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Separation of enantiomers using cellulase (CBH I) silica as a chiral stationary phase

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ABSTRACT

A new chiral stationary phase for high-performance liquid chromatography based on a glycoprotein (celllulase, CBH I) isolated from a culture filtrate of a fungus, *Trichoderma reesei*, was prepared. Chiral acidic and basic drugs were resolved into their enantiomers on this phase. Compared with other similar chiral phases, high enantioselectivity was obtained for β-blocking agents despite low capacity factors. As much as 200 nmol of propranolol in a single injection could be separated into its enantiomers on an analytical column (250 × 5.0 mm I.D.) without loss of resolution. No significant decrease in enantioselectivity was observed after daily use of the phase during a period of 4 months with varying mobile phase compositions. The retention and enantioselectivity of amines increased with increasing pH of the mobile phase, whereas the opposite effect was observed for acids. Addition of organic solvents to the mobile phase both decreased the retention and increased the enantioselectivity for the analytes.

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INTRODUCTION

Mammalian proteins immobilized on a support [1–9] or used as chiral additives to the mobile phase [10] have been utilized successfully as chiral selectors in liquid chromatographic separations of enantiomers. Other proteins, *e.g.*, microbial proteins, might also be of interest as chiral selectors. Recently, a cellulase (CBH I) produced by the fungus *Trichoderma reesei* was immobilized on silica and used as a chiral stationary phase for direct separation of some enantiomeric drugs [11].

We now report on the reproducibility of the preparation of CBH I columns and the sample capacity of the columns. However, our main interest in this study was focused on controlling the enantioselective retention of acidic, basic and uncharged analytes on CBH I–silica by adjusting the pH of the mobile phase and by addition of different kinds and amounts of organic solvents to the mobile phase. A screening of enantiomeric separations of β-blocking agents and analogues and a few other chiral drugs was performed. A brief description of the cellulases and their properties is included.

Cellulase

The degradation of cellulose is a process of major importance in nature [12] and the initial step in the process is effected by enzymes, cellulases, produced mainly by fungi and bacteria. Functionally, cellulases have been divided into two classes, endoglucanases, which attack interior non-crystalline parts of the cellulose chain, and exoglucanases, which attack the chains from the non-reducing end to produce cellobiose. The latter enzymes have also been called cellobiohydrolases. One of the most efficient cellulose-degrading organisms in nature is the mould *Trichoderma reesei*. Some of the *Trichoderma* mutants can produce very large amounts of cellulases in liquid cultures: 20 g/l is not an unusual amount [13]. *T. reesei* produces mainly four different cellulases: two endoglucanases, EG I and EG III, and two cellobiohydrolases, CBH I and CBH II. These four enzymes have a common structural organisation (Fig. 1) with a terminal 36 residue long binding domain connected to the rest of the enzyme (*i.e.*, the core) through a flexible arm. The interconnecting region is rich in serine, threonine and proline residues and is highly glycosylated. The core is catalytically active. The three-dimensional structures of both the binding domain [14] and the CBH II core [15] have been solved. Cellulases are very elongated tadpole-like structures wherein the two functional domains are separated by as much as 100 Å [16].

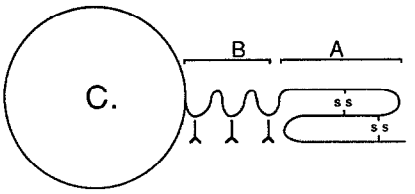


Fig. 1. Structural organization of fungal CBH. Active site is located in the core (C). B is a flexible spacer. A is a 36 amino acids long wedge-shaped peptide that anchors the enzyme to the cellulose fibre, thereby increasing its activity.

CBH I is the quantitatively dominating cellulase of *T. reesei*. It has a molecular weight of 60 000–70 000, an isoelectric point of 3.9, a carbohydrate content of 6% [17] and is stabilized by twelve disulphide bridges [18]. The binding domain of CBH I is located at the C-terminus of the enzyme [19] and the amino terminus of the core is blocked by a pyroglutamoyl moiety [17]. The *Trichoderma* CBH I gene has been characterized [20] and it is therefore possible to produce recombinant proteins with changed properties with the aim of gaining a deeper insight into chiral recognition.

EXPERIMENTAL

Chemicals

Concentrated culture filtrate from the fungus *T. reesei* chain QM9414 was a kind gift from VTT, the Technical Research Centre of Finland (Espoo, Finland). Spherical diol-silica with a particle diameter of 10 μm, pore size 300 Å, area 60 m^2/g and containing 5 $\mu mol/m^2$ of diol was obtained from Perstorp Biolytica (Lund, Sweden). Periodic acid was obtained from Merck (Darmstadt, Germany) and sodium cyanoborohydride from Janssen Chimica (Beerse, Belgium). (*R,S*)-, (*R*)- and (*S*)-propranolol chloride and (*R,S*)-pronethalol were obtained from Imperial Chemical Industries (Macclesfield, UK). (*RR,SS*)- and (*RS,SR*)-labetalol chloride were supplied by Glaxo Group Research (Greenford, UK). Racemic oxprenolol chloride and chlorthalidone

were obtained from Ciba-Geigy (Basle, Switzerland). (*R*)- and (*S*)-alprenolol tartrate, the racemates of the other amino alcohols as chlorides and racemic omeprazole were kindly supplied by Astra Hässle (Mölndal, Sweden). Racemic, (*R*)- and (*S*)-prilocaine chloride, racemic tocainide chloride, mepivacaine chloride and bupivacaine chloride were gifts from Astra Pain Control (Södertälje, Sweden). Racemic mexiletine chloride was obtained from Boehringer Ingelheim (Ingelheim/Rhein, Germany). Racemic benproperine was a gift from Pharmacia (Uppsala, Sweden). (*R,S*)-Warfarin, D- and L-N-CBZ-phenylalanine, D- and L-tryptophan and (*R*)- and (*S*)-1-phenylethanol were purchased from Sigma (St. Louis, MO, USA). (*R*)- and (*S*)-warfarin were kindly supplied by Dr. Istvan Szinai, Central Research Institute for Chemistry of the Hungarian Academy of Sciences (Budapest, Hungary). (*R*)- and (*S*)-ethyl mandelate were from Aldrich (Milwaukee, WI, USA). (+)- and (−)-α-phenylethylsulphamic acid were purchased from ICN Pharmaceuticals (Plainview, NY, USA). (+)- and (−)-trimethylnaphthylethylammonium bromide were a gift from Kabi (Stockholm, Sweden). (*R*)- and (*S*)-naproxen were obtained from Syntex Labs. (Palo Alto, CA, USA). The buffer salts and organic solvents were of analytical or reagent grade. Solute structures are shown in Fig. 2.

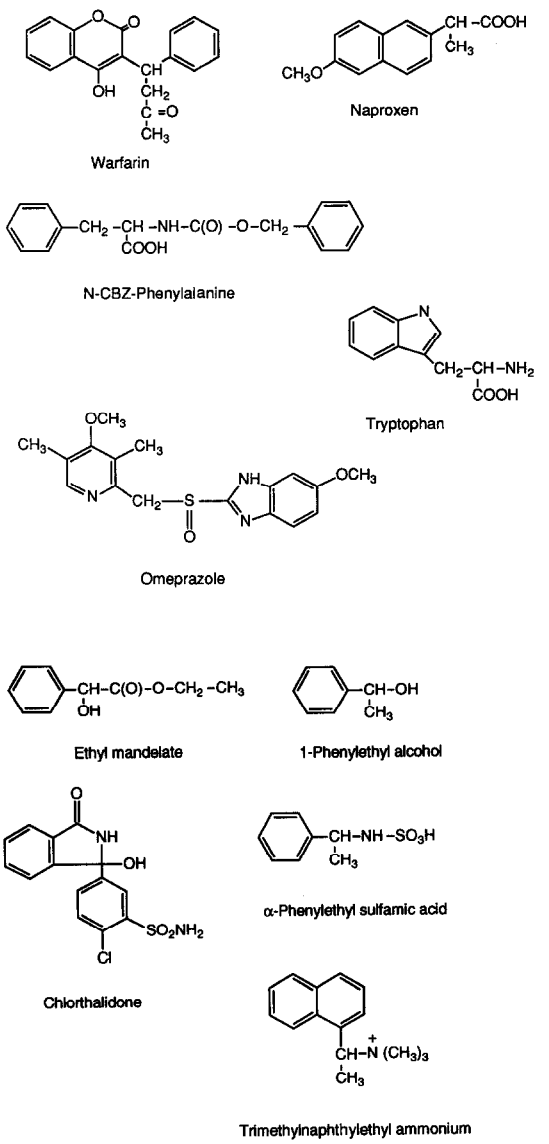


Fig. 2. Solute structures.

Isolation of CBH I from culture filtrate

CBH I was isolated from the crude concentrated culture filtrate of *T. reesei* QM 9414 by gel chromatrography on Sephadex G-25, to remove salts and pigments, followed by two chromatographic steps on DEAE-Sepharose CL-6B at pH 5.0 and 3.7 [17]. After the last chromatographic step the material was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis according to Maizel [21] and the fraction C_1 [17] that contained CBH I displayed no heterogeneity.

Circular dichoroism (CD) experiments

CD measurements were performed by use of samples prepared by diluting 1 ml of a stock solution [obtained by dissolving 52.3 mg of lyophilized CBH I in 10 ml of water purified with a Milli-Q system (Millipore, Bedford, MA, USA)] to a total volume of 5 ml with 0.1 *M* sodium phosphate buffer of pH 2.2, 3.6 or 8.1. After first having recorded the CD spectra of the samples of pH 2.2 and 8.1, the pH of these samples was then adjusted to 3.6 by titration with 1 *M* sodium hydroxide or 1 *M* phosphoric acid, respectively, and the CD measurements were repeated. All CD experiments were performed on a Jasco J-500 A spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) using a 1-mm quartz cell.

Preparation of CBH I columns

A 5-g amount of diol-silica was suspended in 30 ml of water and 0.35 g periodic acid was added. The suspension was first treated on a Sonorex (Berlin, Germany) ultrasonic bath for 1 min and then kept on a rocker table for 2–3 h. The aldehyde silica was washed on a glass filter with water. A 35-ml volume of 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.75 g of CBH I and 0.13 g of sodium cyanoborohydride was added to the wet aldehyde-silica and the suspension was treated in the ultrasonic bath for 1 min. The resulting slurry was agitated on a rocker table for 2 days and then washed with 0.1 *M* phosphate buffer (pH 7.0). The amount of immobilized CBH I was calculated by determining the UV absorbance of the CBH I solution before and after reaction with the aldehyde-silica using a Shimadzu (Kyoto, Japan) UV-160A spectrophotometer. About 30–50% of the CBH I added to the aldehyde-silica became immobilized.

Diol-silica from a single batch and four different batches of CBH I were used to prepare the solid phases. The stationary phases of columns CBH I-A and CBH I-B were made from the same preparation of CBH I. Another preparation of CBH I was used for the solid phases of columns CBH I-C and -E. The solid phases of columns CBH I-D and -F were made from another two CBH I preparations. The solid phases C and E were made on the same occasion and the other solid phases were prepared one at a time.

Columns were prepared by suspending the CBH I-silica in the phosphate buffer and were packed at 350 bar into PTFE-coated (Svefluor, Uppsala, Sweden) stainless-steel columns from Skandinaviska GeneTec (Kungsbacka, Sweden), using an ascending packing technique [22]. The column dimensions for the solid phases CBH I-A, -B, -D, and -E were 250 × 5.0 mm I.D. and for CBH I-C and -F 100 × 4.6 mm I.D.

Chromatographic apparatus

A Model 2150 dual-piston high-performance liquid chromatographic pump (LKB, Bromma, Sweden) and a Lambda-Max Model 481 LC spectrophotometer (Waters Assoc., Milford, MA, USA) connected to a Model BD 41 recorder (Kipp & Zonen, Delft, Netherlands) were used. Chromatographic data were also collected by a JCL6000 chromatographic data system (Jones Chromatography, Hengoed, UK). The injector was a Rheodyne (Cotati, CA, USA) Model 7125. The volume injected was 20 μl in all experiments except those illustrated in Fig. 3 and Table III.

Chromatographic technique

Acetate buffers were prepared from acetic acid and sodium acetate and phosphate buffers from phosphoric acid and sodium hydroxide. Prior to injection, the solutes were dissolved in the mobile phase at concentrations of about 0.1 m*M* unless stated otherwise. The experiments were performed at ambient temperature (21–25°C). The influence of temperature on retention and enantioselectivity was not studied systematically in this work. Preliminary results indicated, however, that the capacity factors and the enantioselectivity may change by about 5% in the temperature range 20–25°C. A comprehensive temperature study is in progress and will be reported separately.

The capacity factors was calculated as $k' = (V_R - V_0)/V_0$, where V_R and V_0 are the retention volumes of the solute and the non-retained compound, respectively. V_0 was obtained from the inflection point of Milli-Q-purified water unless stated otherwise. The enantioselectivity, α, was calculated as k'_2/k'_1, where k'_2 is the capacity facor of the more retained enantiomer. The peak symmetry was calculated in the following way: two tangents to the peak were drawn, and the projection of the point of intersection divided the baseline into two parts, *a*, the front side, and *b*, the rear side. The asymmetry factor, *asf*, was defined as *b*/*a*. The resolution of incompletely resolved peaks was calculated according to ref. 23. At a right-angle to the baseline a line was drawn from the baseline through the valley (the minimum) between the peaks up to a line joining the maxima of the peaks. This distance is defined as *g*. The distance between the intersection of the two lines and the valley is defined as *f*. According to this definition, the ratio $f/g = 1$ corresponds to complete separation. The resolution of completely resolved peaks was calculated by use of the equation

$$R_s = N^{1/2}k'_2(\alpha - 1)/4(1 + k'_2)\alpha$$

although the peaks were asymmetric. *N*, the peak efficiency, was calculated as $16(t_R/w_t)^2$, where t_R is

the retention time of the solute and w_t is the peak width at the baseline, *i.e.*, $a+b$.

RESULTS AND DISCUSSION

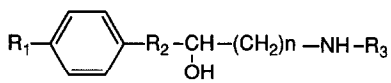
Properties of CBH I silica

Solute structure and enantioselectivity. The enantioselective retention of β-adrenergic blocking

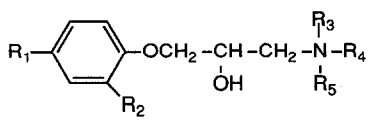
TABLE I

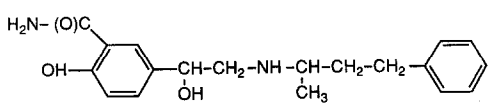
SOLUTE STRUCTURES AND STEREOSELECTIVITY OF β-BLOCKERS

Solid phase: CBH I-B. Mobile phase: 0.065 *M* 2-propanol in phosphate buffer, pH 6.0 (I = 0.01).



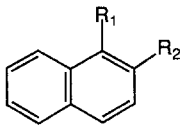
Solute No.	*n*	R_1	R_2	R_3	k'_1	α	*f/g*
1[a]	1	$-(CH_2)_2OCH_3$	$-OCH_2$	$-CH(CH_3)_2$	0.82	2.67	3.1[b]
2	2	$-(CH_2)_2OCH_3$	$-OCH_2$	$-CH(CH_3)_2$	0.51	1.90	0.98
3	3	$-(CH_2)_2OCH_3$	$-OCH_2$	$-CH(CH_3)_2$	0.37	1.50	0.89
4	1	$-OH$	$-OCH_2$	$-CH(CH_3)_2$	0.10	3.95	0.99
5	1	$-OCH_3$	$-OCH_2$	$-CH(CH_3)_2$	0.31	3.81	4.4[b]
6	1	$-CH_2CH_3$	$-OCH_2$	$-CH(CH_3)_2$	0.57	2.65	3.4[b]
7	1	$-OCH_2CH=CH_2$	$-OCH_2$	$-CH(CH_3)_2$	0.50	2.41	0.98
8	1	$-O(CH_2)_2OCH_3$	$-OCH_2$	$-CH(CH_3)_2$	0.26	1.41	0.63
9[c]	1	$-CH_2CONH_2$	$-OCH_2$	$-CH(CH_3)_2$	0.05	4.0	0.94
10[d]	1	$-NHCOCH_3$	$-OCH_2$	$-CH(CH_3)_2$	0.20	1.0	
11	1	$-(CH_2)_2OCH_3$	–	$-CH(CH_3)_2$	0.06	1.0	
12	1	$-(CH_2)_2OCH_3$	$-OCH_2$	$-H$	0.38	1.0	



	R_1	R_2	R_3	R_4	R_5	k'_1	α	*f/g*
13	$-(CH_2)_2C(O)OCH_2CH_3$	$-F$	$-CH(CH_3)_2$	$-H$		1.49	1.0	
14	$-(CH_2)_2OCH_3$	$-Br$	$-(CH_2)_2OC_6H_4$-*p*-$C(O)NH_2$	$-H$		0.70	1.0	
15[e]	$-H$	$-CH_2CH=CH_2$	$-CH(CH_3)_2$	$-H$		0.97	9.88	6.3[b]
16[f]	$-H$	$-OCH_2CH=CH_2$	$-CH(CH_3)_2$	$-H$		0.47	3.27	4.3[b]
17	$-H$	$-Cl$	$-CH_2OC_6H_4$-*p*-$C(O)NH_2$	$-H$		2.96	2.75	4.4[b]
18	$-H$	$-CH_3$	$-(CH_2)_3C_6H_5$	$-H$		1.82	3.77	2.3[b]
19	$-H$	$-H$	$-(CH_2)_2C_6H_4$-*o*-CH_3	$-H$		1.12	1.78	0.92
20	$-H$	$-H$	$-CH(CH_3)_2$	$-CH_3$	$-CH_3$	0.28	1.0	
21	$-H$	$-CH_2CH=CH_2$	$-H$	$-H$		1.56	3.47	4.4[b]
22	$-H$	$-CH_2CH=CH_2$	$-(CH_2)_2OC_6H_4$-*p*-$C(O)NH_2$	$-H$		4.53	4.08	4.7[b]
23	$-H$	$-CH_2CH=CH_2$	$-C_5H_{10}$	see R_3	$-CH_3$	0.41	1.0	
24[g] (*RR/SS*)						0.78	4.17	3.4[b]
25[g] (*RS/SR*)						0.83	1.0	

(Continued on p. 238)

TABLE I *(continued)*



Solute No.	R_1	R_2	k'_1	α	*f/g*
26[h]	$-OCH_2CH(OH)CH_2NHCH(CH_3)_2$	$-H$	2.28	5.18	5.2[b]
27	$-OCH_2CH(OH)CH_2NHCH(CH_3)_2$	$-CH_3$	2.05	1.82	2.1[b]
28[i]	$-H$	$-CH(OH)CH_2NHCH(CH_3)_2$	0.60	1.98	0.97

[a] Metoprolol.
[b] Calculated as R_s.
[c] Atenolol.
[d] Praktolol.
[e] Alprenolol.
[f] Oxprenolol.
[g] Labetalol.
[h] Propranolol.
[i] Pronethalol.

agents and analogues on CBH I-silica is summarized in Table I. An increase in the number of carbon atoms between the hydroxyl and the amino groups resulted in a significant decrease in the enantioselectivity and the capacity factors (Nos. 1–3, Table I). A similar finding with enantioseparations of amino alcohols related to metoprolol on the α_1-AGP phase has been reported by Hermansson and Schill [24]. Complete loss of enantioselectivity was observed for the amino alcohol having four methylene groups between the hydroxylic groups and the amino group. A poorer fit of the solute to the chiral binding site with increasing chain length was suggested as an explanation for the loss of enantioselectivity and this explanation may also hold true for the CBH I-silica. The enantioselectivity of the CBH I-silica is also sensitive to the substitution pattern on the aromatic ring (Nos. 4–10, 13, 15, 16 and 27, Table I). This is illustrated by the difference in enantioselectivity between atenolol (α = 4.0) and praktolol (α = 1.0) (Nos. 9 and 10, Table I). Similar effects were observed on altering the substitution of the amino group of the amino alcohol chain (Nos. 14–23, Table I). The enantioselectivity decreased when the isopropyl group of alprenolol (α = 9.9) was replaced with a hydrogen atom (α = 3.5) (Nos. 15 and 21, Table I).

The enantioselectivities of the local anaesthetic prilocaine and some analogues were also investigated (Nos. 29–34, Table II). Enantioselective retention was observed for a primary or secondary amine with an amide group close to the aromatic ring. No enantioselectivity was observed for tertiary amines in which the chiral carbon atom was contained in a ring structure or when the amide group close to the aromatic ring was replaced with a methoxy group.

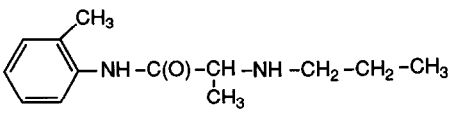
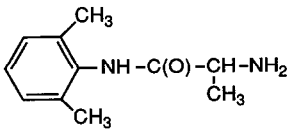
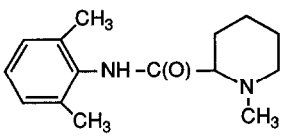
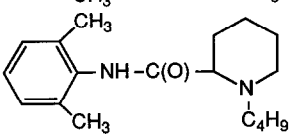
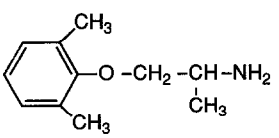
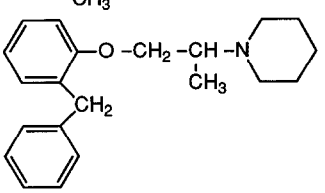
The enantiomers of the weak acid warfarin and the sulphoxide omeprazole were separable in pH intervals where these solutes are mainly uncharged (see Table VI). No enantioselective separation was observed for mono- and divalent carboxylic acids and N-phenylalanine derivatives chromatographed under corresponding conditions.

Capacity. A concentration-independent retention and peak symmetry of (*R*)- and (*S*)-propanolol, respectively, were obtained for injected amounts up to 0.01 nmol (Table III). A possible explanation for this low loading capacity might be a heterogeneous adsorbing surface caused by a low coverage of protein on the CBH I-silica phase. The analyte could thus interact not only with the chiral selector but also with some sites on the silica itself, such as unreacted silanol groups, which are characterized by relatively high equilibrium constants [25]. Hermans-

TABLE II

SOLUTE STRUCTURES AND STEREOSELECTIVITY OF PRILOCAINE AND ANALOGUES

Solid phase: CBH I-A. Mobile phase: 0.065 *M* 2-propanol in phosphate buffer, pH 6.7 (*I* = 0.01).

Solute No.	Formula	k'_1	α	*f/g*
29[a]		0.29	1.49	0.79
30[b]		0.49	1.21	0.26
31[c]		0.38	1.0	
32[d]		0.78	1.0	
33[e]		2.18	1.0	
34[f]		12.0	1.0	

[a] Prilocaine.
[b] Tocainide.
[c] Mepivacaine.
[d] Bupivacaine.
[e] Mexiletine.
[f] Benproperine.

son [26] observed an improvement in peak symmetry when the amount of immobilized α_1-AGP was increased from 76 to 183 mg of protein per gram of silica.

Although the binding isotherm of CBH I-silica is linear only at low sample loads, a large amount of racemic propranolol could be separated into its enantiomers owing to the high enantioselectivity (Fig. 3) (the column contained 75 mg of CBH I per gram of silica). When 10 nmol of racemic propranolol were injected, the retention of (*R*)- and (*S*)-propranolol decreased by 10% compared with the concentration-independent retention. The high selectivity factor for propranolol allowed the injection of as

TABLE III

CAPACITY OF (*R*)- AND (*S*)-PROPRANOLOL ON CBH I-F SOLID PHASE

Mobile phase: 0.065 *M* 2-propanol in acetate buffer, pH 4.7 (I = 0.01). Injected volume: 5 μl. Flow-rate: 1 ml/min. t_0 solute: (+)-norephedrine.

Amount injected (nmol)	(*R*)-		(*S*)-		α	R_s
	k'	*asf*	*k'*	*asf*		
$2.5 \cdot 10^{-3}$	1.06	n.d.	3.21	n.d.	3.03	n.d.
$1.2 \cdot 10^{-2}$	1.06	1.2	3.20	1.2	3.04	4.0
$2.5 \cdot 10^{-2}$	1.03	1.3	3.13	1.3	3.04	4.2
$2.5 \cdot 10^{-1}$	1.01	1.4	3.12	1.5	3.09	4.0

much as 200 nmol (60 μg) of (*R,S*)-propranolol without the loss of peak separation. Retained peak separation at high sample loads is advantageous from an analytical point of view as it increases the detection limit for an enantiomeric impurity in a sample. As the cellulases are also available in large amounts they might therefore afford preparative-scale separations of enantiomers. The retention of both (*R*)- and (*S*)-propranolol decreased at increasing sample loads, the decrease being most pronounced for the more retained enantiomer. On severe overloading, *e.g.*, 500 nmol of racemic propranolol in Fig. 3, the retention of (*S*)-propranolol approaches that of (*R*)-propranolol.

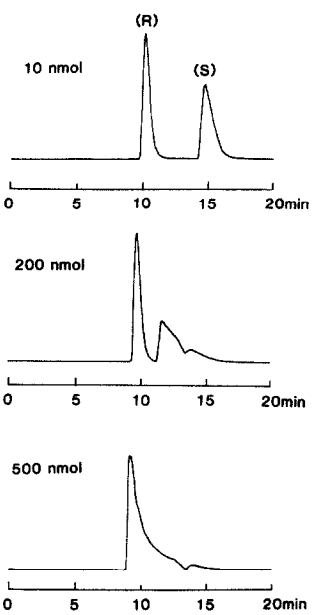


Fig. 3. Loading capacitiy of (*R,S*)-propranolol on the solid phase CBH I-E. Injected volume: 200 μl. Mobile phase: 0.065 *M* 2-propanol in acetate buffer, pH 4.7 (I = 0.01). Flow-rate: 0.5 ml/min.

Reproducibility and stability. Chiral separations obtained on three different CBH I stationary phases are presented in Table IV. Good reproducibility of enantioselectivity, retention and peak symmetry were observed for columns containing CBH I-silica prepared from the same batch of enzyme (columns CBH I-A and CBH I-B) and also from a different batch (column CBH I-E). The retention of the solutes listed in Table IV was low when an acidic mobile phase was used. Small differences in the capacity factors therefore resulted in relatively large differences in the enantioselectivity.

A CBH I column was used daily for 3.5 months at a flow-rate of 1 ml/min and at different pHs (2–8) and with different concentrations of 2-propranol (0.5–6%) in the mobile phase. A 23% change in the capacity factors of warfarin and propranolol was observed during this period, as measured with a reference mobile phase (Table V). The enantioselectivity was almost uninfluenced, but the resolution of propranolol and omeprazole decreased by 4 and 11%, respectively, owing to increased peak tailing. Similar findings were reported recently for an α_1-AGP column [27]. A possible explanation for the retention changes and the decreased peak symmetry might be a gradual loss of CBH I from the support and/or denaturation of the protein by metal ions in the system, as both enantiomers of the solutes were affected equally.

TABLE IV

COMPARISON OF CBH I SOLID PHASES

Mobile phase: 0.065 *M* 2-propanol in acetate buffer, pH 4.7 (I = 0.01). Flow-rate: 0.3 ml/min. V_0 was obtained with (+)-norephedrine.

Solute	CBH I-A				CBH I-B				CBH I-E[a]			
	k'_2	asf_2	α	f/g	k'_2	asf_2	α	f/g	k'_2	asf_2	α	f/g
Propranolol	1.26	1.9	2.6	4.2[b]	1.20	2.4	3.3	5.1[b]	1.21	1.8	2.5	5.0[b]
Alprenolol	0.83	1.9	5.5	4.8[b]	0.87	2.3	7.2	5.8[b]	0.76	2.2	4.6	5.8[b]
(*RR/SS*)-Labetalol	0.47	1.5	1.9	0.94	0.40	2.1	2.5	0.98	0.46	1.9	1.7	0.94
Oxprenolol	0.17	0.9	1.9	0.78	0.16	1.8	2.7	0.90	0.19	n.d.	1.7	0.87
No. 17, Table I	0.82	1.4	2.0	0.98	0.76	1.8	2.5	3.6[b]	0.91	2.0	1.9	3.2[b]
No. 21, Table I	0.48	1.3	2.8	0.99	0.48	1.9	3.0	3.3[b]	0.45	1.8	2.5	2.9[b]

[a] Flow-rate 0.5 ml/min.
[b] Calculated as R_s.

The solid phase used to prepare the CBH I-D column contained about 55 mg of CBH I per gram of silica, whereas the other phases contained about 75 mg/g. The retention and stereoselectivity of the CBH I-D column deviated from those of the other columns, *cf.*, propranolol in Tables IV and V. The retention of propranolol was higher and the stereoselectivity and resolution were lower on the CBH I-D phase than on the other phases. In addition, the enantiomers of warfarin were separable only on the CBH I-D column. CBH I from different batches was, however, used to prepare the solid phases. Variation of CBH I between different batches cannot be excluded as the composition of the culture medium influences the degree of glycosylation of the enzyme. This might account for the differences in the enantioselective retention of propranolol and warfarin.

TABLE V

STABILITY OF CBH I-SILICA

Solid phase: CBH I-D. Mobile phase: phosphate buffer, pH 4.8 (I = 0.01). Flow-rate: 1 ml/min.

Solute	Parameter	Volume of mobile phase (l)				
		Day 12: 5.5	Day 33: 15.5	Day 67: 31.4	Day 88: 41.4	Day 106 49.9
Warfarin	k'_2	6.11	6.05	5.36	4.92	4.65
	asf_2	4.8	4.2	4.5	4.7	5.0
	α	1.26	1.28	1.30	1.28	1.33
	f/g	0.94	0.93	0.94	0.91	0.94
Propranolol	k'_2	4.81	4.88	5.54	5.69	5.85
	asf	3.0	4.2	4.2	4.9	5.4
	α	1.42	1.43	1.42	1.42	1.34
	f/g	1.0	1.0	0.97	0.97	0.96
	R_s	2.1	2.2			
Omeprazole	k'_2	3.47	3.78	3.77	3.64	3.57
	α	1.09	1.10	1.09	1.09	1.09
	f/g	0.36	0.38	0.34	0.32	0.32

TABLE VI

INFLUENCE OF pH

Solid phase: CBH I-D. Mobile phase: phosphate buffer (I = 0.01).

Solute	pK_a[a]	Parameter	pH					
			2.2	3.5	4.7	5.6	6.8	8.1
Acids								
Warfarin	5.0	k'_2	5.58	6.92	6.11	2.45	0.89	0.18
		α	1.27	1.31	1.26	1.14	1.0	1.0
		f/g	0.92	0.94	0.94	0.16		
Naproxen	4.2	k'_2	7.73	8.11	4.24	1.24	0.55	0.14
		α	1.01	1.00	1.00	1.00	1.0	1.0
N-CBZ-phenylalanine	3.7[b]	k'_2	2.61	3.16	1.35	0.50	0.29	0.09
		α	1.00	1.00	1.02	1.0	1.0	1.0
Ampholytes								
Tryptophan	2.38, 9.39[c]	k'_2	0.06	0.15	0.14	0.18	0.24	0.34
		α	1.1	1.0	1.0	1.0	1.0	1.0
Omeprazole	4.0, 8.7[d]	k'_2	0.33	1.47	3.78	4.03	4.01	3.29
		α	1.0	1.0	1.10	1.10	1.06	1.0
		f/g			0.38	0.38	0.16	
Amines								
Prilocaine	7.9	k'_2		0.06	0.28	0.87	1.98	3.72
		α		1.0	1.0	1.0	1.22	1.19
		f/g					0.95	0.87
Propranolol	9.5	k'_2	0.69	1.37	4.81	18.8	106	339
		α	1.0	1.05	1.42	2.34	4.31	3.83
		f/g		0.38	1.0	1.0	1.0	1.0
		R_s			2.1	3.5	5.2	3.2
Uncharged solutes								
Ethyl mandelate		k'_2	0.27	0.30	0.25	0.31	0.37	0.47
		α	1.0	1.0	1.0	1.0	1.0	1.0
1-Phenylethanol		k'_2	0.23	0.26	0.21	0.26	0.34	0.42
		α	1.0	1.0	1.0	1.0	1.0	1.0
Chlorthalidone	9.4	k'_2	1.17	1.29	1.28		1.53	1.60
		α	1.0	1.0	1.0		1.0	1.0
Charged solutes								
α-Phenylethylsulphamic acid	−0.33[e]	k'_2	0.57	0.33	0.03			
		α	1.0	1.0	1.0			
Trimethylnaphthylethylammonium		k'_2	0.43	0.80	1.99		8.97	33.5
		α	1.0	1.0	1.04		1.00	1.02

[a] From ref. 28 unless indicated otherwise.
[b] pK_a of N-acetylalanine [29].
[c] Ref. 30.
[d] Ref. 31.
[e] pK_a of 1-aminoethylsulphonic acid [29].

Further improvement of the CBH I phase requires thorough control of the fermentation conditions and the work-up procedure for CBH I, optimization of the method used to immobilize the protein on the support, the kind of support used and the amount of protein bonded to the support. The influence of the CBH I loading on the chromatographic parameters is under closer investigation.

Influence of mobile phase composition on chiral separation

pH. The influence of mobile phase pH on the enantioselective retention of analytes (amines, carboxylic acids, charged and uncharged solutes) from a CBH I column was investigated (Table VI and Fig. 4). To obtain a variation of the net charge of the protein the study covered a wide pH range, from

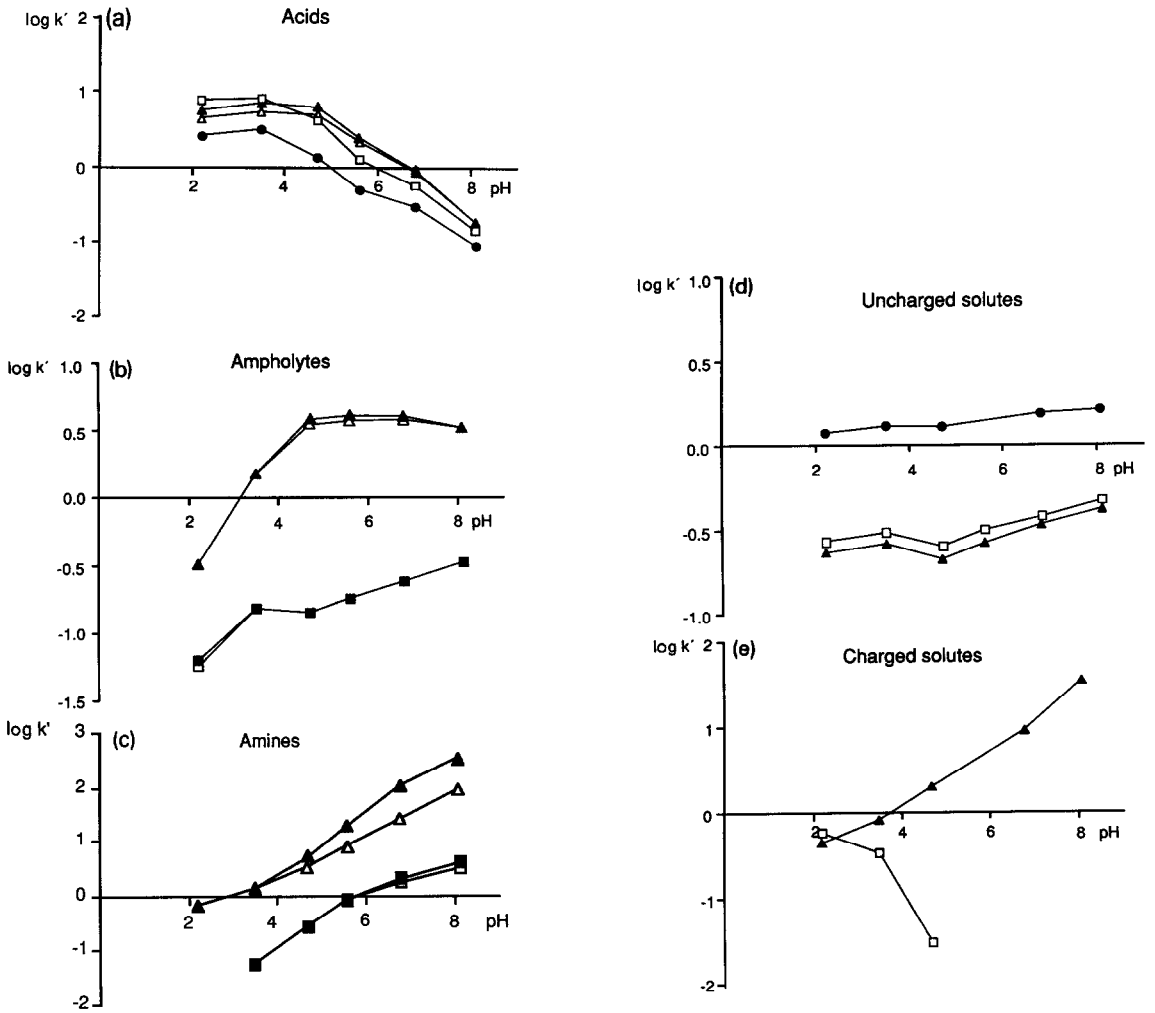


Fig. 4. (a) Influence of pH on retention and enantioselectivity of acids. Solid phase: CBH I-C. Mobile phase: Phosphate buffer (I = 0.01). □ = (*R*)- and (*S*)-naproxen; ● = L- and D-N-CBZ-phenylalanine; ▲ = (*R*)-warfarin; △ = (*S*)-warfarin. (b) Influence of pH on retention and enantioselectivity of ampholytes. Conditions as in (a). ■ = D-Tryptophan; □ = L-tryptophan; ▲ = omeprazole 2; △ = omeprazole 1. (c) Influence of pH on retention and enantioselectivity of amines. Conditions as in (a). ▲ = (*S*)-Propranolol; △ = (*R*)-propranolol; ■ = (*R*)-prilocaine; □ = (*S*)-prilocaine. (d) Influende of pH on retention and enantioselectivity of uncharged solutes. Conditions as in (a). ● = (*R,S*)-Chlorothalidone; □ = (*R*)- and (*S*)-ethyl mandelate; ▲ = (*R*)- and (*S*)-1-phenylethanol. (e) Influence of pH on retention and enantioselectivity of charged solutes. Conditions as in (a). □ = (+)- and (−)-α-phenylethylsulphamic acid; ▲ = (+)- and (−)-trimethylnaphthylethylammonium.

2 to 8. The retention of the acids decreased with increasing pH of the mobile phase. The pH dependence of the retention of the amines and the quaternary amine was opposite to that observed for acids. The retention of the uncharged solutes was almost unaffected by the pH.

The influence of pH on the conformation of CBH I was studied by circular dichroism (CD). The CD spectra showed a significant dependence on pH, indicating conformational changes of the protein (Fig. 5). Different ratios of the CD bands at 210–215 and 230 nm were obtained at pH 2.2, 3.6 and 8.1. After adjustment to pH 3.6 of the samples having initial pH values of 2.2 and 8.1, the CD spectra obtained were identical with that obtained at pH 3.6. The conformational changes occuring in CBH I over this pH range are obviously reversible. It is, open to discussion, however, whether these conformational changes also occur when the CBH I is immobilized on the silica support.

Within the pH range 3.9–8.0, the net charge of the protein is negative, whereas the amines used as analytes are predominantly protonated. The increase in retention observed for the amines and the quaternary amine on increasing the pH of the mobile phase above the isoelectric point of the protein might therefore be partly due to increased attractive electrostatic interactions between the analyte and the protein. As the mobile phase pH approached the pK_a values of the amines (Table VI), their hydrophobicities increases and this might also contribute to increased retention. As was found experimentally, one should have expected less retention of the amines at low pH as compared with that at high pH. At low pH the amines are completely protonated and CBH I also exhibits a positive net charge, *i.e.*, conditions more or less opposite to those obtaining at higher pH. The enantioselectivity of propranolol and prilocaine increasd with pH up to 6.8, but decreased slightly at pH 8.1 despite the increased retention. This behaviour might be ascribed to the kind of conformational changes of CBH I observed in the CD studies of the protein.

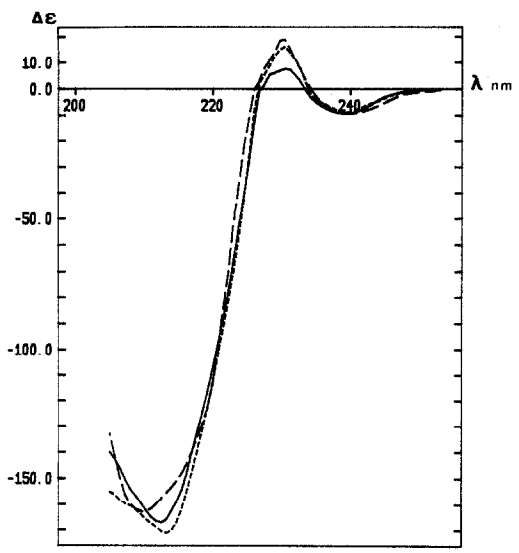


Fig. 5. Circular dicroism spectra of CBH I at (solid line) pH 2.2, (long dashed line) pH 3.6 and (short dashed line) pH 8.1, showing the conformational dependence of pH.

The retention of acids at very low pH is probably due to hydrophobic interactions, but other kinds of interactions, such as hydrogen bonding and various kinds of polar interactions, can, of course, contribute to the net retention. It is not unlikely that the small decrease in retention observed on decreasing the pH from 3.6 to 2.2 is due to conformational changes in the CBH I. The CD studies of CBH I (Fig. 5) confirmed that conformational changes actually occur at low pH values. At high pH the carboxylic groups of the acids exist as carboxylate ions and as the net charge of the CBH I at this pH is also negative a decrease in the retention could be expected. The retention of uncharged solutes, *e.g.*, carboxylic acids, at very low pH, omeprazole in the pH range 5–7.7 or permanently uncharged compounds, was almost independent of mobile phase pH. The highest enantioselectivity of warfarin and omeprazole was obtained at a pH where these solutes are uncharged. In the enantiomeric binding of these solutes to the protein, the presence of a charge on the molecules decreases the enantioselectivity, in contrast to the situation with the chiral separation of amines.

The solute retention on the CBH I silica phase is generally low. By the addition of organic counter ions to the mobile phase it may be possible to increase the retention of ionized acids and amines.

Organic modifier. The influence of the organic solvents 2-propanol, acetonitrile, tetrahydrofuran and methanol on the enantioselective retention and chromatographic performance was studied using

TABLE VII

INFLUENCE OF ORGANIC MODIFIER ON THE SEPARATION OF (*R,S*)-PROPRANOLOL

Solid phase: CBH I-C. Mobile phase: 0.78 *M* organic modifier in acetate buffer, pH 5.5 (I = 0.01). Flow-rate: 1 ml/min. Solute concentration: $5 \cdot 10^{-5}$ *M*.

Organic modifier	k'_1	k'_2	asf_1	asf_2	α	R_s
–	1.27	3.86	3.0	3.1	3.0	3.6
2-Propanol	0.62	2.46	2.3	2.4	3.9	4.0
Acetonitrile	0.62	2.46	2.3	2.6	3.5	3.9
Tetrahydrofuran	0.28	0.94	2.6	2.7	3.4	2.7
Methanol	0.95	3.21	2.6	2.6	3.4	3.7

propranolol as a model compound (Table VII). As might be expected from other studies [32], the retention of (*R*)- and (*S*)-propranolol decreases on addition of an organic solvent to the mobile phase. The enantioselectivity increased irrespective of the solvent used (Table VII) and only minor differences in enantioselectivity could be observed between hydrogen-donating and hydrogen-accepting organic solvents. A slight improvement in the chromatographic performance (*asf*) of (*R*)- and (*S*)-propranolol was observed in the presence of organic solvents (Table VII). Assuming the presence of multiple sites, the solvents probably decrease the overloading of the stationary phase by competing with the analytes for high affinity binding sites [25].

TABLE VIII

INFLUENCE OF CONCENTRATION OF 2-PROPANOL ON THE SEPARATION OF ENANTIOMERS WITH SOLID PHASE CBH I-B

Mobile phase: 2-propanol in phosphate buffer, pH 6.8 (I = 0.01). Flow-rate: 0.9 ml/min.

Solute	Parameter	Concentration of 2-propanol (*M*)			
		0.065	0.26	0.78	1.6
Metroprolol	k'_2	2.62	2.50	2.59	2.09
	asf_2	2.9	2.7	3.4	2.5
	α	2.5	2.7	3.3	4.4
	R_s	4.4	4.1	4.9	5.6
Propranolol	k'_2	33.6[a]		30.7	20.6
	asf_2	3.6		3.7	2.9
	α	4.6		6.1	6.9
	R_s	4.2		5.4	5.9
Prilocaine	k'_2	0.45	0.35	0.29	0.16
	asf_2	1.7	1.7	1.3	1.5
	α	1.8	2.0	2.0	2.1
	f/g	0.96	0.97	0.93	0.70

[a] Solid phase: CBH I-A, flow-rate, 0.3 ml/min.

In a preliminary attempt to optimize the chiral resolution of solutes on the CBH I silica phase, 2-propanol was added to the mobile phase at different concentrations (Tables VIII and IX). The enantioselectivity, the peak symmetry and the efficiency of the amines (Table VIII) were improved on increasing the concentration of the alcohol. The chroma-

TABLE IX

INFLUENCE OF THE CONCENTRATION OF 2-PROPANOL ON THE SEPARATION OF ENANTIOMERS WITH SOLID PHASE CBH I-D

Mobile phase: 2-propanol in phosphate buffer, pH 4.8 (I = 0.01). Flow-rate: 1 ml/min.

Solute		Concentration of 2-propanol (*M*)					
		0	0.13	0.26	0.39	0.52	0.78
Warfarin	k'_2	5.36	3.60	2.95	2.56	2.25	1.80
	asf_2	4.5	2.4	2.6	2.3	2.0	n.d.
	α	1.30	1.33	1.32	1.31	1.31	1.28
	f/g	0.94	0.97	0.97	0.97	0.95	0.90
Omeprazole	k'_2	3.77	2.35	1.87	1.62	1.40	1.10
	α	1.09	1.12	1.14	1.14	1.14	1.13
	f/g	0.34	0.56	0.60	0.58	0.52	0.37

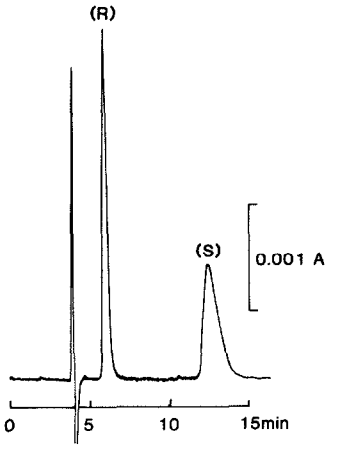


Fig. 6. Separation of (*R,S*)-metoprolol. Solid phase: CBH I-B. Mobile phase: 1.56 *M* 2-propanol in phosphate buffer, pH 6.8 (I = 0.01). Flow-rate: 1 ml/min. Solute concentration: $1.5 \cdot 10^{-4}$ *M*.

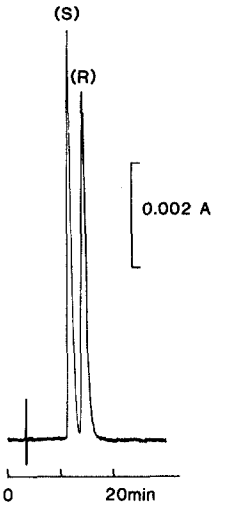


Fig. 8. Separation of (*R,S*)-warfarin. Solid phase: CBH I-C. Mobile phase: 0.26 *M* 2-propanol in phosphate buffer, pH 4.8 (I = 0.01). Flow-rate: 1 ml/min. Solute concentration: $4.6 \cdot 10^{-5}$ *M*.

tographic performance of warfarin and omeprazole (Table IX) was improved by the addition of the organic modifier whereas the enantioselectivity was almost unaffected. This is illustrated by the separation of (*R,S*)-metoprolol, (*R,S*)-prilocaine and

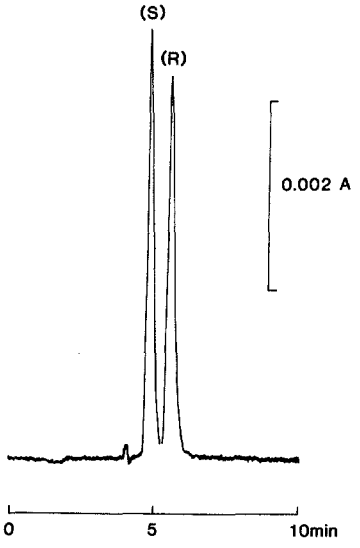


Fig. 7. Separation of (*R,S*)-prilocaine. Solid phase: CBH I-B. Mobile phase: 0.26 *M* 2-propanol in phosphate buffer, pH 6.8 (I = 0.01). Flow-rate: 1 ml/min. Solute concentration: $4.9 \cdot 10^{-5}$ *M*.

(*R,S*)-warfarin in Figs. 6–8. On the α_1-acid glycoprotein column [33,34] the enantioselectivity of solutes generally decreased with increasing concentration of organic solvents in the mobile phase.

Buffer ions and ionic strength. Phosphate buffer (I = 0.01) compared with acetate buffer at pH 4.7 gave a slightly higher stereoselectivity, peak symmetry and resolution (Table X). At pH 4.8, increasing the ionic strength of the phosphate buffer from 0.01

TABLE X

INFLUENCE OF BUFFER IONS ON THE SEPARATION OF ENANTIOMERS

Solid phase: CBH I-D. Mobile phase: buffer, pH 4.7 (I = 0.01). Flow-rate: 1 ml/min. Enantiomer concentration: $2.0 \cdot 10^{-5}$ *M*.

Solute	Parameter	Buffer	
		Acetate	Phosphate
Warfarin	k'_2	5.94	6.11
	asf_2	4.2	4.8
	α	1.26	1.26
	f/g	0.90	0.94
Propranolol	k'_2	4.65	4.81
	asf_2	3.4	3.0
	α	1.37	1.42
	f/g	0.98	1.0

TABLE XI

INFLUENCE OF IONIC STRENGTH ON THE SEPARATION OF ENANTIOMERS

Solid phase: CBH I-D. Mobile phase: phosphate buffer, pH 4.8. Flow-rate: 1 ml/min. Enantiomer concentration: $2.0 \cdot 10^{-5}$ *M*.

Solute	Parameter	Ionic strength	
		0.01	0.1
Warfarin	k'_2	4.92	5.21
	asf_2	4.7	4.5
	α	1.28	1.26
	f/g	0.91	0.91
Propranolol	k'_2	5.69	4.61
	asf_2	4.9	3.4
	α	1.42	1.59
	f/g	0.97	0.97

to 0.1 influenced the retention of the acid and base in different directions but dit not influence the resolution (Table XI). The peak symmetry and the enantioselectivity of the base improved when the ionic strength was increased.

CONCLUSIONS

The enantiomers of β-adrenergic blocking agents were separable with high enantioselectivity on the CBH I-silica phase, *e.g.*, $\alpha = 9.9$ for alprenolol. The observed enantioselectivity factors of β-blocking agents on this phase were higher than those on other similar phases such as α_1-AGP [34] and albumin [35]. The enantiomers of the local anaesthetic prilocaine, the gastric acid inhibitor omeprazole and the anticoagulant warfarin were also separated on this chiral phase.

A CBH I column was used daily for 3.5 months at a flow-rate of 1 ml/min, at different pHs (2–8) and with different concentrations of 2-propanol (0.5–6%) without changes in the enantioselectivity. However, a change in the capacity factors was observed during this period. Further, the column still showed tailing peaks after 3.5 months. Owing to the high enantioselectivity of β-adrenergic blocking agents, the loading capacity for these substances on the CBH I-phase seems to be high. A 60-μg (200-nmol) amount of racemic propranolol was almost completely resolved into the *R* and *S* enantiomers on an analytical column (250 × 5.0 mm I.D.).

The retention and the enantioselectivity of charged analytes were mainly regulated by the pH of the mobile phase. Interestingly, the enantioselectivity of the analytes increased with addition of organic solvents to the mobile phase, although the capacity factors decreased. Increasing the ionic strength from 0.01 to 0.1 improved the enantioselectivity and the peak symmetry and decreased the retention of an amine, whereas the effect on the enantioselective retention of an acid was small.

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