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Chromatographic evaluation of structure selective and enantioselective retention of amines and acids on cellobiohydrolase I wild type and its mutant D214N

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Abstract

The mechanisms of structure selective and enantioselective retentions of amines and acids on two chiral stationary phases based on wild type cellobiohydrolase I (CBH I) and its mutant D214N have been investigated. All the amino alcohols tested had an enantioselective site that overlaps with the catalytically active site of CBH I, whereas the enantioselectivity of prilocaine was not affected by the mutation. The hydroxyl group of the amino alcohols did not seem to be an important contributor to the total binding strength whereas a bromo substituent in the aromatic ring promotes a high enantioselectivity (α=7.05). Interestingly, the chiral recognition site of the acid warfarin overlaps with the binding site of the amino alcohols. Di-*p*-toluoyltartaric acid and dibenzoyltartaric acid were strongly retained probably due to electrostatic attraction, but no enantioselectivity was observed. The difference in retention characteristics for the amino alcohols on the two stationary phases was strongly pH-dependent. A change in elution order of different amino alcohols occurred when changing the pH from 5.0 to 7.0. The difference between the two phases was lower at low pH. The retention times could also be affected by ionic strength and by use of cellobiose as a mobile phase additive but no indication of ion-pair retention of the amines was observed, when adding hexanesulphonate as counter ion to the mobile phase. The temperature dependence of the retention of the enantiomers of propranolol at pH 7.0 on the mutant D214N was similar to what was earlier observed on the wild type CBH I at lower pH.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Cellobiohydrolase stationary phases; Amines; Organic acids; Amino alcohols; Warfarin; Ditoluoyltartaric acid; Dibenzoyltartaric acid

1. Introduction

Cellobiohydrolase I (CBH I) is a cellulolytic enzyme from the fungus *Trichoderma reesei*. It is a glycoprotein, with a molecular mass of 64 000 and an isoelectric point (p*I*) of 3.9. CBH I is structurally organized in three main regions; a core, where the catalytically active site is located and a cellulose binding domain that is attached to the core via a

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linker [1]. CBH I has successfully been used as a chiral selector in chromatography either immobilized on silica [2,3], continuous bed [4] or as a mobile phase additive [5]. The enantioselective interaction of CBH I has also been studied by capillary electrophoresis (CE) [5,6], microcalorimetry [7,8] and molecular modelling (unpublished results). Earlier studies of this chiral selector have indicated that analytes are retained by hydrophobic and electrostatic interactions (see Ref. [9] and references therein). The three-dimensional structure of the CBH I has been elucidated by X-ray crystallography [10]. The active site in CBH I is situated in a 50 Å long tunnel containing binding sites for ten glucose residues and the acidic amino acid residues Glu^{212}, Asp^{214} and Glu^{217} that have been proposed to play a catalytic role [10,11]. From enzymatic and chromatographic studies it has been concluded that the enzymatically active site and the site responsible for chiral recognition of amino alcohols, at least to a part overlap [12]. Moreover it has been shown using site-directed mutagenesis that the carboxylic acid residues involved in the catalytic mechanism also are involved in the enantioselective binding of the enantiomers of the amino alcohols [13]. Furthermore, it has been demonstrated that there are at least two different chiral binding sites on the CBH I molecule for propranolol at pH 7.0 [14]. The dominating chiral binding site is located in the core of the CBH I and the other enantioselective site was found in the cellulose binding domain. The observed enantioselectivity was also affected by achiral binding site(s) on the immobilized CBH I phase [3,15].

The disaccharide cellobiose is the main reaction product from the CBH I catalyzed degradation of cellulose in nature. Cellobiose binds strongly to the active site of CBH I and works thereby as a competitive inhibitor of catalysis [12]. Thus, cellobiose can be used as a mobile phase additive to control the retention and chiral selectivity when the active and the chiral binding sites overlap [4].

Most of the studies on the cellulase-based stationary phases have been performed using enantiomeric amines, especially amino alcohols, as analytes [9]. This study has been focused on the relationship between the solute structure and the structure selectivity as well as enantioselectivity obtained on two chiral stationary phases, e.g. CBH I (wild type) and the mutant D214N [13]. This mutant differs from the wild type in the carboxylic group at the amino acid residue 214 that has been replaced by its isosteric amide counter part by site-directed mutagenesis [16].

The analytes were amines, amino alcohols, mono- and divalent acids. The mechanisms of structure selective and enantioselective interactions were further elucidated by investigating the influence of mobile phase composition (pH, ionic strength, charged and uncharged additives) on the separation. The effect of column temperature on the separation was also investigated.

2. Experimental

2.1. Apparatus

Three different chromatographic systems were used. The first system consisted of a Beckman 114 M Solvent Delivery Module (Palo Alto, CA, USA) pump and a LDC UV III Monitor (Riviera Beach, FL, USA) ultraviolet absorbance detector. The second system was a LDC Analytical pump and a Spectra-Physics UV 2000 (Fremont, CA, USA) detector. The third system consisted of a Jasco PU-980 pump (Tokyo, Japan) and a Merck–Hitachi L-4200 UV–Vis absorbance detector (Tokyo, Japan). In all three chromatographic systems the same volume (20 μl) was injected in Rheodyne Model 7125 or 7725 injectors (Cotati, CA, USA). The detector signals were recorded by Kipp and Zonen recorders (Delft, The Netherlands). The chromatographic columns were thermostated by HETO water baths (Birkerød, Denmark) at 25.0°C unless otherwise stated. The pH of the mobile phases was measured with a Metrohm 632 pH meter equipped with a combined pH glass electrode (Herisau, Switzerland).

2.2. Chemicals

Concentrated culture filtrate from *Trichoderma reesei* strain QM 9414 was obtained from ALKO Research Labs. (Helsinki, Finland). The wild type CBH I was purified as described by Bhikhabhai et al. [17]. The CBH I mutant D214N was made by site-directed mutagenisis and expressed in *Trichoderma reesei* as described by Ståhlberg et al. [16]. The

purity of the proteins were examined by 10% sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

rac-Propranolol was obtained from Imperial Chemical Industries (Macclesfield, UK). (*R*)- and (*S*)-propranolol and *rac*-warfarin 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*-Naproxen was from Syntex Labs. (Palo Alto, CA, USA). *rac*-Ibuprofen, (*R*,*R*)- and (*S*,*S*)-di-*p*-toluoyltartaric acid and (*R*,*R*)- and (*S*,*S*)-dibenzoyltartaric acid were obtained from Fluka (Buchs, Switzerland). *rac*-Chlorthalidone was from Ciba-Geigy Läkemedel (V. Frölunda, Sweden) and *rac*-mexiletine was obtained from Boehringer Ingelheim (Ingelheim/Rhein, Germany). The amino alcohols *rac*-metoprolol, (*S*)-metoprolol, H170/69, *rac*-H170/31 and *rac*-H54/35 were kind gifts from Astra Hässle (Mölndal, Sweden). *rac*-Prilocaine and (*S*)- and (*R*)-prilocaine were supplied from Astra Pain Control (Södertälje, Sweden). D-(+)-Cellobiose was from Sigma.

All mobile phases were prepared with deionized water, purified with a Milli-Q purification system from Millipore (Bedford, MA, USA). Sodium bromide, potassium bromide, acetic acid, phosphoric acid and sodium hydroxide were from Merck (Darmstadt, Germany). Hexanesulphonic acid sodium salt was obtained from Fluka. All chemicals used were of analytical-reagent grade.

2.3. Chromatography

The stationary phases were prepared by immobilization of CBH I and D214N on 10 μm diol silica particles with a pore diameter of 300 Å, as described elsewhere [2]. The protein coupling yields were similar: 41.6 mg CBH I/g silica and 40.9 mg D214N/g silica [13]. The solid-phase was slurry packed into stainless steel columns of 100×2.1 mm (length×I.D.).

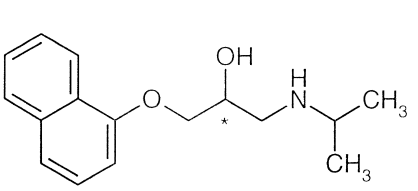
The mobile phases were prepared from 1.00 *M* stock solutions of sodium hydroxide, acetic acid and phosphoric acid and diluted to volume by Milli-Q (Millipore) purified water. The solutes were prepared as stock solutions of 10^{-3} *M* in phosphate buffer pH 7.0 and diluted 100 times in the mobile phases to concentrations of 10^{-5} *M*. The volumetric flow-rate of the mobile phase was 0.2 ml/min in all the experiments.

The chromatograms were evaluated by calculating the retention factor (k') and enantioselectivity (α). k' Was calculated as $k' = (t_R - t_m)/t_m$, where t_R is the retention time of the solute and t_m is the retention time of an unretained solute (Milli-Q water). t_m Was obtained as the inflection point. The enantioselectivity, α, is obtained as $\alpha = k'_2/k'_1$, where k'_2 is the retention factor of the more retained enantiomer and k'_1 is the retention factor of the less retained enantiomer. The selectivity between the two different columns, β, was calculated as $\beta = k'_{\text{CBH I}}/k'_{\text{D214N}}$ where $k'_{\text{CBH I}}$ is the retention factor of more retained enantiomer of the solute on the CBH I column and k'_{D214N} is the retention factor of the more retained enantiomer of the same solute on the mutant D214N column. The selectivity, α^{A} for acids is defined as $\alpha^{\text{A}} = k'/k'_{\text{DBTA}}$ unless stated otherwise. k' is the retention factor of the more retained enantiomer of the solute and k'_{DBTA} is the retention factor of dibenzoyltartaric acid. α^{B} for amines is defined as $\alpha^{\text{B}} = k'/k'_{\text{2-170/31}}$ where k' is the retention factor of the more retained enantiomer of the solute and $k'_{\text{2-170/31}}$ is the retention factor of the more retained enantiomer of the amino alcohol H170/31.

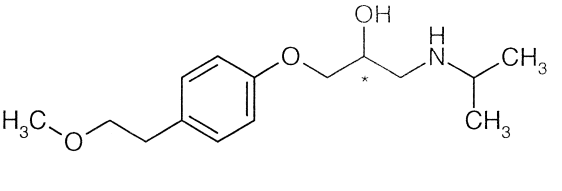
3. Results and discussion

3.1. Solute retention, enantioselectivity and structure selectivity on different CBH phases

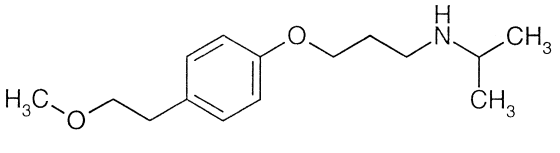
The retention, structure selectivity and enantioselectivity of amines (structures in Fig. 1) obtained on the stationary phases based on the immobilized CBH I and D214N are presented in Table 1. In accordance with previous findings [13] it can be concluded from Table 1 that the CBH I phase often gives a higher retention and enantioselectivity than the mutant D214N. The complexity of the chiral recognition mechanism is illustrated by the effect of the amidation of the Asp^{214} on the retention of various enantiomeric amines. The chiral discrimination (the observed separation factor, α) on the CBH I



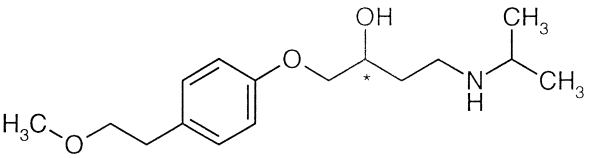
propranolol (pK_a=9.5)



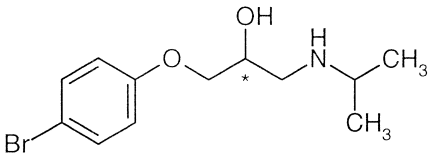
metoprolol (pK_a=9.7)



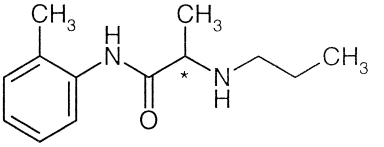
H170/69



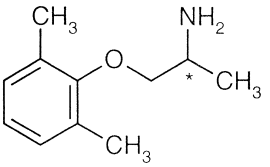
H170/31



H54/35



prilocaine (pK_a=7.9)



mexiletine (pK_a=9.0)

Fig. 1. Structures of basic solutes. pK_a values from Ref. [29].

phase is 1.6 times higher than on the D214N phase for propranolol, metoprolol and H170/31 at pH 7.0 (Table 1). The distance between the hydroxyl and amino functions (cf. metoprolol and H170/31) is of importance as the enantioselectivity is significantly higher for metoprolol than for the methylene analogue (H170/31) on both stationary phases. Interestingly, introducing the methylene group in metoprolol increases the retention (k_2') on the CBH I phase at pH 7.0, whereas a decrease in retention was observed on the D214N phase. These observations support the previous conclusion [13] that the carboxylic function at 214 is involved in retention as well as in structure selectivity and enantioselectivity of amino alcohols.

The enantioselectivity of the amino alcohol with a bromo substituent in the *para* position to the alkanol amine chain (H54/35) is higher than that of propranolol although the retention is lower on the CBH I

Table 1
Influence of pH and ionic strength on retention, structure selectivity and enantioselectivity of amines on CBH I and its mutant D214N

Solute[b]	Parameter	pH 3.0				pH 5.0		pH 7.0			
		CBH I		D214N		CBH I,	D214N,	CBH I		D214N	
		$I=0.01$	$I=0.1$	$I=0.01$	$I=0.1$	$I=0.01$	$I=0.01$	$I=0.01$	$I=0.1$	$I=0.01$	$I=0.1$
Propranolol	$k'_{(S)}$	0.54	0.71	0.53	0.91	10.7	3.10	320	53.5	44.8	17.6
	α	1.00	1.00	1.00	1.00	2.05	1.16	5.01	4.69	3.07	3.02
	β	1.02	0.78			3.46		7.13	3.03		
	α^B					8.13	6.74	11.3	10.5	15.9	18.8
Metoprolol	$k'_{(S)}$	[a]	[a]	[a]	[a]	1.32	0.47	25.1	4.41	3.53	1.16
	α					1.26	1.00	2.22	2.17	1.40	1.44
	β					2.81		7.10	3.80		
	α^B					1.00	1.02	0.89	0.87	1.25	1.23
H54/35	$k'_{(2)}$	[a]	[a]	[a]	[a]	5.10	0.95	203	36.6	14.6	5.70
	α					2.65	1.00	7.05	6.86	2.32	2.29
	β					5.37		13.9	6.43		
	α^B					3.86	2.07	7.19	7.21	5.16	6.06
H170/69	k'	[a]	[a]	[a]	[a]	1.54	0.67	21.9	4.26	3.53	1.54
	β					2.30		6.20	2.77		
	α^B					1.17	1.46	0.78	0.84	1.25	1.64
H170/31	$k'_{(2)}$	[a]	[a]	[a]	[a]	1.32	0.46	28.2	5.08	2.82	0.94
	α					1.10	1.00	1.68	1.61	1.08	1.00
	β					2.87		9.99	5.40		
Prilocaine	$k'_{(R)}$	[a]	[a]	[a]	[a]	0.60	0.64	3.95	0.81	1.98	0.52
	α					1.00	1.00	1.30	1.13	1.30	1.20
	β					0.94		1.99	1.56		
	α^B					0.45	1.39	0.14	0.16	0.70	0.55
Mexiletine	$k'_{(2)}$	[a]	[a]	[a]	[a]	1.27	0.61	18.1	3.25	6.96	2.64
	α					1.00	1.00	1.00	1.00	1.33	1.38
	β					2.08		2.60	1.23		
	α^B					0.96	1.33	0.64	0.64	2.47	2.81

[a] The peaks of the solutes are within the front peaks. All the k' values given in the tables refer to the retention factor of the more retained enantiomer. $\alpha^B = k'/k'_{2\text{-}170/31}$ where k' is the retention factor of the more retained enantiomer of the solute and $k'_{2\text{-}170/31}$ is the retention factor of the more retained enantiomer of H170/31. $\beta = k'_{\text{CBH I}}/k'_{\text{D214N}}$ where $k'_{\text{CBH I}}$ is the retention factor of the more retained enantiomer of the solute on the CBH I column and k'_{D214N} is the retention factor of the more retained enantiomer of the same solute on the mutant D214N column. Mobile phase: sodium phosphate buffer pH 3.0; sodium acetate buffer pH 5.0; sodium phosphate buffer pH 7.0.
[b] Structures in Fig. 1.

(Table 1). Furthermore, the difference in chiral recognition of H54/35 observed on the CBH I and the D214N stationary phase is much larger (ratio of $\alpha_{\text{CBH I}}/\alpha_{\text{D214N}} = 3.0$) than observed for propranolol, metoprolol and H170/31. The reason for the improved enantioselectivity on CBH I when introducing the bromo atom in the aromatic ring has not been elucidated but may be due to steric effects as well as to a change in electron density in the aromatic ring system giving rise to a change in intermolecular interaction with the immobilized enzymes.

It should be noted that the retention of amines on the CBH I type of stationary phases does not seem to require a strong interaction with the hydroxyl group as H170/69 has about the same retention as the second eluted enantiomers of metoprolol and H170/31 (Fig. 1).

Prilocaine has a higher retention on the CBH I phase than on the D214N phase but the observed

chiral recognition was not affected by the amidation of Asp^{214} (Table 1). This indicates that the mechanism of chiral recognition of this solute differs from that of the amino alcohols. The primary amine mexiletine which could not be separated on the CBH I phase gave high enantioselectivity, separation factor 1.33 (pH 7.0) on the D214N phase (Table 1 and Ref. [13]). It has been suggested by others that this is due to a less polar binding site by the amidation of Asp^{214} [13].

The selectivity between different amines (α^{B}), on the CBH I and D214N is also given in Table 1. Interestingly, not only the separation factor (α^{B}) but also the retention order of the amines were different on the CBH I and D214N phases. Metoprolol and H170/69 were less retained than H170/31 on the CBH I phase at pH 7.0, whereas the reverse effect was observed on the D214N phase. A reversed elution order of mexiletine and H170/31 was also observed.

In conclusion, as the structure selectivity as well as the enantioselectivity were effected by the amidation of Asp^{214} in the tunnel it is evident that the active site is involved in the chiral recognition and retention of the analytes. However, conversion of the carboxylic group into an amide at Asp^{214} has a different impact on the binding of analytes (Table 1). The retention of prilocaine and mexiletine were less affected by the amidation, the selectivity between the two phases (β) were 1.99 and 2.6, respectively (pH 7.0; ionic strength, $I = 0.01$). The retention of the amino alcohols were affected differently, β varied between 6.2 and 13.9 at pH 7.0 ($I = 0.01$). The bromo substituted amino alcohol (H54/35) seems to demand a more specific binding compared to the other amino alcohols, since it was more affected by the mutation concerning both the retention (cf. β) and the enantioselectivity.

The CBH I and D214N phases are less efficient in separating enantiomers of acids than of the amino alcohols, (Table 2, structures in Fig. 2). Only the enantiomers of warfarin could be separated on the CBH I phase but no chiral discrimination of any investigated acidic compound could be observed on the D214N phase. Thus, it can be concluded that the chiral binding site of warfarin at least partially

Table 2
Influence of pH and ionic strength on retention, structure selectivity and enantioselectivity of acids on CBH I and its mutant D214N

Solute[b]	Parameter	pH 3.0				pH 5.0		pH 7.0			
		CBH I		D214N		CBH I,	D214N,	CBH I		D214N	
		$I=0.01$	$I=0.1$	$I=0.01$	$I=0.1$	$I=0.01$	$I=0.01$	$I=0.01$	$I=0.1$	$I=0.01$	$I=0.1$
Naproxen	k'	6.83	5.49	5.77	5.33	2.33	2.21	0.31	0.61	0.27	0.57
	β	1.18	1.03			1.05		1.15	1.07		
	α^{A}	0.30	2.08	0.47	2.94	5.06	5.97				
Ibuprofen	k'	4.13	3.58	3.48	3.43	1.41	1.40	[a]	[a]	[a]	[a]
	β	1.19	1.04			1.01					
	α^{A}	0.18	1.36	0.28	1.90	3.06	3.78				
Di-*p*-toluoyltartaric acid	k'	63.7	7.85	35.5	5.59	1.30	1.14	[a]		[a]	
	β	1.80	1.40			1.14					
	α^{A}	2.78	2.97	2.90	3.09	2.83	3.08				
Dibenzoyltartaric acid	k'	22.9	2.64	12.2	1.81	0.46	0.37	[a]	[a]	[a]	[a]
	β	1.88	1.46			1.24					
Warfarin	$k'_{(R)}$	6.62	5.95	3.67	3.49	5.13	2.97	0.46	0.83	0.43	0.78
	α	1.66	1.60	1.00	1.00	1.45	1.00	1.00	1.00	1.00	1.00
	β	1.80	1.70			1.72		1.07	1.06		
	α^{A}	0.29	2.25	0.30	1.93	11.2	3.02				

[a] The peaks of the solutes are within the front peaks. $\alpha^{A} = k'/k'_{DBTA}$ where k' is the retention factor of the more retained enantiomer of the solute and k'_{DBTA} is the retention factor of dibenzoyltartaric acid. Mobile phase: sodium phosphate buffer pH 3.0; sodium acetate buffer pH 5.0; sodium phosphate buffer pH 7.0.

[b] Structures in Fig. 3.

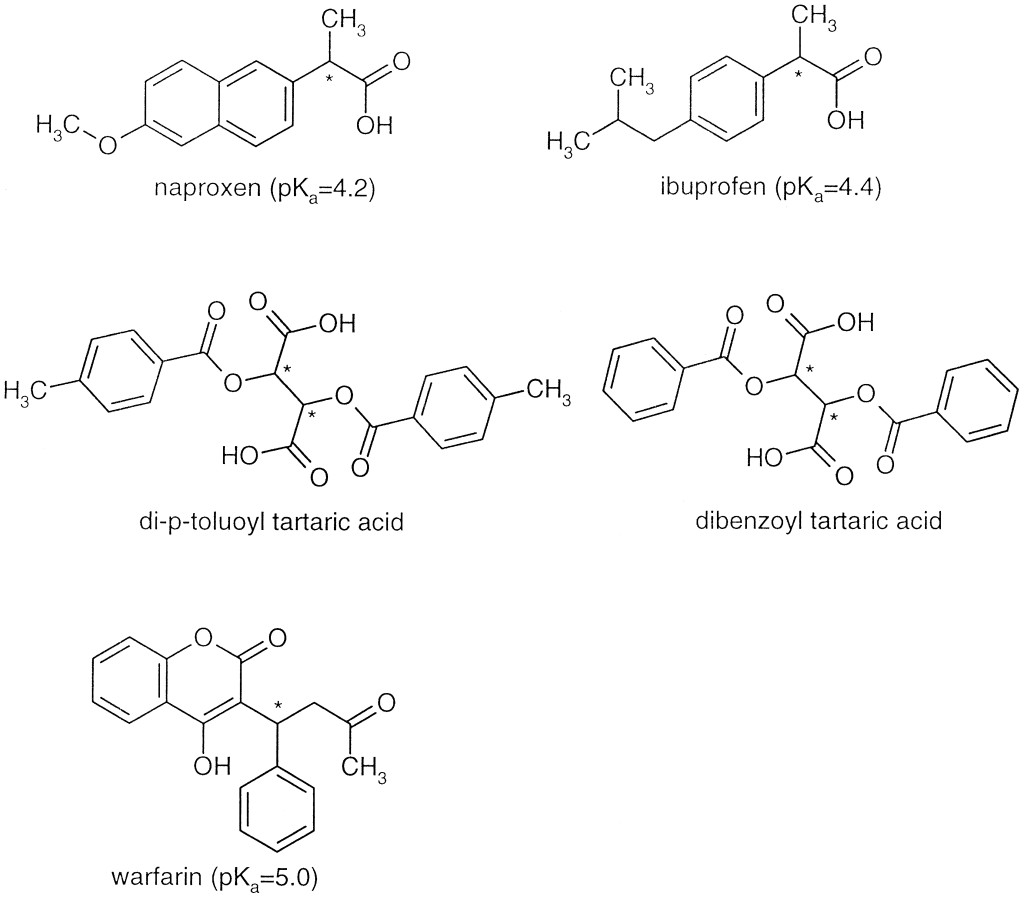


Fig. 2. Structures of acidic solutes. pK_a values from Ref. [29].

overlaps with the chiral discrimination site of the amino alcohols, i.e. enzymatically active site. Chromatograms of *rac*-warfarin from the CBH I and D214N are shown in Fig. 3.

No enantioselectivity for the di-*p*-toluoyltartaric acid and the dibenzoyltartaric acid was observed on either of the phases. However, in CE it has been shown that CBH I promotes chiral recognition of the tartaric acid derivatives [18]. This was probably due to the much lower efficiency of the HPLC system. The pK_a values of the divalent acids differ somewhat depending on the method of calculation (Table 3), however it can be concluded that the di-*p*-toluoyltartaric acid and the dibenzoyltartaric acid are both present as a mixture of mono- and divalent anions at pH 3.0. They were more retained than the monovalent acids, naproxen and ibuprofen at pH 3.0 ($I = 0.01$), which are mainly present in uncharged form (Table 2). The mutation at 214 affects the retention of the di-*p*-toluoyltartaric acid and dibenzoyltartaric acid similarly, as the selectivity between the phases (β) was about the same magnitude, indicating that these analytes also bind at the active site. It seems also to be valid for naproxen and ibuprofen although the β were close to unity.

3.2. Influence of pH and ionic strength on chiral resolution

The pH of the mobile phase is often used to control the retention and enantioselectivity on the CBH I type of chiral stationary phases [3,9]. The retention factors as well as the enantioselectivity of amines were strongly dependent on pH as shown in

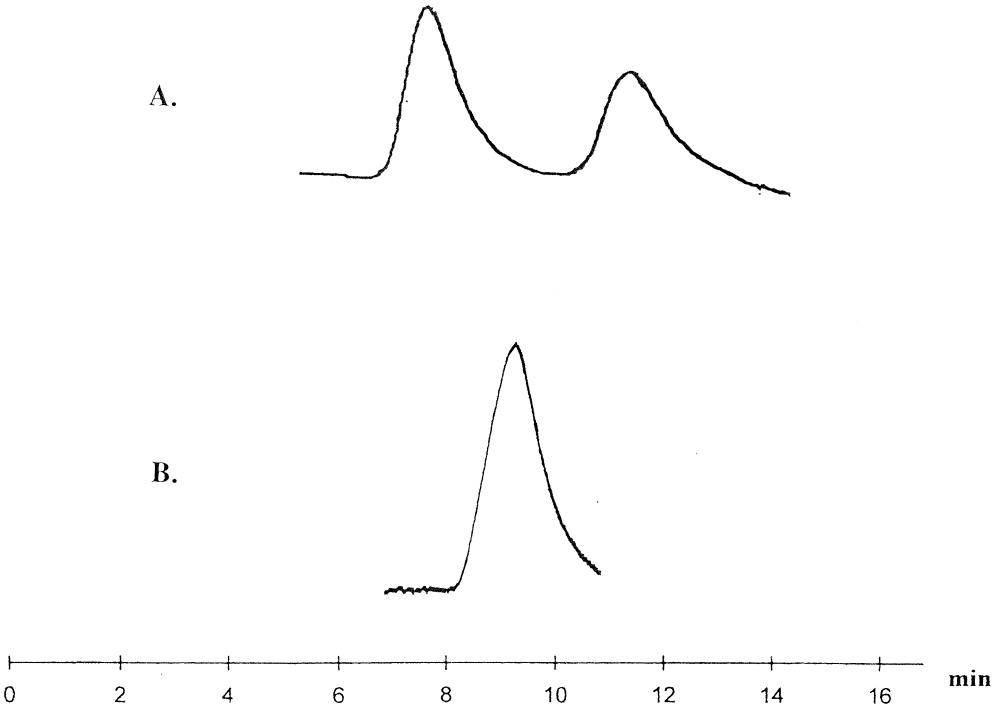


Fig. 3. Chromatograms of *rac*-warfarin on two chiral stationary phases; (A): CBH I, (B) D214N. Mobile phases: sodium phosphate buffer pH 3.0, $I=0.01$.

Table 1. The pK_a values of the amines are above 7.9 which means that they mainly are present as cations in the pH range studied. Thus, the amines should be retained by electrostatic attraction or as a neutral complex (ion-pair) with an ion of opposite charge, i.e. counter ion [19].

Except for propranolol, D214N lost the chiral discrimination properties for amines at pH 5.0. The CBH I was less efficient in chiral resolution at pH 5.0 than pH 7.0 although all amines besides mexiletine and prilocaine were enantioseparated (Table 1). The ratios of retention factors of an analyte observed on the two stationary phases (β) were less at pH 5.0 than at pH 7.0. Contrary to what was observed at pH 7.0, the retention order between the amino alcohols was the same on both stationary phases (cf. α^{B}). Asp^{214} is believed to have the function to control the protonation state of Glu^{212} [16]. It is thus possible that Glu^{212} is still protonated at pH 7.0 in the mutant and that the degree of protonation of the two proteins are more similar at low pH.

The properties of both stationary phases were quite different at pH 3.0 than at higher pH. Propranolol had about the same retention factor on the two phases but no enantioselectivity. Other amines gave a too low retention to be evaluated at pH 3.0.

The influence of buffer ion concentration on the retention of propranolol at pH 3.0 and 7.0 also demonstrate a change in retention mechanism. The retention increases at pH 3.0 whereas it decreases at pH 7.0 when increasing the buffer ion concentration. The decrease in retention factor for amino alcohols at pH 7.0 has been interpreted as a competitive effect

Table 3
Estimated acid dissociation constants

Solute	pK_{a_1}		pK_{a_2}	
	Pallas[a]	ACD[b]	Pallas	ACD
Dibenzoyltartaric acid	0.95	1.85	2.65	3.12
Di-*p*-toluoyltartaric acid	0.95	1.46	2.65	2.74

[a] Pallas for Windows 1.1 (1994) CompuDrug Chemistry Ltd.
[b] ACD Advanced Chemistry Development Inc.

by sodium ion for an electrostatic attraction (ion-exchange) [20]. Of interest is that the D214N phase has a stronger interaction with propranolol than CBH I at pH 3.0 and ionic strength of 0.1 ($\beta<1$).

A more drastic decrease in retention was observed for the divalent acids (di-*p*-toluoyltartaric acid and dibenzoyltartaric acid) than for the monovalent acids naproxen and ibuprofen at increasing pH (Table 2). This was probably due to a more efficient electrostatic repulsion by the stationary phase at a higher pH where the immobilized enzyme as well as the acidic analytes have a negative net charge or due to a more efficient hydration of a divalent anion in the mobile phase. There was no significant difference in the selectivity between the tartaric acid analogues (α^{A}) at pH 5.0 compared to pH 3.0, suggesting that the structure selectivity is not predominantly a result of different pK_a values of the acids. No enantioselectivity was observed for these acids independently of pH.

In the pH range studied, 3.0 to 7.0, warfarin is transformed from an uncharged acid to the corresponding anionic form. Both the retention and enantioselectivity decreased in this pH range. No enantioselectivity for warfarin was observed at pH 7.0 and the two phases had almost the same retention properties, β was close to unity. The interpretation of the pH effect was complicated by the fact that the total net charge of the immobilized protein as well as the charge of the warfarin enantiomers were changed. Furthermore, the influence of pH on the local charge in the binding sites is difficult to predict. Large pH changes in the mobile phase could also induce conformational changes of the protein, i.e. a change of binding sites properties [3].

Different effects of buffer ion concentration on retention were found at low and high pH (Tables 1 and 2). The retention of the amines decreased at increasing ionic strength of the mobile phase at high pH, while the retention of propranolol increased at increasing ionic strength at pH 3.0. The reverse behaviour was observed for the acids; increasing ionic strength led to decreased retention at pH 3.0, and increased retention at pH 7.0. At pH 3.0 a high concentration of dihydrogenphosphate gave a more efficient competition for electrostatic attraction with the stationary phase, resulting in a lower retention factor at high ionic strength for the acidic solutes. Increased retention at high ionic strength might be due to an increased influence of hydrophobic interactions [21].

3.3. Influence of cellobiose on chiral resolution on CBH I

As discussed previously, cellobiose, by its strong binding to the enantioselective site of CBH I, has been used as a mobile phase additive to decrease retention of amino alcohols [4]. In the present study, the effect of cellobiose was examined at pH 3.0 and 5.0 on the CBH I stationary phase, for the amino alcohol propranolol and the acid warfarin in order to characterize the binding sites of these solutes (Figs. 4 and 5).

The enantioselectivity of propranolol decreased rapidly at an increasing amount of cellobiose in the mobile phase at pH 5.0. However, the effect of cellobiose on retention ceased at cellobiose concentrations above the point where enantioselectivity is lost. This residual retention was most probably a result of non-stereoselective binding site(s) on the protein and/or the silica support, not affected by cellobiose. The retention of (*S*)-propranolol at pH 5.0 was still decreasing at cellobiose concentrations above the level where the retention of (*R*)-propranolol levelled off. As cellobiose binds to the active site, this suggests that both enantiomers bind to the same stereoselective site, and the (*R*)-form has a lower affinity than the (*S*)-form. The addition of cellobiose had no significant effect on the retention of propranolol at pH 3.0. There was no indication of enantioselectivity at this pH and the inability of cellobiose to decrease retention was probably due to the fact that the interaction with the chiral recognition site was negligible compared to the non-stereoselective site(s). The affinities to the non-stereoselective site(s) seem to be pH-dependent, as the residual retention of propranolol at high cellobiose concentrations at pH 5.0 was significantly higher than the retention at pH 3.0. Some of the non-stereoselective site(s) are probably unreacted silanol groups (cf. Ref. [3]).

The enantioselectivity of warfarin decreased at increasing concentrations of cellobiose in the mobile phase at both pH 3.0 and 5.0 (Fig. 5). This observation in combination with the incapability of the

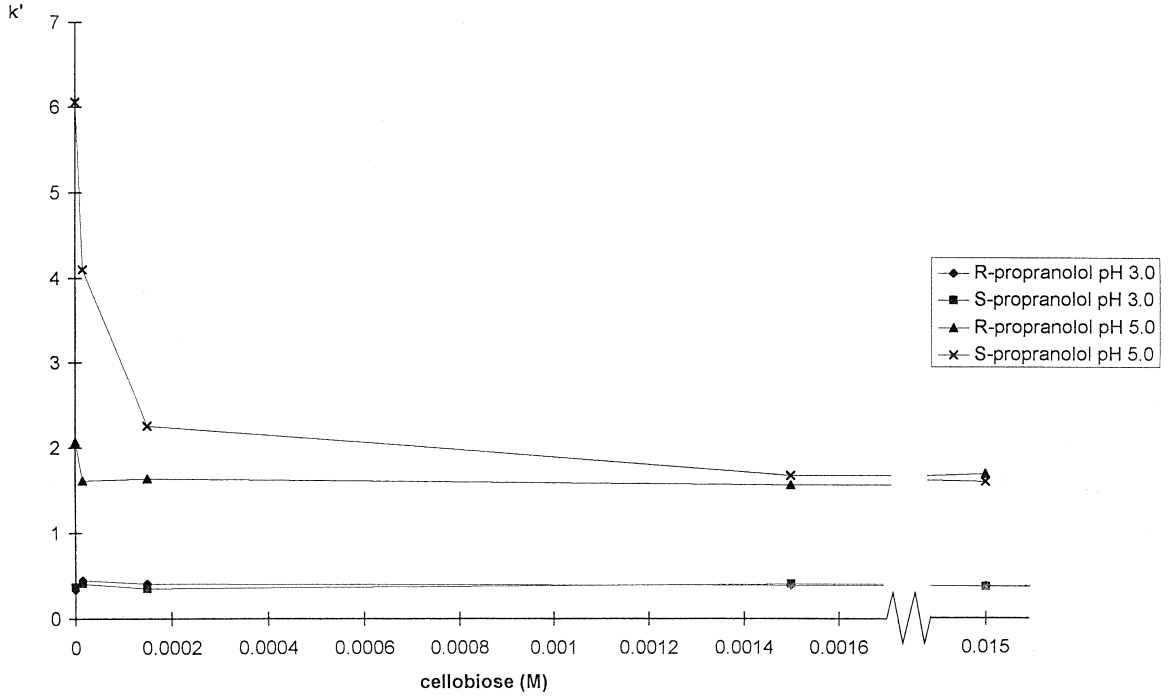


Fig. 4. Chromatographic retention factors for the enantiomers of propranolol as a function of cellobiose concentration. Mobile phases: sodium phosphate buffer $I=0.01$ (pH 3.0) and sodium acetate buffer $I=0.01$ (pH 5.0). Stationary phase: CBH I silica.

D214N phase to resolve the enantiomers of warfarin, strongly indicates that the chiral binding sites of this solute and the amino alcohols overlap. This was quite unexpected considering the differences in physico–chemical characteristics of these substances (i.e. propranolol is an secondary amino alcohol with $pK_a=9.5$ and warfarin is an enolic acid with $pK_a=5.0$). The residual retention of warfarin (i.e. retention at high cellobiose concentrations) did not differ much between pH 3.0 and 5.0. Thus, the non-stereoselective binding of warfarin to CBH I does not seem to be affected by pH in the examined interval, although the degree of ionization of this substance ($pK_a=5.0$) changes drastically. The trends in change of the retention factors of the separate enantiomers of warfarin followed the same pattern as propranolol. The decline of k' for the first eluted enantiomer ((*S*)-warfarin) levelled off at a much lower competitor concentration than the decrease of the retention factor of the (*R*)-warfarin.

3.4. Influence of bromide and hexanesulphonate on the retention and selectivity

Charged modifiers are frequently used in liquid chromatography in order to optimize chiral resolution on protein based stationary phases [22,23]. Addition of dimethyloctylamine increased the retention of the last eluted enantiomer of naproxen on α_1-acid glycoprotein, whereas a decrease was observed for the first enantiomer. An interesting observation on the CBH I phase was that sodium and potassium ions had a different effect on the chiral resolution of amino alcohols [20]. The effect of charged modifiers on the enantioseparation has been proposed to be due to ion-pair retention of the enantiomeric analyte or competition with the analyte according to an ion-exchange mechanism [19,21,24,25]. A change in enantioselectivity by the addition of modifiers in the mobile phase has previously been suggested to be due to competition with

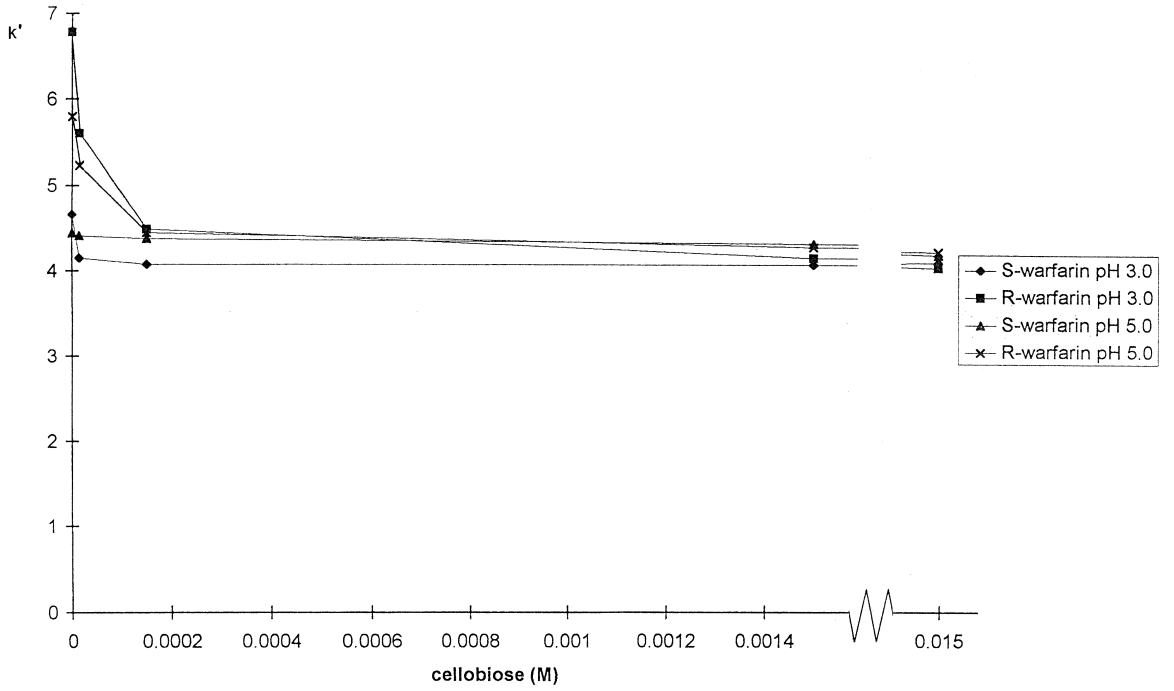


Fig. 5. Chromatographic retention factors for the enantiomers of warfarin as a function of cellobiose concentration. Mobile phases: sodium phosphate buffer $I=0.01$ (pH 3.0) and sodium acetate buffer $I=0.01$ (pH 5.0). Stationary phase: CBH I silica.

one of two or more binding sites for the enantiomeric analyte on the protein [15,26]. Additives have also been claimed to improve enantioselectivity by allosteric effects [27].

Thus, it would be of interest to investigate anions (bromide and hexanesulphonate) as counter ions and co ions for regulating retention and selectivity of amines and acids on the CBH I phase (Tables 4–7).

The drastic decrease in retention of the tartaric acid derivatives at increased ionic strength (Table 2) and the observed decrease in retention of these solutes by the addition of bromide and hexane-

Table 4
Separation of acids on CBH I with sodium bromide as mobile phase additive

Solute	Parameter	0 m*M* NaBr	1 m*M* NaBr	5 m*M* NaBr	10 m*M* NaBr
Di-*p*-toluoyltartaric acid	k'	7.63	7.25	6.92	6.18
	α^{Aa}	2.99	2.97	3.02	2.94
Dibenzoyltartaric acid	k'	2.55	2.44	2.29	2.10
Warfarin	$k'_{(R)}$	5.93	5.85	5.61	5.80
	α^{b}	1.65	1.66	1.65	1.64

[a] $\alpha^{A}=k'_{\text{di-}p\text{-toluoyltartaric acid}}/k'_{\text{DBTA}}$.
[b] $\alpha=k'_{(R)}/k'_{(S)}$. Mobile phase: sodium bromide in sodium phosphate buffer pH 3.0 ($I=0.1$).

Table 5
Separation of acids on CBH I and its mutant D214N with sodium hexanesulphonate as mobile phase additive

Solute	Parameter	pH 3.0						pH 7.0					
		0 m*M*		1.0 m*M*		10 m*M*		0 m*M*		1.0 m*M*		10 m*M*	
		CBH I	D214N	CBH I	D214N	CBH I	D214N	CBH I	D214N	CBH I	D214N	CBH I	D214N
Naproxen	k'	5.49	5.33	5.27	5.15	4.61	4.47	0.61	0.57	0.41	0.43	0.37	0.39
	β	1.03		1.02		1.03		1.07		0.95		0.95	
	α^{Ab}	2.08	2.94	2.53	3.07	3.39	4.38						
Ibuprofen	k'	3.58	3.43	3.30	3.21	2.81	2.59	0.31	0.30	[a]	[a]	[a]	[a]
	β	1.04		1.03		1.08		1.03					
	α^{A}	1.36	1.90	1.59	1.91	2.07	2.54						
Di-*p*-toluoyltartaric acid	k'	7.85	5.59	6.13	5.14	3.68	2.87	[a]	[a]	[a]	[a]	[a]	[a]
	β	1.40		1.19		1.28							
	α^{A}	2.97	3.09	2.95	3.06	2.71	2.81						
Dibenzoyltartaric acid	k'	2.64	1.81	2.08	1.68	1.36	1.02	[a]	[a]	[a]	[a]	[a]	[a]
	β	1.46		1.24		1.33							
Warfarin	$k'_{(R)}$	5.95	3.49	5.55	3.29	5.01	2.87	0.83	0.78	0.60	0.58	0.55	0.52
	α	1.60	1.00	1.67	1.00	1.70	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	β	1.70		1.69		1.75		1.06		1.04		1.06	
	α^{A}	2.25	1.93	2.67	1.96	3.68	2.81						

[a] The peaks of the solutes are within the front peaks.
[b] $\alpha^{A} = k'/k'_{DBTA}$ where k' is the retention factor of the more retained enantiomer of the solute and k'_{DBTA} is the retention factor of dibenzoyltartaric acid. Mobile phase: Sodium hexanesulphonate in phosphate buffer pH 3.0 ($I = 0.1$). Sodium hexanesulphonate in phosphate buffer pH 7.0 ($I = 0.1$).

sulphonate (Tables 4 and 5) indicate an ion-exchange mechanism for these acids. Additional interactions (e.g. hydrophobic interactions) seem also to be of importance as the more hydrophobic di-*p*-toluoyltartaric acid is significantly more retained than its dibenzoyl analogue. However, the selectivity (α^{A}) between the tartaric acid derivatives was not affected by the bromide or hexanesulphonate concentration. Hexanesulphonate was more efficient than bromide in reducing the retention factors of the tartaric acid derivatives (Table 5), probably due to a higher affinity to the protein compared to bromide as it can interact by electrostatic as well as Van der Waals and hydrophobic interactions. The hydrophobic hexanesulphonate can also be used to control the retention of naproxen and ibuprofen at pH 3.0 (Table 5).

Table 6
Influence of mobile phase cation on separation of acids on CBH I

Solute	Parameter	30 m*M* KBr			30 m*M* NaBr			
		$I = 0.1$		$I = 0.1$,	$I = 0.1$		$I = 0.13$	
		pH = 3.0	pH 5.0	pH 3.0	pH = 3.0	pH = 5.0	pH = 3.0	pH = 5.0
Di-*p*-toluoyltartaric acid	k'	8.19	0.53	5.49	5.28	0.43	4.89	0.42
	α^{Ab}	3.08		3.00	2.95		3.13	
Dibenzoyltartaric acid	k'	2.66	[a]	1.83	1.79	[a]	1.56	[a]
Warfarin	$k'_{(R)}$	5.76	3.66	5.99	5.71	3.61	5.91	3.70
	α	1.64	1.48	1.63	1.64	1.50	1.64	1.49
	α^{A}	2.17	26.1	3.27	3.19		3.79	

[a] The peaks of the solutes are within the front peaks.
[b] $\alpha^{A} = k'/k'_{DBTA}$. Mobile phase: sodium or potassium bromide in sodium phosphate pH 3.0 or sodium acetate pH 5.0.

Table 7
Chiral separation of amines on CBH I and its mutant D214N with sodium hexanesulphonate as mobile phase additive

Solute	Parameter	pH 3.0						pH 7.0					
		0 m*M*		1.0 m*M*		10 m*M*		0 m*M*		1.0 m*M*		10 m*M*	
		CBH I	D214N	CBH I	D214N	CBH I	D214N	CBH I	D214N	CBH I	D214N	CBH I	D214N
Propranolol	$k'_{(S)}$	0.71	0.91	0.73	0.84	0.75	0.87	53.5	17.6	48.0	16.2	45.3	15.0
	α	1.00	1.00	1.00	1.00	1.00	1.00	4.69	3.02	4.60	2.76	4.50	2.71
	β	0.78		0.87		0.86		3.03		2.96		3.02	
	α^{B} [b]	7.89	7.58	12.2	9.33	10.7	8.70	10.5	18.7	10.9	15.6	10.7	14.3
Metoprolol	$k'_{(S)}$	[a]	[a]	[a]	[a]	[a]	[a]	4.41	1.16	3.99	1.19	4.00	1.21
	α							2.17	1.44	2.17	1.34	2.11	1.31
	β							3.80		3.35		3.30	
	α^{B} [b]							0.87	1.23	0.91	1.14	0.95	1.15
H54/35	$k'_{(2)}$	[a]	[a]	[a]	[a]	[a]	[a]	36.6	5.70	32.0	5.16	29.5	5.00
	α							6.86	2.29	6.72	2.20	6.64	2.13
	β							6.43		6.20		5.90	
	α^{B} [b]							7.21	6.06	7.27	4.96	6.99	4.76
H170/69	k'	[a]	[a]	[a]	[a]	[a]	[a]	4.26	1.54	3.68	1.43	3.39	1.44
	β							2.77		2.57		2.35	
	α^{B} [b]							0.84	1.64	0.84	1.38	0.80	1.37
H170/31	$k'_{(2)}$	[a]	[a]	[a]	[a]	[a]	[a]	5.08	0.94	4.40	1.04	4.22	1.05
	α							1.61	1.00	1.62	1.00	1.61	1.00
	β							5.40		4.23		4.02	
Prilocaine	$k'_{(R)}$	[a]	[a]	[a]	[a]	[a]	[a]	0.81	0.52	0.66	0.60	0.57	0.63
	α							1.13	1.20	1.14	1.25	>1.00	1.18
	β							1.56		1.10		0.90	
	α^{B} [b]							0.16	0.55	0.15	0.61	0.14	0.60
Mexiletine	$k'_{(2)}$	[a]	[a]	[a]	[a]	[a]	[a]	3.25	2.64	2.73	2.45	2.67	2.30
	α							1.00	1.38	1.00	1.37	1.00	1.35
	β							1.23		1.11		1.16	
	α^{B} [b]							0.64	2.81	0.62	2.36	0.63	2.19

[a] The peaks of the solutes are within the front peaks.
[b] $\alpha^{B} = k'/k'_{2\text{-}170/31}$ where k' is the retention factor of the more retained enantiomer of the solute and $k'_{2\text{-}170/31}$ is the retention factor of the more retained enantiomer of H170/31. Mobile phase: hexylsulphonate in sodium phosphate buffer pH 3.0; hexylsulphonate in sodium phosphate buffer pH 7.0.

Addition of bromide at pH 3.0 and 5.0 had only a small effect on the retention of warfarin (Table 4) indicating that the interaction between warfarin and CBH I is not predominantly dependent on electrostatic attraction.

Contrary to what has been observed for amino alcohols, no significant difference in the retention and selectivity for the tartaric acid derivatives (α^{A}) and enantioselectivity of warfarin was observed when exchanging sodium for potassium as the mobile phase cation (Table 6).

The studies using hexanesulphonate as mobile phase additive did not support an ion-pair retention of amino alcohols on the CBH I type of chiral stationary phases (Table 7). As a matter of fact, the presence of sodium hexanesulphonate gave rise to a slight decrease in the retention of the amines. Hexanesulphonate and/or, as previously observed, sodium ion [20] probably act as a competitor to amines for interaction with the immobilized CBH I and D214N. In general, the presence of hexanesulphonate had no drastic effect on the difference in retention characteristics (β) of amines between the CBH I and the D214N stationary phases. Ion-pair retention at the specific binding site is probably unfavorable as the main retention mechanism seems

to involve an electrostatic attraction to the positively charged form of the solute. Furthermore, there was no evidence of ion-pair retention at the secondary enantioselective site for propranolol (cf. Ref. [14]) although the binding mechanism to this site has not been elucidated.

3.5. Influence of temperature on chiral resolution

An extraordinary temperature effect on the chiral separation of propranolol has been observed on CBH I using acetate buffer pH 5.5 [7]. The retention of (*S*)-propranolol was found to increase with increasing column temperature whereas the reverse was observed for (*R*)-propranolol. Thus, the chiral separation is improved at higher temperature as enantioselectivity is improved. Complementary studies by microcalorimetry at pH 5.0, have shown that both enantiomers bind with an endothermic enthalpy change, the (*S*)-form having the highest positive ΔH, while the chromatographic study gave exothermic enthalpy changes for both enantiomers at this pH [7]. Further investigations by other workers have confirmed a change in the sign of ΔH for the (*S*)-enantiomer between pH 4.7 and 5.5 [28].

A temperature study on the chiral separation was included in this study to compare the retention mechanism on CBH I and D214N (Fig. 6). A phosphate buffer pH 7.0 ($I = 0.1$) was applied as mobile phase. Interestingly, at pH 7.0, not only the (*S*)-propranolol but also the (*R*)-propranolol has a negative slope (i.e. endothermic) of the Van 't Hoff plots for the CBH I. This was contrary to what has been observed previously at lower pH (pH 5.0 and 5.5) [7]. Thus, the sign of the slope of the Van 't Hoff plot of the (*S*)-enantiomer changes somewhere between pH 5.0 and 5.5 [7] and that of the (*R*)-propranolol somewhere between pH 5.5 and pH 7.0. Thus, there seems to be a trend for both enantiomers of increasing value of ΔH at increasing pH, leading to a conversion from exothermic to endothermic reactions. The thermodynamic parameters determined from a chromatographic experiment generally emanate from a complex system of different interactions between the analyte and the stationary phase (i.e. the enantioselective site and non-stereoselective

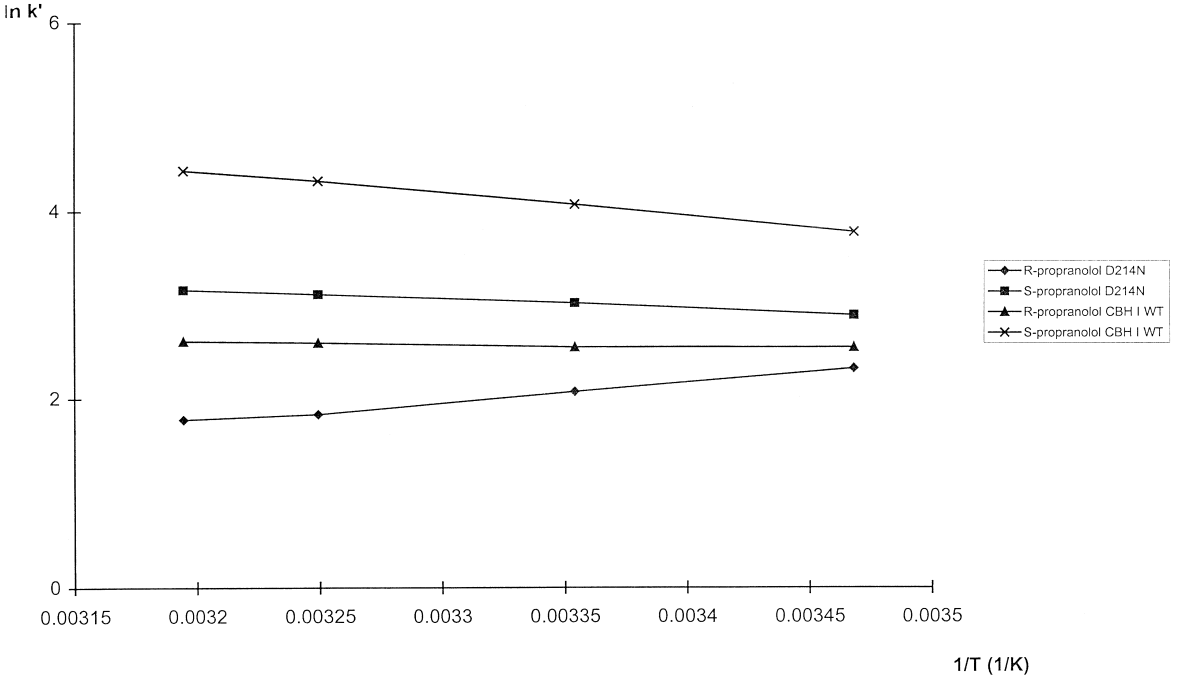


Fig. 6. Van 't Hoff plots of the enantiomers of propranolol on CBH I and D214N stationary phases. Mobile phase: sodium phosphate buffer pH 7.0, $I = 0.1$.

sites on the protein and/or on the silica). The ligand interactions to these sites most likely have different thermodynamic properties as well as different pH dependences leading to a change of sign of the overall ΔH values at increasing pH. The mutant D214N gave Van 't Hoff plots different from that of CBH I. The interaction between the (*S*)-propranolol and D214N is still characterized by an endothermic enthalpy (although smaller in magnitude compared to the wild type) whereas the binding of (*R*)-propranolol is exothermic. Thus, the properties of the D214N mutant at pH 7.0 are similar to the wild type at lower pH. This is in line with the earlier suggestion that Asp^{214} controls the protonation of Glu^{212} in the binding site, and that the degree of protonation of Glu^{212} is higher compared to the wild type at pH 7.0 [16].

The Van 't Hoff plots of prilocaine deviate considerably from linearity, especially on D214N (results not shown). This suggests that the temperature dependence of ΔH and ΔS is larger compared to propranolol. The retention mechanism of prilocaine probably differs from the β-blockers, as the amidation of amino acid residue 214 did not affect the enantioselectivity of this substance (Table 1).

The retention of mexiletine was not significantly affected by a temperature change in this interval on the wild type stationary phase (results not shown). A slight exothermic behaviour was observed for both enantiomers on the mutant, whereas the enantioselectivity was constant.

4. Conclusions

Both amines and acids seem to bind to chiral and achiral binding site(s) on the protein based stationary phases CBH I and D214N silica. The enantioselective binding for amino alcohols as well as for warfarin is at or in the vicinity of the enzymatically active site of the proteins (cf. Ref. [13]). The mutation affected retention, structure selectivity and enantioselectivity for amino alcohols whereas no change in enantioselectivity for prilocaine was observed, i.e. the chiral discrimination mechanism of prilocaine differs from that of the amino alcohols. The hydroxyl group of the amino alcohols did not seem to be an important contributor to the total binding strength.

Except for warfarin, no enantioselectivity could be observed for the mono- or divalent acids on the immobilized protein phases although retention was effected by the mutation. Thus, the binding sites of these acids overlap with the active site of CBH I.

The pH of the mobile phase is the main regulator of retention, selectivity and enantioselectivity on the CBH I type of stationary phases. The retention order between the amino alcohols was different on the two phases at pH 7.0, while it was the same at pH 5.0. The enantioselective retention for amines was lost at low pH, whereas warfarin gave enantioselective retention at low pH but not at pH 7.0. The non-stereoselective binding of warfarin was not affected by pH in the range from 3.0 to 5.0, whereas it was enhanced for propranolol.

The possibility to use charged modifiers to optimize the chiral resolution on these chiral stationary phases is limited. Addition of hexanesulphonate did not promote any ion-pair retention for the amines. However, addition of bromide and hexanesulphonate as co-ion enables regulation of retention for anionic solutes without affecting the selectivity between the tartaric acid analogues.

Cellobiose is an efficient mobile phase additive to decrease retention at the chiral binding site for propranolol and warfarin. This is further evidence that the enantioselective binding sites of these solutes overlap.

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