Journal of Chromatography, 454 (1988) 303–310
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 796

DETERMINATION OF CYCLIC GLUCANS BY ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION

KYOKO KOIZUMI*, YOKO KUBOTA, TOSHIKO TANIMOTO and YASUYO OKADA
Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyuban-cho, Nishinomiya 663 (Japan)
(Received June 8th, 1988)

SUMMARY

Anion-exchange chromatography with pulsed amperometric detection was applied to the determination of cyclodextrins (CDs), branched CDs and cyclosophoraoses. These cyclic glucans with degree of polymerization 6–25 were well resolved in each series by using simple isocratic elution with 150 m*M* sodium hydroxide solution containing 140–200 m*M* sodium acetate. The separation mode was not only simple anion exchange, but also involved some hydrophobic interactions and, moreover, inclusion interactions also seemed to take part in the retention. The detector response per glucose unit of these cyclic glucans was almost the same, regardless of the molecular weight and linkage form. The limit of determinaton of the cyclic glucans was 5–10 pmol and the detection limit was 2.5–5 pmol with a signal-to-noise ratio of 3.

INTRODUCTION

In recent years, research on cyclodextrins (CDs) has made remarkable progress, and particularly research on the pharmaceutical application of CDs is very active at present. The use of CD-containing drugs such as alprostadil-α-CD, limaprost-α-CD (both from Ono Pharmaceutical, Japan) and benexate hydrochloride-β-CD (Shionogi, Japan) in therapy has been authorized by the Japanese Government. In Europe piroxicam-β-CD, developed by Chiesi (Italy), is now registered by the Italian Drug Authorities[1]. Moreover, studies on the preparation and application of branched CDs are continuing energetically[2–11]. The microanalysis of CDs and branched CDs is necessary in this connection.

Recently, a sensitive high-performance liquid chromatographic (HPLC) procedure for carbohydrates using an anion-exchange method and triple-pulse amperometric detection (PAD) at a gold electrode has been developed[12–15]. PAD can detect not only reducing aldoses and ketoses but also non-reducing sugars such as xylitol and sucrose.

In this work we examined anion-exchange chromatography for the determination of CDs and branched CDs, using PAD, and also investigated the chromatographic behaviour and detector response of another series of cyclic glucans, cyclosophoraoses [CyS A–I, cyclic (1→2)-β-D-glucans] for comparison.

0021-9673/88/$03.50

EXPERIMENTAL

Materials

The CDs were gifts from Sanraku (Fujisawa, Japan) and purified by recrystallization from hot water. Maltosyl (G_2)-, maltotriosyl (G_3)-, maltotetraosyl (G_4)- and maltopentaosyl (G_5)-CDs were synthesized from CDs and maltooligosaccharides [degree of polymerization (DP) 2–5] by the reverse action of *Pseudomonas* isoamylase[6] or *Klebsiella aerogenes* pullulanase[16]. Glucosyl (G_1)-CDs were prepared from G_2- or G_3-CDs by hydrolysis with purified *Rhizopus delemar* glucoamylase GIII[17]. These branched CDs were purified by high-performance liquid chromatography (HPLC) on YMC-Pack ODS columns[18] to a high state of purity. The CySs were isolated from culture filtrates of *Agrobacterium* and *Rhizobium*[19] and purified by reversed-phase HPLC[20].

Sodium hydroxide and sodium acetate used for preparation of eluents were of analytical-reagent grade. The eluents were 150 m*M* sodium hydroxide solution containing 140–300 m*M* sodium acetate as a modifier to elute oligo- and polysaccharides; 300 m*M* sodium hydroxide solution was prepared by dilution of carbonate-free 50% sodium hydroxide solution with deionized water (18 MΩ cm) purified using a NANO-pure II (Barnstead, Newton, MA, U.S.A.) and diluted with the same volume of sodium acetate solution, prepared with 18 MΩ cm deionized water and filtered through a 0.2-μm membrane filter.

Apparatus and column

HPLC was performed on a Dionex BioLC Model 4000i system equipped with a Model PAD II pulsed amperometric detector consisting of an amperometric flow-through cell with a gold working electrode and a silver – silver chloride reference electrode and a potentiostat. The sample loop size was 50 μl. The column used was a Dionex HPIC-AS6 (10 μm) (250 × 4 mm I.D.) equipped with an AG6 guard column (50 × 4 mm I.D.) (all from Dionex, Sunnyvale, CA, U.S.A.). A Chromatopac C-RIA digital integrator (Shimadzu, Kyoto, Japan) was used to calculate peak areas.

Chromatographic conditions and measurements

PAD utilizes a repeating sequence of three applied potentials: E_1 set to a small positive value for sample oxidation, E_2 set near the positive potential limit of the working electrode in the eluent to clean the electrode surface electrochemically and E_3 set near the negative potential limit to reduce gold oxide to gold. The three potentials are applied for specific durations, t_1, t_2 and t_3, respectively. To produce an increase in sensitivity, a decrease in the background current and an overall increase in detectability, the following pulse potentials and durations were optimal for analyses of cyclic glucans at Range 2 (sampling period, 200 ms): $E_1 = 0.10$ V ($t_1 = 300$ ms), $E_2 = 0.60$ V ($t_2 = 120$ ms) and $E_3 = -0.80$ V ($t_3 = 300$ ms). The response time of the PAD II detector was set to 1.0 s. The eluents, prepared daily, were degassed before use and kept under a stream of nitrogen. The sample solutions were prepared using 18 MΩ cm deionized water and filtered through a 0.2-μm membrane filter. All separations were carried out at ambient temperature with a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

Analysis of CDs and branched CDs

Fig. 1a shows the chromatogram of a mixture of α-, β- and γ-CD. The column used was a strongly basic anion-exchange column in the hydroxide form and carbohydrates can be separated by anion exchange with highly alkaline eluents, as carbohydrates have pK values ranging from 12 to 14[21]. In general, retention of a homologous series of carbohydrates on this column increases as the degree of polymerization (DP) increases. However, the elution order of CDs was α-CD (DP 6), γ-CD (DP 8) and β-CD (DP 7). This result indicates that there are some hydrophobic interactions on this column, but the interaction may be weaker than that on the ODS column; the retention order of CDs on the ODS column is γ-CD < α-CD ≪ β-CD[22]. The ion-exchange resin used for the column consists of a 10-μm substrate coated with a monolayer of anion-exchange latex. It is said[23] that the ion-exchange capacity is less than 1% of that of ordinary anion-exchange resin.

Fig. 1 shows the elution profiles of (b) branched α-CDs, (c) branched β-CDs and

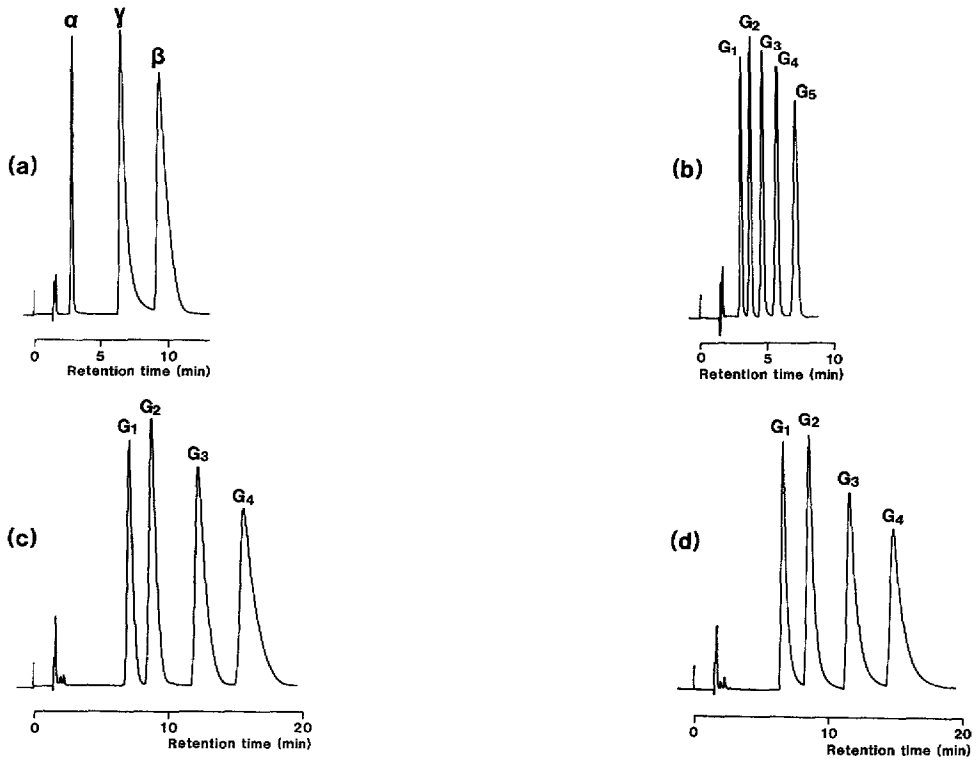

Fig. 1. Elution profiles of (a) α-, β- and γ-CD, (b) branched α-CDs, (c) branched β-CDs and (d) branched γ-CDs on a HPIC-AS6 column (250 × 4 mm I.D.). α = α-CD; β = β-CD; γ = γ-CD; G_1 = glucosyl; G_2 = maltosyl; G_3 = maltotriosyl; G_4 = maltotetraosyl; G_5 = maltopentaosyl. Chromatographic conditions: eluent, 150 mM sodium hydroxide–200 mM sodium acetate; flow-rate, 1 ml/min; detector, PAD II; meter scale, $10 \cdot 10^3$ nA; temperature, ambient (25 ± 1°C). Sample size: α-CD, 6.25; β-CD, 18.75; γ-CD, 12.5; branched α-CD, 3; branched β-CD, 10; branched γ-CD, 7.5 nmol.

(d) branched γ-CDs. For a series of branched CDs the retention increases as the length of the branch increases. G_1-CD to G_4-CD in each series showed similar elution profiles to that on C_{18}-bonded silica whereas the elution order of G_5-α-CD was different from that on C_{18}-bonded silica, which was abnormal, G_5-α-CD eluting before G_3-α-CD[18].

Although the peak shapes of α-CD and branched α-CDs were all good, those of the β-CD and especially the γ-CD series were poor. These peaks showed tailing, the extent of which increased with decrease in the sample concentration, particularly with the γ-CD series. As the peak shapes of CySs, having much longer retention times (t_R), are excellent, as shown in Fig. 3, the peak shape is independent of t_R. This phenomenon may arise from inclusion of benzyltrialkylammonium in the stationary phase with the cyclic glucan. For this interaction the cavity of γ-CD may be of the most suitable size (cavity diameter = 9.5 Å)[24] and the cavity size of β-CD (7.8 Å)[24] may also be suitable, whereas that of α-CD (5.7 Å)[24] is too small and those of CySs must be too large.

For control of retention sodium acetate was added to the eluent. The effect of sodium acetate concentration on the capacity factors for CDs and branched CDs is shown in Fig. 2. Changes in the sodium acetate concentration did not affect the mutual elution order of branched CDs, whereas the elution order of β-CD and γ-CD in each series changed with the sodium acetate concentration in the eluent.

PAD at a gold electrode is applicable to the sensitive detection of the HCOH group in carbohydrates. Although the exact oxidation reaction mechanism is not clear, a high pH is required in order to obtain adequate sensitivity. pH 13 is the optimum for detection. On the basis of this information, quantitative analyses of CDs and branched CDs by anion-exchange chromatography with PAD were carried out using 150 m*M* sodium hydroxide–200 m*M* sodium acetate as the eluent. First the relative detector response was evaluated from chromatograms of standard mixtures of α-, β- and γ-CD,

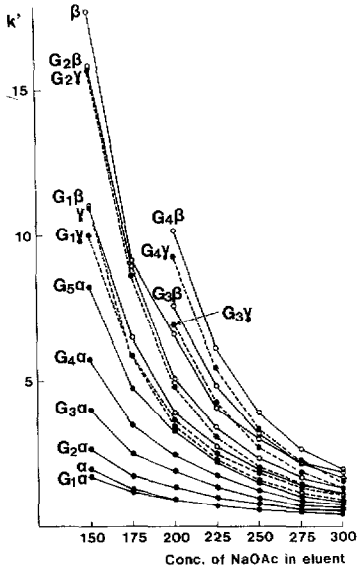

Fig. 2. Relationship between sodium acetate (NaOAc) concentration in the eluent and the capacity factor of each CD and branched CD. Sample size, 5 nmol each. Chromatographic conditions other than eluent as in Fig. 1.

TABLE I

RELATIVE DETECTOR RESPONSE OF CDs AND BRANCHED CDs

Chromatographic conditions as in Fig. 1. The amounts of CDs and branched CDs used were 5 nmol each.

CD	*R.D.R.**	*CD*	*R.D.R.*	*CD*	*R.D.R.*	*CD*	*R.D.R.*
α-	1.0	α-	1.0	β-	1.0	γ-	1.0
β-	1.7	G_1-α-	1.4	G_1-β-	1.1	G_1-γ-	1.2
γ-	2.0	G_2-α-	1.6	G_2-β-	1.5	G_2-γ-	1.5
		G_3-α-	2.0	G_3-β-	1.8	G_3-γ-	1.6
		G_4-α-	2.3	G_4-β-	2.0	G_4-γ-	1.8
		G_5-α-	2.6				

* R.D.R. = Relative detector response.

and each series of branched α-, β- and γ-CDs (5 nmol each) (Table I). The results indicated that the sensitivity of detection does not decrease with increasing molecular weight. The detector response per HCOH group in CDs and branched CDs varied only in limited ranges.

The linearity of the detector response was investigated by injection of progressive dilutions of a mixture of α-, β- and γ-CD and a mixture of branched α-CDs under the same chromatographic conditions as in Fig. 1. The calibration graph of peak height *vs.* concentration of α-CD was linear in the range 10 pmol–1 nmol ($r = 0.999$), whereas those of β-CD and γ-CD were linear in the range 25 pmol–500 pmol ($r = 0.996$ and 0.995, respectively). The detection limits for α-, β- and γ-CD were 5, 10 and 10 pmol, respectively, with a signal-to-noise ratio of 3. The reason for the poorer sensitivities for β- and γ-CD appears to be retardation of the retention time (t_R) accompanying peak broadening with decreasing CD concentration (Table II). This phenomenon could be explained by a relative increase in the surface area accessible to the aforesaid interaction with β- and γ-CD. A reduction in t_R by using 150 m*M* sodium hydroxide–300 m*M* sodium acetate as the eluent avoided these effects and increased the sensitivities for β- and γ-CD to the same level as for α-CD or even higher; the detection limit for γ-CD was 2.5 pmol.

Table III gives quantitative results for branced α-CDs. The limits of determination and detection of G_1-, G_2- and G_3-α-CD, which have shorter t_R and give very sharp peaks, were lower. The reproducibility of the peak heights at 1 nmol, 200 pmol and 50 pmol was good.

TABLE II

VARIATION OF RETENTION TIME WITH CONCENTRATION OF CD

Chromatographic conditions as in Fig. 1.

Concentration (pmol)	*Retention time (min)*		
	α-CD	*β-CD*	*γ-CD*
500	2.92	10.47	7.07
50	2.92	11.13	8.23
25	2.92	11.32	8.92

TABLE III
CALIBRATION GRAPHS AND DETECTION LIMITS FOR BRANCHED α-CDs
Chromatographic conditions as in Fig. 1.

CD	t_R *(min)*	*Calibration graph*					*Detection limit (pmol) for S/N = 3***
		Linear region (pmol)	*r**	*C.V.** (n = 5) at*			
				1 nmol	*200 pmol*	*50 pmol*	
G_1-α-	3.0	10–1000	0.999	0.92	1.13	1.36	5
G_2-α-	3.6–3.7	10–1000	0.999	0.87	1.18	2.07	5
G_3-α-	4.4–4.5	10–1000	0.999	1.04	1.17	2.05	5
G_4-α-	5.4–5.6	20–1000	0.999	0.87	0.98	2.05	10
G_5-α-	6.7–6.9	20–1000	0.999	1.18	2.31	2.81	10

* r = Correlation coefficient.
** C.V. = Coefficient of variation.
*** S/N = Signal-to-noise ratio.

Analysis of CySs

For comparison, analyses of CyS A–I, having a different linkage form [β-(1→2)] from CDs [α-(1→4)] and much higher DPs (17–25), were performed. Fig. 3 shows the elution profile of CyS A–I. The elution pattern was different from those obtained on amino-bonded silica with acetonitrile–water as eluent and also on C_{18}-bonded silica with methanol–water as eluent[20]: in the former instance there was an increased retention with increasing molecular size of CyS, and in the latter probably an increased retention with decreasing solubility in water, the elution order of CyS-C and -D, and CyS-E and -F being reversed. On this anion-exchange column with 150 m*M* sodium hydroxide solution containing sodium acetate as the eluent, the elution order of CyS-D and -E was reversed. Moreover, in spite of their much larger molecular sizes, CySs moved faster on this column (Fig. 4).

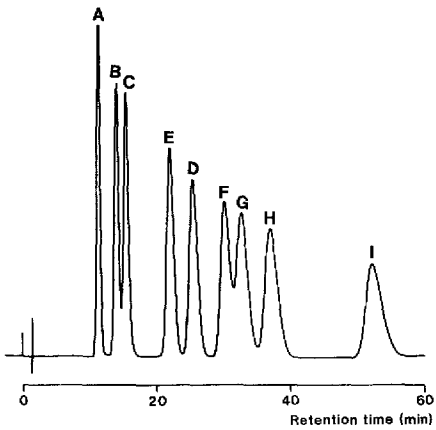

Fig. 3. Separation of CyS A–I. DP: A = 17; B = 18; C = 19; D = 20; E = 21; F = 22; G = 23; H = 24; I = 25. Chromatographic conditions: eluent, 150 m*M* sodium hydroxide–140 m*M* sodium acetate; other conditions as in Fig. 1. Sample size, 5 nmol each.

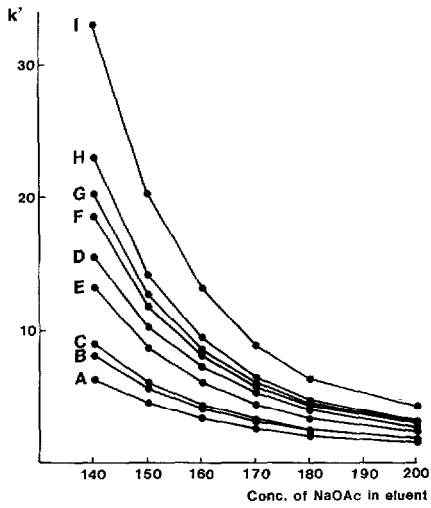

Fig. 4. Relationship between sodium acetate (NaOAc) concentration in the eluent and the capacity factor of each CyS. Sample size, 5 nmol each. Chromatographic conditions other than eluent as in Fig. 1.

The retention times and relative detector responses of CyS A–I under the same chromatographic conditions as in Table I are summarized in Table IV and compared with the data for CDs. The detector responses per glucose unit of β- and γ-CD and CySs are almost the same.

Under the same chromatographic conditions as in Table IV, the CySs gave calibration graphs having good linearity (all with $r = 0.999$) in the ranges 5–500 pmol for CyS A–H and 10–500 pmol for CyS I. The detection limits were 2.5 pmol for CyS A–H and 5 pmol for CyS I.

TABLE IV

COMPARISON OF RETENTION TIMES (t_R) AND RELATIVE DETECTOR RESPONSES OF CySs WITH THOSE OF CDs

Chromatographic conditions as in Fig. 1. The amounts of CDs and CySs used were 5 nmol each.

Glucan	t_R *(min)*	*R.D.R.**	*DP***	*R.D.R. per Glc unit*
α-CD	2.92	0.61	6	0.7
β-CD	10.47	1.00	7	1.0
γ-CD	7.07	1.10	8	1.0
CyS-A	3.83	2.54	17	1.1
CyS-B	4.19	2.59	18	1.0
CyS-C	4.33	2.88	19	1.1
CyS-D	5.70	3.09	20	1.1
CyS-E	5.13	3.04	21	1.0
CyS-F	6.04	3.34	22	1.1
CyS-G	6.23	3.66	23	1.1
CyS-H	6.26	3.68	24	1.1
CyS-I	8.08	3.38	25	1.0

* R.D.R. = Relative detector response.
** DP = Number of glucose (Glc) units.

CONCLUSION

The combination of anion-exchange separation with a highly alkaline eluent and PAD provides a powerful new technique for the analysis of cyclic glucans. As cyclic glucans have no reducing end, they are stable to the highly alkaline eluent. For the separation of cyclic glucans, 150 m*M* sodium hydroxide solution having the optimum pH for detection by PAD can be used as the eluent and therefore no post-column addition of a strong base is needed. The sensitivity of detection of β-CD by PAD is 20 times that using a refractive index detector of high sensitivity, used together with a pump-minimized pulsating flow[25].

ACKNOWLEDGEMENTS

The authors are indebted to Professor S. Hizukuri (Kagoshima University) for the preparation of branched CDs, and also thank Dr. A. Amemura (Osaka University) and Dr. M. Hisamatsu (Mie University) for the preparation of CySs.

REFERENCES

1 J. Szejtli and J. Pagington, *Cyclodextrin News*, 2, No. 8 (1988) 6–7.
2 S. Kobayashi, N. Shibuya, B. M. Young and D. French, *Carbohydr. Res.*, 126 (1984) 215–224.
3 Y. Sakano, M. Sano and T. Kobayashi, *Agric. Biol. Chem.*, 49 (1985) 3391–3398.
4 J. Abe, Y. Takeda, S. Hizukuri, K. Yamashita and N. Ide, *Carbohydr. Res.*, 131 (1984) 175–179.
5 K. Koizumi, T. Utamura, M. Sato and Y. Yagi, *Carbohydr. Res.*, 153 (1986) 55–67.
6 J. Abe, N. Mizowaki, S. Hizukuri, K. Koizumi and T. Utamura, *Carbohydr. Res.*, 154 (1986) 81–92.
7 S. Kitahata, Y. Yoshimura and S. Okada, *Carbohydr. Res.*, 159 (1987) 303–313.
8 Y. Yoshimura, S. Kitahata and S. Okada, *Carbohydr. Res.*, 168 (1987) 285–294.
9 K. Koizumi, Y. Okada,Y. Kubota and T. Utamura, *Chem. Pharm. Bull.*, 35 (1987) 3413–3418.
10 P. Fügedi, P. Nánási and J. Szejtli, *Carbohydr. Res.*, 175 (1988) 173–181.
11 Y. Okada, Y. Kubota, K. Koizumi, S. Hizukuri,T. Ohfuji and K. Ogata, *Chem. Pharm. Bull.*, 36 (1988) 2176–2185.
12 R. D. Rocklin and C. A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577–1590.
13 G. G. Neuberger and D. C. Johnson, *Anal. Chem.*, 59 (1987) 203–204.
14 K. Ohsawa, Y. Yoshimura, S. Watanabe, H. Tanaka, A. Yokota, K. Tamura and K. Imaeda, *Anal. Sci.*, 2 (1986) 165–168.
15 M. R. Hardy, R. R. Townsend and Y. C. Lee, *Anal. Biochem.*, 170 (1988) 54–62.
16 S. Hizukuri, S. Kawano, J. Abe, K. Koizumi and T. Tanimoto, *Biotech. Appl. Biochem.*, in press.
17 J. Abe, H. Nagano and S. Hizukuri, *J. Appl. Biochem.*, 7 (1985) 235–247.
18 K. Koizumi, Y. Kubota, Y. Okada, T. Utamura, S. Hizukuri and J.-I. Abe, *J. Chromatogr.*, 437 (1988) 47–57.
19 M. Hisamatsu, A. Amemura, K. Koizumi, T. Utamura and Y. Okada, *Carbohydr. Res.*, 121 (1983) 31–40.
20 K. Koizumi, Y. Okada, T. Utamura, M. Hisamatsu and A. Amemura, *J. Chromatogr.*, 299 (1984) 215–224.
21 J. A. Rendleman, *Carbohydrates in Solution*, Advances in Chemistry Series, No. 117, American Chemical Society, Washington, DC, 1973, p. 51.
22 K. Koizumi, T. Utamura, T. Kuroyanagi, S. Hizukuri and J.-I. Abe, *J. Chromatogr.*, 360 (1986) 397–406.
23 J. Weiss, in E. L. Johnson (Editor), *Handbook of Ion Chromatography*, Dionex Co., Sunnyvale, CA, 1986, p. 26.
24 J. Szejtli, *Cyclodextrins and Their Inclusion Complexes*, Akadémiai Kiadó, Budapest, 1982, p. 25.
25 K. Koizumi, Y. Kubota, Y. Okada and T. Utamura, *J. Chromatogr.*, 341 (1985) 31–41.