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Separation of enantiomers using α-chymotrypsin-silica as a chiral stationary phase

I. Marle*, A. Karlsson** and C. Pettersson

Department of Analytical Pharmaceutical Chemistry, Uppsala University, Biomedical Center, Box 574, S-751 23 Uppsala (Sweden)

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ABSTRACT

Direct separations of enantiomeric acids and N-substituted amino acids using α-chymotrypsin adsorbed or covalently immobilized on silica as the chiral stationary phase are presented. Phosphate buffer was used as the mobile phase. α-Chymotrypsin covalently bound to an aldehyde-activated silica resulted in a stable phase as opposed to α-chymotrypsin adsorbed on silica. The covalently bound stationary phase maintained the chiral discrimination ability even after more than 4000 column volumes of mobile phase had passed through the column. An increase in the amount of α-chymotrypsin from 110 to 140 mg/g silica was found to affect the retention by a factor of two without significantly influencing the enantioselectivity. The effect of pH, ionic strength and charged modifiers was studied in order to find optimum conditions for chiral separations. The chiral resolution could be optimized by adjusting the pH and by addition of octanesulphonate to the mobile phase.

INTRODUCTION

The enantioselective discriminating properties of proteins and enzymes have been utilized in the development of chiral separation systems for use in liquid chromatography [1]. A large number of enantiomeric drugs have been resolved on chiral stationary phases based on immobilized bovine [2] and human serum albumin [3], α_1-acid glycoprotein [4], ovomucoid [5] and cellulase [6].

The endopeptidase α-chymotrypsin, known to catalyse the enantioselective hydrolysis of a broad range of amides and esters [7], was recently immobilized on a glutaraldehyde-activated hydrophilic polymer that had been chemically bound to silica. The resulting chiral stationary phase was used for the separation of enantiomeric amino acids and amino acid derivatives [8]. Wainer and co-workers studied the stereochemical recognition of enantiomeric free and derivatized amino acids [9] and enantiomeric dipeptides [10] on the immobilized α-chymotrypsin phase. They suggested that the interaction occurred both at the enzymatic active site of the α-chymotrypsin and at other hydrophobic sites on the enzyme.

α-Chymotrypsin immobilized on different kinds of solid phases was hydrolytically active and the resolution obtained of racemic amino acid esters or amides was actually a separation of L-amino acids and intact D-amino acid esters or amides [8,9,11].

In this paper, we report on different immobilization techniques used for the preparation of α-chymotrypsin silica phases, *i.e.*, adsorption and covalent immobilization via reactive epoxy, aldehyde and tresyl groups, and on their applicability as chiral stationary phases [12]. The purpose of this investigation was to evaluate enantioselectivity and chromatographic performance of the α-chymotrypsin silica phases and to study the effect of the mobile

Correspondence to: Dr. Curt Pettersson, Department of Analytical Pharmaceutical Chemistry, Biomedical Center, Uppsala University, P.O. Box 574, S-751 23 Uppsala, Sweden.

* Present address: Analytical Quality Control, Astra Pharmaceutical Production AB, S-151 85 Södertälje, Sweden.

** Present address: Analytical Chemistry, Astra Hässle AB, S-431 83 Mölndal, Sweden.

phase composition (pH, ionic strength and charged modifiers) on enantioselective retention.

EXPERIMENTAL

Apparatus

The pump used was a model 110 A (Altex Scientific, Berkeley, CA, USA) equipped with a pulse damper (Touzard et Matignon, Vitry, France). The injector was a Model 7125 (Rheodyne, Cotati, CA, USA) with a 20-μl loop. The detector was a SpectroMonitor D (LDC Analytical, Riviera Beach, FL, USA) with variable wavelength. The recorder was a Model BD 40 (Kipp & Zonen, Delft, Netherlands).

The separation columns were made of stainless steel with a polished inner surface, equipped with modified Swagelok connectors and stainless-steel frits (2-μm porosity) or stainless-steel sieve filters (2-μm porosity) supported by spreaders (HPLC Teknik, Robertsfors, Sweden).

The chromatographic systems were thermostated at 20.0 ± 0.1°C by a Type 02 PT 923 water-bath (Heto, Birkerød, Denmark).

The pH meter was a Model E 632 (Methrohm, Herisau, Switzerland).

Chemicals

LiChrospher Si 100 (10 μm, 100 Å), LiChrospher Si 300 (10 μm, 300 Å), LiChrosorb Si 100 (10 μm, 100 Å), LiChrosorb DIOL (5 μm, 100 Å), (*R*)- and (*S*)-N-(1-phenylethyl)phthalamic acid and periodic acid (pro analysi) were obtained from E. Merck (Darmstadt, Germany). LiChrosorb DIOL (10 μm, 100 Å) activated with 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride) was kindly supplied by L. Hansson of Hafslund Nycomed Innovation (Ideon, Malmö, Sweden). Bovine α-chymotrypsin (three times crystallized and lyophilized), D- and L-tryptophan, D- and L-N-Boc-phenylalanine (Boc = *tert.*-butoxycarbonyl), D-and L-N-CBZ-phenylalanine (CBZ = carbobenzoxy), DL- and L-N-acetyltryptophan, racemic warfarin and (*R*)- and (*S*)-1-phenylethanol were obtained from Sigma (St. Louis, MO, USA). (2*R*,3*R*)- and (2*S*,3*S*)-dibenzoyltartaric acid and (2*R*,3*R*)- and (2*S*,3*S*)-di-*p*-toluoyltartaric acid were purchased from Fluka (Buchs, Switzerland). Racemic 2-(4-chlorophenoxy)propionic acid and 3-glycidoxypropyltrimethoxysilane were obtained from Janssen Chimica (Beerse, Belgium). (*S*)-2-(4-Chlorophenoxy)propionic acid and racemic 2-(4-iodophenoxy)propionic acid were kindly supplied by the Department of Organic Pharmaceutical Chemistry, University of Uppsala, Sweden. Racemic ketoprofen was obtained from Kabi Pharmacia (Helsingborg, Sweden) and racemic ibuprofen from Astra Läkemedel (Södertälje, Sweden). (*R*)- and (*S*)-naproxen were supplied by Syntex Laboratories (Paolo Alto, CA, USA). (*R*)- and (*S*)-propranolol hydrochloride were supplied by Imperial Chemical Industries. Macclesfield, UK). (1*S*,2*R*)-Ephedrine hydrochloride were obtained from Serva (Heidelberg, Germany) and (1*R*,2*S*)-ephedrine hydrochloride from Academical Pharmacy (Uppsala, Sweden). N,N-Dimethyloctylamine (DMOA) was supplied by ICN Pharmaceuticals (Plainview, NY, USA) and sodium 1-octanesulphonate by Eastman Kodak (Rochester, NY, USA).

All other substances were of analytical-reagent grade and used without further purification.

The solute structures are given in Fig. 1.

Column preparation

The solid phases used for *in situ* immobilization of α-chymotrypsin (α-CHT) were packed by a slurry technique using chloroform (LiChrospher Si 100 and Si 300, LiChrosorb Si 100 and DIOL) or dichloromethane (tresyl chloride-activated LiChrosorb DIOL) as suspending medium and hexane as packing solvent. After packing the frits were exchanged for sieve filters to minimize the effects of protein denaturation [13] during the immobilization of the enzyme.

α-CHT covalently immobilized on silica in batch experiments (α-CHT-A and -B phases below) were packed at 200 bar using sodium phosphate buffer (pH 6.5, $I = 0.1$) as suspending medium.

α-CHT adsorbed on silica

The LiChrospher Si 100, LiChrosorb Si 100 and LiChrospher Si 300 columns (150 × 4.6 mm I.D.) were equilibrated with sodium phosphate buffer ($I = 0.1$). A mobile phase containing 2.00 g/l of α-CHT in the buffer was applied at 1 ml/min until the front boundary had reached the plateau. Depending on the experimental conditions (Table I) the breakthrough volume correspondend to 30–100 column volumes of α-CHT solution. Finally, the

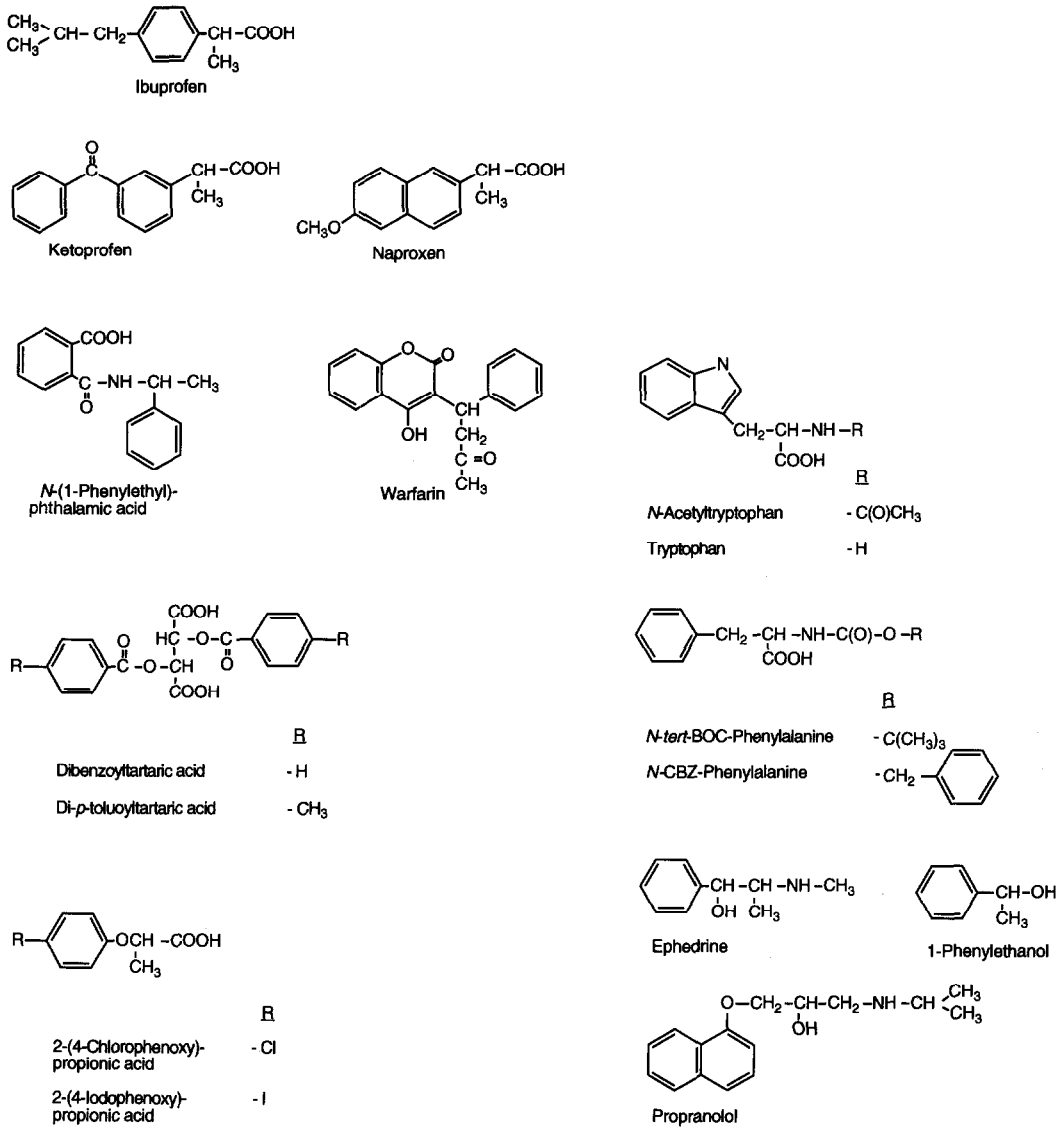

Fig. 1. Structures of solutes.

columns were equilibrated with sodium phosphate buffer (pH 5.3, $I = 0.1$).

The amount of α-CHT adsorbed was calculated by integration between Vm, obtained from the retention of sodium nitrate before the adsorption of α-CHT, and the breakthrough volume of α-CHT [14].

α-CHT-A: α-chymotrypsin immobilized on epoxide-activated silica

Monomeric epoxide-activated LiChrospher Si 100 was prepared according to Herman *et al.* [15]. The immobilization of α-CHT to epoxy-silica followed the description given by Hermansson [16]. The reaction scheme is given in Fig. 2a.

The amount of α-CHT immobilized on the silica of the α-CHT-A and -B phases was determined by

TABLE I

INFLUENCE OF PORE DIAMETER, SPECIFIC SURFACE AREA AND pH ON ADSORPTION OF α-CHT

Solid phase	Pore diameter (Å)	Specific surface area[a] (m^2/g)	pH	Amount of α-CHT adsorbed (mg/g)
LiChrospher Si 100	100	250	5.3	200
LiChrospher Si 100	100	250	7.4	300
LiChrosorb Si 100	100	300	7.4	220
LiChrospher Si 300	300	250	5.3	40
LiChrospher Si 300	300	250	7.4	130

[a] Ref. 25.

UV-spectrophotometry at 280 nm as the difference between the amount of α-CHT added to the reaction vessel and the amount of enzyme remaining in the supernatant after reaction and centrifugation.

The α-CHT-A phase was packed into a 100 × 3.0 mm I.D. column.

α-CHT-B: α-chymotrypsin immobilized on aldehyde-activated silica

LiChrosorb DIOL was oxidized to its aldehydic form by periodic acid [17]. A 4.0-g amount of LiChrosorb DIOL was suspended in 100 ml of water–methanol (4:1, v/v) and 2.0 g of periodic acid dissolved in 10 ml of the water–methanol mixture were added. The reaction mixture was stirred at room temperature for 48 h, then the aldehyde-activated silica was washed with an excess of water and dried.

α-CHT was immobilized on the aldehyde-activated silica mainly according to the method reported by Ohlson *et al.* [18]. A sample of 300 mg of α-CHT was dissolved in 15 ml of sodium carbonate buffer (pH 10, $I=0.2$) and 2 g of aldehyde-activated silica were added. The suspension was stirred at room temperature for 20 h. The α-CHT-silica was isolated by centrifugation and washed with water. The phase was finally mixed with suspending medium and stored in a refrigerator. The reaction scheme is given in Fig. 2b.

The α-CHT-B phase was packed into a 100 × 3.0 mm I.D. column.

α-CHT-C: in situ immobilization of α-chymotrypsin on aldehyde-activated silica

A column (150 × 4.6 mm I.D.) packed with LiChrosorb DIOL was equilibrated with water–methanol (4:1, v/v), 44 column volumes of 0.058 *M* periodic acid in the water–methanol mixture were pumped through the column and finally it was washed with 20 column volumes of sodium phosphate buffer (pH 6.3, $I=0.1$).

α-CHT was immobilized on the aldehyde-activated silica by applying a mobile phase consisting of 2.00 g/l of α-CHT in sodium carbonate buffer (pH 10, $I=0.1$) to the column at 1 ml/min until the front boundary had reached the plateau, corresponding to about 90 column volumes of α-CHT solution (Fig. 2b). The column was then washed with sodium phosphate buffer (pH 5.3, $I=0.1$) until a stable baseline was obtained.

The amount of α-CHT immobilized to the aldehyde-activated silica was determined as described above for α-CHT adsorbed on silica.

α-CHT-D: in situ immobilization of α-chymotrypsin on tresyl-activated silica

A column packed with tresyl-activated silica (150 × 3.0 mm I.D.) was equilibrated with coupling buffer, *i.e.*, sodium phosphate buffer (pH 7.1, $I=0.5$) containing 0.5 *M* NaCl [19]. A mobile phase containing 2.00 g/l of α-CHT in the coupling buffer was applied to the column at 1 ml/min until the breakthrough was complete, corresponding to 87 column volumes of α-CHT solution. The column was washed with 30 column volumes of coupling buffer and 30 column volumes of tris(hydroxymethyl)aminomethane (Tris)–HCl buffer (pH 8.0, $I=0.08$) to remove residual tresyl groups. It was finally equilibrated with sodium phosphate buffer (pH 5.3, $I=0.1$). The reaction scheme is given in Fig. 2c.

The amount of α-CHT immobilized on the tresyl-activated silica was determined by UV spectrophotometry at 282 nm. Eluate fractions were collected during the breakthrough and during the column washing. The amount of α-CHT immobilized was calculated as the difference between the amount of α-CHT that was introduced to the column and the amount found in the eluate fractions.

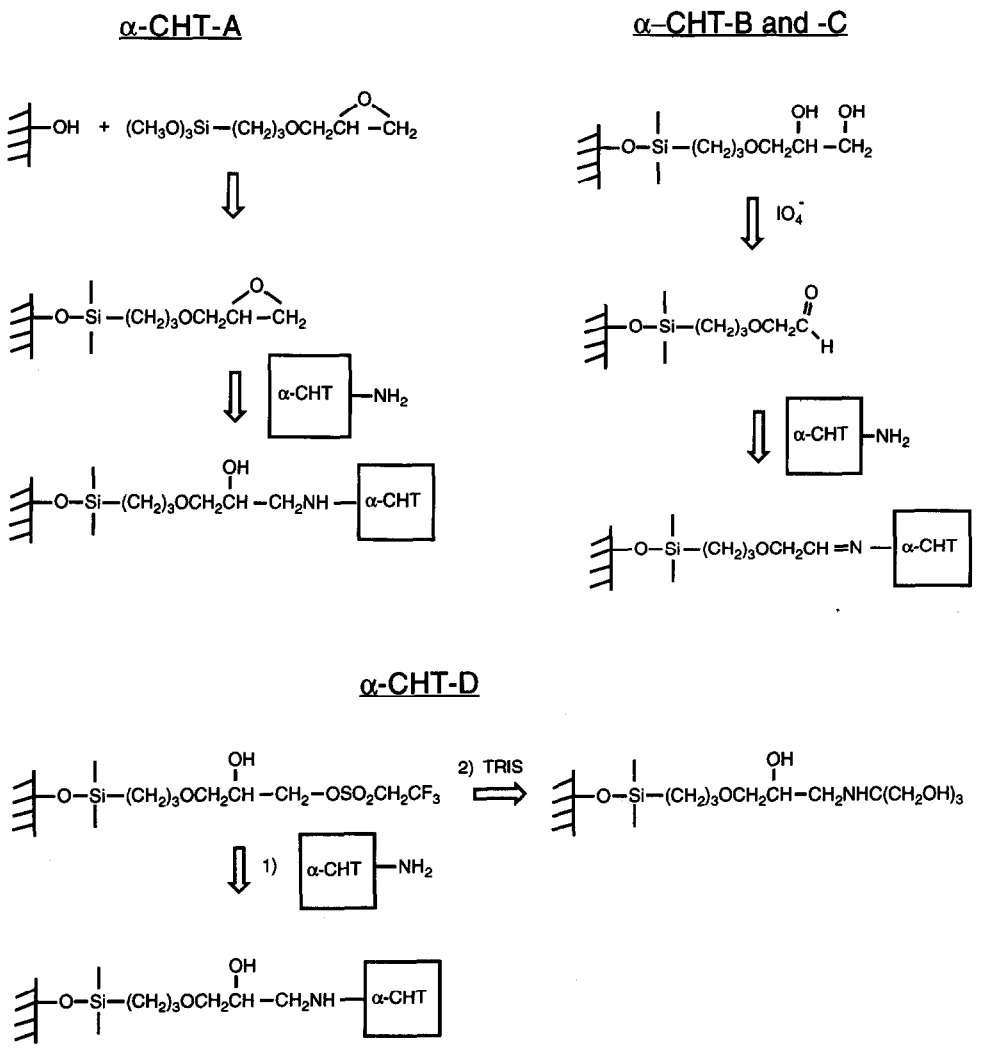

Fig. 2. Reaction schemes of the different methods for covalent immobilization of α-CHT on silica.

Chromatographic technique

The mobile phases were sodium phosphate buffers with an ionic strength of 0.1. The solutes were injected dissolved in the mobile phase at concentrations of about 0.1 m*M*. The flow-rate was 1 ml/min. V_0 was obtained by injection of sodium nitrate, which was assumed to be non-retained. The peak asymmetry factor, *asf*, was calculated as described previously [20].

RESULT AND DISCUSSION

α-Chymotrypsin adsorbed on silica phases

Different types of silicas were investigated in order to evaluate the chiral recognition and stability of α-CHT-coated silica phases (Table I). The amount α-CHT adsorbed on LiChrospher silica was affected by both the pore diameter of the solid phase and the mobile phase pH. Previously, maximum protein adsorption was observed at the isoelectric point of proteins, *i.e.*, bovine serum albumin on silica [21] and bovine serum albumin, lysozyme and α-CHT on porous glass [22]. It was not feasible

to study the adsorption of α-CHT at the isoelectric point, p*I* 8.1–8.3 [23], owing to the limited stability of silica at such a high pH [24]. However, in accordance with the observations for the adsorption of α-CHT on porous glass, it was found that a pH close to the p*I* favoured the adsorption of α-CHT on silica (Table I).

According to the manufacturer, LiChrospher Si 100 has the same specific surface area (250 m^2/g [25]) as LiChrospher Si 300 but a smaller mean pore diameter. A higher degree of exclusion of α-CHT (molecular size 50 × 40 × 40 Å [26]) from LiChrospher Si 100 than from LiChrospher Si 300 would have been expected. However, the LiChrospher Si 100 phase showed greater adsorption of the enzyme (Table I). A larger amount of isolated (free) silanol groups of the 100 Å silica than of the 300 Å silica [27,28] is probably the reason for the difference observed. Further, the adsorption of α-CHT on LiChrosorb Si 100 having a specific surface area of 300 m^2/g [25] was significantly lower than that on LiChrospher Si 100. However, the surface area accessible to α-CHT depends not only on the specific surface area but also of the pore structure, *i.e.*, pore size and pore distribution, which may be different for the two silica phases.

The α-CHT-coated silica phases gave enantioselective retention for various solutes, *e.g.*, naproxen (Fig. 3).

The coating of the silica with α-CHT was performed at pH 7.4, as this resulted in a sorbent with a high chiral capacity owing to the large amount of protein adsorbed (Table I). The chiral separation of naproxen ($\alpha = 1.68$) was effected when 120 column volumes (0.30 l) of the mobile phase, phosphate buffer (pH 5.3), had passed through the column.

Unfortunately, the leakage of α-CHT from the coated silica phases was relatively high. This is illustrated by the continuous decrease in the capacity factors on coated LiChrospher Si 100 shown in Fig. 4. Almost no enantioselectivity was observed when 1200 column volumes (3 l) of phosphate buffer pH 5.3 had passed through the column.

In conclusion, although chiral separations are possible on α-CHT-coated phases, they do not appear to be useful for quantitative analyses of enantiomers, owing to instability.

α-CHT covalently immobilized on silica

Immobilization technique and chromatographic performance. Three different silica phases with reactive epoxy (A), aldehyde (C) or tresyl (D) groups were used to immobilize α-CHT covalently (Table II). The highest stereoselectivities and shortest retention times were generally obtained when the α-CHT was immobilized on epoxy-silica, α-CHT-A. The high enantioselectivity was due to a higher loading of the enzyme on this phase. α-CHT covalently immobilized *in situ* on tresyl-activated silica resulted in increased retention and low stereoselectivity. Immobilization of α-CHT on aldehyde-(α-CHT-C) and tresyl-activated silica (α-CHT-D) gave about the same loading of the enzyme (Table II). However, the enantioselectivity of the α-CHT-C phase was higher than that of the α-CHT-D phase,

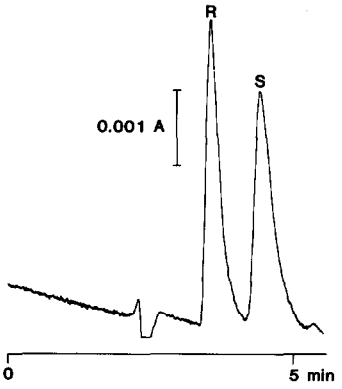

Fig. 3. Separation of (*R,S*)-naproxen. Solid phase: α-CHT adsorbed on LiChrospher SI 100 at pH 7.4. Mobile phase: phosphate buffer (pH 5.3).

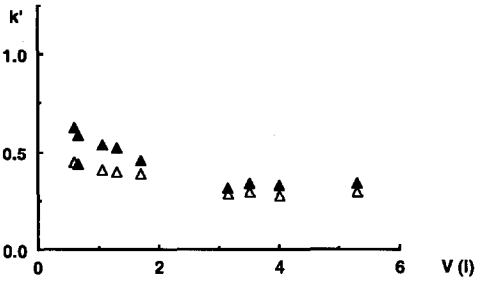

Fig. 4. Stability of an α-CHT-coated silica phase. Solid phase: α-CHT adsorbed on LiChrospher SI 100 at pH 5.3. Mobile phase: phosphate buffer (pH 5.3). Solutes: △ = (*R*)-naproxen; ▲ = (*S*)-naproxen.

TABLE II

ENANTIOSELECTIVE RETENTION OF α-CHT-SILICA PHASES

Mobile phase: Phosphate buffer (pH 5.3).

Solute	Parameter	A[a] (200 mg/g)[b]	C[a] (140 mg/g)[b]	D[a] (130 mg/g)[b]
N-(1-Phenylethyl)phthalamic acid	k'_2	0.89	1.3	1.8
	α	1.6	1.7	1.3
	asf	1.8	3.3	1.8
Naproxen	k'_2	2.7	10	16
	α	2.4	1.8	1.2
	asf	1.6	3.8	1.4
Warfarin	k'_2	2.5	11	61
	α	1.4	1.3	1.0
	asf	–	2.9	1.3
Di-*p*-toluoyltartaric acid	k'_2	1.7	4.4	1.7
	α	2.9	1.7	1.4
	asf	1.8	5.2	1.8

[a] For details, see Experimental.
[b] Amount of α-CHT immobilized.

whereas the retention using the α-CHT-C phase was generally lower than that using the α-CHT-D phase. The chromatographic performance of the α-CHT-A and -D phases was higher than that of the α-CHT-C phase, which gave strongly tailing peaks. In addition to the larger amount of α-CHT immobilized on the α-CHT-A phase than on the -C and -D phases, the different immobilization procedures and the different silica matrices used could also add to the discrepancies observed.

Although the chromatographic properties of the α-CHT-A phase were superior to those of the α-CHT-C phase, the latter chiral phase is to be preferred owing to its greater stability.

Stability and reproducibility. The stability of two batches of the α-CHT-C phase was investigated, columns 1 and 4 in Fig. 5. The retention times for the naproxen enantiomers decreased by about 10% on both columns but the stereoselectivity was unaffected even after 10 l (>4000 column volumes) of the mobile phase has passed through the columns. A small loss of α-CHT due to hydrolysis of the imino bond between the protein and the silica might be the reason for the slight decrease in retention. The uninfluenced enantioselectivity indicates that the solute retention is dominated by adsorption on α-CHT sites, *i.e.*, negligible adsorption on the matrix and the spacer. Using a highly adsorbing matrix, the enantioselectivity was shown to decrease with decreasing amount of chiral selector [29].

The α-CHT-C phases could be prepared with reproducible enantioselectivity (Fig. 5). Almost no

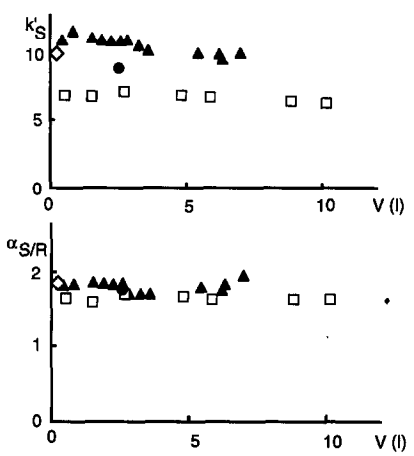

Fig. 5. Stability and reproducibility of solid phase α-CHT-C. Mobile phase: phosphate buffer (pH 5.3). Solutes: (*R*)- and (*S*)-Naproxen. ▲ = column 1 (140 mg α-CHT/g silica); ◇ = column 2 (130 mg/g); ● = column 3 (130 mg/g); □ = column 4 (110 mg/g).

differences in the enantioselectivity of naproxen were observed for the four columns investigated. The capacity factors were, as expected, dependent on the loading of the enzyme on the stationary phase. Increasing the amount of α-CHT from 110 to 140 mg/g of silica resulted in an increase in k' by a factor of two. This result may support the suggestion, given in a study using cyanogen bromide-activated Sepharose 4B as the matrix, that the last fraction of α-CHT is immobilized in a conformation other than the first one [30] and will result in a higher binding affinity for chiral solutes. The conformational change would be due either to a heterogeneity in the support material or to induced heterogeneity because of interactions of enzyme which attaches later to those protein molecules already established on the surface [30].

Solute structure and stereoselectivity

The α-CHT silica phases separated enantiomeric mono- and divalent acids, 1-phenylethanol, tryptophan and N-substituted amino acid derivatives, Tables III–V (for solute structures, see Fig. 1). All these solutes have an aromatic group in the vicinity of the asymmetric carbon atom, indicating that charge-transfer, hydrophobic and/or steric interactions may be important for chiral recognition on the α-CHT phase. The carboxylic acids were mainly ionized at the pH used (5.3 and 6.4) and may thus interact by electrostatic attraction with the α-CHT phase, as it has a positive net charge below pI 8.1–8.3 [23]. The high enantioselectivities obtained on α-CHT silica allowed the resolution of various enantiomers, despite the low column efficiency (Figs. 6–9). The small separation factors obtained for the enantiomers of propranolol and ephedrine indicate that the α-CHT silica phases are less suitable for separating enantiomeric amino alcohols.

Regulation of retention and enantioselectivity by the mobile phase composition

pH and ionic strength. The influence of pH on enantioselective retention is presented in Table V. On increasing the pH a decrease in retention of the carboxylic acids and an increase in retention for the amines was observed. This might be due to a change in hydrophobic and/or electrostatic interactions. However, native α-CHT can exist in at least two conformational states, one enzymatically active and the other inactive, and the fraction of α-CHT present in the active conformation increases from 45% to 85% when the pH is increased from 3 to 7 (I=0.1) [31,32].

Covalent immobilization of α-CHT on silica, Sepharose or synthetic polymer most often changes the physical properties of the free enzyme resulting in, for example, a decrease in the specific activity, a displacement of the pH of maximum hydrolytic activity, an increase in the thermal stability and decreased autolysis [33–37]. It seems reasonable to assume that the fraction of immobilized α-CHT being hydrolytically active will undergo similar conformational changes as the native enzyme on pH

TABLE III

SOLUTE STRUCTURE AND ENANTIOSELECTIVITY WITH α-CHT-C SOLID PHASE

Mobile phase: phosphate buffer (pH 5.3).

Solute	Column[a]	k'_2	α
2-(4-Iodophenoxy) propionic acid	1	1.26	1.16
Di-*p*-toluoyl tartaric acid	2	4.43	1.67
N-(1-Phenylethyl) phthalamic acid	2	1.31	1.7
Warfarin	2	10.8	1.31
1-Phenylethanol	1	0.72	1.2

[a] See caption to Fig. 5.

TABLE IV

SOLUTE STRUCTURE AND ENANTIOSELECTIVITY WITH α-CHT-B[a] SOLID PHASE

Mobile phase: phosphate buffer (pH 6.4)

Solute	k'_2	α
Tryptophan	0.77	1.4
N-Acetyltryptophan	2.71	1.92
N-CBZ-phenylalanine	5.25	1.62
N-Boc-phenylalanine	2.42	2.6
Ketoprofen	2.43	1.0
Ibuprofen	1.4	1.0
Naproxen	6.22	1.54

[a] 130 mg α-CHT/g silica.

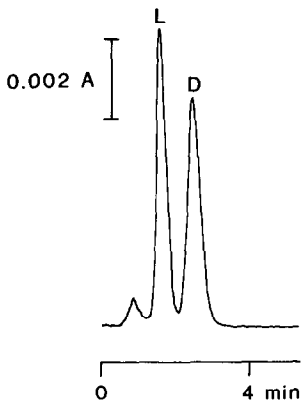

Fig. 6. Separation of DL-N-acetyltryptophan. Solid phase: α-CHT-B. Mobile phase: phosphate buffer (pH 6.4).

changes. The increase in retention at pH 5.3 for the uncharged 1-phenylethanol and the weak acid warfarin ($pK_a = 5.0$ [38]) indicated a modification of the retaining properties of the α-CHT silica phase by pH. Further, the change in enatioselectivity at pH 5.3 for several solutes also supports the proposal of conformational changes.

Ionic strength variations in the range 0.01–1.0 influence the binding properties and the enzymatic activity of native α-CHT [31]. The effect of the ionic strength in the range 0.025–0.1 on the enatioselective retention of some solutes on α-CHT silica is given in Table VI. Propranolol gave stereoselective retention on the α-CHT silica phase only at low buffer ion concentrations, whereas the enantiomers

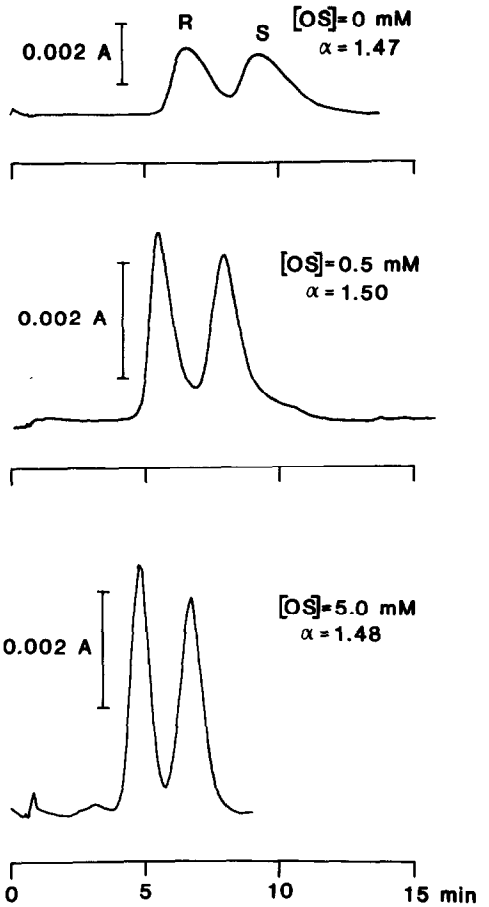

Fig. 8. Influence of octanesulphonate (OS) on enantioselective resolution. Solid phase: α-CHT-B. Mobile phase: OS in phosphate buffer (pH 5.0). Solute: (*R,S*)-naproxen.

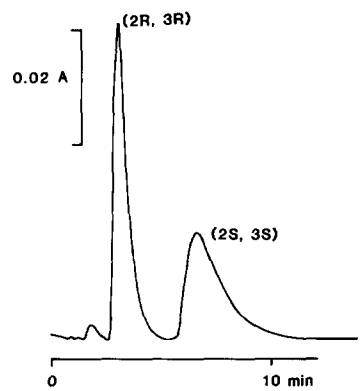

Fig. 7. Separation of (2*R*,3*R*;2*S*,3*S*)-di-*p*-toluoyltartaric acid. Solid phase: α-CHT-A. Mobile phase: phosphate buffer (pH 3.8).

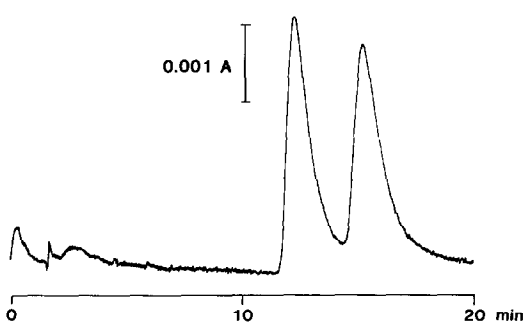

Fig. 9. Separation of the enantiomers of warfarin. Solid phase: α-CHT-C (110 mg α-CHT/g silica). Mobile phase: phosphate buffer (pH 5.4).

TABLE V

INFLUENCE OF pH ON ENANTIOSELECTIVE RETENTION

Solid phase: α-CHT-A. Mobile phase: phosphate buffer.

Solute	pH 3.85		pH 5.28		pH 6.40	
	k'_2	α	k'_2	α	k'_2	α
2-(4-Chlorophenoxy) propionic acid	0.50	1.0	0.28	1.0	0.18	1.0
Di-benzoyl tartaric acid	2.54	2.05	0.46	2.3	0.23	2.1
Di-*p*-toluoyl tartaric acid	9.13	2.85	1.71	3.0	0.81	2.2
Naproxen	4.15	2.13	2.72	2.45	1.61	2.5
N-(1-Phenylethyl) phthalamic acid	1.30	1.3	0.89	1.6	0.58	1.6
Warfarin	1.97	1.37	2.51	1.38	1.5	1.0
N-CBZ-phenylalanine	3.37	1.36	–	–	2.06	1.98
N-Boc-phenylalanine	1.7	2.0	1.58	2.4	1.14	2.4
N-Acetyltryptophan	1.5	1.4	1.58	1.7	1.28	1.9
Tryptophan	0.17	1.4	0.37	1.7	0.45	1.5
1-Phenylethanol	0.29	1.0	0.41	1.1	0.40	1.1
Propranolol	1.12	1.04	3.9	1.0	5.3	1.0
Ephedrine	0.28	1.1	1.4	1.1	1.6	1.1

of naproxen and di-*p*-toluoyltartaric acid were separated at both low and high buffer ion concentrations.

Charged modifiers. Addition of organic cations and anions to the mobile phase has been used to regulate retention and stereoselectivity on protein chiral stationary phases, *e.g.*, immobilized α_1-acid glycoprotein [4] and albumin [39].

Octanesulphonate (OS) in the mobile phase decreased the retention times of (*R*)- and (*S*)-naproxen but had almost no effect on the enantioselectivity when α-CHT-silica was used as the stationary phase (Fig. 8). OS improved the column efficiency and the peak symmetry and thus increased the chiral resolution.

Chiral separations using a cationic modifier, dimethyloctylamine (DMOA), in the mobile phase were also investigated (Table VII). The capacity factors for the enantiomeric acids were not affected by the DMOA, indicating a negligible retention of the acids as ion pairs with DMOA. Interestingly, contrary to the improved enantioselectivity of chiral carboxylic acids using α_1-acid glycoprotein-silica as stationary phase [4], DMOA had no effect on

TABLE VI

INFLUENCE OF IONIC STRENGTH (*I*) ON ENANTIOSELECTIVE RETENTION

Solid phase: α-CHT-A. Mobile phase: phosphate buffer (pH 5.3).

Solute	I = 0.025		I = 0.050		I = 0.10	
	k'_2	α	k'_2	α	k'_2	α
Naproxen	1.68	2.2	1.92	2.2	2.20	2.3
Di-*p*-toluoyl tartaric acid	1.21	4.3	1.28	4.0	1.49	3.2
Propranolol	11.4	1.1	7.95	1.0	5.4	1.0

TABLE VII

INFLUENCE OF DIMETHYLOCTYLAMINE (DMOA) ON ENANTIOSELECTIVE RETENTION

Solid phase: α-CHT-A. Mobile phase: DMOA in phosphate buffer (pH 5.4).

Solute	DMOA (m*M*)								
	0			0.20			2.0		
	k'_2	α	*asf*	k'_2	α	*asf*	k'_2	α	*asf*
Naproxen	1.99	2.2	1.6	2.04	2.3	1.6	2.03	2.2	1.7
Di-*p*-toluoyl tartaric acid	1.30	3.3	1.6	1.30	3.5	1.8	1.58	3.3	1.6
N-(1-Phenylethyl) phthalamic acid	–	–	–	0.65	1.7	1.7	0.58	1.9	1.7
N-Boc-phenylalanine	–	–	–	1.02	2.3	2.1	1.35	3.3	1.8
N-CBZ-phenylalanine	–	–	–	2.08	1.11	1.8	2.14	1.57	1.9
Tryptophan	–	–	–	0.26	1.4	1.9	0.22	1.9	1.6
Propranolol	4.98	1.0	1.8	2.87	1.0	2.4	1.75	1.0	1.9

the enantioselectivity for the anionic solutes naproxen, di-*p*-toluoyltartaric acid and N-(1-phenylethyl)phthalamic acid (Table VII). However, the significant changes in the enantioselectivity for the amino acid derivatives when DMOA was present in the mobile phase suggested that DMOA may interact with the immobilized enzyme, giving rise to altered binding properties. The decrease in the retention of propranolol with increasing concentration of DMOA might be due to a competition by propranolol enantiomers and DMOA for the same adsorption sites on the stationary phase.

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