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Chiral separation by a monofunctionalized cyclodextrin derivative: From selector to permethyl-β-cyclodextrin bonded stationary phase

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ABSTRACT

Preparation of (6-monoureido-6-monodeoxy) permethylated β-cyclodextrin bonded chiral stationary phase from permethylated 6-monoamino-6-monodeoxy-β-cyclodextrin is described. The optimized chiral stationary phase was evaluated by using HPLC separation of racemates of coumarin derivatives. Column characterization was performed by solid-state ^{13}C, ^{15}N, ^{29}Si NMR using cross-polarization at the magic angle spinning. The development process was supported by CE experiments where the complex formation between cyclodextrins and warfarin was investigated. The results demonstrate good enantio-discrimination for coumarin derivatives.

1. Introduction

Cyclodextrin (CD) technology offers various solutions for the separation of drug enantiomers in chemical, pharmaceutical and biological research [1]. Yet, there appears to be a continuous challenge in finding optimal conditions for the separation within reasonable analysis time. Most CD derivatives used in chiral separations are mixtures of high variety of randomly substituted homologues and regioisomers. Hence, reproducibility is often related to batch-to-batch reproducibility of chemical synthesis of the applied CDs. Even small differences in the degree of substitution or isomer distribution can influence the result of the separations [2–6]. Application of single isomer cyclodextrin derivatives turns advantageous in the batch-to-batch reproducibility of reactions. However, in the preparation of single isomer CD derivatives it is difficult to achieve the desired purity at reasonable yield in a series of selective, multi-step reactions [7].

Our aim was to apply permethylated 6-monoamino-6-monodeoxy-β-cyclodextrin [8] (PMMABCD) as chiral selector for the development of a permethylated 6-monoureido-6-monodeoxy β-cyclodextrin (UPMBCD) silica-bonded chiral stationary phase (CSP) and demonstrate its chromatographic properties. Warfarin and related coumarin derivatives (see Fig. 1) were chosen to test enantiomeric separation properties of the new CSP.

2. Experimental

2.1. Materials

Sodium azide-1-^{15}N, 3-isocyanatopropyltriethoxysilane, hexamethyldisilazane, n-hexane, trimethylchlorosilane, methanol, DMF, toluene, acetone, tetrahydrofurane, pyridine, racemic warfarin sodium-chlatrate and achiral test substances (phenol, toluene, aniline, ethyl-benzene) were purchased from Sigma–Aldrich (Milwaukee, USA). Pd/C (10% Pd), acetonitrile (HPLC gradient grade), phosphoric acid, boric acid, acetic acid and triethylamine (analytical grade) were from Merck (Darmstadt, Germany). Hypersil Silica 5 μm mean pore diameter 100 Å, surface area 280 m^2/g, pore volume 0.61 cm^3/g (high-purity, "B"-type silica) was from Thermo Hypersil Keystone (Runcorn, Cheshire, UK). Nucleodex-β-PM column was from Macherey-Nagel (Düren, Germany). CD-Screen column (containing 4-nitrophenyl groups) is a product of ChiroQuest Ltd. (Budapest, Hungary). Empty 250 mm × 4.0 mm I.D. HPLC columns were from Sugiyama Shoji Co., Ltd. (Yokohama, Japan). 6^I-O-tosyl-β-cyclodextrin, permethylated 6-monoamino-6-monodeoxy-β-cyclodextrin and heptakis (2,3,6-tri-O-methyl)-

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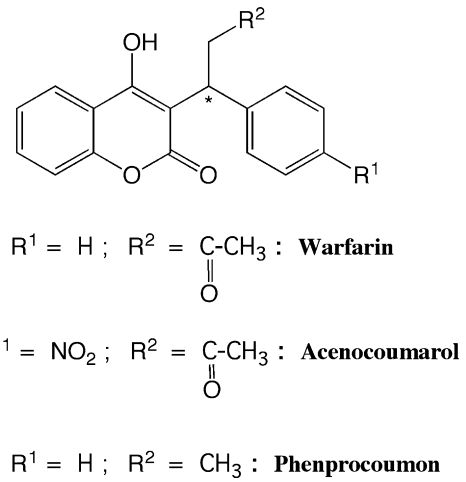

Fig. 1. The molecular structure of coumarin-type anticoagulants.

β-cyclodextrin (TRIMEB) are products of CycloLab Ltd. (Budapest, Hungary). *rac*-Acenocumarol and *rac*-phenprocoumon were from Promochem (Wesel, Germany). (*S*)-warfarin and (*R*)-warfarin enantiomers were prepared as previously described [9].

2.2. Synthesis of UPMBCD-silica

The preparation of ^{15}N-labelled PMMABCD has been described [10]. In order to overcome sensitivity problems in natural abundance for ^{15}N NMR experiments, 50 at% ^{15}N-labeling of the selector was performed starting from Na^{15}N=N=N. The silylating agent was prepared by the reaction of PMMABCD (2.1 g, 1.5 mmol) with equimolar 3-isocyanatopropyltriethoxysilane (0.37 ml, 0.37 g, 1.5 mmol) in distilled, dry tetrahydrofurane (10 ml) at room temperature. Elemental analysis of silylating agent found: C, 52.64; H, 8.15; N, 1.61; Si, 1.59%. Calculated: C, 52.04; H, 8.01; N, 1.69; Si, 1.69%. After removing the solvent the product was used without further purification in the bonding process.

6.6 g silica was suspended in 50 ml toluene with ultrasonication, traces of water were removed by azeotropic destillation. 1 g of the silylating agent was dissolved in 5 ml pyridine and this solution was added dropwise to the cool suspension of the silica, while vigorous stirring was applied. The reaction mixture was gently stirred at 105 °C for 20 h. The product was filtered, washed with toluene, acetone and methanol, in this order. The surface coverage of the obtained dry silica gel (7.06 g) was 0.30 μmol/m^2. In order to improve peak shape an end-capping procedure was performed by applying hexamethyldisilazane (0.7 ml, 0.54 g, 3.3 mmol) and trimethylchlorosilane (0.7 ml, 0.59 g, 5.4 mmol) in dry tetrahydrofurane (50 ml) as silylating agents at 50 °C for 1 h. The filtrate was washed with acetone, 25% (v/v) methanol and acetone again in order to remove ammonium chloride.

2.3. Chromatographic experiments

The isomeric purity of the PMMABCD selector was determined by HPLC with Evaporative light scattering detector (Polymer Laboratories Ltd., Church Stretton, UK). A special HPLC column developed for cyclodextrin analysis (CD-Screen) thermostated to 25 °C was used. The mobile phase was acetonitrile—0.1 M triethylamine formate buffer (pH 4) with solvent gradient: the acetonitrile content increased from 5% to 90% during 20 min. The flow rate was 1 ml/min. The isomeric purity of PMMABCD was 96.5%, the detected impurity was identified as permethyl-β-cyclodextrin. UPMBCD column performance was evaluated under reversed phase conditions. Agilent 1100 HPLC system with diode array detector at 283 nm was used for the liquid chromatographic measurements. The test solution for analytical separation was racemic warfarin (3×10^6 M) dissolved in 20% aqueous methanol. The (*S*)-warfarin peak was identified with the pure (*S*)-enantiomer.

2.4. Capillary electrophoresis

Capillary electrophoresis was performed with an Agilent Capillary Electrophoresis ^{3D}CE system applying bare fused silica capillary of 64.5 cm total and 56 cm effective length with bubble cell and 50 μm I.D. (Agilent Technologies, Santa Clara, CA, USA). On-line UV absorption at 209 and 308 nm was detected by DAD UV–vis detector. The capillary was thermostated at 20 °C. Britton–Robinson buffer prepared from 40 mM borate, 40 mM acetate and 40 mM phosphate in a mixture of 1:2:2 (v/v/v) was applied as background electrolyte (BGE) at pH values adjusted by NaOH. Between measurements, the capillary was rinsed by 1 M NaOH, 0.1 M NaOH and distilled water, subsequently for 1 min and with BGE for 8 min. Warfarin samples were dissolved in absolute ethanol and further diluted with distilled water. Racemic warfarin (2×10^{-6} M) was spiked with the pure (*R*) enantiomer (10^{-6} M) and injected by 5×10^3 Pa pressure for 3 s. Runs were performed in the positive-polarity mode with 30 kV. The quality of the selector can be characterized by selectivity (α) and resolution (R_S) of the enantiomers to be separated. The mentioned parameters are given by the following equations [11]:

$$\alpha_{1,2} = \frac{\mu_1}{\mu_2} \quad (1)$$

$$R_S = \frac{1.18(t_1 - t_2)}{w(0.5)_1 + w(0.5)_2} \quad (2)$$

where μ is the apparent mobility of the enantiomers (1,2 in lower index), $w(0.5)$ is the peak width at half height, t is the migration time. In order to calculate the apparent complex stability constant (K_i) of warfarin enantiomers to the selector CD, the mobilities of the analytes in the absence ($\mu_{0,i}$) and in the presence ($\mu_{x,i}$) of CD in five concentrations in the range of 5–20 mM and 15–40 mM (c_x) for PMMABCD and TRIMEB, respectively, were determined. By plotting $(\mu_{x,i} - \mu_{0,i})$ vs. $(\mu_{x,i} - \mu_{0,i})/c_x$, the absolute value of slope of the regression line equals the stability constant [12].

2.5. Nuclear magnetic resonance spectroscopy

Solid-state ^{13}C, ^{15}N, ^{29}Si NMR experiments were performed on a 600 MHz Varian NMR SYSTEM^{TM} using zirconia rotors in Varian/Chemagnetics narrow bore 3.2 mm HXY triple resonance MAS probe operated in HX double resonance mode. Cross-polarization at the magic angle spinning (CP/MAS) was applied to enhance ^{13}C, ^{15}N and ^{29}Si sensitivity. MAS rates of 7 kHz were chosen and proton high power decoupling (SPINAL scheme) was applied during 20 ms acquisition time. CP contact times of 1, 2, 3 ms were applied for the ^{13}C, ^{15}N and ^{29}Si experiments, respectively. Repetition delay was 5 s. Spectral windows of ^{13}C: 125 kHz, ^{15}N: 69 kHz, ^{29}Si: 62.5 kHz were used and digital resolutions varied in the range 1.9–3.8 Hz. Carbon chemical shifts are referenced to adamantane (δ = 38.6, 29.5 ppm). Nitrogen-15 shifts are referenced to the nitromethane chemical shift scale (δ = 0 ppm) using solid ^{15}N-glycine as a secondary reference (δ = −350 ppm). ^{29}Si shifts are referred to tetramethylsilane (δ = 0 ppm).

The solid-state NMR measurements have been performed on dried silica samples.

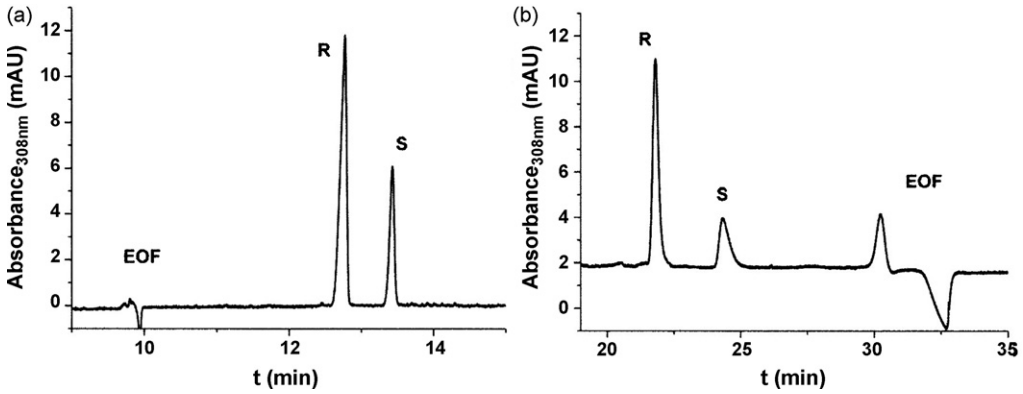

Fig. 2. Enantioseparation of warfarin (enantiomer ratio *R*:*S* = 2:1) by capillary electrophoresis. Buffer: 40 mM Britton–Robinson containing selector (a) 25 mM TRIMEB pH 5.5 and (b) 10 mM PMMABCD pH 4.0.

3. Results and discussion

3.1. Selector testing

3.1.1. CE test

Although capillary electrophoresis (CE) has proven successful in separations of warfarin enantiomers with CDs as chiral selectors [13,14], the stability constants are rarely presented [15]. In order to determine enantio-discrimination of chiral selectors quantitatively, we performed comparative CE investigations for two soluble methylated β-CD derivatives: TRIMEB and PMMABCD (Fig. 2). The apparent K'_S and K'_R binding constants of warfarin enantiomers have been determined from electropherograms obtained for various CD concentrations.

Optimal conditions were found at pH = 5.5 for TRIMEB (25 mM; R_S = 5.16; α = 1.05), and pH = 4.0 for PMMABCD (10 mM; R_S = 4.27; α = 1.10). The apparent binding constants K'_S and K'_R are summarized in Table 1. The results clearly indicate that both permethylated CDs well discriminate warfarin enantiomers. Stronger binding with (*R*)-warfarin was found for both selectors in good agreement with HPLC results between 3 < pH < 5 (see Section 3.3). We note that in a different approach using negatively charged highly sulfated-β-CD, comparable results were found (R_S = 4.07) [14]. CE experiments with a model ureido permethyl-CD selector gave similar results too (data not shown). Although the binding of warfarin to β-CD was stronger (K' = 802 ± 81 M^{-1}) than to permethylated derivatives, we did not found selectivity for β-CD complexation. Our findings indicate enantiorecognition to be the consequence of permethylation of the cyclodextrin selector.

3.2. Column preparation

Permethylated cyclodextrins have been widely used as chiral stationary phases [16,17]. There are two main strategies for immobilization of the CD selector. Method 1: the silica surface is first covered with a silylating agent having reactive groups, e.g. glycidyl, amino, isocyanato, alkene or alkyne [18–21]. In the following step the selector reacts with a certain portion of these groups. The amount of unreacted spacers depends on the space filling of the selector molecule. Disadvantages of this method are the surface inhomogeneity and possible non-specific interactions of the analyte with this layer. Method 2: the silylating agent is reacted with the selector molecule prior to the immobilization process. The resulted product is then attached to the silica surface. Although high amount of silanol groups remains unreacted by this method, one can achieve a chemically uniform coverage. In order to avoid interfering interactions, the new CSP was prepared according to Method 2. Fig. 3 outlines the chemical procedure for the preparation of UPMBCD-bonded stationary phase.

Table 1
Resolution (R_S), selectivity (α) and apparent complex stability constants of warfarin enantiomers to BCD derivatives (K'_R and K'_S) determined by CE.

	β-CD	TRIMEB	PMMABCD
R_S	<0.5	5.16	4.27
α	~1.00	1.05	1.10
K'_R (M^{-1})	802 ± 81	48 ± 5	121 ± 13
K'_S (M^{-1})	802 ± 81	42 ± 4	85 ± 8
Elution order	n.a.	*R*,*S*	*R*,*S*

Conditions in Section 2.4.

3.3. Column characterization

Immobilization of the chiral selector (PMMABCD) as substituted urea (UPMBCD) has been proved by multinuclear ^{13}C, ^{29}Si, ^{15}N solid-state NMR using magic angle spinning (MAS) and ^{1}H cross-polarization (CP) [22,23]. Combination of these techniques for the characterization of dried CSPs has become a successful approach when investigating the surface modification reaction of the silica [24]. Figs. 4–6 show the spectra acquired in these investigations. Characteristic signals of the C–H groups of the CD appeared in the

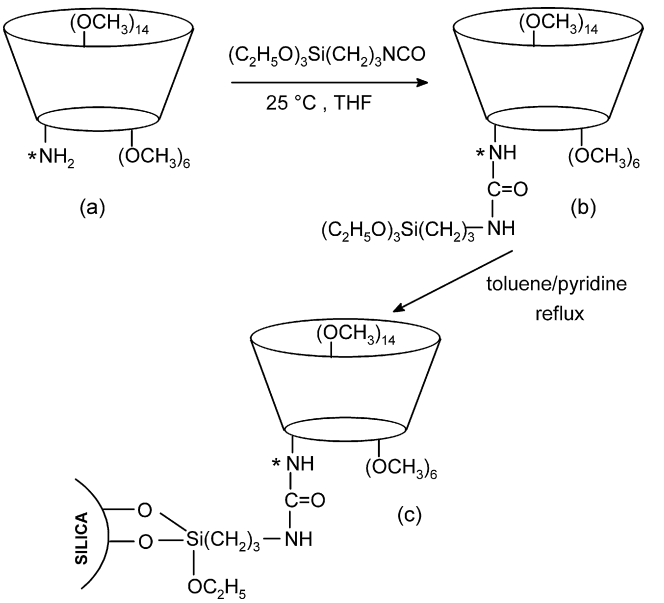

Fig. 3. Reaction scheme for surface modification of the silica support. The asterisk (*) indicates the position of ^{15}N-labeling.

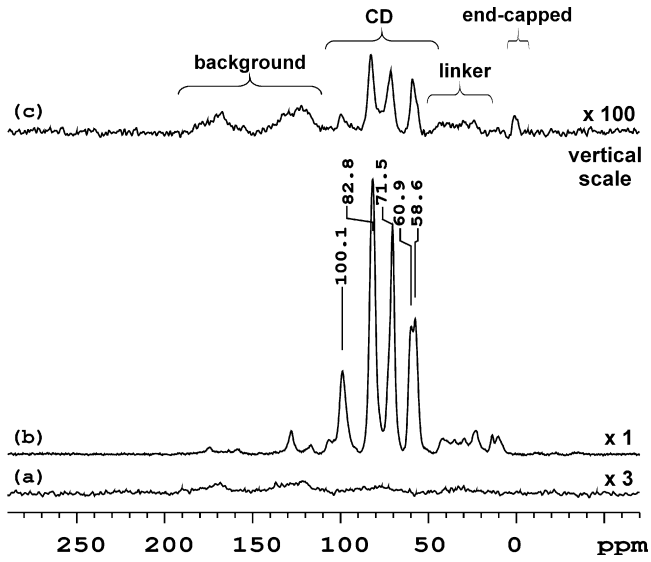

Fig. 4. Solid-state ^{13}C-CP/MAS NMR of (a) the unmodified silica, (b) the chiral silylating agent, (c) the UPMBCD modified CSP.

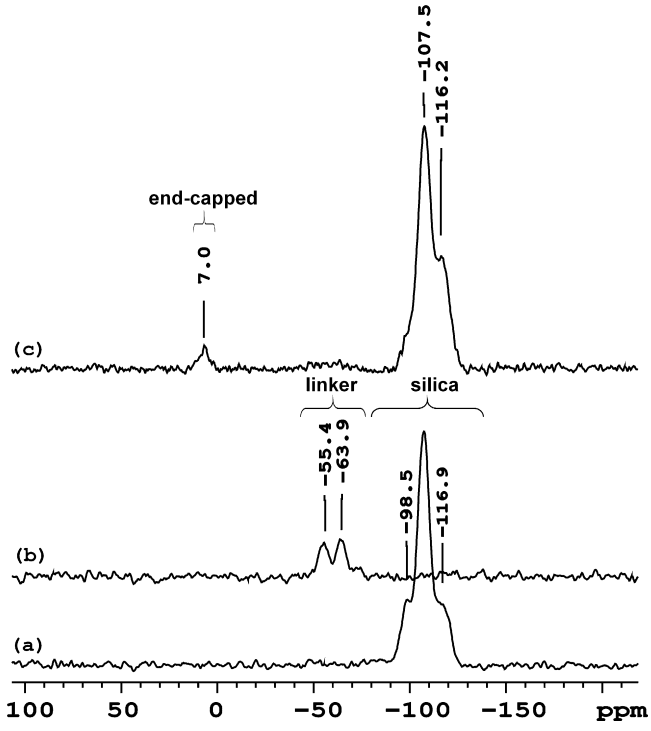

Fig. 5. Solid-state ^{29}Si-CP/MAS NMR of (a) the unmodified silica, (b) chiral silylating agent, (c) UPMBCD modified CSP. (Spectra were recorded under identical CP/MAS experimental conditions).

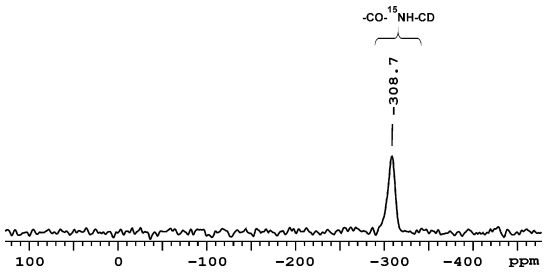

Fig. 6. Solid-state ^{15}N-CP/MAS NMR characterization of the CSP functionalized by the silylating agent prepared from ^{15}N-labeled (50 at%) UPMBCD.

^{1}H–^{13}C-CP/MAS (δ 50–110 ppm). ^{13}C NMR also detects the lower intensity CH_2– groups (δ 10–50 ppm) of the linker part of the modifier as well as the terminal –O–$SiMe_3$ moieties (δ 0–10 ppm) of the end-capped silica. The rest of the carbon signals appearing as broad peaks, are due to carbon background.

^{1}H–^{29}Si-CP/MAS NMR provides information about the polysiloxane network of the dried CSP (Fig. 5). Intensity changes were found for peaks centered at δ −98, −107, −116 ppm (compare Fig. 5a and c) which stem from the various HO–Si$^{(IV)}$–O(2–3) sites. This indicates the redistribution of the degree of cross-linking on the surface of the silica network [25,26] owing to immobilization of the chiral UPMBCD selector. The $SiMe_3$ groups of the capped surface silanols of the CSP appear well separated at around δ +7 ppm, allowing their detection with better sensitivity and selectivity compared to ^{13}C NMR (Fig. 4). A broad, low intensity signal appears in the range δ −30 to −80 ppm, corresponding to the surface bonded $(O)_2Si(OEt)$–CH_2– moieties.

Complete selectivity for the immobilized UPMBCD could be achieved by using ^{1}H–^{15}N-CP/MAS NMR (Fig. 6). This method detects only the nitrogen atoms of the CSP, the corresponding resonance shown at δ −308 ppm stems exclusively from UPMBCD bound to silica gel. Hence, solid-state NMR data prove the successful immobilization.

3.4. Chromatographic separations

The silica-bonded UPMBCD was packed into an analytical column, tested by HPLC separation of chiral and achiral substances in reversed phase mode and compared with another, commercially available one (Nucleodex-β-PM).

Excellent chiral separation of warfarin was achieved on the UPMBCD column at pH 3 as compared to Nucleodex-β-PM (Fig. 7). In the latter case, permethyl-BCD is bonded to the silica support via tioether linkage, which fact can be a reason of the different chromatograms. In each case, increasing pH value of the mobile phase caused the retention of warfarin dramatically decreased together with loosing resolution of the enantiomers. (*S*)-warfarin shows

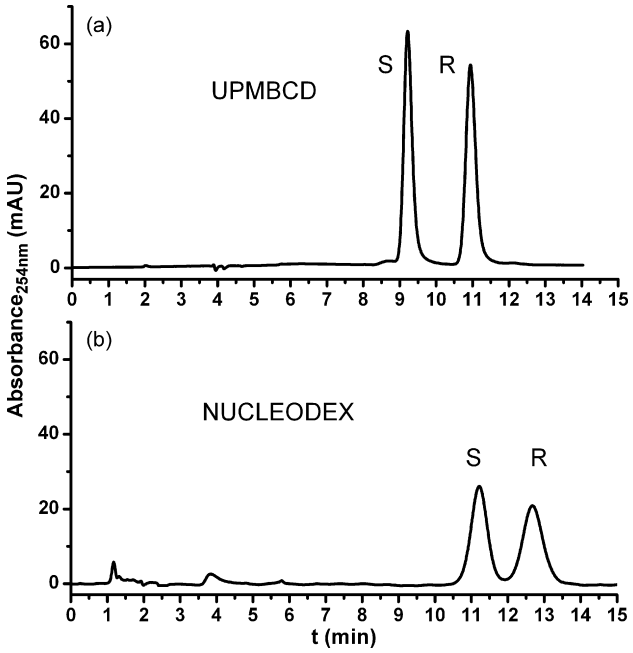

Fig. 7. Separation of racemic warfarin on UPMBCD (a) and Nucleodex-β-PM (b) column. Chromatographic conditions: see Section 2.3, eluent: (a) methanol-TEAP buffer 35:65 (v/v), pH 3 and (b) methanol-TEAP buffer 55:45 (v/v), pH 3, column temperature 25 °C, detection: UV 283 nm.

Table 2
Chiral separation of test compounds[a].

Substance	Retention time of the first eluted peak (min)	α	Resolution	Solvent in the mobile phase
Warfarin	9.2	1.19	3.71	35% methanol
Acenocoumarol	24.7	1.18	2.9	35% methanol
Phenprocoumon	20.3	1.07	1.5	35% methanol
Naproxen	22.2	1.19	4.14	35% methanol
Phenoprop	11.9	1.18	2.36	35% methanol
Clopidogrel	11.6	1.16	3.61	20% acetonitrile
Tropic acid	4.9	1.09	2.06	5% acetonitrile
cis-Chrysantemic acid	7.2	1.14	3.05	35% methanol
cis-Permetrinic acid	17.7	1.13	2.23	15% acetonitrile
trans-Permetrinic acid	21.9	1.19	4.35	15% acetonitrile

[a] Mobile phase: 0.1% triethylammonium phosphate buffer (pH 3)—methanol or acetonitrile, flow rate 0.7 ml/min, column temperature 25 °C, detection: UV 254 nm

weaker interaction with the permethylated CD ring than the *R* isomer. This observation is in accordance with the results obtained in CE experiments in the acidic pH range (Fig. 2). Good resolution could also be achieved by the UPMBCD column for other coumarin derivatives (Table 2).

The results obtained on the UPMBCD column and on Nucleodex-β-PM show separation factors (α) not very different, thus the difference between resolutions (R_S) can be attributed to different number of theoretical plates.

We have also tested the UPMBCD column with some achiral test substances (routinely used for characterization of reversed phase columns in the Engelhardt test) and have compared the data obtained with those of the commercially available permethylated β-cyclodextrin column. The results are presented in Fig. 8.

As seen, the commercial Nucleodex-β-PM column is more retentive than UPMBCD for the basic test material. The peak of aniline (peak 4) is strongly distorted probably due to strong silanophilic interactions. The mean theoretical number of plates is threefold higher for UPMBCD than for the commercial column. The asymmetry factors are near to 1.0 and the basic analyte elutes in the range of non-polar compounds. The good peak shape and the reversed elution order of phenol and aniline indicate negligible interactions with residual silanols, which can be interpreted as the result of efficient end-capping procedure.

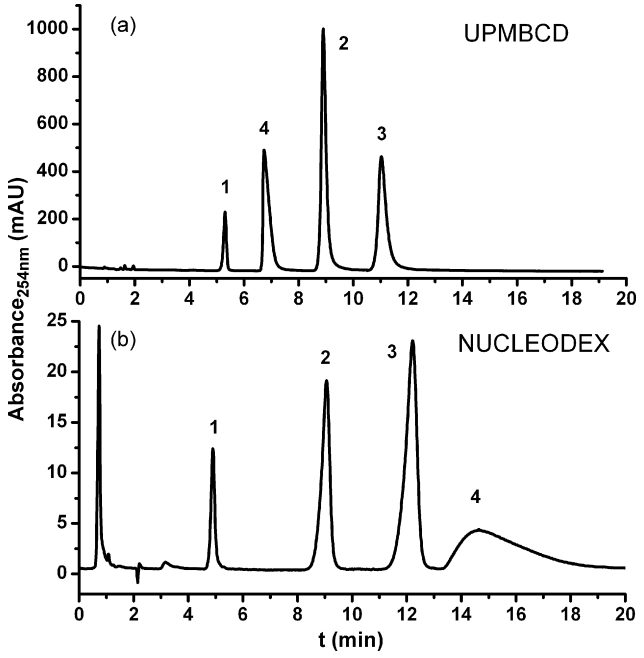

Fig. 8. Separation of achiral test mixture on UPMBCD (a) and Nucleodex β-PM (b) column. 1: phenol, 2: toluene, 3: ethyl-benzene, 4: aniline. Chromatographic conditions: see Section 2.3. Eluent: methanol–water 60:40 (v/v), flow rate 0.8 ml/min.

Some other chiral substances were also successfully separated ($R_S > 2$) on the UPMBCD column, as shown in Table 2.

4. Conclusion

We have developed an optimized permethylated β-cyclodextrin based chiral stationary phase, taking into account the most critical factors, such as the quality of the silica, residual silanols, chemical homogeneity of the silylating agent and the derivatized surface. The immobilized UPMBCD CSP proved to be useful for the stereoselective analysis of coumarin derivatives. In particular, separation of warfarin, a commercial racemic anticoagulant, presents a good example for the practical importance of the new CSP. The UPMBCD-bonded CSP is characterized by high number of theoretical plates, enhanced enantioselectivity and excellent peak shape compared to a commercially available column.

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