Resolution of racemic drugs on a new chiral column based on silica-immobilized cellobiohydrolase Characterization of the basic properties of the column

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Abstract

The basic properties of a new chiral column based on silica-immobilized cellobiohydrolase 1 (CBH 1) were studied using 32 different drugs and endogenous compounds as model compounds. All the compounds were resolved with high separation factors and high resolution. The stability of the CBH 1 column was studied by pumping two different mobile phases through the column, one of pH 6 and the other of pH 7. A 34-l volume of mobile phase, corresponding to about 38 000 column volumes, was pumped through the column. The enantioselectivity, the resolution, the separation efficiency and the retention of the test compounds were unchanged after the passage of 38 000 column volumes of mobile phase. The most important tool for affecting the enantioselectivity, resolution and retention is the pH of the mobile phase. Uncharged modifiers such as acetonitrile and 1- and 2-propanol were also used. Unique effects were obtained for a series of drugs and endogenous compounds, related to epinephrine, by increasing the 2-propanol concentration in the mobile phase from 0 to 20%. The capacity factor for the most retained enantiomer could be selectively increased by increasing the 2-propanol concentration in the mobile phase, resulting in a substantial improvement in the enantioselectivity. For one compound, octopamine, the capacity factor of the most retained enantiomer increased by almost 100%, resulting in a very large increase in the separation factor from 1.9 to 3.3. The resolution increased from 4.8 to 7.8. The relationship between the molecular structure and the enantioselectivity was also studied. The data indicate that the steric bulk on a basic nitrogen influences the enantioselectivity to a large extent, in addition to the degree of ring substitution. Further, the Chiral-CBH column seems to be very well suited for the resolution of preferentially basic drugs of different character.

1. Introduction

Proteins of different types are an important group of compounds in nature. Enzymes transform a substrate into a product, drugs and endogenous compounds are transported in the body as complexes with proteins such as albumin

and α_1-acid glycoprotein (AGP). The proteins are asymmetric molecules which means that they can bind a low-molecular-mass ligand, such as a drug, stereoselectively. This property of proteins has been utilized for the chromatographic resolution of enantiomers by immobilization of the protein on a matrix. One of the most frequently used chiral stationary phases (CSPs) today is a column based on the protein AGP. The extreme-

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ly broad applicability of the AGP column has been documented in a large number of publications (e.g., [1–6]). This broad applicability of AGP can be assigned to the unique primary, secondary and tertiary structure that nature has built into the protein.

Other protein phases with a narrower applicability are bovine serum albumin and the human serum albumin columns [7,8]. The latest addition to this category of CSPs is the ovomucoid column [9] and a column based on cellobiohydrolase 1 [10–13].

Cellobiohydrolase 1 (CBH 1) is a protein with very interesting properties concerning the ability to discriminate between enantiomers. CBH 1 is very well suited for use as a chiral selector in liquid chromatographic columns owing to the very high stability of the enzyme. The reason for this high stability might be that nature designed the enzyme to work extracellularly. CBH 1 has a molecular mass of 64 000. It consists of 497 amino acid residues, stabilized by 12 disulphide bridges. The carbohydrate content is about 6%. Recently the three-dimensional structure of the active site of CBH 1 has been elucidated by X-ray crystallography [14]. The binding site is a tunnel with the dimensions $4 \times 7 \times 40$ Å. There are seven acidic amino acid residues, four tryptophan residues and also tyrosine, serine, threonine, arginine and histidine lining the tunnel. This gives the prerequisites for obtaining stereoselective binding of a broad range of chiral solutes.

In this paper, a new chiral column, Chiral-CBH, based on cellobiohydrolase, is described. The properties of the Chiral-CBH column differ significantly from those of the CBH 1 column referred to above, owing to large differences in the surface chemistry of the base silica and large differences in the immobilization technique.

2. Experimental

2.1. Apparatus

The chromatographic system consisted of an LKB Model 2150 HPLC pump (Pharmacia,

Uppsala, Sweden), a Kontron (Eching/Munich, Germany) HPLC 360 autosampler equipped with a 20-μl loop and a Spectra 100 variable-wavelength UV detector (Spectra-Physics, San Jose, CA, USA). The experimental data were collected and analysed on a Kontron 450 MT2 data system, which also controlled the autosampler. pH was measured with an Orion SA520 pH meter connected to a Ross Model 8102 combination electrode. The column used was a commercially available Chiral-CBH (ChromTech, Hägersten, Sweden) with the dimensions 100×4.0 mm I.D. (5-μm particles). A Chiral-CBH guard column (ChromTech) was coupled in front of the analytical column. The dimensions of the guard column were 10×3.0 mm.

2.2. Chemicals

2-Propanol and acetonitrile, of HPLC grade, were obtained from Lab-Scan (Dublin, Ireland). The drug compounds were obtained from different companies. The salts used for the preparation of mobile phases were of analytical-reagent grade.

2.3. Chromatographic conditions

The experiments were performed at room temperature. The flow-rate was 0.9 ml/min and the UV detector was operated at 210 nm. The sample concentrations were in the range 0.02–0.05 mg/ml. Mobile phases containing phosphate buffers were prepared from sodium dihydrogenphosphate and the pH was adjusted with sodium hydroxide. Disodium EDTA was added before adjusting the pH. Thereafter an appropriate amount of the organic modifier was added, followed by water to the final volume. Mobile phases containing acetate buffers were prepared from sodium acetate and the pH was adjusted with acetic acid. Disodium EDTA was added before adjusting the pH. Thereafter an appropriate amount of the organic modifier was added, followed by water to the final volume. The hold-up volume (V_m) was measured by injection of distilled water.

3. Results and discussion

3.1. Binding of solutes to CBH 1

CBH 1 is a very stable enzyme with a molecular mass of 64 000 and an isoelectric point of 3.9. This means that the CBH 1 molecule has a net negative charge in the pH range normally used in chromatographic experiments (4–7). In the chromatography of basic compounds in this pH range, ionic binding between the solute and the protein is an important type of interaction, in order to retain basic solutes on this column. Ionic binding has, in addition to ion-pair adsorption (ion pairs are neutral species and are bound by hydrophobic interaction and hydrogen bonding), been demonstrated to be very important for the retention of basic drugs on an AGP column [1,2,6]. From the three-dimensional structure of CBH 1 it can be seen that the binding site is a tunnel with the dimensions 4 × 7 × 40 Å [14]. The binding site contains seven acidic amino acid residues and aspartic and glutamic acid [14]. This means that it seems very likely that the ion-exchange mechanism is an important retention mechanism on the CBH 1 column. Further, the binding site (the tunnel) also contains four tryptophan residues and also other amino acid residues such as tyrosine, serine, threonine, arginine and histidine, which means that both hydrogen bonding and hydrophobic interaction are very important for the retention of the solutes. From the above it follows that the enantioselectivity and the retention of the solutes can be affected by the pH of the mobile phase and also the nature and concentration of an uncharged organic modifier such as 1- or 2-propanol or acetonitrile. pH is a very important factor since it affects both the degree of charge of the solute and the chiral bonding properties of the protein. An uncharged modifier can also strongly influence the stereoselective binding of the solutes.

3.2. Retention, enantioselectivity and resolution versus the concentration of uncharged organic modifier

Unique effects were observed on the reten- tion, the enantioselectivity and the resolution for a series of epinephrine analogues on increasing the 2-propanol concentration, as demonstrated in Table 1. As can be seen, the capacity factor of the most retained octopamine enantiomer is almost doubled by increasing the 2-propanol concentration from 0 to 20%, whereas the capacity factor for the first-eluted enantiomer is almost unchanged. This means that the sepa- ration factor and the resolution increase substan- tially on increasing the 2-propanol concentration. The separation factor of octopamine increases from 1.90 to 3.32 and the resolution increases from 4.8 to 7.8. Similar effects, but not so dramatic, were also observed for the analogues norepinephrine, normethanephrine, phenyl- ethanolamine and epinephrine, as demonstrated in Table 1. To our knowledge, such effects have never been observed previously on any other chiral column. From Table 1 it can be seen that the enantioselectivity for all epinephrine ana- logues increases on increasing the 2-propanol concentration. The largest increase in the capaci- ty factors was observed for compounds contain- ing a primary amino group, whereas the capacity factors for metanephrine and epinephrine, con- taining a secondary amino group, demonstrated a deviatiant behaviour. The capacity factors for both enantiomers of metanephrine decreased slightly and a very small increase in the capacity factor was observed for the last-eluted enantio- mer of epinephrine. Replacing 2-propanol with 5% acetonitrile in the same buffer resulted in lower enantioselectivity and lower retention for all compounds.

The unique behaviour of increasing retention with increasing 2-propanol concentration cannot easily be explained. A reasonable explanation could be that the addition of 2-propanol induces a reversible change of the secondary structure, which means that the binding site changes character and exposes other amino acid residues in the binding site (the tunnel), giving a higher affinity of the last-eluted enantiomer. As the retention of the least retained enantiomer is only slightly affected, it is reasonable to assume that the enantiomers are retained at different loca- tions, i.e., different subsites, in the 40 Å long

Table 1
Effects of uncharged organic modifiers on k', α and R_s for epinephrine analogues

Modifier concentration	Octopamine				Metanephrine				Normetanephrine				Epinephrine				Norepinephrine				Phenylethanolamine			
	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s
None	3.04	5.79	1.90	4.83	2.83	3.30	1.17	0.91	3.68	6.30	1.72	3.67	2.25	2.97	1.32	1.62	3.60	6.29	1.74	3.75	–	–	–	–
0.5% 2-PrOH	3.01	6.04	2.01	5.23	2.68	3.23	1.20	1.08	3.57	6.43	1.80	3.98	2.19	2.98	1.36	1.83	3.53	6.41	1.82	3.96	2.25	2.96	1.31	1.95
5%2-PrOH	3.03	7.57	2.50	6.81	2.40	3.02	1.26	1.43	3.28	6.68	2.04	4.82	2.08	3.08	1.48	2.26	3.40	7.19	2.12	4.99	2.18	3.06	1.40	2.44
10% 2-PrOH	3.02	8.61	2.85	7.52	2.20	2.82	1.29	1.57	3.06	6.84	2.23	5.20	2.04	3.12	1.53	2.48	3.27	7.60	2.32	5.51	2.19	3.19	1.46	2.64
20% 2-PrOH	3.31	11.0	3.32	7.80	2.19	2.83	1.29	1.52	3.03	7.42	2.45	4.87	2.27	3.37	1.49	2.21	3.43	8.40	2.45	5.30	2.51	3.74	1.49	2.77
5%CH$_3$CN	2.35	4.27	1.82	3.48	1.95	2.24	1.15	0.83	2.60	3.93	1.51	2.19	1.76	2.34	1.33	1.74	2.68	4.37	1.63	3.07	1.83	2.27	1.24	1.56

Column: Chiral-CBH, 100 × 4.0 mm I.D. Mobile phase: 10 mM sodium phosphate buffer (pH 6.0)–50 μM disodium EDTA, having different concentrations of modifiers.

Fig. 1. Capacity factors, k', versus 2-propanol concentration. Column, Chiral-CBH (100 × 4.0 mm I.D.); mobile phases, 10 mM sodium phosphate buffer (pH6.0)–50 μM disodium EDTA containing 0–20% 2-propanol. (A) Samples: □ = k'_1 talinolol; ◆ = k'_2 talinolol; ■ = k'_1 atenolol; ◇ = k'_2 atenolol. (B) Samples: 1 = k'_1 cathinone; 2 = k'_2 cathinone; 3 = k'_1 laudanosine; 4 = k'_2 laudanosine; 5 = k'_1 dobutamine; 6 = k'_2 dobutamine.

binding site (the tunnel) of CBH 1. The decrease in the capacity factor observed for metanephrine with increasing modifier concentration might be the result of competition for hydrogen bonding and/or hydrophobic interaction.

When the unique properties reported above were observed, we were also interested in establishing whether that kind of effect could be observed for other classes of compounds with other characteristics. Five different basic compounds of different character were included in the study: the amino ketone cathinone, two amino alcohols, the two well known β-blockers atenolol and talinolol, the secondary amine dobutamine and the tertiary amine laudanosine. The structures are shown in Table 3.

Fig. 1A and B demonstrate the influence of the 2-propanol concentration on the retention of the five model compounds. As can be seen in Fig. 1A, the capacity factors of the enantiomers of atenolol are affected to a very limited extent by increasing the concentration of 2-propanol to 20%, i.e., a behavior similar to that observed for metanephrine. The retention of the talinolol enantiomers is strongly reduced. From Fig. 1B it can be seen that the amino ketone cathinone demonstrates a behaviour similar to that of

atenolol. The capacity factor of cathinone is almost constant in the concentration range of 2-propanol used in the study. This behaviour may indicate that hydrophobic interaction is not very important in the retention process for these solutes. The capacity factors of dobutamine and laudanosine are reduced to a relatively large extent. It is very interesting that the capacity factor of the most retained enantiomer of dobutamine decreases more rapidly than that of the least retained enantiomer. For laudanosine the reversed situation occurred.

The relative influence of a modifier on the retention of the enantiomers of a solute is very important for the enantioselectivity, as is clearly demonstrated in Fig. 2. As can be seen, the enantioselectivity of atenolol, talinolol and laudanosine increases strongly at 2-propanol concentrations up to 10%. At higher concentrations the enantioselectivity is almost unaffected for atenolol and talinolol. Similar effects have been observed previously on the AGP column for carboxylic acids such as tiaprofenic acid and some weak acids such as methylphenobarbital and some barbituric acid derivatives [2–4], and also on the CBH 1 column for some basic drugs [11]. The separation factor for dobutamine is
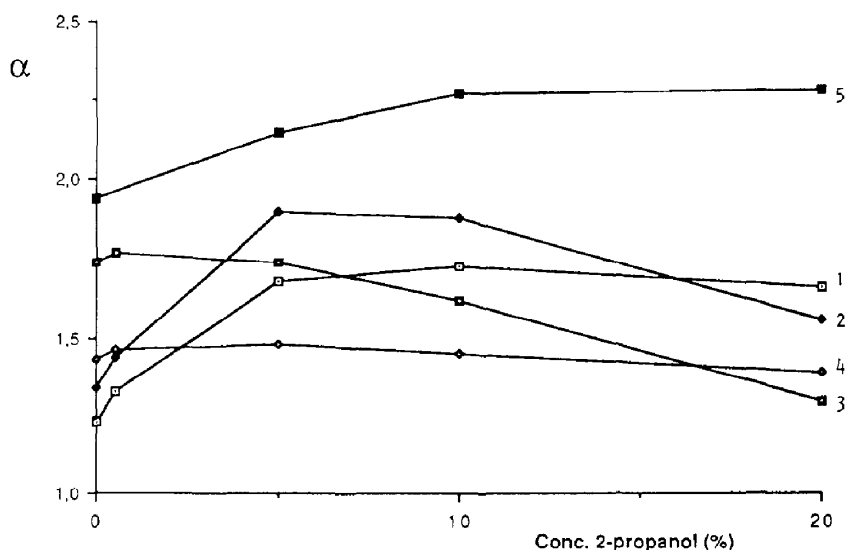
Fig. 2. Effect on the separation factor, α, of increasing the 2-propanol concentration. Conditions as in Fig. 1. Samples: 1 = talinolol; 2 = laudanosine; 3 = dobutamine; 4 = cathinone; 5 = atenolol.
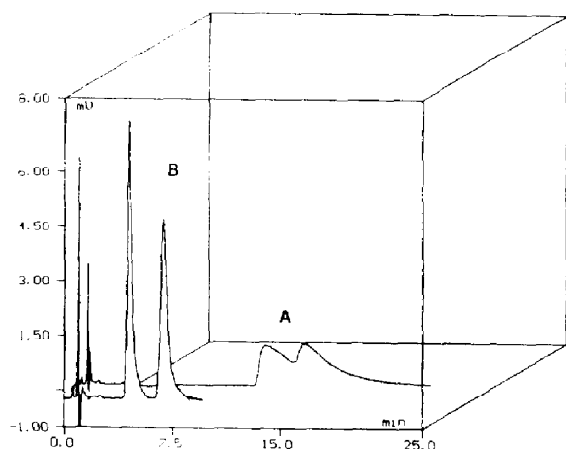
Fig. 3. Improvement of the chromatographic performance by addition of 2-propanol to the mobile phase. Column, Chiral-CBH (100 × 4.0 mm I.D.); sample, talinolol; mobile phase. (A) 10 mM sodium phosphate buffer (pH 6.0)–50 μM disodium EDTA and (B) 5% 2-propanol in 10 mM sodium phosphate buffer (pH 6.0)–50 μM disodium EDTA.
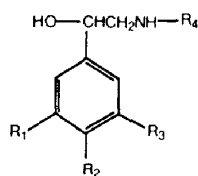
olution continues to increase up to a 2-propanol concentration of 10% in the mobile phase.

The reason for the very strong increase in the resolution on increasing the 2-propanol concentration is that the enantioselectivity increases with increasing modifier concentration. However, the separation efficiency and the peak symmetry are also highly affected by the 2-propanol concentration, as can be seen in Fig. 3. Chromatography of talinolol using a phosphate buffer of pH 6 without 2-propanol results in a low separation efficiency, low enantioselectivity and only partial resolution. However, addition of 5% 2-propanol gives baseline resolution, as can be seen from Fig. 3. This is the result of higher enantioselectivity and better chromatographic performance when 2-propanol was added to the mobile phase.

The reason for the better chromatographic performance obtained for talinolol with 2-propanol in the mobile phase might be due to masking of a high affinity site by 2-propanol. This is supported by the fact that the retention is drastically reduced in the concentration range where the improvement of the chromatographic performance is obtained. It is very interesting to compare the behaviour of the two analogues

reduced on increasing the modifier concentration, whereas the separation factor for cathinone remains almost constant. The resolution increases for all compounds with increasing 2-propanol concentration up to 5%. For two of the compounds, laudanosine and atenolol, the res-

Table 2
Chromatographic properties of structural analogues of epinephrine

HO—CHCH$_2$NH—R$_4$

Solute	R$_1$	R$_2$	R$_3$	R$_4$	k_1'	α	R_s
Phenylethanolamine	H	H	H	H	2.18	1.40	2.44
Octopamine	H	OH	H	H	3.03	2.50	6.81
Norepinephrine	H	OH	OH	H	3.40	2.12	4.99
Epinephrine	H	OH	OH	CH$_3$	2.08	1.48	2.26
Normetanephrine	H	OH	CH$_3$O	H	3.28	2.04	4.82
Metanephrine	H	OH	CH$_3$O	CH$_3$	2.40	1.26	1.43
Isoprenaline	H	OH	OH	CH(CH$_3$)$_2$	1.80	1.14	0.75
Metaproterenol	OH	H	OH	CH(CH$_3$)$_2$	2.80	1.00	–
Terbutaline	OH	H	OH	C(CH$_3$)$_3$	1.61	1.18	0.86

Column: Chiral-CBH, 100 × 4.0 mm I.D. Mobile phase: 5% 2-propanol in 10 mM sodium phosphate buffer (pH 6.0)–50 μM disodium EDTA.

talinolol and atenolol with increasing 2-propanol concentration in the mobile phase (see Fig. 1A). The retention of atenolol is affected to a very limited extent by the 2-propanol concentration in the range 0–20% compared with the effect obtained for talinolol. This may indicate that they are bound in a different way, or to different subsites in the 40 Å long binding site.

3.3. Influence of solute structure on retention and enantioselectivity

A series of analogues of epinephrine were utilized as model compounds in order to study the influence of the molecular structure on the enantioselectivity and the retention. The compounds were chromatographed using a mobile phase of 5% 2-propanol in 10 mM sodium phosphate buffer (pH 6.0) containing 50 μM disodium EDTA. The results are summarized in Table 2. Phenylethanolamine, octopamine and norepinephrine have a primary amino group in the side-chain but they differ in the ring substitution. Phenylethanolamine has an unsubstituted ring, octopamine has a hydroxy group in the *para* position and norepinephrine has two hydroxy groups in *meta* and *para* positions to the side-chain. The retention increases in the order-phenylethanolamine $(k'_1 = 2.18) <$ octopamine $(k'_1 = 3.03) <$ norepinephrine $(k'_1 = 3.40)$, i.e., the retention increases with increasing number of hydroxy groups in the aromatic ring. This indicates that the hydroxy groups are involved in hydrogen bonding with hydrogen bonding groups in the binding site. The highest enantioselectivity of the above three compounds is obtained for octopamine with an α value of 2.5 and a resolution, R_s, of 6.8.

Comparison of norepinephrine with epinephrine, with a methyl group on the basic nitrogen, demonstrates that the introduction of the methyl group strongly influences both the retention and the enantioselectivity. The separation factor is reduced from 2.12 to 1.48. The retention is also reduced by about 40%. The same effects were observed for the pair normetanephrine and metanephrine. Higher enantioselectivity and retention were observed for normetanephrine.

which has a primary amino group in the side-chain. This might be due to the higher affinity in the ionic binding because the positively charged nitrogen atom will come closer to the anionic amino acid residue in the binding site. It can be seen that an even larger substituent, i.e., an isopropyl or an isobutyl group, instead of a methyl group on the basic nitrogen gives a further decrease in both the enantioselectivity and the retention. However, these observations are valid for this series of compounds and it is probably too early to draw any general conclusions concerning the relationship between the molecular structure and the enantioselectivity. More data have to be collected. For example, it has been observed for alprenolol and its desisopropyl analogue that the dealkylated analogue gave a lower enantioselectivity and higher retention [11]. However, as can be seen from Table 2, most of the compounds can be resolved with very high R_s values.

By chromatography of a series of analogues with different steric bulk on a basic nitrogen on the AGP column, it was observed that the enantioselectivity for many series of compounds increases with increasing steric bulk on the basic nitrogen atom [3,15].

3.4. Applicability of the Chiral-CBH column

The applicability of the Chiral-CBH column was tested by injecting a large number of drug compounds of different character. The molecular structures, the capacity factors of the least retained enantiomers, the separation factors (α) and the resolution (R_s) are summarized in Table 3. As can be seen, high enantioselectivity and very high resolution were obtained. Fig. 4A–D show chromatograms of four different types of drugs; atenolol, cimetidine sulphoxide, norepinephrine and toliprolol, resolved on a CBH 1 column. As can be seen, a very good chromatographic performance was obtained. With the present knowledge, our data indicate that the CBH 1 column is very useful for the resolution of a wide variety of basic drug compounds. In addition, among the basic drugs the column demonstrated excellent properties for amino
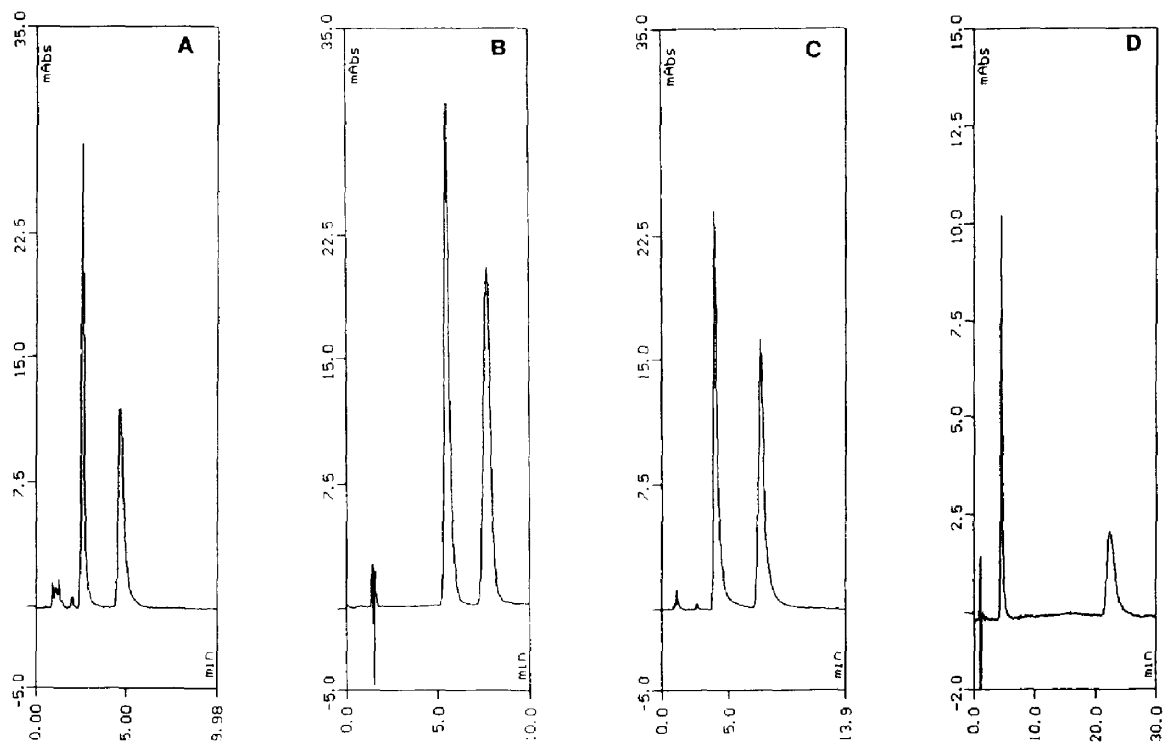
Fig. 4. Resolution of enantiomers on the Chiral-CBH column. (A) Sample, atenolol; column, Chiral-CBH (100 × 4.0 mm I.D.); mobile phase, 5% 2-propanol in 10 mM sodium phosphate buffer (pH 6.0)–50 μM disodium EDTA. (B) Sample, cimetidine sulphoxide; column, Chiral-CBH (150 × 4.0 mm I.D.); mobile phase, 10 mM sodium phosphate buffer (pH 6.0)–50 μM disodium EDTA. (C) Sample, norepinephrine; conditions as in (A). (D) Sample, toliprolol; conditions as in (A).

alcohols, which also was demonstrated on the first-generation CBH 1 column [10–13]. All the β-blockers that were tested were easily resolved, as can be seen from Table 3.

3.5. Stability of the CBH column

Stability studies were performed using a 100 × 4.0 mm I.D. column with a guard column (10 × 3.0 mm I.D.) in front of the analytical column. Two different mobile phases were used: 5% 2-propanol in 10 mM phosphate buffer (pH 6.0) containing 50 μM disodium EDTA and 5% 2-propanol in 10 mM phosphate buffer (pH 7.0) containing 50 μM disodium EDTA. The reason for choosing these mobile phases for the stability tests was that most of the separations that have been performed on the CBH column so far have been performed in the pH range 5–7. EDTA was added to the mobile phase in order to

complex the metal ions present. The mobile phase was pumped at a flow-rate of 0.9 ml/min, to obtain the same linear velocity as on a column having an I.D. of 4.6 mm and using a flow-rate of 1.0 ml/min.

In total 34 l of mobile phase were pumped through the column at pH 6.0, which corresponds to about 38 000 column volumes. It also corresponds to the continuous use of the column for 8 h per day, 5 days per week for 16 weeks. The guard column was exchanged after every 4 l of mobile phase (corresponding to about 4500 column volumes) had passed through the column and thereafter the three test compounds, cathinone, metoprolol and octopamine, were injected.

Fig. 5A shows the capacity factors of the most retained enantiomers of the three test compounds and Fig. 5B shows the resolution, R_s, versus the volume of mobile phase pumped

Table 3
Examples of baseline-resolved drugs and endogenous compounds

Substance	Formula	k'_1	α	R_s	Mobile phase[a]
Acebutolol	$CH_3(CH_2)_2CONH$—(ring, $COCH_3$)—$OCH_2CHCH_2NHCH(CH_3)_2$, OH	3.77	4.16	7.33	1
Atenolol	NH_2COCH_2—(ring)—$OCH_2CHCH_2NHCH(CH_3)_2$, OH	1.69	2.26	4.19	2
Betaxolol	(cyclopropyl)$-CH_2O(CH_2)_2$—(ring)—$OCH_2CHCH_2NHCH(CH_3)_2$, OH	3.82	3.63	6.45	1
Bisoprolol	$(CH_3)_2OCHCH_2OCH_2CH_2$—(ring)—$OCH_2CHCH_2NHCH(CH_3)_2$, OH	3.21	3.95	7.82	2
Carbuterol	HO—(ring, $NHCONH_2$)—$CHCH_2NHC(CH_3)_3$, OH	2.92	1.66	4.06	4
Cathinone	(ring)—$C-CH$, O NH_2 CH_3	2.36	1.54	3.29	2
Cimetidine sulphoxide[b]	$CH_3NHCNHCH_2CH_2SCH_2$—(imidazole, H_3C), NCN, O	3.04	1.54	3.28	3
Dobutamine	HO, HO—(ring)—$CH_2CH_2NHCHCH_2CH_2$—(ring)—OH, CH_3	5.62	1.78	3.41	2
Dropropizine	(ring)—N(piperazine)$N-CH_2CHOHCH_2OH$	4.16	1.49	2.85	4
Epanolol	(ring, $N≡C$)—$OCH_2CHCH_2NH(CH_2)_2NHCCH_2$—(ring)—$OH$, OH, O	9.52	2.22	3.64	5

Table 3 (*continued*)
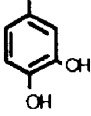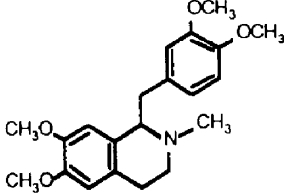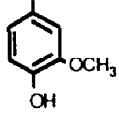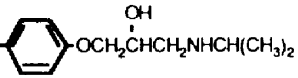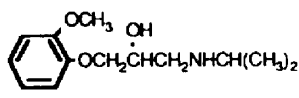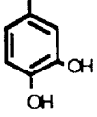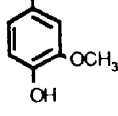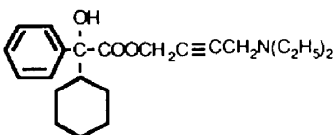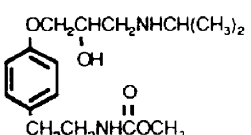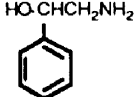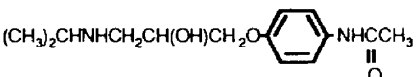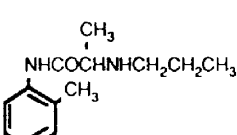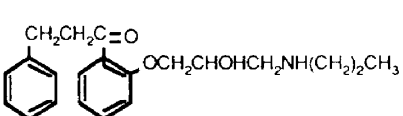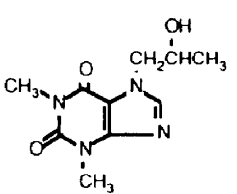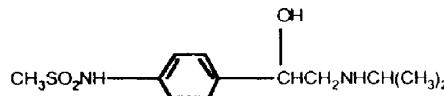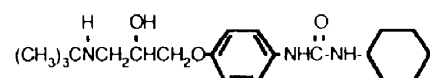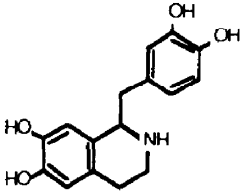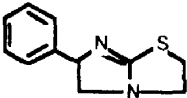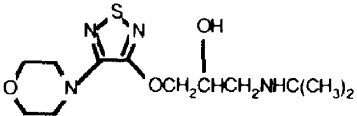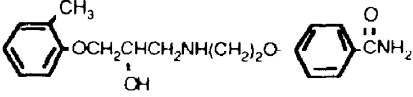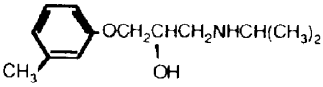
Substance	Formula	k'_1	α	R_s	Mobile phase[a]
Epinephrine	HO·CHCH$_2$NHCH$_3$ ...	4.26	1.69	4.00	6
Laudanosine	...	2.82	2.00	3.00	2
Metanephrine	HO·CHCH$_2$NHCH$_3$ ...	8.52	1.38	2.65	4
Metoprolol	CH$_3$OCH$_2$CH$_2$—...—OCH$_2$CHCH$_2$NHCH(CH$_3$)$_2$	3.59	3.16	7.15	2
Moprolol	...OCH$_2$CHCH$_2$NHCH(CH$_3$)$_2$	4.72	1.91	4.21	2
Norepinephrine	HO·CHCH$_2$NH$_2$ ...	3.40	2.12	4.99	2
Normetanephrine	HO·CHCH$_2$NH$_2$ ...	3.28	2.04	4.82	2
Octopamine	HO·CHCH$_2$NH$_2$ ...	3.03	2.50	6.81	2

Table 3 (*continued*)

Substance	Formula	k'_1	α	R_s	Mobile phase[a]
Oxybutynine		5.20	2.08	3.42	2
Pamatolol		4.01	2.31	5.34	2
Phenylethanolamine		4.62	1.54	3.26	6
Practolol		8.66	1.36	2.51	7
Prilocaine		3.16	1.48	2.90	4
Propafenone		7.06	2.04	4.10	1
Proxyphylline		1.22	1.63	2.47	4
Sotalol[b]		6.66	1.26	2.52	4
Talinolol		3.12	1.77	3.14	2

Table 3 (continued)

Substance	Formula	k_1'	α	R_s	Mobile phase[a]
Tetrahydropapaveroline		3.17	1.76	3.45	8
Tetramisole		2.45	1.58	3.29	9
Timolol		1.55	4.10	5.34	9
Tolamolol		3.46	2.07	3.68	5
Toliprolol		3.95	6.05	10.8	2

Column: Chiral-CBH, 100×4.0 mm I.D.

[a] Mobile phases: $1 = 5\%$ 2-propanol in 10 mM sodium acetate buffer (pH 5.5)–50 μM disodium EDTA; $2 = 5\%$ 2-propanol in 10 mM sodium phosphate buffer (pH 6.0)–50 μM disodium EDTA; $3 = 10$ mM sodium phosphate buffer (pH 6.0)–50 μM disodium EDTA; $4 = 5\%$ 2-propanol in 10 mM sodium phosphate buffer (pH 7.0)–50 μM disodium EDTA; $5 = 5\%$ 2-propanol in 10 mM sodium acetate buffer (pH 5.0)–50 μM disodium EDTA; $6 = 5\%$ 2-propanol in 10 mM sodium phosphate buffer (pH 6.5)–50 μM disodium EDTA; $7 = 5\%$ acetonitrile in 10 mM sodium phosphate buffer (pH 7.0)–50 μM disodium EDTA; $8 = 5\%$ acetonitrile in 10 mM sodium acetate buffer (pH 5.5)–50 μM disodium EDTA; $9 = 10\%$ 2-propanol in 10 mM sodium phosphate buffer (pH 6.0)–50 μM disodium EDTA.

[b] Sotalol and cimetidine sulphoxide were resolved on a column with the dimensions 150×4.0 mm I.D.

through the column. As can be seen, both the retention and the resolution are almost constant. No deterioration of the column properties was observed. Most likely the column could be run with an additional 38 000 column volumes or more without a negative influence on the chromatographic properties of the analytical column. Fig. 6 shows two chromatograms of cathinone, obtained before the stability studies and after 38 000 column volumes of mobile phase had been pumped through the column.

As was mentioned above, an additional stability study was also performed using a higher pH of the buffer (7.0). The difference in this study was that the guard column was exchanged after the passage of 7–8 l of mobile phase. Cathinone and prilocaine were used as test compounds. Fig. 7A and B show the capacity factors of the most
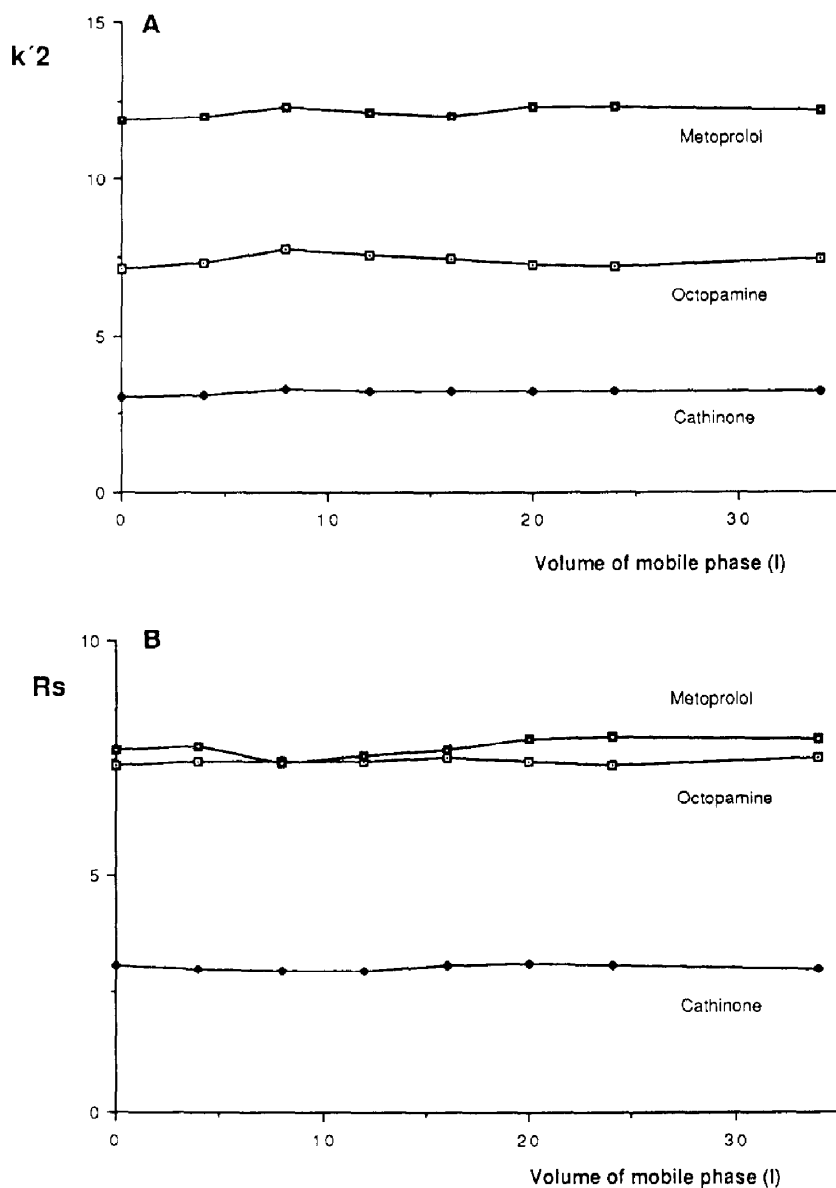
Fig. 5. (A) Capacity factors of the most retained enantiomer versus the volume of mobile phase pumped through the column. Column, Chiral-CBH (100×4.0 mm I.D.) with a Chiral-CBH guard column (10×3.0 mm I.D.); mobile phase, 5% 2-propanol in 10 mM sodium phosphate buffer (pH 6.0)–50 μM disodium EDTA. (B) Resolution versus the volume of mobile phase pumped through the column. Conditions as in (A).

retained enantiomer and the resolution, R_s, versus the volume of mobile phase pumped through the column. In total 22 l, corresponding to about 24 500 column volumes of mobile phase, were pumped in this stability study. As can be seen, the retention and the resolution of

the basic test compounds were almost constant and no negative influence on the chromatographic performance was observed. Therefore, it is reasonable to assume that there is no difference between the stability of the column at pH 6 and 7. The guard column is used in order to trap
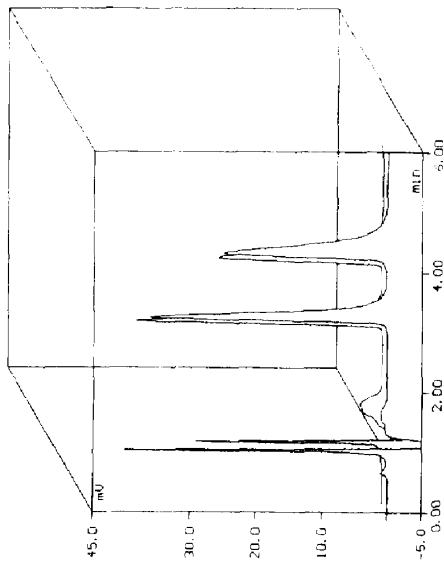
Fig. 6. Chromatograms of cathinone obtained before and after (front chromatogram) the passage of 34 l of mobile phase. Conditions as in Fig. 5A.
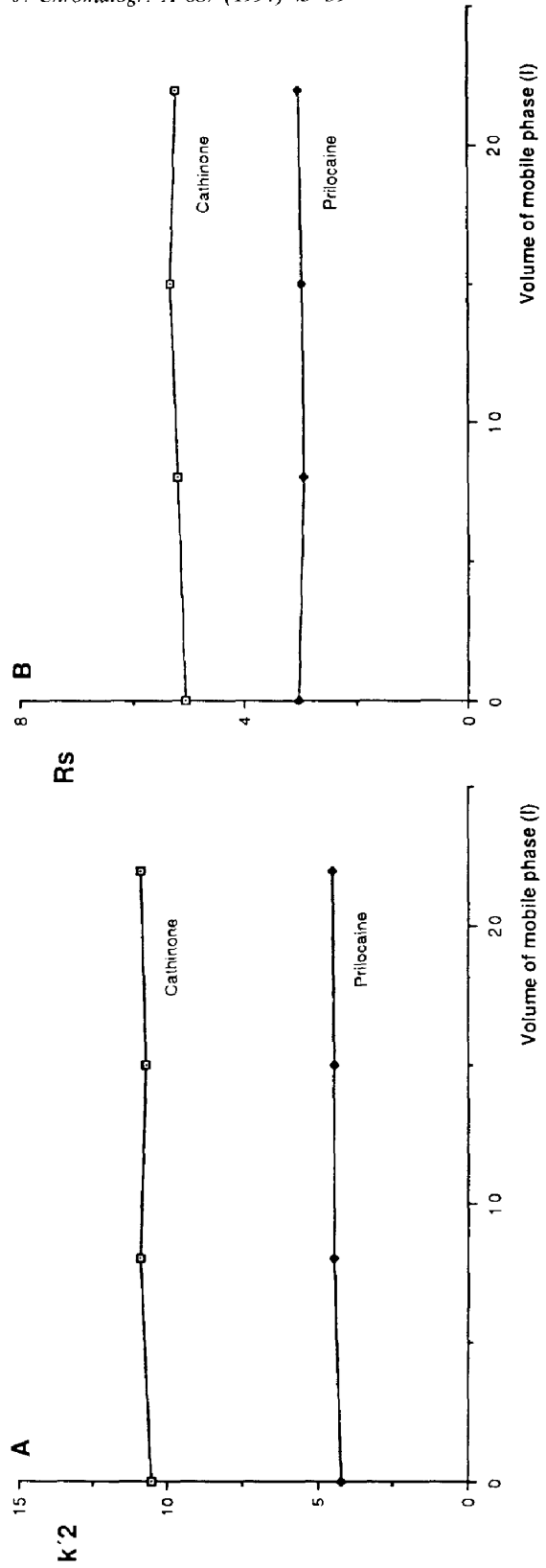
Fig. 7. (A) Capacity factors of the most retained enantiomer versus the volume of mobile phase pumped through the column. Column, Chiral-CBH (100 × 4.0 mm I.D.) with a Chiral-CBH guard column (10 × 3.0 mm I.D.); mobile phase, 5% 2-propanol in 10 mM sodium phosphate buffer (pH 7.0)–50 µM disodium EDTA. (B) Resolution versus the volume of mobile phase pumped through the column. Conditions as in (A).

compounds and metal ions that are bound to the column with high affinity.

On exchanging the guard column regularly the analytical column can be used for very long periods of time without a reduction of its chromatographic properties, as has been demonstrated in the stability studies. In the study performed at pH 6 the guard column was exchanged after 4 l of mobile phase. However, at pH 7 the guard column was exchanged after about 7–8 l of mobile phase. The stability studies demonstrated that the guard column should be replaced after about 7–8 l of mobile phase irrespective of the pH of the mobile phase. When 7–8 l of mobile phase had been pumped through the column, the resolution was decreased to some extent, which is the result of a small decrease in the separation efficiency caused by the guard column. However, the analytical column was totally unaffected by the large volumes of mobile phase that were pumped through the column, as is demonstrated in Figs. 5–7.

From this experiment, it can be concluded that the guard column traps something from the mobile phase that affects the migration of the sample on the guard column. Obviously, it must be present in the mobile phase at a very low concentration and it also has a very high affinity to the CBH 1 guard column. This conclusion is supported by the fact that the chromatographic properties of the analytical column are totally unaffected. As was mentioned above, it can be hypothesized that it is metal ions that are trapped on the guard column.

References

[1] J. Hermansson, J. Chromatogr., 269 (1983) 71.
[2] J. Hermansson and G. Schill, in P.A. Brown and R.A. Hartwick (Editors), High Performance Liquid Chromatography (Monographs on Analytical Chemistry Series), Wiley-Interscience, New York, 1988, pp. 337–374.
[3] J. Hermansson, Trends Anal. Chem., 8 (1989) 251.
[4] J. Hermansson and I. Hermansson, J. Chromatogr., 666 (1994) 181.
[5] I. Fitos, J. Visy, M. Simonyi and J. Hermansson, J. Chromatogr., 609 (1992) 163.
[6] J. Hermansson and A. Grahn, J. Chromatogr., in press.
[7] S. Allenmark, B. Bomgren and H. Borén, J. Chromatogr., 264 (1983) 63.
[8] E. Domenici, C. Bertucci, P. Salvadori, G. Felix, S. Motellier and I. Wainer, Chromatographia, 29 (1990) 170.
[9] T. Miwa, T. Mayakawa, M. Kayano and Y. Miyake, J. Chromatogr., 408 (1987) 316.
[10] P. Erlandsson, I. Marle, L. Hansson, R. Isaksson, C. Pettersson and G. Pettersson, J. Am. Chem. Soc., 112 (1990) 4573.
[11] I. Marle, P. Erlandsson, L. Hansson, R. Isaksson, C. Pettersson, G. Pettersson, J. Chromatogr., 586 (1991) 233.
[12] I. Marle, S. Jönsson, R. Isaksson, C. Pettersson, J. Chromatogr., 648 (1993) 333.
[13] R. Isaksson, C. Pettersson, G. Pettersson, S. Jönsson, I. Marle, J. Stålberg and J. Hermansson, Trends Anal. Chem., in press.
[14] C. Divne, J. Ståhlberg, T. Reinikainen, L. Ruohonen, G. Pettersson, J.K.C. Knowles, T. Teeri and A. Jones, Science, in press.
[15] U. Norinder and J. Hermansson, Chirality, 5 (1991) 422.