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Cellobiohydrolase I as a chiral additive in capillary electrophoresis and liquid chromatography

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Abstract

Cellobiohydrolase I (CBH I) was used as a chiral selector in free solution in capillary electrophoresis, applying the partial filling technique. The enantiomers of the amino alcohols oxprenolol and propranolol could be completely resolved with selector plugs of only 1.0 cm in plain acetate buffer at pH 5.0. Although warfarin migrates in the same direction as CBH I at this pH, its enantiomers were separated and detected at the anodic end of the capillary, well resolved from the protein plug. Furthermore, propranolol was enantioseparated at pH 3.0, where the protein has a positive net charge and migrates in the same direction as this amino alcohol. Addition of 2-propanol significantly improved the peak shape of overloaded peaks. CBH I was also used as a complexing agent in the mobile phase in HPLC for chiral separation of *rac*-propranolol. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Chiral selectors; Buffer composition; Mobile phase composition; Cellobiohydrolase I; Amino alcohols; Oxprenolol; Propranolol; Warfarin; β-blockers

1. Introduction

The cellulolytic enzyme cellobiohydrolase I (CBH I) from the fungus *Trichoderma reesei* has been used as a chiral selector immobilized to support particles in liquid chromatography [1–3]. It also proved to be useful as a stationary phase in capillary affinity gel electrophoresis as a co-gel with bovine serum albumin (BSA) [4,5]. A similar cellulase from the fungus *Aspergillus niger*, was used as a complexing agent in capillary electrophoresis for enantioseparation of pindolol [6].

A general feature of proteins is their ability to absorb ultraviolet light in a broad range of wavelengths, which may cause severe disturbances in the UV detection. To overcome this problem in electrophoresis, the partial filling technique was developed [7], where only a part of the capillary was filled with the selector, leaving the detection window free of proteins. The use of this technique has later been reported by others [8–12].

The primary aim of this study is to further emphasize the earlier findings (cf. [4–7]) of the usefulness of CBH I as a selector in capillary electrophoretic enantioseparations of drugs. The use of the partial filling technique with selector plugs migrating both away from and towards the detector is investigated. If the solute and the selector have different migration directions, the selector will never pass the detector and thus not give rise to any

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disturbances. If there is a sufficient mobility difference between the sample and the selector, it is also possible to separate samples which migrate in the same direction as the protein. Anodic or cathodic detection is used depending on the migration direction of the solute. Furthermore, we report on a comparison between CBH I as chiral selector in the mobile phase in liquid chromatography and in the background electrolyte in CE.

2. Experimental

2.1. Apparatus

The capillary electrophoresis was performed using a Beckman P/ACE system 2050 (Beckman Instruments, Palo Alto, CA, USA). The detection was carried out with ultraviolet absorption at 214 nm or 300 nm. The capillary measured 27 cm (effective length 20 cm)×50 μm I.D.

The chromatography was carried out using an LDC Analytical pump (Riviera Beach, FL, USA) and a Spectra Physics UV 2000 (Fremont, CA, USA) ultraviolet absorbance detector. The samples were injected in a Rheodyne Model 7125 injector (Cotati, CA, USA) equipped with a 20-μl loop. A HETO water bath (Birkerød, Denmark) was used to thermostat the chromatographic system. A Cecil 3000 series scanning spectrophotometer (Cambridge, UK) was used to quantify the CBH I solutions. A Metrohm 632 pH meter (Herisau, Switzerland) was used to measure the pH of the buffer solutions.

2.2. Chemicals

rac-, (*R*)-, (*S*)-Propranolol, *rac*-warfarin and *rac*-N-carbobenzoxy(CBZ)-phenylalanine were purchased from Sigma (St. Louis, MO, USA). (*R*)- and (*S*)-warfarin were gifts from Dr. Istvan Szinai, Central Research Institute for Chemistry of the Hungarian Academy of Sciences (Budapest, Hungary). *rac*-Oxprenolol and *rac*-alprenolol were obtained from Astra Hässle (Mölndal, Sweden). *rac*-Remoxipride, *rac*-prilocaine and *rac*-ibuprofen were from Astra (Södertälje, Sweden). *rac*-Trimethyl(naphthylethyl)-ammonium bromide was a gift from Kabi (Stockholm, Sweden). (+)-, (−)-Naproxen were from Syntex (Palo Alto, CA, USA) and (+)-, (−)-di-*p*-toluoyl-tartaric acid were purchased from Fluka (Buchs, Switzerland). Mesityloxide was obtained from Aldrich (Steinheim, Germany). Acrylamide, N,N,N′,N′-tetramethylethylenediamine and ammonium persulfate were of electrophoresis reagent purity grade and were bought from Bio-Rad (Richmond, CA, USA). γ-Methacryloxypropyltrimethoxysilane was from Sigma. Gromsil diol-silica particles with a diameter of 5 μm and a pore size of 60 Å were from Grom Analytic HPLC (Herrenberg-Kayh, Germany). All other substances were of analytical grade and used without further purification. The water was of Millipore quality.

The solute structures are shown in Fig. 1.

3. Methods

CBH I from *Trichoderma reesei* strain QM9414 was purified from the culture filtrate as described by Bhikhabhai et al. [13]. The protein was transferred to the desired buffer by size exclusion chromatography on a Sephadex PD-10 column. The concentrations of CBH I in the solutions were determined by UV absorbance at 280 nm (ϵ=78 800 $M^{-1}cm^{-1}$ [14]).

The capillaries were polyacrylamide coated as previously reported [15] in order to suppress electroosmosis and protein wall adsorption. The separations were performed at a constant voltage of 15.0 kV. The electroosmotic mobilities of the coated capillaries were measured by injection of mesityloxide and were always lower than $8\cdot10^{-6}$ $cm^2/V\,s$. The CBH I-plugs were injected by pressure (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa). The linear flow-rate at this pressure was determined for all the different protein solutions by measuring the time needed for the protein to reach the detection window. In this way the metric length of each applied protein plug was calculated. Both ends of the capillary were submerged in neat buffer during the run. Between runs, the capillary was rinsed with buffer. The reproducibility of one of the electrophoretic systems was tested by injection of ten consecutive samples, which yielded a relative standard deviation in observed mobility of 0.64%. The solutes were dissolved in buffer and diluted 100 times in water before pressure injection (0.5 p.s.i.) for 5 s. The

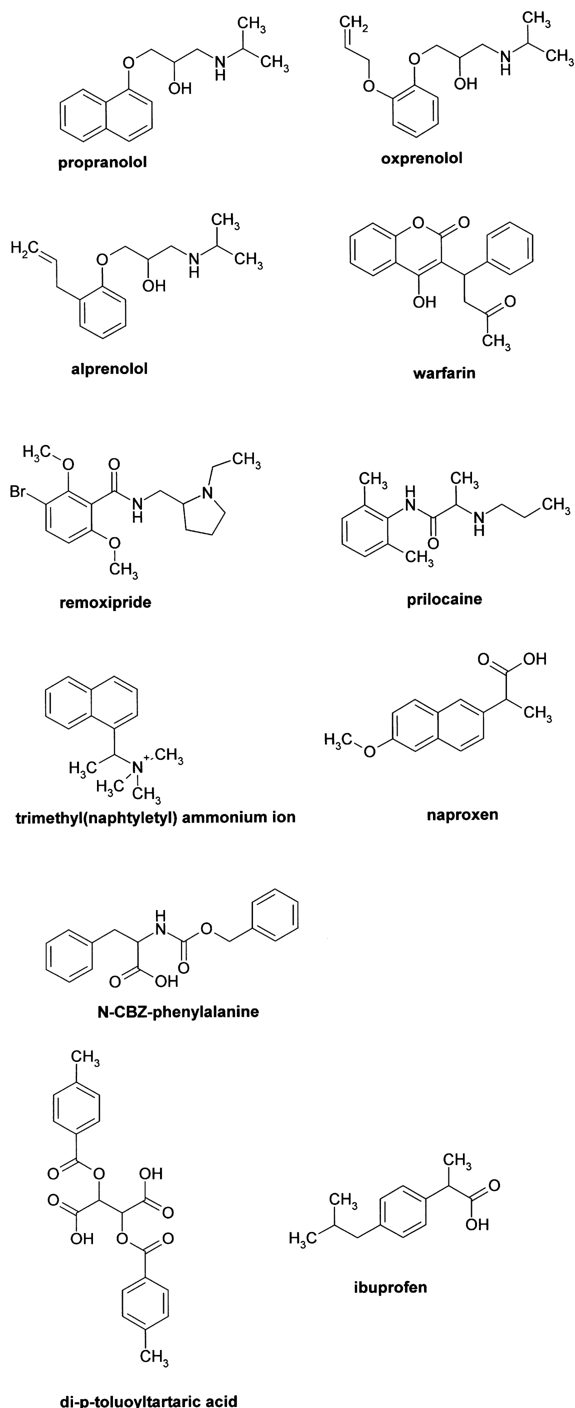

Fig. 1. Solute structures.

injected solute concentration was 10^{-5} *M* unless otherwise stated. The thermostat was set at 25.0°C. The detection was carried out on the cathodic side for basic solutes and on the anodic side for acidic compounds.

The diol silica particles for the HPLC-stationary phase were suspended in chloroform and packed into a stainless steel column of 100×0.2 mm I.D. The column was enclosed in a jacket with circulating water for temperature control at 25.0°C. The flow-rate was 0.2 ml/min.

4. Results and discussion

The use of CBH I as a chiral complexing agent in the mobile phase in HPLC was investigated. The stationary phase consisted of diol-silica particles with a pore size of 60 Å. The small pores were chosen in order to volumetrically exclude CBH I from the particle inner surface and minimize protein adsorption (cf. [16]). The mobile phase was a 65 μ*M* CBH I solution in acetate buffer at pH 5.0. The protein unavoidably passes the detector and cause severe background disturbance. A low protein UV absorption is necessary for sample detection. This restriction limits the number of possible solutes to those absorbing in the high wavelength region. It was possible to detect propranolol at 310 nm after injection of a concentration of 2 μ*M* (Fig. 2a).

The same CBH I solution (65 μ*M*) was also used in CE, applying the partial filling technique (Fig. 2b). The protein has a negative net charge at pH 5.0 (isoelectric point, p*I*=3.9 [2]) and it migrates away from the detector. This enables detection of the solute at a shorter wavelength without interference. Compared to the above mentioned HPLC method, this makes it possible to analyze a much broader range of solutes in CE. A difference in retention order between the propranolol enantiomers in CE and HPLC was obtained as expected.

The dependence of enantioselectivity on CBH I concentration was investigated. It was possible to resolve the enantiomers of propanolol using a 1-cm long selector plug in the whole range of concentrations studied (0.10–0.80 m*M*) (Table 1). An enantioseparation of racemic propranolol at a CBH I concentration of 0.80 m*M* is shown in Fig. 3.

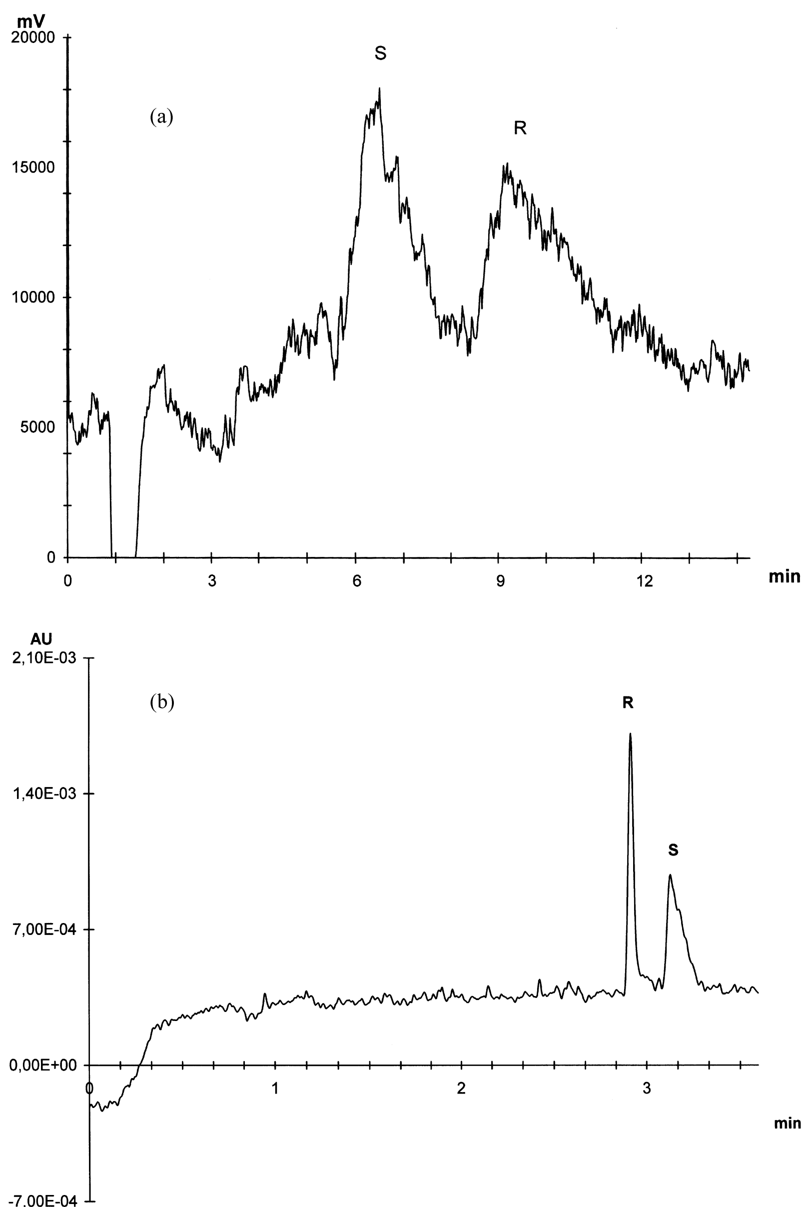

Fig. 2. Chiral separation of *rac*-propranolol in (a) HPLC using CBH I as a mobile phase additive; mobile phase: 65 μ*M* CBH I in acetate buffer (pH 5.0) I=0.02; stationary phase: diol-silica with 60 Å pores. (b) CE using CBH I as a selector; BGE: acetate buffer (pH 5.0) I=0.02. Plug: 2.8 cm of 65 μ*M* CBH I in BGE.

Increasing selectivity factors (α^*) accompanied by decreasing apparent mobilities were observed when the protein concentration was raised. The α^* value of oxprenolol was lower than for propranolol and this substance could only be separated at high protein concentration. The separation times can probably be lowered significantly by using a shorter capillary, because the protein plug is very short relative the total capillary length.

In an earlier study, a concentrated phosphate buffer (0.4 *M*), pH 5.1, containing 2-propanol was used to separate the enantiomers of different amino

Table 1
Influence of CBH I concentration on chiral separation; plug: 1.0 cm of CBH I in BGE

		Concentration of CBH I (m*M*)							
		Acetate buffer (pH 5.0), I=0.02				Phosphate buffer (pH 5.0), I=0.02			
		0.10	0.20	0.40	0.80	0.095	0.19	0.38	0.76
rac-Propranolol	μ_1 (cm^2/V s)·10^4	2.11	2.12	1.84	1.76	2.06	2.07	1.98	1.82
	μ_2 (cm^2/V s)·10^4	2.01	1.96	1.65	1.55	2.05	2.05	1.90	1.74
	α^*	1.05	1.08	1.11	1.13	1.01	1.01	1.04	1.05
	f/g	0.70	0.67	1.0	1.0	0.25	0.10	0.95	1.0
rac-Oxprenolol	μ_1(cm^2/V s)·10^4	2.13	1.85	1.90	1.86	2.05	2.04	2.03	1.88
	μ_2(cm^2/V s)·10^4	2.13	1.85	1.85	1.77	2.05	2.04	2.01	1.80
	α^*	1.00	1.00	1.03	1.05	1.00	1.00	1.01	1.04
	f/g			1.0	1.0			0.43	1.0

Selectivity factor (α^*) is defined as $\alpha^*=\mu_1/\mu_2$, where μ_1 and μ_2 are the observed mobilities of the first and second eluted enantiomer respectively.
Kaiser factor was calculated as f/g. A straight line is drawn between the two peak maxima; g is defined as the distance from this line to the base line through the valley between the two peaks, f is the distance from the same line to the valley [19]. I=ionic strength.

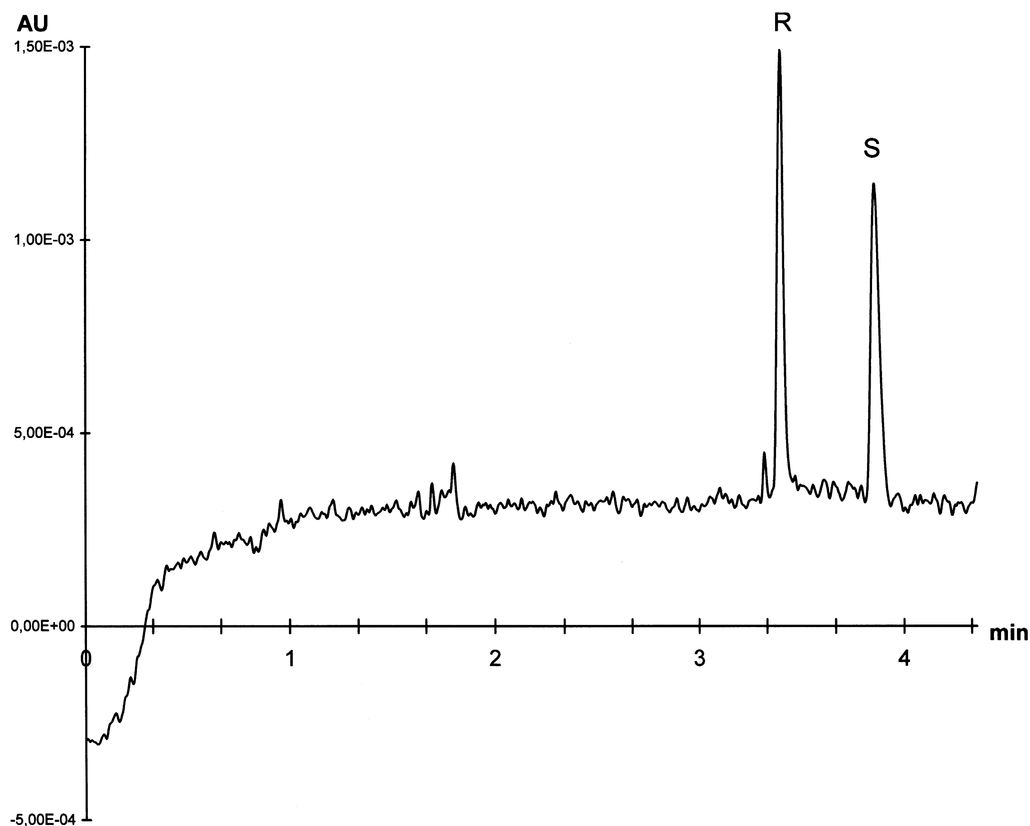

Fig. 3. Chiral separation of *rac*-propranolol; BGE: acetate buffer (pH 5.0), I=0.02; plug: 1.0 cm of 0.80 m*M* CBH I in BGE.

Table 2
Influence of 2-propanol on chiral separation

		2-Propanol (% v/v)			
		0	5	10	20
rac-Propranolol	μ_1 (cm^2/V s)·10^4	1.97	1.70	1.59	1.06
	μ_2 (cm^2/V s)·10^4	1.81	1.63	1.50	0.995
	α^*	1.09	1.05	1.06	1.07
	N_1	48 000	80 500	68 200	65 000
	N_2	96 600	132 000	137 000	47 000
rac-Oxprenolol	μ_1 (cm^2/V s)·10^4	2.01	1.72	1.61	1.08
	μ_2 (cm^2/V s)·10^4	1.96	1.69	1.57	1.08
	α^*	1.02	1.02	1.02	1.01
	f/g	1.0	1.0	0.95	0.11

BGE: phosphate buffer (pH 5.0) I=0.02; plug: 3.0 cm of 0.15 m*M* CBH I in BGE
Conditional plate number (N) was calculated as $N=16\, t_{mig}^2/w_t^2$, where t_{mig} is the migration time of the peak and w_t is the base width of the same peak in time units.

alcohols with CBH I as the selector [7]. Our experiments show that chiral separations of oxprenolol and propranolol are possible in a 0.02 *M* phosphate buffer (pH 5.0) without organic additives (Table 1). By lowering the buffer concentration, the current is reduced and it is possible to use higher field strengths (i.e. faster separations) without the risk of excessive joule heating. The selectivity factors are somewhat higher when acetate buffer is used instead of phosphate, but the improvement of α^* at higher protein concentrations was the same in both cases.

In a liquid chromatographic experiment on a silica bed with immobilized CBH I as chiral stationary phase, addition of 2-propanol to the mobile phase increased the selectivity factors and improved the resolution of the amino alcohols due to more symmetrical peaks [2]. Addition of 2-propanol to a phosphate buffer containing 0.15 m*M* CBH I resulted in decreased mobilities for the propranolol and oxprenolol enantiomers (Table 2). It is probable that this was mainly caused by an increase in electrolyte viscosity and/or an altered degree of ionization of the solute. Except for somewhat smaller values, the addition of 2-propanol gave no significant trend in selectivity factors. The efficiencies (N) of the propranolol enantiomers pass a maximum value at increasing concentration of 2-propanol. The *S*-enantiomer has a higher plate number than the *R*-form up to the 10% level of 2-propanol, whereas the *R*-peak is more efficient at 20% 2-propanol. It is important to note that these plate numbers are conditional and do not describe the separation power of the whole capillary, since the separations only take place in a 3.0-cm plug.

Table 3
Effect of sample concentration on peak shape[a]

Concentration of *rac*-propranolol (·10^5 *M*)	Asf[b]	
	R-Propranolol	*S*-Propranolol
0.52	1.3	3.9
1.0	1.6	4.6
5.2	2.9	10
10	3.6	11
52	5.0	19
100	12[c]	

[a] BGE: phosphate buffer (pH 5.0) I=0.02; Plug: 3.0 cm of 0.15 m*M* CBH I in BGE.
[b] Asymmetry factor Asf, was measured at 10% peak height as reported by Snyder and Kirkland [20].
[c] No sign of enantioseparation.

The effect of sample concentration on peak shape was studied (Table 3). At high sample concentration, the asymmetry of the *S*-peak increases more than the *R*-peak. This is probably due to the higher affinity of the *S*-enantiomer to CBH I (i.e. overloading and slow mass transfer kinetics [17,18]). When a sample concentration of 0.53 m*M* is injected into this system, the second peak of propranolol is severely overloaded (Fig. 4a). Addition of 20% 2-propanol sharpens the *S*-peak considerably (Fig. 4b). 2-Propanol decreases the affinity of propranolol to CBH I probably by competition for hydrophobic binding sites and/or by a more favourable solvation of the free solute molecule. This decreases the degree of

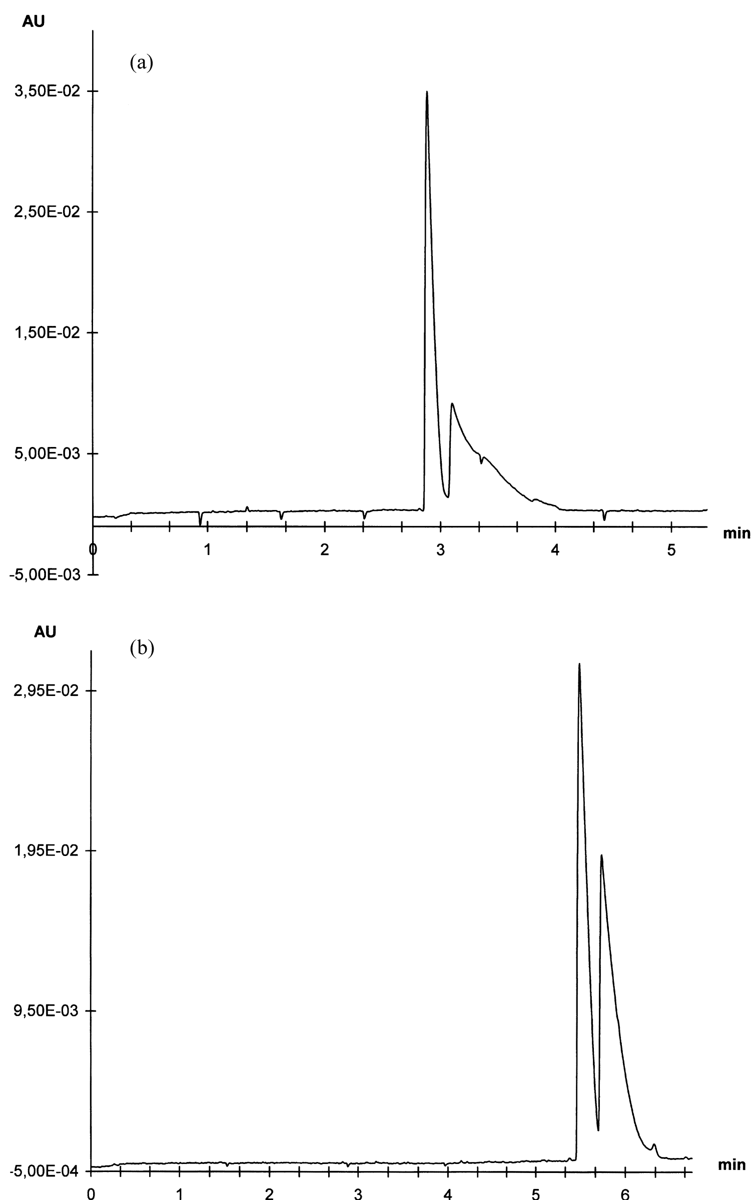

Fig. 4. Chiral separation of *rac*-propranolol. Sample concentration: 0.52 m*M*; plug: 3.0 cm of 0.15 m*M* CBH I in BGE. (a) BGE: phosphate buffer (pH 5.0) I=0.02. (b) BGE: 20% 2-propanol in phosphate buffer (pH 5.0) I=0.02.

Table 4
Chiral separation and plug length

		Applied plug length (cm)		
		1.0	1.9	4.1
rac-Oxprenolol	μ_1 (cm^2/V s)·10^4	1.80	1.79	1.80
	μ_2 (cm^2/V s)·10^4	1.80	1.79	1.76
	α*	1.00	1.00	1.02
	f/g			0.85
rac-Alprenolol	μ_1(cm^2/V s)·10^4	1.87		
	μ_2 (cm^2/V s)·10^4	1.83		
	α*	1.02		
	f/g	0.95		
rac-Warfarin	μ_1 (cm^2/V s)·10^4	−0.87	−0.88	−0.90
	μ_2(cm^2/V s)·10^4	−0.87	−0.88	−0.89
	α*	1.00	1.00	1.02
	f/g			0.83

BGE: acetate buffer (pH 5.0) I=0.02; plug: 75 μM CBH I in BGE.

overloading. Thus, addition of 2-propanol enables analysis of high sample concentrations with good peak shape. This can be beneficial when solutes with low detectability are determined or when minute amounts of impurities need to be quantified. Peak distortion as a result of sample overloading was not discussed in the previous CE study which used CBH I as a selector [7], although high analyte concentrations were injected.

The separation of the enantiomers of basic and acidic substances with a 72 μM CBH I solution in acetate buffer (pH 5.0) at different plug lengths was tested (Table 4). Alprenolol was almost baseline resolved already at a plug length of 0.97 cm (f/g= 0.95), whereas oxprenolol needed a plug of 4.1 cm to obtain an f/g value of 0.85. Remoxipride, prilocaine and trimethyl-(naphthylethyl)ammonium ion were also tested under the same conditions, but no sign of enantioselectivity was observed, even with a plug length of 16 cm.

Acidic substances migrate in the same direction as CBH I at pH 5 (i.e. towards the anode). A sufficient difference in electrophoretic mobility between the solute and selector is a prerequisite for an nondistorted detection of the acidic enantiomers. Naproxen, N-CBZ-phenylalanine, di-p-toluyl-tartaric acid and ibuprofen were all tested for enantioselectivity without success. However, it was possible to separate the enantiomers of warfarin in combination with anodic detection (Fig. 5).

At pH 3.0, the protein has a positive net charge and migrates towards the cathode, like the amino alcohols. According to the same principles, valid for

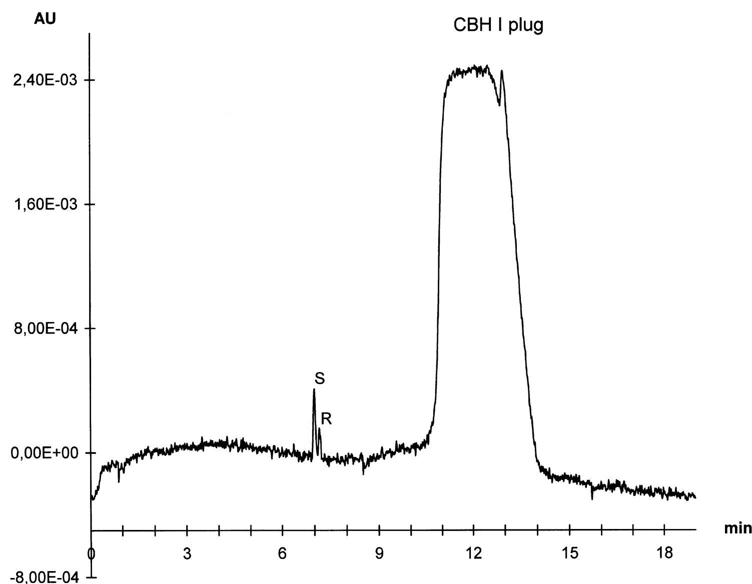

Fig. 5. Chiral separation of 33% (R)-warfarin in (S)-warfarin; BGE: acetate buffer (pH 5.0) I=0.02; plug: 4.1 cm of 75 μM CBH I in BGE; anodic detection.

Table 5
Chiral separation of *rac*-propranolol at pH 3.0

	Applied plug length (cm)					
	3.0	5.0	6.0	7.0	8.0	10
μ_1(cm^2/V s)·10^4	1.80	1.86	1.78	1.80	1.81	1.83
μ_2(cm^2/V s)·10^4	1.78	1.83	1.75	1.76	1.76	1.77
α*	1.01	1.02	1.02	1.02	1.03	1.03
f/g	0.26	0.60	0.60	0.61	0.72	1.0

BGE: phosphate buffer (pH 3.0) I=0.02; plug: 0.77 m*M* CBH I in BGE.

the separation of the acidic substances at pH 5.0 propranolol migrates away from the protein plug before it reaches the detection window. It was possible to separate the enantiomers of propranolol using a 0.77 m*M* CBH I solution at this pH (Table 5). When CBH I was used as a chiral stationary phase in HPLC, no indication of enantioseparation could be seen under similar conditions [unpublished results]. This was probably due to the low retention and the low efficiency found in chromatography.

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References

[1] P. Erlandsson, I. Marle, L. Hansson, R. Isaksson, C. Pettersson, G. Pettersson, J. Am. Chem. Soc. 112 (1990) 4573.
[2] I. Marle, P. Erlandsson, L. Hansson, R. Isaksson, C. Pettersson, G. Pettersson, J. Chromatogr. 586 (1991) 233.
[3] J. Mohammad, Y.M. Li, M. El-Ahmad, K. Nakazato, G. Pettersson, S. Hjertén, Chirality 5 (1993) 464.
[4] H. Ljungberg, S. Nilsson, J. Liq. Chromatogr. 18 (1995) 3685.
[5] S. Nilsson, L. Schweitz, M. Petersson, Electrophoresis 18 (1997) 884.
[6] S. Busch, J.C. Kraak, H. Poppe, J. Chromatogr. 635 (1993) 119.
[7] L. Valtcheva, J. Mohammad, G. Pettersson, S. Hjertén, J. Chromatogr. 638 (1993) 263.
[8] Y. Tanaka, S. Terabe, J. Chromatogr. A 694 (1995) 277.
[9] T.J. Ward, C. Dann III, A.P. Brown, Chirality 8 (1996) 77.
[10] A. Amini, C. Pettersson, D. Westerlund, Electrophoresis 18 (1997) 950.
[11] S. Fanali, C. Desiderio, J. High Resolut. Chromatogr. 19 (1996) 322.
[12] C. Desiderio, C.M. Polcaro, P. Padiglioni, S. Fanali, J. Chromatogr. A 781 (1997) 503.
[13] R. Bhikhabhai, G. Johansson, G. Pettersson, J. Appl. Biochem. 6 (1984) 336.
[14] J. Ståhlberg, G. Johansson, G. Pettersson, Biochim. Biophys. Acta 1157 (1993) 107.
[15] S. Hjertén, J. Chromatogr. 347 (1985) 191.
[16] C. Pettersson, T. Arvidsson, A.L. Karlsson, I. Marle, J. Pharm. Biomed. Anal. 4 (1986) 221.
[17] I. Marle, S. Jönsson, R. Isaksson, C. Pettersson, G. Pettersson, J. Chromatogr. 648 (1993) 333.
[18] T. Fornstedt, G. Zhong, Z. Bensetiti, G. Guiochon, Anal. Chem. 68 (1996) 2370.
[19] R. Kaiser, Chromatographie in der Gasphase, I, Gaschromatographie, Bibliographisches Institut, Mannheim, 1960, p. 35.
[20] L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley Interscience, New York, 2nd ed., 1979, p. 222.