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ANALYSES OF BRANCHED CYCLODEXTRINS BY HIGH-PERFORMANCE LIQUID AND THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The chromatographic behaviour of 6-O-α-D-glucosyl-, 6-O-α-maltosyl and 6-O-α-maltotriosyl derivatives of α-, β- and γ-cyclodextrin (CD) and of 6,6‴-di-O-α-D-glucosyl-β-CD on three kinds of high-performance liquid chromatographic (HPLC) columns and four kinds of thin-layer chromatographic (TLC) plates was investigated. The order of elution of these branched CDs on a Hibar LiChrosorb NH_2 column with acetonitrile–water followed the order of increasing molecular size. On the other hand, their elution profiles on a Hibar LiChrosorb RP-18 column with methanol–water and on an Asahipak GS-320 column with water were different from that on the NH_2-bonded silica, that is, the member of the γ-CD series eluted first among the three CD derivatives having the same side-chains, then the α-CD derivative and the β-CD derivative considerably behind that. The two systems differed from one another in the elution sequence of the members in each series. TLC analyses were performed on silica gel 60 TLC plates and HPTLC plates with 1-propanol–ethyl acetate–water–25% ammonia solution, on NH_2 HPTLC plates with acetonitrile–water and on Si 50000 HPTLC plates with 1-butanol–pyridine–water, and their utility as a more convenient method than HPLC for the analysis of CDs was recognized. The analysis on the NH_2 HPTLC plate may be used as a pilot for HPLC analysis on NH_2-bonded silica.

INTRODUCTION

Recently, we obtained ten branched cyclodextrins (CDs, cyclomaltaoses)[1,2]: 6-O-α-D-glucosylcyclomaltohexaose (G_1-α-CD), 6-O-α-maltosylcyclomaltohexaose (G_2-α-CD), 6-O-α-maltotriosylcyclomaltohexaose (G_3-α-CD), 6-O-α-D-glucosylcyclomaltoheptaose (G_1-β-CD), 6-O-α-maltosylcyclomaltoheptaose (G_2-β-CD), 6-O-α-maltotriosylcyclomaltoheptaose (G_3-β-CD), 6,6‴-di-O-α-D-glucosylcyclomaltoheptaose (2G-β-CD), 6-O-α-D-glucosylcyclomaltooctaose (G_1-γ-CD), 6-O-α-malto-

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sylcyclomaltooctaose (G_2-γ-CD) and 6-O-α-maltotriosylcyclomaltooctaose (G_3-γ-CD).

In this work their chromatographic behaviour on three high-performance liquid chromatographic (HPLC) columns of different separation modes and on four kinds of thin-layer chromatographic (TLC) plates was investigated and compared with that of the unbranched CDs, α-, β- and γ-CD, to find useful methods for the analyses of these CDs.

EXPERIMENTAL

Apparatus

A high-sensitivity refractive index (RI) monitor, Shodex RI SE-31 (Showa Denko, Tokyo, Japan) or RID-300 (JASCO, Tokyo, Japan), was used in conjunction with a Tri Rotor SR-1 pump (JASCO) and a U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) or Familic-300S (JASCO) and a Model 7520 injector (Rheodyne, Cotati, CA, U.S.A.). The columns used were Hibar LiChrosorb NH_2 (250 $\times$ 4 mm I.D.), Hibar LiChrosorb RP-18 (250 $\times$ 4 mm I.D.) (both from Merck, Darmstadt, F.R.G.) and Asahipak GS-320 (500 $\times$ 7.6 mm I.D.) (Asahi Kasei, Tokyo, Japan). HPLC analyses at constant temperature were conducted with TU-300 column oven (JASCO).

Materials

Three branched CDs, G_1-α-CD, G_1-β-CD and 2G-β-CD, were isolated from the mother liquor of a large-scale preparation of β-CD using *Bacillus ohbensis* cyclomaltodextrin glucanotransferase[1]. Maltosyl- and maltotriosyl-CDs were synthesized from CDs and maltose or maltotriose with the reverse action of *Pseudomonas* isoamylase[2]. G_1-γ-CD was prepared from G_3-γ-CD by hydrolysis with *Rhizopus delemar* glucoamylase[11]. α-, β- and γ-CD were commercially available.

Silica gel 60 TLC plates, silica gel 60 F_{254} HPTLC plates, NH_2 F_{254s} HPTLC plates and Si 50000 F_{254s} HPTLC plates (all from Merck) were used for TLC analyses after cutting into 20 $\times$ 5 cm sections (TLC plates) and 10 $\times$ 5 cm sections (HPTLC plates).

Analytical-reagent-grade organic solvents were dried and freshly distilled before use. Water used in solvent preparations was distilled, deionized and redistilled.

Procedure

HPLC. For HPLC analysis on an NH_2-bonded silica column standard mixtures of the α-, β- and γ-CD series were individually prepared, containing CD, G_1-CD, G_2-CD and G_3-CD in the each series. Analyses on a Hibar LiChrosorb RP-18 column and on an Asahipak GS-320 column were performed by the use of a standard mixture of α-, β- and γ-CD, and three standard mixtures of their glucosyl (G_1), maltosyl (G_2) and maltotriosyl (G_3) derivatives as samples. 2G-β-CD was separately analysed in all instances. The amount of each CD applied to a column was 15–43 μg. HPLC analysis on a C_{18}-bonded silica column was performed at a constant temperature and the other analyses were conducted at room temperature. The flow-rate was always 1 ml/min.

TLC. Si 50000 HPTLC plates were pre-washed with solvent (1-butanol–

pyridine–water, 6:4:3) and dried at 100°C for 1 h followed by drying *in vacuo* overnight. Preconditioning was unnecessary for silica gel 60 and NH_2 plates. Standard mixtures for the each series, containing 2% of CD and 0.5% each of branched CDs, and a 0.5% solution of 2G-β-CD were prepared. The sample solution applied was *ca.* 1 μl to a silica gel 60 TLC plate, *ca.* 0.4 μl to silica gel 60 HPTLC and NH_2 HPTLC plates and *ca.* 0.2 μl to an Si 50000 HPTLC plate. Appropriate compositions of the solvent system were used (see Figs. 4, 5 and 6).

The solvent for NH_2 plates was prepared fresh daily by mixing acetonitrile with water in the ratio of 3:2 (v/v). The other solvents were prepared the previous night and could be used for 3–4 days. The plate was developed at room temperature two to five times in a closed glass tank thoroughly saturated with the solvent vapour. After each development the Si 50000 plate was dried with air, followed by vacuum drying for 2 h, and the other plates were dried at 40–50°C for 30–60 min. CDs and branched CDs on the chromatograms were detected by spraying with 5% (v/v) concentrated sulphuric acid in ethanol and heating at 100°C.

RESULTS AND DISCUSSION

High-performance liquid chromatography

Analysis on an NH_2-bonded silica column. A comparison of a Hibar LiChrosorb NH_2 column with an ERC-NH-1171 column (Erma Optical Works, Tokyo, Japan), which was very useful for the simultaneous analysis of series of linear D-glucooligosaccharides and -polysaccharides [degree of polymerization (DP) from 2 to about 35][3] led us to choose the former for the analyses of CDs and branched CDs whose DPs vary in the limited range of 6 to 11.

Fig. 1 shows the HPLC elution profiles of three series of CDs and branched CDs. The elution sequence with NH_2-bonded silica and acetonitrile–water follows the order of increasing molecular size[4,5]. The branched CD was eluted more slowly than unbranched CD with the same DP: the retention times (t_R) of G_1-α-CD (DP 7) and G_1-β-CD (DP 8) were longer than those of β-CD (DP 7) and γ-CD (DP 8), respectively. The t_R values of branched CDs having the same DP were almost the same: G_2-α-CD and G_1-β-CD (both DP 8), G_3-α-CD, G_2-β-CD and G_1-γ-CD (all DP 9) and G_3-β-CD and G_2-γ-CD (both DP 10), respectively, had almost identical t_R values. 2G-β-CD, which has two glucosyl branches, was eluted more slowly than the isomer G_2-β-CD, having one maltosyl branch.

Analysis on a C_{18}-bonded silica column. Fig. 2 shows chromatograms of CDs (α, β and γ), glucosyl-CDs (G_1-α, G_1-β and G_1-γ), maltosyl-CDs (G_2-α, G_2-β and G_2-γ), diglucosyl-β-CD (2G-β) and maltotriosyl-CDs (G_3-α, G_3-β and G_3-γ), on a Hibar LiChrosorb RP-18 column. Although a baseline separation of α-CD and γ-CD could be achieved on the Hibar LiChrosorb RP-18 column (57814.6 theoretical plates per metre) used in the previous study[1], it was difficult on the same type column but with a smaller number of theoretical plates (55235.8 per metre) under the same conditions. As C_{18}-bonded silica is a reversed phase, the separation mechanism is probably an example of hydrophobic chromatography, *i.e.*, increased retention with decreasing solubility in water[6]. Previously we examined this assumption in terms of the relationship between the solubilities and the t_R values of α-, β- and γ-CD[4]. In all instances, the elution profiles of three branched CDs having the same branch were similar to those of three unbranched CDs. It is predictable from the t_R values in Fig.

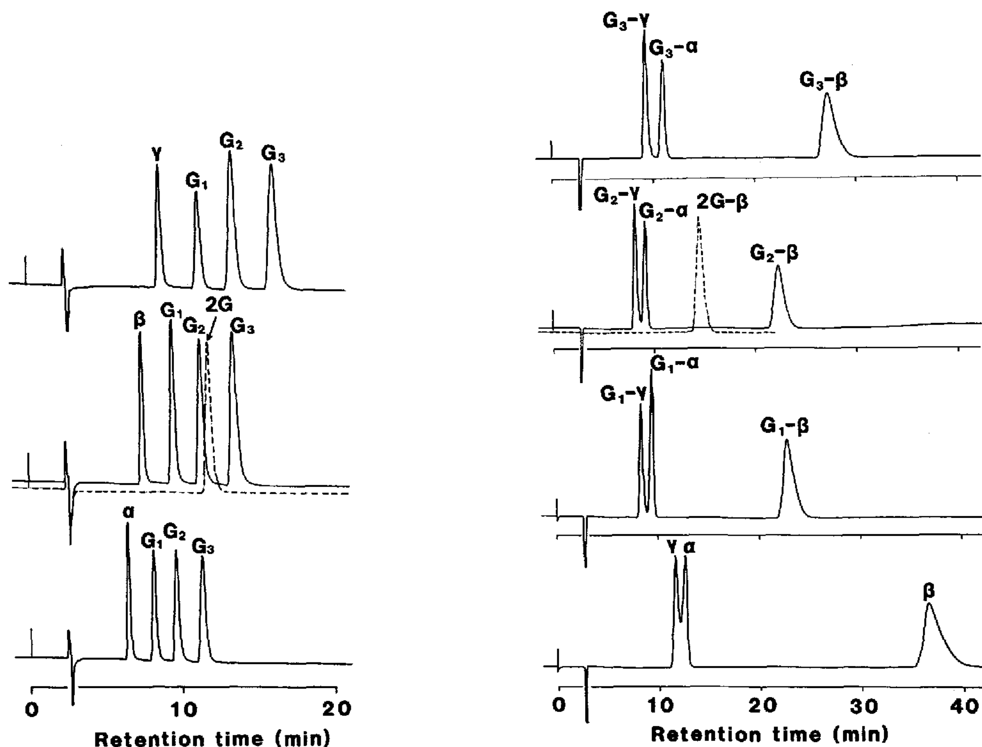

Fig. 1. Chromatograms of individual standard mixtures of three series of cyclodextrins (CDs) and branched CDs on NH_2-bonded silica. In each series the mixture contained α-, β- or γ-CD and its 6-O-α-D-glucosyl (G_1), 6-O-α-maltosyl (G_2) and 6-O-α-maltotriosyl (G_3) derivatives. The dotted line shows a chromatogram of 6,6‴-di-O-α-D-glucosyl-β-CD (2G). Chromatographic conditions: column, Hibar LiChrosorb NH_2 (250 × 4 mm I.D.); eluent, acetonitrile–water (60:40); flow-rate, 1 ml/min; detector, Shodex RI SE-31 at 4 × 10^{-5} RI units full-scale; temperature, ambient.

Fig. 2. Chromatograms of a standard mixture of α-, β- and γ-CD and standard mixtures of their glucosyl, maltosyl and maltotriosyl derivatives, respectively, on C_{18}-bonded silica. α, α-CD; β, β-CD; γ,γ-CD; G_1, glucosyl; G_2, maltosyl; G_3, maltotriosyl; 2G, diglucosyl. Chromatographic conditions: column, Hibar LiChrosorb RP-18 (250 × 4 mm I.D.); eluent, methanol–water (8:92); temperature, 28.3°C; flow-rate, 1 ml/min; detector, RID-300 at 2 × 10^{-5} RI units full-scale.

2 that all branched CDs are more soluble in water than are their mother CDs, the maltosyl derivatives are the most soluble, the solubilities of the glucosyl derivatives are slightly lower and those of the maltotriosyl derivatives are the lowest of the three branched CDs. Diglucosyl-β-CD may be distinctly more soluble than the other branched β-CDs.

The dependences of the capacity factor (k') of the CDs and branched CDs on the methanol concentration in the eluent and the column temperature are shown in Tables I and II, respectively. The k' values decreased with increase in methanol concentration or column temperature.

The effect of methanol concentration on k' was appreciable, a 1% increase in the former resulting in a significant decrease in k', especially for longer retained β-CD series. On elution with 11% methanol the elution sequence of α-CD, γ-CD and

2G-β-CD changed, that is, 2G-β-CD was eluted faster than α-CD and, moreover, γ-CD.

In general, the k' on C_{18}-bonded silica is more affected by temperature than those on NH_2-bonded silica and the others. The change in k' caused by a 5°C increase in the column temperature was roughly comparable to that given by a 1% increase in the methanol concentration in the eluent, and the effect of temperature on the k' of unbranched CDs was greater than that of branched CDs. Performing HPLC at an elevated temperature was preferable to an increase in methanol concentration for the simultaneous analysis of CDs and branched CDs.

Analysis on an Asahipak GS-320 column. A series of Asahipak GS columns were packed with vinyl alcohol polymer gel, which carries many uniformly distributed hydroxy groups and can be used for HPLC in the dual mode of gel-permeation chromatography and adsorption chromatography. The exclusion limit (pullulan) of GS-320 is 40 000. In addition to water, ethanol and methanol at concentrations up to 100% and acetonitrile up to 50% can be used as eluents.

The elution pattern of CDs and branched CDs with Asahipak GS-320 and water resembled that with the C_{18}-bonded silica and methanol–water, namely, the γ-CD series eluted at first, then the α-CD series and the β-CD series last (Fig. 3). The elution sequence in each series was in order of decreasing molecular size: G_3-CD → G_2-CD → G_1-CD → CD. 2G-β-CD eluted faster than G_2-β-CD.

Addition of methanol to the eluent changed the elution profile. The changes in t_R with variation of the methanol concentration in the eluent are given in Table III. Usually, on HPLC analysis with an Asahipak GS-320 column with methanol–

TABLE I

EFFECT OF METHANOL CONCENTRATION IN THE ELUENT ON THE CAPACITY FACTORS (k') OF CYCLODEXTRINS

Chromatographic conditions other than the eluent as in Fig. 2.

Cyclodextrin	*Methanol concentration in eluent (%)*			
	8	*9*	*10*	*11*
α-CD	3.54	2.64	2.01	1.85
G_1-α-CD	2.35	1.72	1.20	0.81
G_2-α-CD	2.29	1.57	1.07	0.70
G_3-α-CD	2.91	1.99	1.36	0.87
β-CD	12.26	9.27	6.75	5.41
G_1-β-CD	7.17	5.21	3.75	2.52
G_2-β-CD	7.01	4.94	3.43	2.29
2G-β-CD	4.17	2.88	1.97	1.30
G_3-β-CD	8.75	6.05	4.13	2.70
γ-CD	3.17	2.37	1.72	1.68
G_1-γ-CD	1.95	1.41	1.00	0.68
G_2-γ-CD	1.88	1.32	0.91	0.59
G_3-γ-CD	2.30	1.57	1.08	0.68

TABLE II

EFFECT OF COLUMN TEMPERATURE ON THE CAPACITY FACTORS (k') OF CYCLODEXTRINS

Chromatographic conditions other than temperature as in Fig. 2.

Cyclodextrin	*Column temperature (°C)*		
	28.3	*33.3*	*38.3*
α-CD	3.54	2.64	1.74
G_1-α-CD	2.35	1.72	1.19
G_2-α-CD	2.29	1.64	1.16
G_3-α-CD	2.91	2.08	1.42
β-CD	12.26	8.95	6.01
G_1-α-CD	7.17	5.30	3.83
G_2-β-CD	7.01	5.11	3.59
2G-β-CD	4.17	3.19	2.33
G_3-β-CD	8.75	6.28	4.40
γ-CD	3.17	2.36	1.59
G_1-γ-CD	1.95	1.45	1.05
G_2-γ-CD	1.88	1.40	1.00
G_3-γ-CD	2.30	1.67	1.14

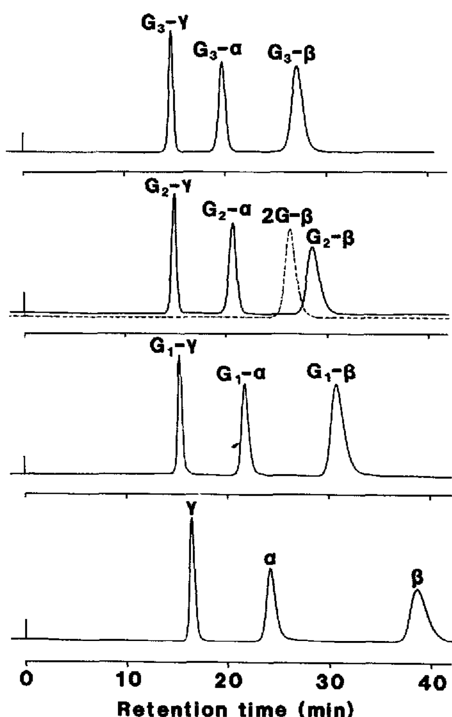

Fig. 3. Chromatograms of a standard mixture of α-, β- and γ-CD and standard mixtures of their glucosyl, maltosyl and maltotriosyl derivatives, respectively, on an Asahipak GS-320 column (500 × 7.6 mm I.D.). See legend to Fig. 2 for composition of mixture. Chromatographic conditions: eluent, water; other conditions as in Fig. 1.

water the t_R values of the compounds that elute prior to water (t_R = 17.22 min) are little affected by the concentration of methanol. On the other hand, the t_R values of compounds that are retained longer than water on the column decrease with an increase in methanol concentration. An increase in methanol concentration decreased the retention of the α-CD and β-CD series, whereas the retention of the γ-CD series, which eluted prior to water, increased slightly.

Thin-layer chromatography

For the separation of oligosaccharides, TLC has been used as a more convenient method than HPLC[3,7–10]. In this work we attempted to separate the CDs and the branched CDs by TLC using several kinds of plates: silica gel 60 TLC, silica gel 60 F_{254} HPTLC, NH_2 F_{254s} HPTLC, Si 50000 F_{254s} HPTLC, CN F_{254s} HPTLC, RP-8 F_{254s} TLC, RP-18 F_{254s} TLC and silica gel 60 WF_{254s} TLC plates (all from Merck), and found that the first four were useful.

The concentration of unbranched CDs in the standard mixture of each series was four times that of branched CDs, as the former travelled comparatively long distances on the plates and, moreover, the sensitivity of the former for 5% sulphuric acid in ethanol reagent was lower than that of the latter.

Analyses on silica gel 60 plates. In our previous study[1] a satisfactory separation of β-CD, G_1-β-CD and 2G-β-CD was obtained on a silica gel 60 plate by duplicate developments with 1-propanol–ethyl acetate–water–25% ammonia solution (6:2:5:3). A better separation of CD, G_1-CD, G_2-CD and G_3-CD in each series was achieved by choosing a solvent of lower R_F value, coupled with a larger number of multiple developments. In this solvent system lower proportions of water and 25% ammonia

TABLE III

EFFECT OF METHANOL CONCENTRATION IN THE ELUENT ON THE RETENTION TIMES (t_R, min) OF CYCLODEXTRINS

Chromatographic conditions other than eluents as in Fig. 3. t_R of water = 17.22 min.

Cyclodextrin	*Methanol concentration of eluent (%)*			
	0	*5*	*10*	*15*
α-CD	24.18	20.52	18.41	18.37
G_1-α-CD	21.72	18.75	17.55	17.15
G_2-α-CD	20.60	17.95	16.92	16.61
G_3-α-CD	19.66	17.46	16.52	16.21
β-CD	38.68	35.10	31.90	30.12
G_1-β-CD	30.70	28.83	26.83	25.73
G_2-β-CD	28.52	26.59	25.05	23.90
2G-β-CD	26.30	24.56	23.33	22.30
G_3-β-CD	27.10	25.40	24.27	22.95
γ-CD	16.38	16.43	16.53	16.76
G_1-γ-CD	15.26	15.35	15.53	15.75
G_2-γ-CD	14.80	15.03	15.16	15.41
G_3-γ-CD	14.64	14.77	14.96	15.17

solution resulted in a lowering of the R_F value. Fig. 4 (left) shows a chromatogram of CDs and branched CDs on a silica gel 60 plate developed five times with 1-propanol–ethyl acetate–water–25% ammonia solution (6:2:4:2). Each run took about 6 h at room temperature. In each series the unbranched CDs had the highest R_F values and the R_F values of branched CDs decreased with increase in the length of the side-chain. The R_F value of 2G-β-CD was lower than that of the isomer G_2-β-CD and similar to that of G_3-α-CD having the same molecular size.

On a silica gel 60 HPTLC plate, complete separation of the CD and branched CDs in each series was achieved even with a single development with the same solvent mixture as mentioned above. The best separation was obtained with four replicate developments of 2.5 h each [Fig. 4 (right)]. Analysis using the HPTLC plate showed considerable advantages: the separation was much more rapid and a smaller amount of sample was required. Silica gel 60 HPTLC plates with a concentration zone (Merck) were also tested, but no improvement was obtained.

Analysis on an NH_2 HPTLC plate. The separation on the HPTLC plate pre-coated with aminoalkyl-bonded silica gel 60 was expected to be comparable to that on an NH_2-bonded silica column. Five replicate 30-min developments with

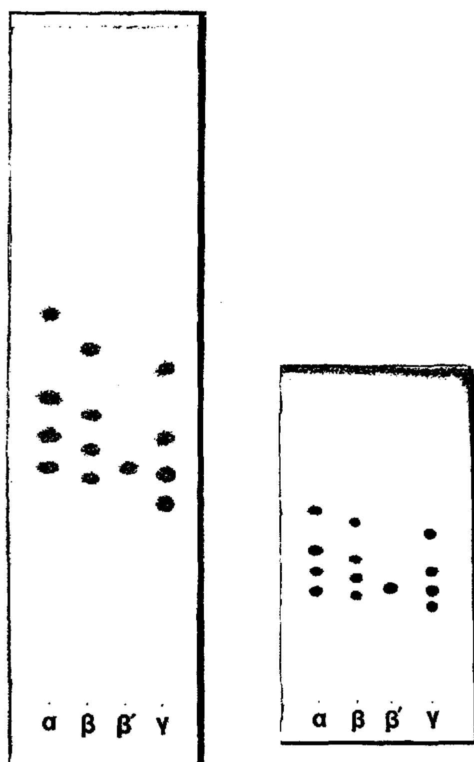

Fig. 4. Chromatograms of CDs and branched CDs on silica gel 60 plates developed with 1-propanol–ethyl acetate–water–25% ammonia solution (6:2:4:2). Left, TLC plate (20 × 5 cm), five replicate 6-h developments; right, HPTLC plate (10 × 5 cm), four replicate 2.5-h developments. α, α-CD series; β, β-CD series; γ, γ-CD series; each series contained CD, glucosyl-CD, maltosyl-CD and maltotriosyl-CD (from the top); β', diglucosyl-β-CD.

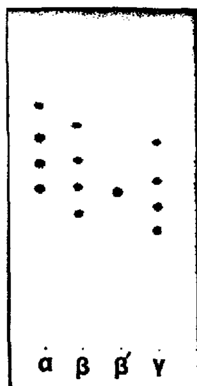

Fig. 5. Chromatogram of CDs and branched CDs on an NH_2 HPTLC plate (10 × 5 cm) developed with acetonitrile–water (3:2). The development was repeated five times. The time required for one development was 30 min. See legend to Fig. 4 for samples.

acetonitrile–water (3:2) gave an analogous chromatogram (Fig. 5) to that on a Hibar LiChrosorb NH_2 column with the same solvent system. Therefore, this TLC analysis may be used as a convenient pilot for HPLC analysis on NH_2-bonded silica.

Analysis on an Si 50000 HPTLC plate. Si 50000 HPTLC plates are coated with synthetic porous silica with a uniform large pore size of 50 000 Å. As Si 50000 has a very low surface activity, it can be used as a stationary phase support for partition chromatography. Consequently, the chromatographic behaviour of the CDs and the branched CDs on this plate differed from that on silica gel 60 and NH_2 plates. Fig. 6 (left) shows a separation of the CD and branched CDs in each series on an Si 50000 HPTLC plate by a single development with 1-butanol–pyridine–water (6:4:3). The application of a suitable amount of sample (as small as possible) gave a satisfactory separation in a single development. On this plate the β-CD series had the highest R_F

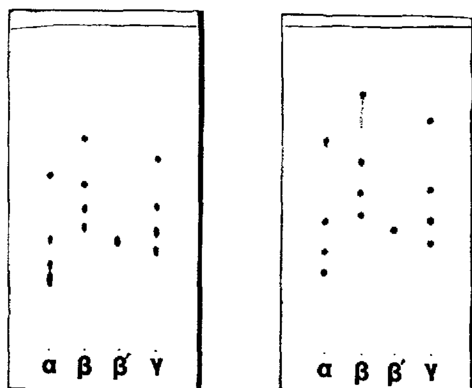

Fig. 6. Chromatograms of CDs and branched CDs on Si 50000 HPTLC plates (10 × 5 cm) developed with 1-butanol–pyridine–water. Left, single development for 1.5 h with 1-butanol–pyridine–water (6:4:3); right, two replicate 1.5-h developments, the first with 1-butanol–pyridine–water (6:3:2) and the second with the same system in the proportions 6:4:3. See legend to Fig. 4 for samples.

value and the R_F value of the α-CD series was the lowest, but the sequence in each series was the same as those on the other plates.

Duplicate developments of 1.5 h each with 1-butanol–pyridine–water in the proportion 6:3:2 for the first development and 6:4:3 for the second decreased the spot width of branched CDs, but caused tailing of the spots of α-CD and β-CD [Fig. 6 (right)]. Developments with the same solvent system with different proportions and with other solvent systems, *e.g.*, 1-butanol–acetic acid–water and 1-propanol–ethyl acetate–water, did not give better results.

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