3)_2$	$CH_2CH_2OCH_3$	H	H
3	1	$CH(CH_3)_2$	H	$CH_2CH_2OCH_3$	H
4	1	$CH(CH_3)_2$	H	H	$CH_2CH_2OCH_3$
5	2	$CH(CH_3)_2$	H	H	$CH_2CH_2OCH_3$
6	3	$CH(CH_3)_2$	H	H	$CH_2CH_2OCH_3$
7	1	$CH_2CH_2CH_3$	H	H	$CH_2CH_2OCH_3$
8	1	$C(CH_3)_3$	H	H	$CH_2CH_2OCH_3$
9	1	$CH(CH_3)_2$	H	H	OCH_3
10	1	$CH(CH_3)_2$	H	H	C(O)H
11	1	$CH(CH_3)_2$	H	H	NO_2

Fig. 1. Solute structures.

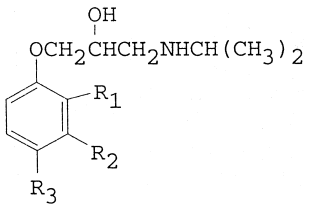
using statistical experimental design and PLS [12,13], see below.

The influence of solute structure on enantioselective retention using the two chiral stationary phases, Chiral-AGP and Chiral-CBH, is given in Tables 1–4. For both the stationary phases the same mobile phase pH and column temperature were used but acetonitrile was the mobile phase modifier used on the Chiral-AGP phase while 2-propanol was used on Chiral-CBH.

For metoprolol and its positional isomers the enantioselectivity increased in position of the substituent, *para*=none<*meta*<*ortho*, when using the CBH phase. For the AGP phase the enantioselectivity increase in order of the substituent in, none< *para*<*meta*<*ortho*, position. Interestingly, as high a separation factor as 12.4 was obtained for the solute having the substituent in the *ortho* position using the Chiral-CBH stationary phase, Table 1. For solutes differing in substituent at the nitrogen atom the same pattern was observed using the both chiral stationary phases. Enantioselectivity increase with increasing bulkiness of the alkyl group, *n*-propyl<isopropyl< *tert*.-butyl, Table 2. Also for solutes having different numbers of methylene groups between the nitrogen atom and the stereogenic centre the same pattern in enantioselectivity was obtained for the two protein phases, Table 3. Enantioselectivity decreased with the number of methylene groups, Table 3. For the solute structure, solute 12 lacking any methylene group, which include a primary alcohol function a much higher enantioselectivity was observed using the CBH phase. When altering substituents in the *para* position the Chiral-CBH phase gave high separation factors for electron donating as well as for electron withdrawing substituents, Table 4. For the Chiral-AGP phase higher separation factors were obtained for electron donating substituents, low or no separation was obtained for solutes with electron withdrawing substituents, Table 4. Even the pattern

Table 1
Solute structure and enantioselective retention[a]

A.



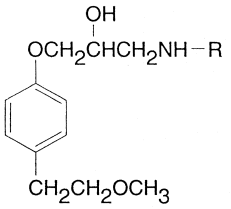
Sample No.	R_1	R_2	R_3	A		B	
				k_1	α	k_1	α
1	H	H	H	14.0	1.13	4.22	3.40
2	$CH_2CH_2OCH_3$	H	H	19.2	1.93[b]	4.53	12.4
3	H	$CH_2CH_2OCH_3$	H	27.3	1.62	3.21	5.61
4	H	H	$CH_2CH_2OCH_3$	8.18	1.41	5.21	3.37

[a] Solid phase: Chiral-AGP. Mobile phase: phosphate buffer (pH 7.4, $c=20$ m*M*)–acetonitrile (99:1). Column temperature: 20°C. Solid phase: Chiral-CBH. Mobile phase: phosphate buffer (pH 7.4, $c=20$ m*M*)–2-propanol (92:8). Column temperature: 20°C.
[b] =2% ACN.

in retention differs for the two stationary phases for all kind of solutes, Table 4. Note that the retention of electron donating substituents in the *para* position were higher using the Chiral-AGP phase while the opposite was observed using the Chiral-CBH phase for electron withdrawing substituents. In conclusion, for all the solutes higher enantioselectivities were obtained on the Chiral-CBH phase. Separation factors between 1.2 and 12.4 were obtained using the Chiral-CBH phase and between 1.1 and 1.9 using the mobile phase conditions given in Table 1.

Table 2
Solute structure and enantioselective retention[a]

B.



Sample No.	R	A		B	
		k_1	α	k_1	α
7	$CH_2CH_2CH_3$	8.80	1.21	2.73	2.60
4	$CH(CH_3)_2$	8.18	1.41	5.21	3.37
8	$C(CH_3)_3$	8.91	1.57	1.43	3.68

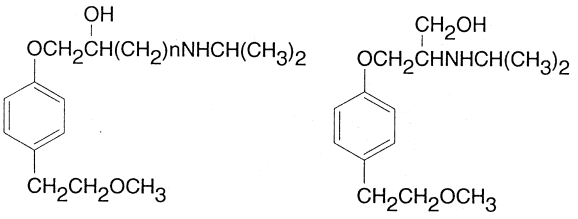
[a] Conditions as in Table 1.

3.2. Statistical evaluation and effects of the mobile phase variables on enantiomeric retention on Chiral-AGP and Chiral-CBH

The influence of three mobile phase variables were

Table 3
Solute structure and enantioselective retention[a]

C.

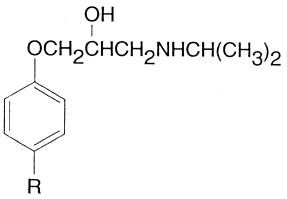


Sample No.	*n*	A		B	
		k_1	α	k_1	α
12	0	5.36	1.35	2.63	2.04
4	1	8.18	1.41	5.21	3.37
5	2	9.00	1.18	7.89	2.00
6	3	9.53	1.11	6.66	1.16

[a] Conditions as in Table 1.

Table 4
Solute structure and enantioselective retention[a]

D.



Sample No.	R	A		B	
		k_1	α	k_1	α
4	$CH_2CH_2OCH_3$	8.18	1.41	5.21	3.37
9	OCH_3	9.97	1.44	5.44	5.09
10	C(O)H	3.67	1.00	18.6	2.23
11	NO_2	5.64	1.05	26.7	3.87

[a] Conditions as in Table 1.

studied for the 12 solutes on each chiral stationary phase, Tables 5 and 6. Type of organic modifier, i.e., methanol, acetonitrile and 2-propanol, concentration of organic modifier, mobile phase buffer pH, ionic strength, column temperature and mobile phase pH were studied univariately for enantioresolution of metoprolol as screening experiments. From these studies the following parameters were chosen for the experimental design using the Chiral-AGP phase, ionic strength 20–120, mobile phase pH 5.1–7.4 and column temperature 20 to 40°C, Table 5. For the Chiral-CBH phase the content of 2-propanol (5–11%, v/v), the mobile phase pH (5.7–6.7) and the column temperature (10–30°C) were the mobile phase variables, Table 6.

The loading plots of the optimised mathematical models are given in Fig. 2. A variable located together with a response, e.g., all the capacity factors and mobile phase pH in Fig. 2A indicate that an increase in the variable increase the value of the response. If instead a response is orthogonally located to a variable, e.g., the separation factor (a5) and column temperature in Fig. 2B an increase in the variable decreases the value of the response. The closer a descriptor variable is to the origin the less is the effect on the response.

The optimised statistical model included the three linear terms and one interaction term, io*pH, for the AGP phase. The three linear terms and one quadratic term were used to optimise the statistical model using Chiral-CBH. All the solutes, except for the *para*-substituted methoxy compound show the same behaviour when altering the mobile phase parameters for the Chiral-AGP stationary phase. An increase of

Table 5
Experimental design – Chiral-AGP (X=solute No.)

Parameter	Abbreviation	Type	Settings
Ionic strength	io	Factor	20–120
pH	pH	Factor	5.1–7.4
Column temperature	Te	Factor	20–40°C
Retention factors for the first eluted enantiomer (k_1)	KXa	Responses	–
Retention factors for the last eluted enantiomer (k_2)	KXb	Responses	–
Separation factors ($\alpha = k_2/k_1$)	aX	Responses	–

Table 6
Experimental design – Chiral-CBH (X=solute No.)

Parameter	Abbreviation	Type	Settings
Content of 2-propanol	ISO	Factor	5–11% (v/v)
pH	pH	Factor	5.7–6.7
Column temperature	Te	Factor	10–30°C
Retention factors for the first eluted enantiomer (k_1)	KXa	Responses	–
Retention factors for the last eluted enantiomer (k_2)	KXb	Responses	–
Separation factors ($\alpha = k_2/k_1$)	aX	Responses	–

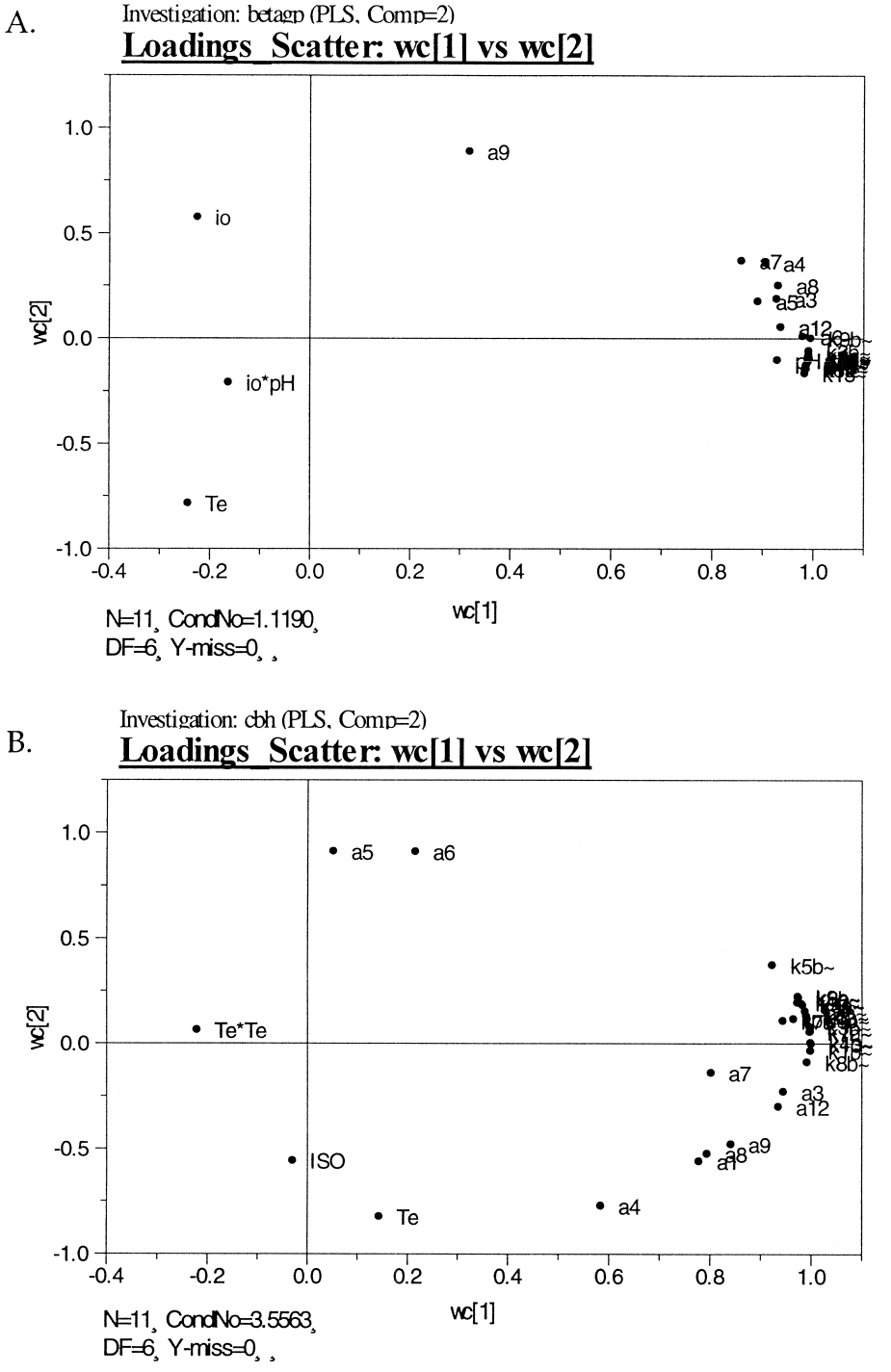


Fig. 2. Loading plot that correlates the descriptor variables to the responses, retention and selectivity factors. (A) Chiral-AGP, (B) Chiral-CBH.

mobile phase pH increases the enantioselectivities and so also the capacity factors. The ionic strength, column temperature and interaction term, io*pH, decrease the chiral recognition, Fig. 2A. The significant statistical effects for the optimised model using the Chiral-AGP phase are given in Table 7.

A more complex pattern was obtained for the Chiral-CBH phase, Fig. 2B. As for the Chiral-AGP phase increased mobile phase pH gave in general higher enantioselective retentions. However, the loading of column temperature and content of 2-propanol were confusing for some of the solutes. It seems like an increase in both these parameters can give an increase or decrease in enantioselectivities depending on solute structure. The variable giving the quadratic term could not be determined from the actual design, however, the quadratic behaviour will decrease the capacity factors as it have an opposite location in the loading plot, Fig. 2B. In order to establish which of the variables influenced the quadratic effect further experiments are needed. The variables giving statistical significant effects for the optimised model using the Chiral-CBH phase are given in Table 7.

Table 7
Variables giving significant effects on the responses

Response	Variables giving significant effects	
	Chiral-AGP	Chiral-CBH
k1a	–	ISO, pH, Te*Te
k1b	–	pH, Te, Te*Te
k3a	io, pH, Te, io*pH	ISO, pH, Te*Te
k3b	io, pH, Te, io*pH	ISO, pH, Te, Te*Te
k4a	io, pH, Te, io*pH	ISO, pH, Te*Te
k4b	io, pH, Te, io*pH	pH, Te, Te*Te
k5a	io, pH, Te, io*pH	ISO, pH, Te*Te
k5b	io, pH, Te, io*pH	ISO, pH, Te, Te*Te
k6a	io, pH, Te, io*pH	ISO, pH, Te, Te*Te
k6b	io, pH, Te, io*pH	ISO, pH, Te, Te*Te
k7a	io, pH, Te, io*pH	ISO, pH
k7b	io, pH, Te, io*pH	pH, Te, Te*Te
k8a	io, pH, Te, io*pH	ISO, pH, Te*Te
k8b	io, pH, Te, io*pH	pH, Te, Te*Te
k9a	io, pH, Te, io*pH	ISO, pH, Te, Te*Te
k9b	io, pH, Te, io*pH	ISO, pH, Te*Te
k12a	io, pH, Te, io*pH	ISO, pH, Te, Te*Te
k12b	io, pH, Te, io*pH	pH, Te, Te*Te
a1	–	PH, Te
a3	Te, pH	PH, Te
a4	Te, pH	ISO, pH, Te
a5	PH	ISO, pH, Te
a6	Te, pH	ISO, pH, Te
a7	Te, pH	ISO
a8	Te, pH	PH, Te
a9	io, Te	PH, Te
a12	PH	pH, Te, Te*Te

3.3. Reversal of retention order of amino alcohol enantiomers on Chiral-CBH

As the optimised model for Chiral-CBH shows that the separation factors of the two solutes 5 and 6 will decrease with an increase in column temperature and content of 2-propanol, Fig. 2B, these solutes were interesting to study further. The rather low separation factor for solute No. 6 indicated that a reversal of the retention order of the enantiomers was possible to achieve when altering these two variables. As the two enantiomers of solute No. 6 are not available in pure forms we use an analytical Chiral-CBH column to, in a semipreparative way, separate the two enantiomers. The mobile phase used was phosphate buffer (pH 6)–2-propanol (4:1), the column temperature was set to 20°C and a 40-μg amount was injected. The two enantiomers were given signs, I or II, depending on the retention order in the semipreparative system. Solutions with higher concentrations of II were prepared and injected using mobile phases with different concentrations of 2-propanol, Figs. 3 and 4, and also by using different

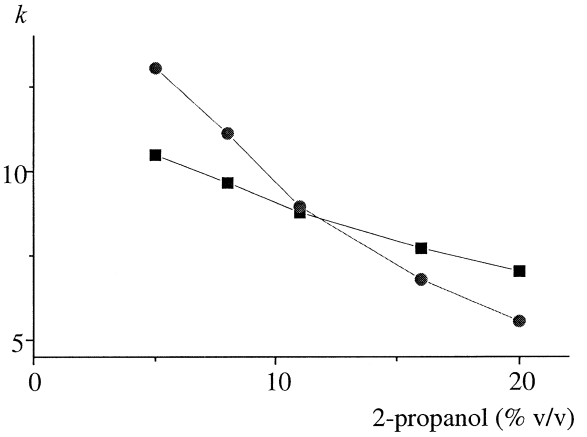


Fig. 3. Effect of 2-propanol concentration on enantioselective retention. Solid phase: Chiral-CBH. Mobile phase: phosphate buffer (pH 6.2) with *X*% (v/v) 2-propanol. Column temperature: 20°C. Solute: solute No. 6.

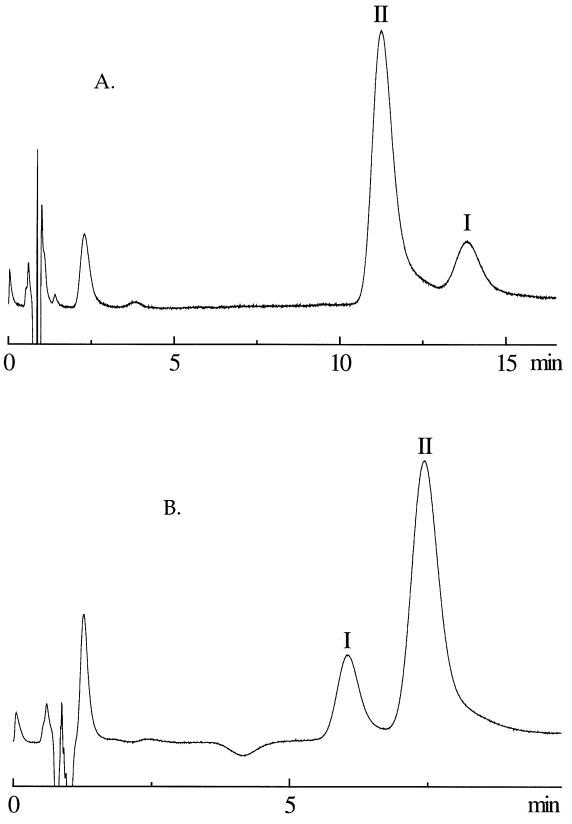


Fig. 4. Reversal of retention order by altering the 2-propanol concentration in the mobile phase. Solid phase: Chiral-CBH. Mobile phase: (A) phosphate buffer (pH 6.2)–2-propanol (95:5), (B) phosphate buffer (pH 6.2)–2-propanol (4:1). Column temperature: 20°C. Solute: solute No. 6.

column temperatures, Figs. 5 and 6. The effect of 2-propanol concentration on enantioselectivity was studied in the range 5 to 20% (v/v) at a mobile phase pH of 6.2. Enantiomer II eluted first at low concentrations of 2-propanol in the mobile phase but eluted last at higher concentrations, Figs. 3 and 4. At a concentration of about 10% (v/v) the two enantiomers coeluted, Fig. 3.

The influence of the column temperature on enantioselective retention was studied in the range 10 to 40°C using a mobile phase with a pH of 6.2 and a concentration of 8% (v/v) 2-propanol. Using low column temperatures, enantiomer II eluted first but a reversal of the retention order was obtained using a higher column temperature, Figs. 5 and 6. An increased column temperature will therefore move

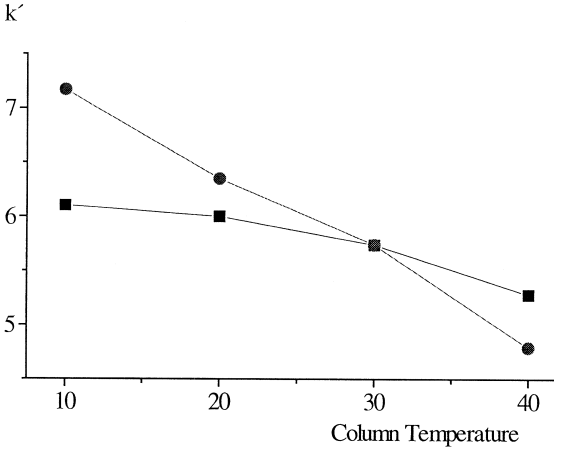


Fig. 5. Influence of column temperature on enantioselective retention. Solid phase: Chiral-CBH. Mobile phase: phosphate buffer (pH 6.2)–2-propanol (92:8). Solute: solute No. 6.

the equilibrium towards chromatographic conditions present at a high concentration of 2-propanol in the mobile phase. Explanations for the reversal in retention order by addition of 2-propanol and by changing the column temperature might be conformational changes of the protein immobilised on the silica surface and/or that the two enantiomers are retained by different retention mechanisms, i.e., adsorption to different sites. Reversal in retention order of enantiomers has previously been presented in normal-phase [16] and reversed-phase chromatography [17]. In the reversed-phase example [17] reversal in the retention order of mosapride and its main metabolite was obtained when changing mobile phase pH and column temperature using Chiral-AGP as the chiral stationary phase.

4. Conclusions

Two protein-based chromatographic silica supports, Chiral-AGP and Chiral-CBH, were tested regarding enantioselective retention. A set of 12 closely structurally related amino alcohols was used as the test compounds. All the racemic solutes were separated and the highest enantioselectivities expressed as separation factors were obtained using the Chiral-CBH column. The experiments were performed using statistical experimental design and

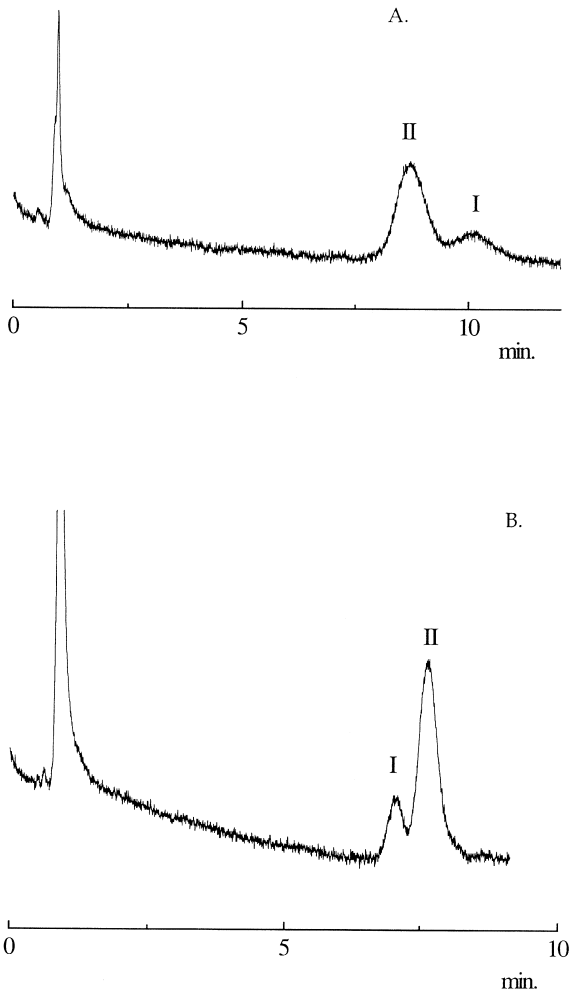


Fig. 6. Reversal of retention order by changing the column temperature. Solid phase: Chiral-CBH. Mobile phase: phosphate buffer (pH 6.2)–2-propanol (92:8). Solute: solute No. 6. (A) 10°C, (B) 40°C.

correlations between descriptor variables and chromatographic responses were evaluated using partial least-squares as regression method. The effect of changes in mobile phase buffer pH and column temperature on enantioselective retention is shown for the two chiral supports as is the effect of ionic strength for the Chiral-AGP column and and the effect of 2-propanol concentration when using the Chiral-CBH column. Minor changes in the solute structure, e.g., type of alkyl group attached to the nitrogen atom, position of substituent in the aromatic ring and distance between the stereogenic centre and the nitrogen atom have a large impact on enantioselectivity. Reversal in retention order when altering the modifier concentration and column temperature is shown.

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