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Discrimination between enantioselective and non-selective binding sites on cellobiohydrolase-based stationary phases by site specific competing ligands

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Abstract

A systematic study was performed to investigate the influence of cellobiose or lactose on the enantioselective retention behaviour of some β-blockers in liquid chromatography using Cellobiohydrolase (CHB) I from *Trichoderma reesei* or Cellobiohydrolase 58 from *Phanerochaete chrysosporium* immobilized on silica as stationary phases. The results revealed that the retention could be described by the function

$$k'_x = k'_{ns,x} + \frac{k'_{es,x}}{1 + \frac{[\text{competitor}]}{K_d}}$$

where the observed capacity factor corresponds to the sum of an enantioselective mode being influenced by a site specific competing ligand (competitor) and a non-selective mode unaffected by the competitor. A non-constrained non-linear least-square regression gave in all cases virtually identical nondisplacable capacity factors (k'_{ns}) for both enantiomers of the same drug. The experimental capacity factors ($k'_{x,C}$) of the enantiomers all show a close fit to the adapted function. The K_d values calculated for the competitor were also virtually identical for each pair of enantiomers and were in accordance with K_i data determined for the competitors in classical enzyme kinetics experiments, demonstrating that one unique site; namely, the catalytic site, was responsible for the enantioselective binding. Similar results were obtained with the resolution of *rac*-alprenolol and *rac*-metoprolol on CBH I phase.

Keywords: Chiral stationary phases, LC; Enantiomer separation; Cellobiohydrolase; β-Blockers

1. Introduction

Some proteins have found use as chiral stationary phases (CSPs) due to their ability to discriminate enantiomers: bovine serum albumin (BSA) [1], α_1-acid glycoprotein (AGP) [2], ovomucoid [3], α-chymotrypsin [4] and cellobiohydrolase (CBH) [5]. Much work has been done to investigate the binding and selective mechanism of enantiomeric compounds to the protein stationary phases in liquid chromatography. The retention behaviour of enantiomers in a chiral selective environment has also been studied intensively. Immobilized BSA has been investigated thoroughly by Allenmark and co-workers [6–12],

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who proposed that there were two kinds of binding sites on the surfaces of the proteins, namely non-selective sites with lower affinities and an enantioselective site with higher affinity. By studying the adsorption isotherms for a few pairs of enantiomers on the BSA using a bi-Langmuir isotherm model, Guiochon and et al. could estimate the number of non-selective and enantioselective sites on BSA, showing that at most one enantioselective site exists for each BSA molecule for the kind of enantiomers studied [13].

Cellulases, new members in the group of protein CSPs, were found to be the best choice for chiral analysis of β-adrenergic receptor antagonists [5]. Later, a large numbers of compounds were analysed and CHIRAL-CBH columns became commercially available [14]. This encouraged studies on the mechanism of chiral discrimination by cellulases [15–19]. The *Trichoderma reesei* cellulases including two endoglucanases (Endoglucanase I and II) and two exoglucanases (Cellobiohydrolase I and II) have a common two-domain organization with a large catalytic domain connected to a small cellulose-binding domain (CBD) via a glycosylated linker peptide [20]. The two functional domains of CBH I can be separated by papain cleavage [21]. Almost all of the chiral selectivity originates from the catalytic domain, as found by comparison of the chiral separation using intact and fragmented CBH I as CSPs [16]. Solution of the three-dimensional structure of the catalytic domain of CBH I revealed a striking feature: an extensive cellulose-binding tunnel initially estimated to contain seven glucosyl binding subsites [22]. Cellobiose, the main product released by CBH I in hydrolysis of cellulose [23,24], binds to $+1$ and $+2$ subsites in the tunnel of CBH I [22,25], resulting in strong product inhibition of the enzyme activity ($K_d = 18.5\ \mu M$) [26]. Addition of cellobiose to the mobile phase impaired the protein's chiral selectivity, indicating that the binding sites for the cellobiose and the analytes overlap at least partially [27]. Studies of the enzyme inhibition by propranolol and alprenolol combined with enantioseparation data for these compounds on the cellulase-CSPs confirmed that the enzymatically active site was involved in chiral recognition [18]. Of three mutant proteins obtained by site-direct mutagenesis of catalytically important carboxylic groups [22] to their corresponding amides, D214N retained a considerable enantioselectivity, whereas E212Q and E217Q are virtually inactive, despite the fact that the active site was isomorphous with the wild-type protein [28]. This influence on the enantioselectivity paralleled that observed for the catalytic activity of the mutants [19].

CBH 58 from *Phanerochaete chrysosporium*, an obvious counterpart to CBH I from *T. reesei*, was recently found to be a similarly excellent chiral selector for β-blockers. In addition, it can separate some basic compounds which were not resolved on a CBH I-CSP (data not published). Sequence data clearly establish that CBH 58 is a close homologue of CBH I [29–32] with the same structural organization. The crystal structure of the CBH 58 catalytic domain shows that it is essentially isomorphous with CBH I except for the absence of some loops covering part of the tunnel and an additional tyrosine at the end of the tunnel [33].

A recent study of the thermodynamics and mass transfer kinetics of the retention of the *R*- and *S*-enantiomers of propranolol on CBH I immobilized on silica reported that the experimental data fitted the bi-Langmuir isotherm model, indicating that the protein-based adsorbent offered two different classes of binding sites. Both the thermodynamics and mass transfer kinetics of the binding were heterogeneous, meaning that the protein/adsorbent contained a large number of non-chiral low energy adsorption sites with rapid mass transfer kinetics and a small number of enantioselective high energy adsorption sites with slower mass transfer kinetics [34]. A related study on the chiral separation of propranolol enantiomers on CBH I-CPS reported the interaction enthalpy and entropy of the non-selective and enantioselective binding mode of both *R*- and *S*-propranolol on immobilized CBH I [35].

Generally, the binding of a chiral ligand X to a chromatographic medium with m classes of non-selective sites and n classes of enantioselective sites is described by the following expression

$$B_x = [\mathrm{X}]\left(\sum_1^m \frac{c_{i,\mathrm{ns}}}{K_{\mathrm{d},i} + [\mathrm{X}]} + \sum_1^n \frac{c_{i,\mathrm{es}}}{K_{\mathrm{d},i} + [\mathrm{X}]}\right) \quad (1)$$

where B_x is the amount of chiral ligand bound to the CSP, [X] the concentration of chiral ligand and c the

binding capacity for the ligand ''X''. At a ligand concentration [X]<<the dissociation constants $K_{d,i}$, i.e., in the linear chromatography regime, Eq. (1) is simplified into

$$B_x = [X]\left(\sum_1^m \frac{c_{i,ns}}{K_{d,i}} + \sum_1^n \frac{c_{i,es}}{K_{d,i}}\right) \quad (2)$$

Since

$$B_x = k'_x[X] \quad (3a)$$

$$k'_x = \frac{B_x}{[X]} \quad (3b)$$

the total capacity factor k'_x can be expressed as

$$k_x = \sum_1^m \frac{c_{i,ns}}{K_{d,i}} + \sum_1^n \frac{c_{i,es}}{K_{d,i}} \quad (4)$$

Using the definitions

$$k'_{ns,x} = \sum_1^m \frac{c_{i,ns}}{K_{d,i}}$$

$$k'_{es,x} = \sum_1^n \frac{c_{i,es}}{K_{d,i}} \quad (5b)$$

Eq. (4) is simplified to

$$k'_x = k'_{ns,x} + k'_{es,x} \quad (6)$$

Assuming a single class of enantioselective sites that is also able to bind a site specific competing substance (competitor), as suggested by the reported effect of cellobiose, the apparent $k'_{es,x}$ in Eq. (6) is affected by the competitor and expressed as $k'_{es,x}\left(1 + \frac{[competitor]}{K_d}\right)$ so that the retention behaviour of a racemic solute should depend on the competitor concentration

$$k'_x = k'_{ns,x} + \frac{k'_{es,x}}{1 + \frac{[competitor]}{K_d}} \quad (7)$$

In the present work, Eq. (7) is applied to data from selected combinations of four series representing two different proteins, three pairs of enantiomers of β-blockers and two competitors. This new model allows a simple estimate of the non-selective and selective contribution to the retention of the analytes when these proteins were used as chiral selectors.

2. Materials and methods

2.1. Chemicals

R-, *S*- and *rac*-propranolol hydrochloride, D-(+)-cellobiose and D-(+)-lactose were purchased from Sigma (St. Louis, MO, USA). *R*- and *S*-alprenolol hydrochloride, *rac*-metoprolol hydrochloride were supplied by Astra Hässle (Mölndal, Sweden). 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). Sodium cyanoborohydride was from Janssen (Beerse, Belgium). Periodic acid (HIO_4), acetic acid, phosphoric acid, ammonium and sodium hydroxide were purchased from Merck (Darmstadt, Germany). All chemicals used were of analytical grade. The water used was Milli-Q purified water.

2.2. Experimental apparatus

The chromatographic system consisted of LKB 2150 HPLC pump (LKB-Produkter, Bromma, Sweden), a LDC/Milton Roy Spectromonitor D equipped with a 1-μl cell (LDC/Milton Roy Company, FL, USA), a Model BD40 recorder (Kipp and Zonen, The Netherlands) and a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, USA) equipped with a 20-μl loop.

A pH meter Model E 623 (Metro, Wheres, Switzerland) equipped with a combined pH glass electrode and a spectrophotometer, Shimadzu UV-160A (Shimadzu, Kyoto, Japan) were also used.

2.3. Preparation of the proteins and the columns

CBH I was purified from the concentrated culture filtrate from the fungus *T. reesei* strain QM 9414 obtained from ALKO Research Labs. (Helsinki, Finland) as described earlier [36]. CBH 58 was purified from culture filtrates of *P. chrysosporium* strain K3 [37]. The purity of the proteins was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

The preparation of CBH I- and CBH 58-CSPs and the columns were carried out as described in earlier papers [15,17]. The coupling yields were 42 mg of

protein/g silica for CBH I and 54 mg of protein/g silica for CBH 58, respectively.

2.4. High-performance liquid chromatography (HPLC) procedure

The mobile phase buffers (sodium phosphate, pH 6.0, $I=0.01$, with cellobiose or lactose from 0 to 7.3 mM) were applied to the CBH-CSPs at a constant flow-rate of 0.2 ml/min and a group of β-blockers including propranolol, alprenolol, metoprolol were chosen as solutes. Enantiomers of propranolol and alprenolol were used in the case of high cellobiose or lactose concentrations in order to obtain an accurate retention time for each enantiomer. The chromatography experiments were performed at ambient temperature ~22°C.

The first and second experiment series were carried out by using CBH I-CSP (CBH I column A) or CBH 58-CPS (CBH 58 column) to separate the enantiomers of *rac*-propranolol in the mobile phase of sodium phosphate buffer, pH 6.0 ($I=0.01$) with various concentrations of cellobiose (0 to 1.46 mM). Eighteen months later, the third series was run to separate the enantiomers of *rac*-propranolol (1 nmol), *rac*-alprenolol (1 nmol) and *rac*-metoprolol (0.2 nmol) on a newly packed CBH I column B (using the same batch of CBH I-CPS as in column A) in the same mobile phase with various concentrations of cellobiose (0 to 2.92 mM). The fourth series was done to study the resolution of *rac*-propranolol using the CBH 58 column in the same mobile phase including various concentrations (0 to 2.92 mM) of cellobiose or lactose.

All the experiments were done in duplicate.

2.5. Non-linear regression analysis of retention data

The capacity factors of the analytes were fitted to the function by non-linear regression using the program Axum 5.0 (MathSoft)

3. Results and discussion

Eq. (7) is valid if there are two independent classes of binding sites, non-selective and enantioselective, on the chiral adsorbent and only the selective sites are influenced by the competitor. The equation contains two terms: k'_{ns}, corresponding to the non-selective binding, and k'_{es}, to the enantioselective binding of an enantiomer to a protein phase. The terms, enantioselective sites and non-selective sites, are introduced so as to demarcate the different binding properties of the enantiomers on the CSPs [38]. Our work is also in agreement with an earlier study of the retention of the enantiomers of mandelic acid and *N*-benzoylalanine on BSA-CSP (anion exchanger) [39], in which the adsorption isotherms of the enantiomers were well accounted for by a bi-Langmuir equation. One of the isotherms corresponds to non-selective interactions that are the same for both enantiomers and the other to the chiral selective interaction. All of the compounds tested can be resolved into their enantiomers on the protein-based adsorbents (Table 1). The chiral resolution is completely lost at high competitor concentration, showing that k'_{es} represents an interaction that can be totally suppressed by cellobiose or lactose, whereas k'_{ns} is unaffected by sugar competitors for all pairs of enantiomers studied (Table 2).

The theoretical capacity factors (k'_x), calculated as the sum of k'_{ns} and k'_{es} for each enantiomer from the non-linear regression, according to the equation are almost the same as those obtained from the chromatography (k'_C) without competitors in the mobile phases (Table 2). The good fit of the adapted function to the data points, together with the small variations in the calculated K_d for the competitor, supports the model with a single class of structurally well-defined selective sites together with non-selective sites. The experimental capacity factor is the sum of the contributions originating from non-selective and enantioselective sites for an enantiomer. This allowed us to calculate the apparent enantioselectivity $[\alpha_C=(k'_{ns}+k'_{es,2})/(k'_{ns}+k'_{es,1})]$ and true enantioselectivity ($\alpha_T=k'_{es,2}/k'_{es,1}$). It is easy to see that the apparent enantioselectivity is always somewhat lower than the true enantioselectivity [35]. Any factors that can effect a relative decrease in the k'_{ns} will thus increase the apparent enantioselectivity, which is expected.

A comparison of the k'_{ns} and k'_{es} for propranolol on CBH I-A, -B and CBH 58 CSPs indicates that they differ for different CSPs (Table 2), showing that the

Table 1
Influence of competitors to the enantioselectivity on CBH I- and CBH 58-CSPs[a]

Column	CBH I-A	CBH I-B			CBH 58		
Series	First	Third			Second	Fourth	
Solute[b]	Propranolol	Propranolol	Alprenolol	Metoprolol	Propranolol	Propranolol	Propranolol
Competitor	Cellobiose	Cellobiose	Cellobiose	Cellobiose	Cellobiose	Cellobiose	Lactose
Competitor concentration = 0							
$k'_{1,C}$	12.1	14.2	6.35	3.12	54.6	48.6	48.6
$k'_{2,C}$	59.3	50.7	38.4	5.18	116	99	99
α	4.90	3.57	6.05	1.66	2.12	2.04	2.04
Competitor concentration = 14.6 m*M*							
$k'_{1,C}$	9.14	12.3	4.61	2.54	54.6		42.7
$k'_{2,C}$	37.1	33.4	21.6	3.68	110		80.9
α	4.06	2.72	4.68	1.45	2.01		1.89
Competitor concentration = 73 m*M*							
$k'_{1,C}$		10.1	3.82	2.30		32.9	25.5
$k'_{2,C}$		16.2	9.36	2.67		62.1	42.6
α		1.60	2.45	1.16		1.88	1.67
Competitor concentration = 146 m*M*							
$k'_{1,C}$	6.40	10.3	3.20	2.20	31.2		20.3
$k'_{2,C}$	11.9	14.5	6.08	2.20	56.3		31.4
α	1.86	1.41	1.90	1.00	1.81		1.55
Competitor concentration = 730 m*M*							
$k'_{1,C}$		10.1	3.22	2.13		14.2	14.6
$k'_{2,C}$		11.1	3.72	2.13		19.5	17.7
α		1.09	1.16	1.00		1.37	1.21
Competitor concentration = 1460 m*M*							
$k'_{1,C}$	5.39	10.1	3.30	2.08	13.5		13.1
$k'_{2,C}$	5.93	10.8	3.58	2.08	16.7		14.7
α	1.10	1.07	1.08	1.00	1.24		1.12
Competitor concentration = 2920 m*M*							
$k'_{1,C}$		10.1	3.24	2.05		12.9	12.4
$k'_{2,C}$		10.4	3.40	2.05		14.5	13.5
α		1.03	1.05	1.00		1.13	1.09
Competitor concentration = 7300 m*M*							
$k'_{1,C}$						12.3	11.4
$k'_{2,C}$						12.8	11.7
α						1.04	1.03

[a] Mobile phase: sodium phosphate, pH 6.0 ($I = 0.01$) with competitor (cellobiose or lactose) of various concentrations.
[b] Solute: *rac*-propranolol, 0.001 mmol or 0.0002 mmol; *R*-propranolol or *S*-propranolol, 0.0002 mmol; D-, L-alprenolol, 0.001 mmol; D-alprenolol or L-alprenolol, 0.0005 mmol; *rac*-metoprolol, 0.0002 mmol.

non-selective sites as well as the enantioselective sites are unique on each CSP for the same solute. CBH I-A and -B can be regarded as two different phases, since they were packed and the chromatographies were performed at different occasions. Although CBH 58 Column used in the fourth series was the same column as in the second series, it may be regarded as a different CSP because it was stored

Table 2
Experimental and calculated parameters[a]

Column	CBH I-A	CBH I-B			CBH 58		
Series	First	Third			Second	Fourth	
Solute	Propranolol	Propranolol	Alprenolol	Metoprolol	Propranolol	Propranolol	Propranolol
Competitor	Cellobiose	Cellobiose	Cellobiose	Cellobiose	Cellobiose	Cellobiose	Lactose
$k'_{ns,1}$[b]	5.4±0.2	9.95±0.17	3.18±0.08	2.09±0.02	9.1±4.7	11.3±0.8	11.6±0.7
$k'_{ns,2}$[b]	5.1±0.1	9.92±0.58	3.02±0.14	2.02±0.04	7.8±6.1	11.0±1.1	11.8±1.0
$k'_{es,1}$[b]	6.6±0.3	4.30±0.35	3.17±0.17	1.02±0.05	47.4±5.1	37.5±1.3	38.0±1.2
$k'_{es,2}$[b]	54.2±0.1	41.0±1.14	35.4±0.3	3.16±0.09	110.7±6.6	88.2±1.8	88.5±1.9
$K_{d,(1)}$[b] (mM)	19.2±3.6	13.7±4.4	12.4±2.7	13.0±2.5	139±59	94.7±14	48.1±6.2
$K_{d,(2)}$[b] (mM)	21.0± 0.1	17.7±1.9	15.9±0.5	16.1±1.8	120.3±29.4	97.6±8.6	43.3±3.6
k'_1[b]	12.1±0.3	14.2±0.4	6.34±0.19	3.11±0.1	56.5±6.9	48.8±1.6	49.5±1.4
$k'_{1,C}$[b]	12.1	14.2	6.35	3.12	54.6	48.6	48.6
k'_2[b]	59.3±0.1	50.9±0.6	38.4±0.31	5.18±0.1	118.5±9.0	99.2±2.1	100.3±2.1
$k'_{2,C}$[b]	59.3	50.7	38.4	5.18	116	99.0	99.0

[a] The parameters in Table 2 were calculated from the mathematical model: $k'_x = k'_{ns,x} + \dfrac{k'_{es,x}}{1 + \dfrac{[\text{competitor}]}{K_d}}$ except $k'_{1,C}$ and $k'_{2,C}$.

[b] $k'_{ns,1}$: non-selective binding capacity factor of the first eluted enantiomer; $k'_{es,1}$: enantioselective binding capacity factor of the first eluted enantiomer; $k'_{ns,2}$: non-selective binding capacity factor of the second eluted enantiomer; $k'_{es,2}$: enantioselective binding capacity factor of the second eluted enantiomer; $K_{d,(1)}$: dissociation constant for the competitor to the protein calculated from the first eluted enantiomer; $K_{d,(2)}$: dissociation constant for the competitor to the protein calculated from the second eluted enantiomer; k'_1: capacity factor of the first eluted enantiomer; k'_2: capacity factor of the second eluted enantiomer; $k'_{1,C}$: capacity factor of the first eluted enantiomer from chromatography; $k'_{2,C}$: capacity factor of the second eluted enantiomer from chromatography.

in the refrigerator for one and a half year after which some changes may occur, e.g., the protein may become denatured or released to some extent [15]. Actually, one can see in Table 2 that for propranolol, the k'_{ns} increased while k'_{es} decreased with time so that the ratio of k'_{es}/k'_{ns} decreased (36% and 41% for the *R*- and *S*-forms on CBH I-CSP, 64% and 56% for the *R*- and *S*-forms on CBH 58-CSP, respectively), i.e., the apparent selectivity (α_C) decreased, but $k'_{es,1}$ and $k'_{es,2}$ decrease in parallel so that the intrinsic true selectivity ($\alpha_T = k'_{es,2}/k'_{es,1}$) remains almost unchanged, indicating a quantitative loss of enantioselective sites rather than a change in the mode of interaction.

It has been reported that the hydrophobic cavity of BSA and the enzymatic active site of CBH I should contain the chiral selective sites [39,18]. Any other compounds which can bind reversibly to the active site of the protein may compete with the chiral compounds for the binding site and can thus be used as selective competitors. Cellobiose binds specifically in the active site of CBH I [22,25] where propranolol also binds [40], i.e., cellobiose and propranolol compete for the same binding site. The affinity of cellobiose for CBH I ($K_i = 18.5$ μM at pH 5.0, [26]) is stronger than that of propranolol ($K_i =$ 490 μM for the *R*-form and $K_i = 100$ μM for the *S*-form at pH 5.0, [18]), so cellobiose can easily displace the propranolol enantiomers. As shown in Table 1, the retention of the enantiomers decreased successively with increasing concentration of cellobiose in the mobile phases, in accordance with the equation where the apparent k'_{es} is expressed as $k'_{es}/(1+[\text{competitor}]/K_d)$. It is apparently not difficult to deduce from the equation that in the case of very high displacer concentration, the second term, $k'_{es}/(1+[\text{competitor}]/K_d)$, becomes negligible so that k' asymptotically approaches k'_{ns}. This is also in agreement with the study of adsorption of propranolol enantiomers on CBH I-CSP [34]. Anyway, the k'_C for each enantiomer converge to a common value on the non-linear regression diagram (Fig. 1) and the apparent selectivity approaches 1 (Table 1).

Two other β-blockers, namely, alprenolol and

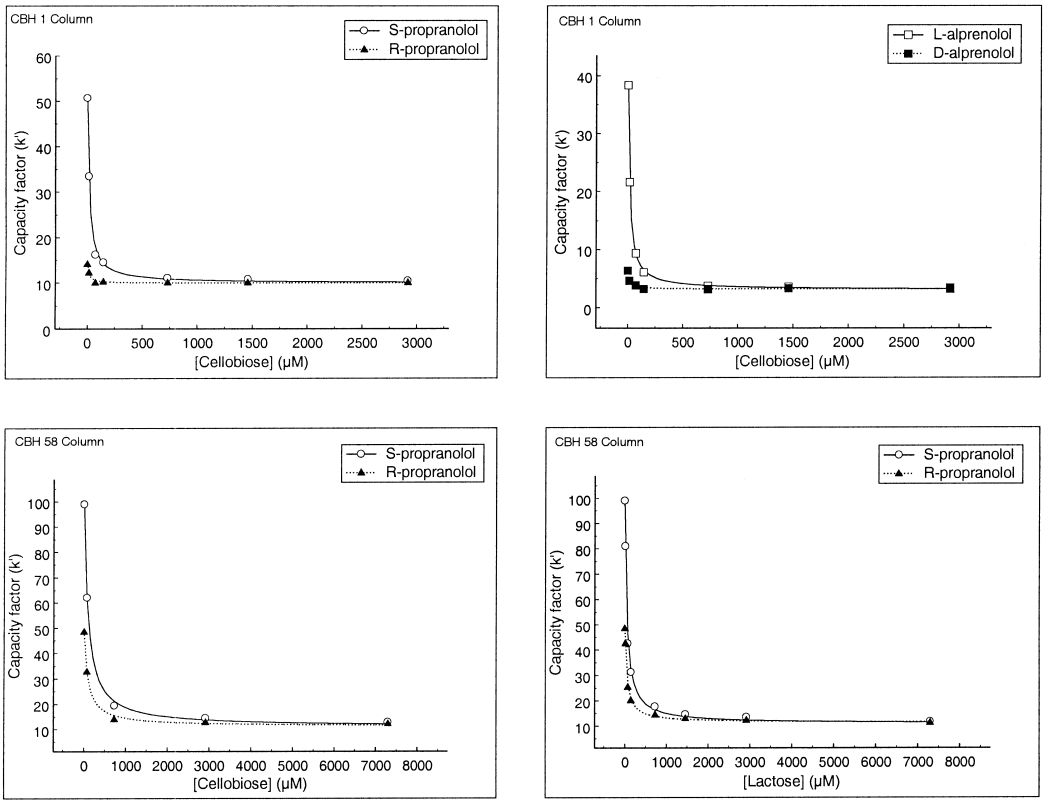

Fig. 1. Discrimination of enantiomers of β-blockers on CBH I-CSP and CBH 58-CSP using cellobiose or lactose as competitors by non-linear regression according to the function

$$k'_x = k'_{ns,x} + \frac{k'_{es,x}}{1 + \frac{[\text{competitor}]}{K_d}}$$

The lines demonstrate the regressions calculated from the model and the spots represent the data from the chromatographic experiments.

metoprolol, were also analysed in this study to test whether the model is generally valid and the result confirmed our expectation. The retention of these two pairs of enantiomers on CBH I-CSP were generally lower than those of the enantiomers of propranolol, but the effect of cellobiose was the same, all chiral resolution being lost at high competitor concentration where only the non-selective adsorption remains (Table 1 and Fig. 1).

One benefit of Eq. (7) is the possibility to calculate K_d for a competitor–protein combination as an alternative to the classical way using the compound as an inhibitor in enzyme kinetics. As shown in Table 2, the K_d for cellobiose with CBH I and CBH 58, and the K_d for the binding of lactose to CBH 58 were calculated individually by non-linear regression. They are in good agreement with the corresponding data obtained from enzyme kinetics (for cellobiose and CBH I, $K_d = 18.5$ μ*M* at pH 5.0; and for CBH 58, $K_d = 110$ μ*M* at pH 5.0. For lactose and CBH 58, $K_d = 60$ μ*M* at pH 5.0), (Szabó, BMC Uppsala University, data not published). In the third series, even though different β-blockers were analysed using the same CBH I-CSP and cellobiose as competitor, the K_d value for cellobiose and CBH I obtained from each non-linear regression were consistent, proving that the same sites were involved. If different K_d values were obtained for a certain combination of competitor and CSP, it could be suspected that there exist alternative binding sites for

the enantiomers to be displaced. For example, CBH I has totally 11 glucosyl subsites through the whole tunnel [25]. Cellobiose, as a product, naturally binds strongly to +1 and +2 subsites, but possibly, it could also bind to the other subsites, although with a clearly lower affinity. These tentative cellobiose binding subsites would, in principle, allow the enantiomers to interact with different binding sites that were still subject to competition by cellobiose, but in such a case, a dramatic difference in K_d for cellobiose may be observed. Interestingly, the discovery of a compound that could be shown to bind specifically to a ''new'' subsite of the enzyme could provide a reporting strategy to determine the affinity for cellobiose to that site.

4. Conclusions

In this work, two competitors were used and three chiral compounds were analysed on two protein phases. The fact that all data fit Eq. (7) indicates that the same mechanism is involved in all cases. This approach should generally, when applicable, serve to define the properties of CSPs.

For a particular chiral compound on a protein phase, the retention capacities from the non-selective interactions, k'_{ns}, are, by definition, the same for the pair of enantiomers, whereas the retention capacities from the enantioselective interactions, k'_{es}, are obviously different, allowing a separation. The interactions between an enantiomer and both the non-selective and enantioselective sites on the protein-CSP constitute the total retention capacity of the enantiomer. In this study, cellobiose or lactose which preferably bind to the active site of CBH I-type enzymes were applied alternatively to the mobile phases as selective competitors to enhance the elution of the enantiomers from the protein phases. These selective competitors only affected the interactions between the enantiomers and their enantioselectives sites on the protein without interfering with the non-selective adsorption. The consistency of the K_d for a selected enzyme–competitor combination generally observed here proved that a single class of sites on the adsorbent was responsible for the chiral resolution of the analytes investigated.

This selective competition can be applied in further investigations of the retention mechanism of the chiral compounds on CSPs, since the effects of changes in pH, ionic strength or adding organic solvent as modifier; usage of mutant protein phases etc., can be resolved with respect to the selective and non-selective adsorption. It is also obvious that the apparent selectivity can be increased by minimising the relative degree of non-selective adsorption, as well as by increasing the discriminating power of the enantioselective site.

5. Symbols

- k'_x, capacity factor of an enantiomer calculated from the regression
- $k'_{ns,x}$, non-selective binding capacity factor of an enantiomer
- $k'_{es,x}$, enantioselective binding capacity factor of an enantiomer
- K_d, dissociation (inhibition) constant of the competitor to the protein
- $k'_{x,C}$, capacity factor of an enantiomer calculated from the chromatography

5.1. Subscripts

- ns, non-selective site
- es, enantioselective site
- x, $x=1$, 2 for the less and more retained enantiomer, respectively

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