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Retention behavior and chiral recognition of β-cyclodextrin-derivative polymer adsorbed on silica for warfarin, structurally related compounds and Dns-amino acids

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ABSTRACT

Warfarin enantiomers that have previously been reported to be difficult to separate on cyclodextrin bonded high-performance liquid chromatographic supports can be easily and completely resolved on a stationary phase obtained by deposition on silica of an epichlorohydrin–β-cyclodextrin polymer derivative. The separation of other hydroxy-coumarin analogues and Dns-amino acids is also demonstrated. Studying the influence of the pH and methanol content of the aqueous mobile phase allows the conditions required to separate these compounds to be optimized.

INTRODUCTION

Cyclodextrins (CD) have been succesfully used as chiral liquid chromatography stationary phases for the separation of enantiomers. First, cross-linked β-CD–polymers beads were used to resolve the enantiomers of mandelic acid derivatives [1] and indole alkaloids [2] in conventional chromatography. Then, the grafting of β-CD to silica by Armstrong [3] gave rise to Cyclobond stationary phases. These supports exhibit excellent properties, allowing the enantiomeric resolution of numerous compounds such as alkaloids, amino acid derivatives, organometallic compounds, mandelic acid and drugs [3–6]. However, no chiral stationary phase is universally effective for the resolution of all types of enantiomers. For example warfarin, a 3-substituted 4-hydroxycoumarin derivative, could not be resolved on a β-CD bound column [7].

There are a number of requirements for chiral recognition by the β-CD cavity. In addition to inclusion of a part of the molecule, the chiral center or one of its substituents must be near and interact with the mouth of the β-CD cavity. So, computer models of *d* and *l* isomers of the anticoagulant warfarin demonstrate that the phenyl group of the drug is too far from the CD rim to achieve differential complexation [7].

To extend the range of chiral recognition separation, derivatization of the secondary hydroxyl groups of β-CD has been carried out in order to provide additional interactions. Several isomer separations that are not possible on high-performance

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liquid chromatographic (HPLC) supports bearing native CD residues have been achieved on acetylated [4] or hydroxypropyl-substituted β-CD phases [8].

β-CD silica supports can also be obtained by non-covalent coating of β-CD polymers to the mineral surface. In a previous study [9] we grafted native β-CD to polyethyleneimine to prepare a water-soluble polymer easily adsorbed at the silica surface, the amine functions providing a tightly adsorbed coating as a result of strong interactions with silanol groups. This liquid chromatographic support was able to separate structural isomers but no specific drug chiral recognition was observed for warfarin, which was eluted as a single peak, as on Cyclobond phases [7].

The present work deals with the properties of a new HPLC support for enantiomer separations that we have described recently [10]. It is based on adsorption onto silica of a soluble epichlorohydrin–β-CD polymer bearing ammonium substituents. The efficiency of these supports in resolving warfarin and related coumarin enantiomers was examined. Moreover the separation of Dns-amino acids (Dns = dansyl = 5-dimethylaminonaphthalene-1-sulphonyl) already described on immobilized native [11] or modified β-CD [12], was investigated to compare the chromatographic properties of our β-CD polymer phase with previous ones.

EXPERIMENTAL

Materials

β-CD (Ringdex B) was a gift from Orsan (Paris, France). The coumarin anticoagulants, sodium warfarin (Merrel Dow, Bourgoin-Jallieu, France), coumachlor and acenocoumarol (Ciba-Geigy, Rueil-Malmaison, France) and phenprocoumon (Hoffman-La Roche, Basle, Switzerland) were used as supplied by the manufacturers and are listed in Table I.

Dns-amino acids were obtained from Sigma (St. Louis, MO, USA).

LiChrospher Si-100, 5 μm particle diameter, was obtained from Merck (Darmstadt, Germany).

Preparation of the enantiomers of warfarin

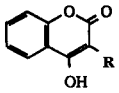
Warfarin was resolved by the method of West *et al.* [13] to yield optically pure *R*-warfarin ($[\alpha]_D^{25} = +149.0$) and *S*-warfarin ($[\alpha]_D^{25} = -148.7$) (concentration = 1 *M*, 0.5 *M* sodium hydroxide).

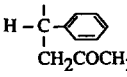
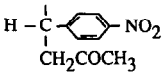
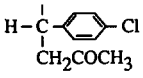
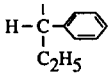
Preparation of the chromatographic supports

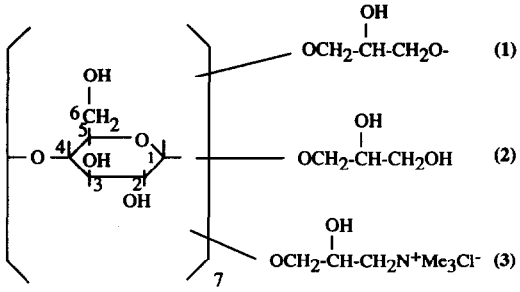
Several soluble epichlorohydrin–β-CD polymers bearing ammonium substituents (EP–β-CD-N^+) were obtained as previously described [10]. Firstly we prepared EP–β-CD derivatives by reacting epichlorohydrin and β-CD. By varying the molar ratio of these compounds, β-CD polymers with different amounts of dihydroxypropyl groups as substituents and hydroxypropyl groups as bridges linking β-CD were obtained. It has been previously determined by ^{13}C-NMR spectra that the hydroxypropyl substitutions take place at the C-2, C-3 and even C-6 positions of β-CD [14,15]. Then, by reacting the polymers with 2,3-epoxypropyltrimethylammonium chloride we obtained EP–β-CD-N^+ derivatives containing about one hydroxypropylammonium group (N^+) per β-CD moiety. The supposed structure is illustrated in Fig. 1.

TABLE I

COUMARIN ANTICOAGULANTS AND THEIR FORMULAE



Drug	R
Warfarin D,L-3-(α-Acetonyl-4-benzyl)-4 hydroxycoumarin	
Acenocoumarol D,L-3-(α-Acetonyl-4-nitrobenzyl)-4 hydroxycoumarin	
Coumachlor D,L-3-(α-Acetonyl-4-chlorobenzyl)-4 hydroxycoumarin	
Phenprocoumon D,L-3-(α-Ethyl-benzyl)-4 hydroxycoumarin	



(1) hydroxypropyl bridge
(2) dilhydroxypropyl substituent
} *OHP GROUPS*

(3) hydroxypropylammonium substituent *N+ GROUP*

Fig. 1. Proposed structure of EP-β-CD-N^+.

The number of ammonium substituents was determined by anion argentimetric titration. The number of β-CD cavities was evaluated by spectroscopic measurement of the decoloration of phenolphthalein solutions at pH 10.5, which form a very stable complex with β-CD [16].

The 2-hydroxy-3-trimethylammonium β-CD derivative (β-CD-N^+), compound **6**, was prepared as described previously [17], with 1.1 ammonium substituents per β-CD residue.

Solutions of compounds **1–6** in distilled water (6%, v/v), were adsorbed on LiChrospher Si-100 at room temperature, stirred with a vortex mixer for 24 h, then filtered, washed and dried under vacuum.

The composition of compounds **1–6** and the carbon content of supports are summarized in Table II.

TABLE II

COMPOSITION OF β-CD DERIVATIVES AND CHARACTERIZATION OF SUPPORTS

EP–β-CD-N^+ polymer	N^+–β-CD ratio	OHP[a]–β-CD ratio	Support carbon content (%)
1	1.76	12.6	4.67
2	0.93	9.3	4.46
3	1.39	7.6	4.11
4	1.16	5.8	4.07
5	1.46	1.2	2.07
β-CD-N^+			
6	1.1	0	1.15

[a] OHP is the sum of mono- and dihydroxypropylgroups present in the CD polymer.

Chromatographic experiments

Stainless-steel columns (10 cm × 4.6 mm I.D.) were filled with the supports by a slurry packing technique. Phosphate buffer (0.1 *M*) of various pH was used as eluent. Methanol was used as organic modifier. The flow-rate was 1 ml/min. Samples of 20 μl of 10 μ*M* coumarin derivatives and dansyl amino acid solutions were injected.

The apparatus was the same as described previously [9].

RESULTS AND DISCUSSION

Influence of the OHP–β-CD ratio on the chromatographic behavior of coumarin derivatives

Fig. 2a, b and c presents the chromatographic profiles of racemic warfarin on columns **IV–VI** filled with the supports obtained by impregnation of silica with 6% solutions of compounds **4–6**, respectively. A mixture of 20% methanol and 80% phosphate buffer (pH 4) was used as mobile phase. Note that the chromatographic profile of warfarin depends on the value of OHP–β-CD ratios and also on the amount of β-CD derivative deposited (from carbon content results in Table II). An increase in warfarin retention was observed with an increase in the amount of β-CD derivative adsorbed. So excellent separation of warfarin enantiomers (resolution factor R_s = 2.0) is observed in Fig. 2a, while poor resolution is observed in Fig. 2b and neither

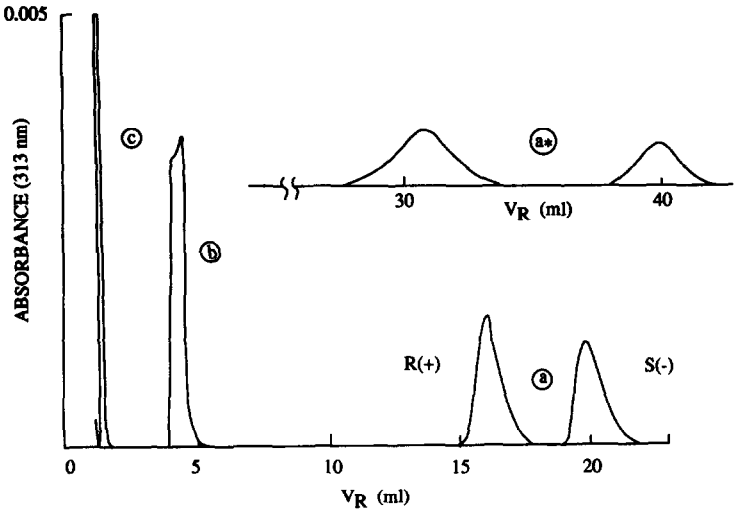


Fig. 2. Elution profiles (V_R = elution volume) of racemic warfarin on 10 cm × 4.6 mm I.D. columns **IV–VI**, filled respectively with LiChrospher Si-100 coated with β-CD derivatives **4–6**. Silica was impregnated with 6% solutions of (a) EP–β-CD-N^+ derivative **4**, (b) EP–β-CD-N^+ derivative **5** and (c) β-CD-N^+ derivative **6** and (a*) a 12% solution of β-CD derivative **4**. Eluent: methanol–0.1 *M* phosphate buffer (pH 4) (20:80).

retention nor separation in Fig. 2c, which corresponds to a support without an OHP group. So there is a minimum OHP–β-CD ratio that is needed to obtain sufficient adsorption of the β-CD derivative on silica and consecutive resolution of warfarin enantiomers.

An additional experiment presented in Fig. 2a* was done on column **IV*** filled with silica impregnated with a 12% solution of compound **4**. Stronger retentions of warfarin isomers are noticed on this support. The result of elemental analysis (C = 7.82%) indicates that saturation of silica was not reached when impregnated with a 6% solution of β-CD derivative (Table II, carbon content results). But as it appears that the separation properties of column **IV** are high enough to assure a complete resolution of warfarin enantiomers, the subsequent experiments were done on this column.

Moreover we injected separately the enantiomers of warfarin. The *R*(+) isomer is eluted first (Fig. 1a), thus the presence of OHP groups on β-CD reinforces the *S*(−) isomer complexation.

Fig. 3 shows the separation on column **IV** of three other coumarin anticoagulant enantiomers which all contain one phenyl group substituent on the chiral C atom. They are well resolved with factors of resolution which are not related to their retention volumes: warfarin and phenprocoumon R_s values are respectively 2.0 and 0.8, while the latter compound has a retention volume 4.5 times greater than that of warfarin. The R_s values of acenocoumarol and coumachlor are 0.5 and 1.5, respectively. The diversity of the R_s values demonstrates the influence of the various substituents near the chiral center, a nitro group being less favorable than a chloro group.

We can conclude that the presence of hydroxypropyl groups bonded to β-CD is responsible for the additional interactions with the chiral group outside the cavity,

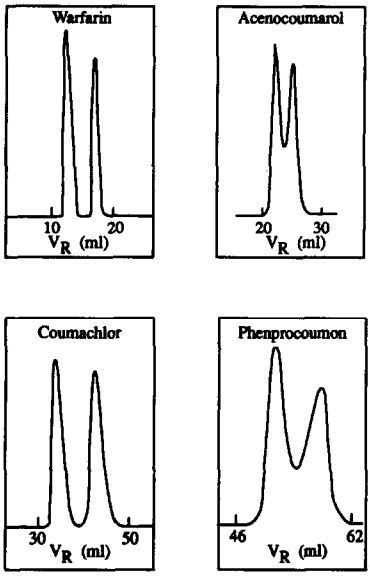


Fig. 3. Separation of structurally related coumarin isomers on column **IV**. Eluent: methanol–pH 4 phosphate buffer (20:80).

resulting in the ability of these derivatives to bind preferentially to one of the enantiomers. This assumption is supported by a recent paper [8] describing the separation of coumachlor (R_s = 0.6) on *S* and rac-2 hydroxypropyl bonded support [mobile phase: triethylammonium acetate buffer (pH 7)–acetonitrile (95:5)].

However, it can be seen that the presence of OHP groups at OHP–β-CD ratios higher than 5.8 does not change the elution profiles of warfarin, acenocoumarol and coumachlor. This is not the case for phenprocoumon, the enantiomers of which could not be resolved on column **III** (Fig. 4b), and the retention time of which remains about the same as on column **IV** (Fig. 4a). The role of the number of OHP sub-

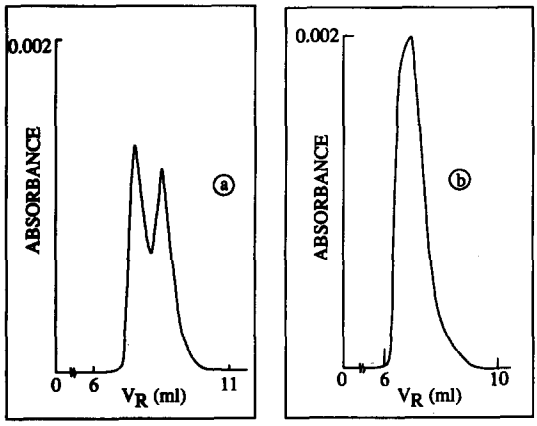


Fig. 4. Racemic phenprocoumon elution profiles on columns **III** (a) and **IV** (b) filled respectively with silica coated with β-CD derivatives **3** and **4**. Eluent: methanol–pH 4 phosphate buffer (40:60).

stituents per β-CD is demonstrated, the chiral recognition of phenprocoumon disappearing when it exceeds a value of *ca.* 7. A steric hindrance, resulting from the higher degree of substitution on β-CD, probably prevents the separation of phenprocoumon enantiomers.

The rest of the study was therefore performed on supports made from the impregnation of silica with a 6% solution of the β-CD derivative No. **4** (OHP–β-CD = 5.5).

Effect of methanol content and pH of the mobile phase on the separation of coumarin derivative enantiomers

The capacity factors (k') and resolutions of the coumarin derivatives were measured by changing the methanol–phosphate buffer ratio in the mobile phase (at pH 4), from 10:90 to 60:40. We observed a decrease in the capacity factor of the enantiomers as the percentage of methanol increased. A plot of k' *vs.* percentage methanol for the *R*- and *S*-warfarin enantiomers is shown in Fig. 5 in order to illustrate this general behavior.

At the same time a decrease in the resolution factor was observed (Table III), and the enantiomers could not be resolved with mobile phases containing more than 60% methanol. Thus an increase in the methanol content causes a loss of β-CD inclusion properties, the organic modifier reducing the interaction between the solute and the CD cavity. This behavior is usually observed on β-CD bonded columns [18].

Significant decreases of retention and selectivity were observed when the pH of the mobile phase was changed from 4 to 5.5, as shown in Fig. 6 for a fixed 30% methanol content. A smaller change occurred between pH 5.5 and 7. At pH 7 only phenprocoumon enantiomers were separated when the methanol content of the mobile phase was 30%. With mobile phases containing smaller amounts of methanol, warfarin and coumachlor isomers were also separated at this pH, but with a resolution less than that obtained at pH 4 with a methanol content giving similar retention.

The studied coumarin derivatives are only slightly soluble in aqueous solution

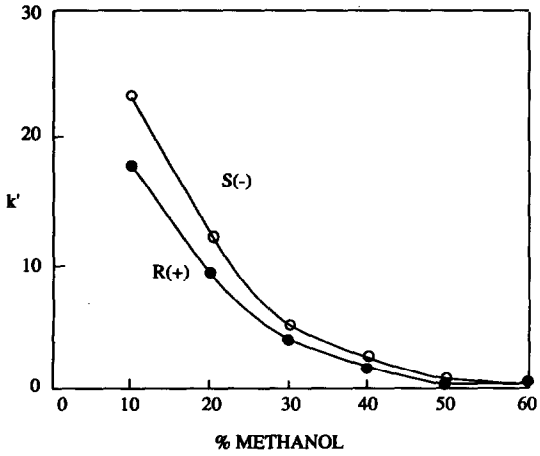


Fig. 5. Variation in the capacity factors of warfarin enantiomers on column IV as a function of the methanol content of the mobile phase (at fixed pH 4).

TABLE III

EFFECT OF METHANOL CONTENT IN THE pH 4 MOBILE PHASE ON THE COLUMN IV RETENTION AND RESOLUTION PARAMETERS OF COUMARIN DERIVATIVES

Compounds	Capacity factors		Separation factor ($\alpha = k'_2/k'_1$)	Resolution factor (R_s)	Mobile phase methanol content (%)
	k'_1	k'_2			
Warfarin	9.00	11.90	1.32	2.0	20
	4.30	5.40	1.26	1.3	30
	1.82	2.28	1.25	0.8	40
	0.14	0.14	1.00	0	60
Acenocoumarol	15.64	17.85	1.14	0.6	20
	8.23	8.97	1.09	0.4	30
	3.15	3.40	1.08	0.2	40
	0.36	0.36	1.00	0	60
Coumachlor	23.20	32.02	1.38	1.5	20
	8.88	11.14	1.25	1.1	30
	3.00	3.43	1.14	0.7	40
	0.32	0.32	1.00	0	60
Phenprocoumon	35.78	40.43	1.13	0.9	20
	13.33	15.14	1.13	0.8	30
	4.50	5.04	1.12	0.5	40
	0.54	0.54	1.00	0	60

in their non-ionized form. Their pK_a values are in the range 4.3–5.1 [19]. Inclusion of the drug in the hydrophobic cavity of β-CD is reinforced at low pH as the non-ionized moieties are preferentially complexed by β-CD [20]. This phenomenon is responsible for the increased retentions observed at pH 4. At higher pH, the increased hydration

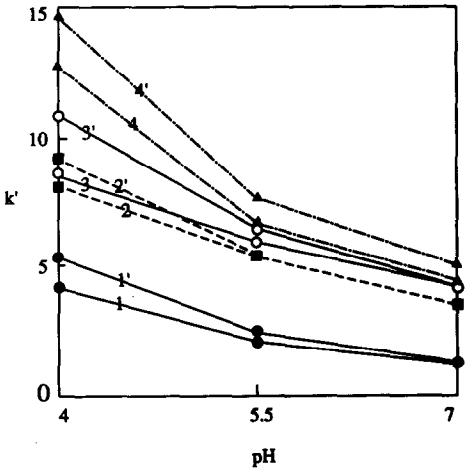


Fig. 6. Variation in the capacity factors of the coumarin derivatives on column IV as a function of the pH of the mobile phase, at fixed methanol content (30%). The curves relative to the first and second isomers of warfarin are respectively, 1 and 1′ (●), acenocoumarol 2 and 2′ (■), coumachlor 3 and 3′ (○), and phenprocoumon 4 and 4′ (▲).

of the ionized moiety is unfavorable for complex formation, and this explains the lower retention and resolution of the coumarin derivatives.

In order to explain the origin of the relative retention of these anticoagulants on the supports impregnated with β-CD derivatives, we compared their relative capacity factors with the apparent partition coefficients (*n*-octanol to water) previously reported by Otagiri *et al.* [19], these being 7.94, 10.9 and 17.1 for acenocoumarin, warfarin and phenprocoumon, respectively. We noticed that these are in good agreement for warfarin and phenprocoumon. Conversely the value of the acenocoumarol partition coefficient indicates that this compound is less hydrophobic than warfarin, while its retention volume on column **IV** is higher. Otagiri's previous results have shown that the complexation constant of native β-CD with these drugs increases with their partition coefficient [19]. We assume that the nitro substituent on the phenyl ring of acenocoumarol is responsible for an additional interaction with the hydroxypropyl groups outside the cavity, as it may be possible for the oxygen of the nitro group which has electron pairs to form hydrogen bonds [21].

Separation of dansyl amino acid enantiomers

Column **IV** exhibits enantioselectivity for certain dansyl amino acids (Dns-amino acids) using water–methanol mobile phases. Fig. 7 presents the chromatograms obtained under optimized mobile phase conditions. As can be seen, separations were achieved for threonine, leucine and phenylalanine D,L pairs with R_s values of, respectively, 1.18, 0.95 and 1.28. The L-enantiomers elute first. Their capacity coefficients increase with increasing hydrophobicity of the side chain on the chiral carbon atom, as previously reported [11,12].

Excellent enantioselectivities have been found on native β-CD bonded columns, the dimethylaminonaphthyl group penetrating tightly in the cavity and the chiral center with its substituents being close to the hydroxyl groups on the CD rim where hydrogen bonds can be formed with the carboxylate and amine moieties [11]. It has been reported recently [12] that modification of the hydroxyl groups on the β-CD molecule with a hydrophobic substituent results in a reduction in the ability to form

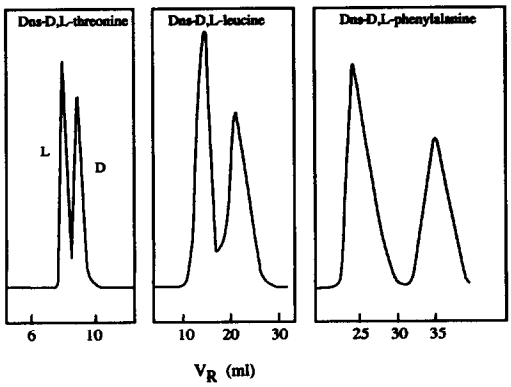


Fig. 7. Separation of Dns-amino acids enantiomers on column **IV**, using methanol–phosphate buffer mixtures as mobile phases. Methanol–pH 4 buffer (20:80) for Dns-thréonine, methanol–pH 5.5 buffer (30:70) for Dns-leucine and Dns-phenylalanine.

TABLE IV

EFFECT OF METHANOL CONTENT IN THE pH 4 MOBILE PHASE ON THE COLUMN IV RETENTION AND RESOLUTION PARAMETERS OF Dns-AMINO ACIDS

Compounds	Capacity factors		Separation factor ($\alpha = k'_2/k'_1$)	Resolution factor (R_s)	Mobile phase methanol content (%)
	k'_1	k'_2			
Threonine	4.7	5.43	1.15	1.18	20
	2.28	2.57	1.13	0.40	30
	0.57	0.57	1.00	0	50
Tryptophan	10.00	10.00	1.00	0	30
	3.50	3.50	1.00	0	40
Leucine	15.78	20.43	1.29	1.00	30
	8.00	10.96	1.37	0.80	40
	3.14	4.57	1.45	0.80	50
	0.93	1.39	1.49	0.54	60
Phenylalanine	18.64	28.64	1.54	1.50	30
	10.50	15.00	1.43	1.50	40
	4.28	6.64	1.58	0.88	50
	1.16	1.80	1.55	0.70	60

an inclusion complex with all Dns-amino acids and consequently a reduction in their ability to resolve their enantiomers. This was interpreted as being due to a change in the depth of the cavity in which the phenylcarbamoyl or propylcarbamoyl substituents of β-CD may penetrate [12]. Owing to their hydrophilicity, the hydroxypropyl substituents of the EP–β-CD-N^+ polymers have no tendency to penetrate the hydrophobic cavity of β-CD. This explains why the enantiomers of Dns-leucine are separated on column **IV** (Fig. 7) while they were not on previous 6-*O*-phenyl or propyl carbamoylated β-CD stationary phases [12].

Moreover, we have noticed, as have previous authors [11,12], that Dns-D,L-tryptophan cannot be resolved under any mobile phase conditions while its retention volume is superior to that of Dns-threonine (Table IV). It has been suggested that the side chain on the chiral atom of Dns-tryptophan and the Dns group are included at the same extent, preventing a chiral recognition, such a mechanism being supported by the fact that this enantiomeric resolution could be achieved by replacing β-CD by larger γ-CD [12].

Table IV shows the capacity coefficients and resolution factors for different Dns-D,L-amino acids as a function of the methanol content of the mobile phase at fixed pH 4. k' and R_s values decreased with the increase of methanol content, as in the case of coumarin derivatives. However, note that for a methanol content lower than 30%, Dns-leucine and -phenylalanine were retained more strongly and the peaks were asymmetric, leading to a loss of resolution.

Table V compares the retention and separation parameters of the Dns-amino acids at pH 4, 5.5 and 7, with a 30% methanol content. Increasing the pH of the mobile phase lowers the k' values. Dns-threonine was resolved only at pH 4 because its k' value was too low at higher pH. The other amino acids were resolved even at pH 7. pH 5.5 leads to better resolution, pH 4 giving stronger retentions and large peaks.

TABLE V

EFFECT OF MOBILE PHASE pH ON THE COLUMN IV RETENTION AND RESOLUTION PARAMETERS OF Dns-AMINO ACIDS AT FIXED 30% METHANOL CONTENT

Compounds	Capacity factors		Separation factor ($\alpha = k'_2/k'_1$)	Resolution factor (R_s)	Mobile phase, pH
	k'_1	k'_2			
Threonine	2.28	2.57	1.13	0.40	4.0
	1.40	1.40	1.00	0.00	5.5
	0.80	0.80	1.00	0.00	7.0
Leucine	10.07	15.07	1.50	0.95	5.5
	6.85	11.14	1.63	0.85	7.0
Phenylalanine	16.10	25.00	1.55	1.28	5.5
	11.85	18.28	1.54	1.20	7.0

In conclusion, our columns can be used for optical resolution of Dns-D,L-amino acids. The presence of hydrophilic hydroxypropyl substituents on the β-CD polymer does not prevent the separation of their enantiomers.

Finally it is important to note the reproducibility of the above experiments. We noticed that after about 6 l of eluents of different pH and methanol content there was a decrease in all the retention volumes. The carbon content of the support of column IV decreased to 2.8%, indicating a loss of polymer adsorbed onto silica. In these conditions all the enantiomers were still resolved with a decrease of R_s. The cross-linking of the polymer adsorbed on silica is under study in order to avoid the leaching of the active adsorbed layer.

β-CD polymer-coated support represents a good candidate for enantiomeric separations as it is cheap and very easy to use. Therefore its applicability to preparative chromatography is under investigation.

CONCLUSIONS

In summary, the described β-CD derivative polymers have been shown to be convenient for the preparation of stationary phases able to recognize enantiomers. It is observed that the chiral recognition properties of the packings are different to those of native β-CD supports because of the presence of hydroxy and dihydroxy substituents on the β-CD, allowing the separation of warfarin and related compounds.

REFERENCES

1 A. Harada, M. Furue and S. Nozakura, *J. Polym. Sci.*, 16 (1978) 189.
2 B. Zsadon, L. Décsei, M. Szilasi, F. Tudos and J. Szejtli, *J. Chromatogr.*, 270 (1983) 127.
3 D. W. Armstrong, *J. Liq. Chromatogr.*, 7 (1984) 353.
4 T. J. Ward and D. W. Armstrong, *J. Liq. Chromatogr.*, 9 (1986) 407.
5 W. L. Hinze, T. E. Riehl, D. W. Armstrong, W. DeMond, A. Alak and T. Ward, *Anal. Chem.*, 57 (1985) 237.
6 D. W. Armstrong, Y. I. Han and S. M. Han, *Anal. Chim. Acta*, 208 (1988) 275.
7 D. W. Armstrong, T. J. Ward, R. D. Armstrong and T. E. Beesley, *Science*, 232 (1986) 1132.
8 A. M. Stalcup, S. Chang, D. W. Armstrong and J. Pitha, *J. Chromatogr.*, 513 (1990) 181.

9 B. Sébille, N. Thuaud, J. Piquion, N. Behar, *J. Chromatogr.*, 409 (1987) 61.
10 A. Deratani, B. Sébille, N. Thuaud, G. Lelièvre, *Patent pending,* France, 1990.
11 W. L. Hinze, T. E. Riehl, D. W. Armstrong, W. DeMond, A. Alak and T. Ward, *Anal. Chem.*, 57 (1985) 239.
12 K. Fujimura, S. Suzuki, K. Hayashi and S. Masuda, *Anal. Chem.*, 62 (1990) 2198.
13 B. D. West, S. Preis, C. H. Schroeder and K. P. Link, *J. Am. Chem. Soc.*, 83 (1961) 2676.
14 N. Wiedenhof, J. N. J. J. Lammers and C. L. van Panthaleon van Eck, *Staerke*, 21 (1969) 119.
15 A. Harada, M. Furue and S. Nozakura, *Polymer J.*, 13 (1981) 777.
16 R. P. Rohrbach, L. J. Rodriguez, E. M. Eyring and J. F. Wojcik, *J. Phys. Chem.*, 81 (1977) 944.
17 A. Deratani, G. Lelièvre, T. Maraldo and B. Sébille, *Carbohydr. Res.*, 192 (1989) 215.
18 D. W. Armstrong, W. DeMond and B. Czech, *Anal. Chem.*, 57 (1985) 481.
19 M. Otagiri, J. G. Fokkens, G. E. Hardee and J. H. Perrin, *Pharm. Acta Helv.*, 53 (1978) 241.
20 M. Otagiri, T. Miyaji, K. Uekama and K. Ikeda, *Chem. Pharm. Bull*, 24 (1976) 1146.
21 W. A. Schroeder, *J. Am. Chem. Soc.*, 73 (1951) 1122.