Carbohydrate Research, 215 (1991) 67–80
Elsevier Science Publishers B.V., Amsterdam

High-performance liquid chromatography of mono- and oligo-saccharides on a graphitized carbon column

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(Received June 26th, 1990; accepted in revised form November 8th, 1990)

ABSTRACT

Chromatographic behavior of several mono- and di-saccharides and cyclomaltaoses on Hypercarb, a graphitized carbon column, was investigated. Monosaccharides were weakly retained on this column and were eluted with water within 1.8–3.6 min at 1 mL/min of flow rate and 30°. Nevertheless, each peak of D-xylose, D-glucose, D-galactose, and L-fucose was split into anomer peaks. Disaccharides were adequately eluted with 15:85 methanol–water or 4:96 acetonitrile–water, and each showed two peaks for the anomers. These peaks each coalesced into a single peak upon the addition of mM sodium hydroxide to the eluent. Simultaneous separation of nine glucodisaccharides was achieved on this column by gradient elution with mM sodium hydroxide solution containing 1.5–5.0% acetonitrile. Detection was by a pulsed amperometric detector. Cyclomaltohexaose (αCD), cyclomaltoheptaose (βCD), cyclomaltooctaose (γCD), and their glucosyl derivatives (G-αCD, G-βCD and G-γCD) were analyzed by using 13–15% aq. acetonitrile as the eluent. Interestingly their retention times were increased with increases in temperature, and the sharpness of their peaks was also shown to be enhanced. Furthermore, positional isomers of glucosyl-inositol and of dimaltosyl-βCD, neither of which can be separated on conventional bonded phases, were well resolved on this column.

INTRODUCTION

Hypercarb, a graphitized carbon h.p.l.c. column, has been used for the analyses of samples such as pharmaceutical products, peptides, amino acids[1], and phenols[1,2], and it has been demonstrated to have unique ability of resolving isomeric and closely related compounds. However, little has been known about the chromatographic behavior of carbohydrates, especially underivatized ones, on this column. In this paper we report some information obtained from studies on h.p.l.c. of several mono- and di-saccharides and cyclomaltaoses (cyclodextrins, CDs) on Hypercarb.

EXPERIMENTAL

Materials. — Kojibiose, sophorose, and laminaribiose were gifts. Nigerose was prepared from its peracetate by deacetylation with 0.5% sodium methoxide in methanol. The other disaccharides and all monosaccharides were obtained from commercial sources and were purified by semi-preparative h.p.l.c., if necessary. CDs and a mixture

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of glucosyl-inositols were kindly donated by Sanraku, Ltd. Glucosyl-α and -βCDs were prepared as previously reported[3]. Glucosyl-γCD was a gift. A mixture of dimaltosyl-βCDs is commercially produced by Ensuiko Sugar Refining Co., Ltd. (Yokohama, Japan). Reagent-grade organic solvents used for chromatography were dried, freshly distilled before use, and filtered through a 0.45-μm membrane filter. Water used in eluent preparations was distilled, deionized, and redistilled. The sodium hydroxide solutions, which were used as the eluent and as the pH modifier of the effluent in chromatography with pulsed amperometric detection, were prepared by dilution of carbonate-free 50% sodium hydroxide solution with deionized water (18 MΩ cm) purified using a NANOpure II device (Barnstead).

Apparatus and columns. — H.p.l.c. analyses were performed with a JASCO 880-PU pump, a Waters U6K universal injector, and a Showa Denko SE-61 r.i. monitor. H.p.l.c. using a gradient-elution method was conducted with a Dionex BioLC Model 4000i system equipped with a Model PAD 2 pulsed amperometric detector consisting of an amperometric flow-through cell with a gold working electrode, a silver–silver chloride reference electrode, and a potentiostat. For the pH modification of column effluent, an anion micromembrane suppressor AMMS-MPIC was used, and, for analyses at constant temperature, a column oven, model SSC 3510C (Senshu Scientific Co.), was used. A Shimadzu Chromatopac C-R3A digital integrator was used for quantitative analysis. The columns used were two Hypercarb columns (100 × 4.6 mm i.d.) of different lots (Shandon) and a YMC-Pack A-312 column (150 × 6 mm i.d.) (YMC).

H.p.l.c. — The eluents used for isocratic elution were water and water containing acetonitrile or methanol. The eluent A and B used for gradient elution were mM sodium hydroxide solution containing 5% acetonitrile and mM sodium hydroxide solution, respectively. A linear gradient method: 0–15 min, 30–100% eluent A, was adopted. H.p.l.c. with an r.i. detector was carried out at constant temperature using a column oven. Samples (30–200 nmol) were loaded, and simultaneous separation of glucodisaccharides (3–5 nmol each) by gradient elution was performed at ambient temperature. As the effluent pH modifier, 1.5M sodium hydroxide solution was used and delivered to an anion micromembrane suppressor at a flow rate of 1 mL/min by pressurizing a reagent reservoir with about 2.5 p.s.i. nitrogen, and saccharides in the pH-modified effluent were detected by p.a.d., using pulse potentials and duration times as previously described[4].

RESULTS AND DISCUSSION

Monosaccharides. — Table I summarizes the retention data for common aldopentoses, aldohexoses, ketohexoses, and a sugar alcohol, as obtained on a Hypercarb column with water at various temperatures. Peaks of anomers were assigned by comparing their integration with known equilibrium proportions of those anomers[5–7] (Table II).

TABLE I

Retention data for popular aldopentoses, aldohexoses, ketohexoses, and a sugar alcohol on Hypercarb with water at various temperatures[a]

Carbohydrate		*Retention time (min) at*			
		15°	*30°*	*45°*	*60°*
L-arabinose	α	1.82	1.80		
	β	s			
D-lyxose		1.95	1.82		
D-xylose	β	2.08	1.90	1.85	1.75
	α	2.45	2.15		
D-ribose	β	2.30	2.10		
	α	s			
D-glucose	β	2.40	2.20	2.05	1.90
	α	2.62	2.33		
D-mannose		2.50	2.25		
D-galactose	β	2.60	2.27	2.10	1.93
	α	3.05	2.60		
L-rhamnose		2.90	2.55		
L-fucose	β	3.35	3.00	2.60	2.40
	α	4.20	3.60	s	
D-fructose	β	2.10	2.00		
L-sorbose	α	2.25	2.05		
D-glucitol		2.40	2.15		

[a] s = shoulder.

As aldopentoses other than D-xylose have the possibility of taking two conformations ($^4C_1$ and $^1C_4$), and, moreover, their retention times (T_Rs) are too short, the separation of anomers is rather difficult. Especially noteworthy is the fact that the peak of D-lyxose, which has an axial OH on C-2, was not split. Also the peaks of aldohexoses having an axial OH on C-2, *e.g.*, D-mannose and L-rhamnose, were difficult to split into peaks of α and β anomers. However, on another Hypercarb column from a different lot, at 10°, L-rhamnose showed split peaks (T_R = 3.5 and 4.45 min), and a shoulder appeared on the peak of D-mannose. This fact suggested that the graphite structure of the carbon surface was slightly different between the two columns, which resulted in the difference in sensitivity to a small steric change of the solute.

On the other hand, the anomers of aldohexoses having an axial OH at C-4, *e.g.*, D-galactose and L-fucose, showed the best separation.

TABLE II

Conformations of monosaccharides, configurations of OH on C-1, -2, -3, -4, and -5, and proportion of the α anomer (%) in aqueous solution at equilibrium.

Carbohydrate	*Conformation*	*Configuration of OH on*					*Proportion of α anomer (%)*
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	
α-L-arabinose	$^4C_1$	eq	eq	eq	ax		73.5[a]
β-L-arabinose	$^4C_1$ ($\rightleftharpoons {}^1C_4$)	ax	eq	eq	ax		
α-D-lyxose	$^4C_1$ ($\rightleftharpoons {}^1C_4$)	ax	ax	eq	eq		71.0[b]
β-D-lyxose	$^4C_1$	eq	ax	eq	eq		
α-D-xylose	$^4C_1$	ax	eq	eq	eq		33.0[c]
β-D-xylose	$^4C_1$	eq	eq	eq	eq		
α-D-ribose	$^4C_1$ ($\rightleftharpoons {}^1C_4$)	ax	eq	ax	eq		26.0[d]
β-D-ribose	$^4C_1$	eq	eq	ax	eq		
α-D-glucose	$^4C_1$	ax	eq	eq	eq		36.0[e]
β-D-glucose	$^4C_1$	eq	eq	eq	eq		
α-D-mannose	$^4C_1$	ax	ax	eq	eq		67.0[f]
β-D-mannose	$^4C_1$	eq	ax	eq	eq		
α-D-galactose	$^4C_1$	ax	eq	eq	ax		27.0[g]
β-D-galactose	$^4C_1$	eq	eq	eq	ax		
α-L-rhamnose	$^4C_1$	ax	ax	eq	eq		73.1[a]

of the calcium form at 4° with 30:70 water–acetonitrile as eluent and photometric detection after postcolumn labelling with 2-cyanoacetamide. The present method is easier (eluent is water, column temperature is 11°, and derivatization is unnecessary) and more rapid (the pyranose anomers of L-fucose were resolved within 6 min; R_s = 1.8); therefore, for examination of a rapid anomerization as shown in Fig. 2, this method is well suited.

D-Glucosamine hydrochloride was not retained on Hypercarb, whereas both gluconic and glucuronic acids were retained too strongly on the column for practical chromatography.

Disaccharides. — Disaccharides were adequately eluted from the Hypercarb column with 15:85 methanol–water (Table III) or 4:96 acetonitrile–water (Table IV).

TABLE III

Retention data for some common disaccharides on Hypercarb with 15:85 methanol–water at various temperatures[a]

Carbohydrate	*Retention time (min) at*				
	10°	*15°*	*30°*	*45°*	*60°*

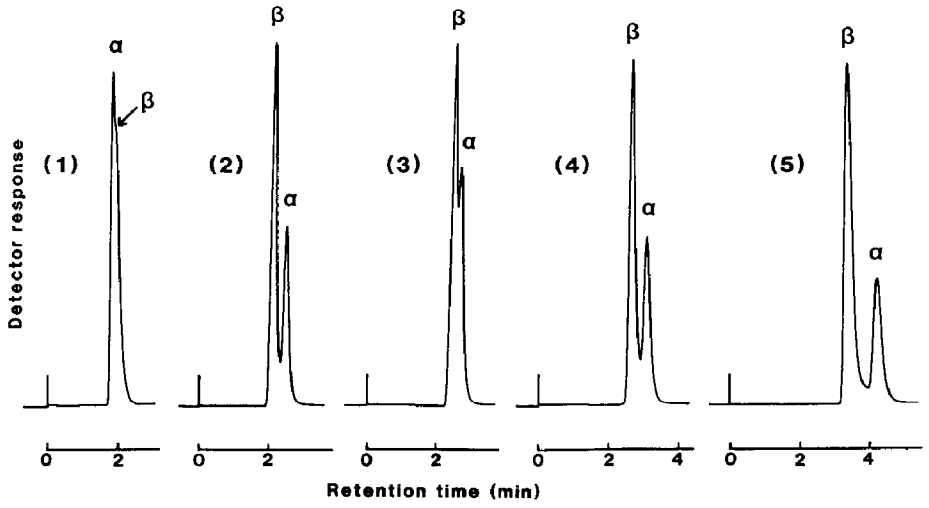

Fig. 1. Chromatograms of some monosaccharides: (1) L-arabinose, (2) D-xylose, (3) D-glucose, (4) D-galactose, and (5) L-fucose. Chromatographic conditions: column, Hypercarb (100 × 4.6 mm i.d.); eluent, water; flow rate, 1 mL/min; detector, SE-61 r.i.; temperature, 15°.

as the rate of interconversion between the α and β anomer is accelerated and the T_Rs become shorter. Therefore, Hypercarb cannot be used for the separation of monosaccharides.

However, this type of chromatography may be used as a convenient method for studying the anomerization of some monosaccharides. Fig. 2 illustrates the time course of anomerization of α-L-fucose in distilled water at 25°. Analysis of aldose anomers by h.p.l.c. was precisely studied on cation-exchange columns by Honda *et al.*[8], and, in their work, the course of anomerization of α- and β-D-glucoses was estimated using a column

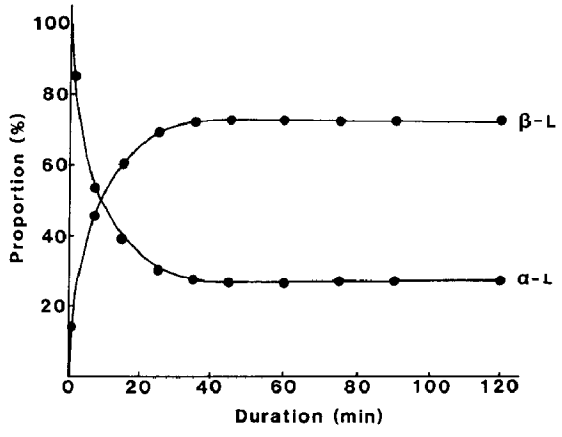

Fig. 2. Time course of anomerization of α-L-fucose in distilled water at 25°. Chromatographic conditions: temperature, 11°; other conditions were as in Fig. 1.

of the calcium form at 4° with 30:70 water–acetonitrile as eluent and photometric detection after postcolumn labelling with 2-cyanoacetamide. The present method is easier (eluent is water, column temperature is 11°, and derivatization is unnecessary) and more rapid (the pyranose anomers of L-fucose were resolved within 6 min: R_s = 1.8); therefore, for examination of a rapid anomerization as shown in Fig. 2, this method is well suited.

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Retention data for some common disaccharides on Hypercarb with 15:85 methanol–water at various temperatures[a]

Carbohydrate	*Retention time (min) at*				
	10°	*15°*	*30°*	*45°*	*60°*
trehalose		3.3	2.6	2.3	2.2
kojibiose		4.2 6.5	3.4 4.7	3.0 3.8	3.0
nigerose		10.9 13.3	7.4 8.7	6.0	5.0
maltose		7.2 9.0	5.2 6.2	4.2	4.0
isomaltose		3.5 5.2	2.8 3.9	2.5 3.2	2.5
sophorose	22.0 23.2	s 19.0	12.0	8.4	6.6
laminaribiose		36.0 41.8	20.0 21.7	13.7	10.8
cellobiose	23.6 28.4	22.2 26.0	15.6 17.0	11.3	9.1
gentiobiose		12.3 22.7	7.8 13.2	6.9	5.8
sucrose		5.7	4.0	3.3	2.9
lactose		6.6 11.0	5.2 7.8	4.2 5.8	4.3

[a] s = shoulder.

TABLE IV

Retention data for some common disaccharides on Hypercarb with 4:96 acetonitrile–water at various temperatures[a]

Carbohydrate	*Retention time (min) at*					
	10°	*15°*	*30°*	*45°*	*60°*	*70°*
trehalose		2.5	2.4	2.2	2.2	
kojibiose		2.7 3.6	2.9 3.6	2.8 3.4	2.7 3.1	2.9
nigerose		5.4 7.8	5.2 7.2	4.8 6.2	5.0	
maltose		4.4 5.0	4.2 4.5	3.8 4.2	3.7	
isomaltose		2.5 3.5	2.5 3.3.	2.4 3.2	2.5	
sophorose		b 10.5	8.8 9.8	7.2 s	6.8	
laminaribiose	24.4	20.6	17.1	13.2	11.3	
cellobiose		13.2 16.0	12.0 14.2	11.2	10.0	
gentiobiose		6.7 11.1	6.2 9.6	5.6 s	5.6	
sucrose		3.6	3.4	3.1	2.9	
lactose		4.3 6.3	4.3 5.8	4.2 5.4	4.1 4.8	4.1 s

[a] b = band broadening due to the partial separation of anomers, s = shoulder.

and their peak shapes were generally better with the latter. Fig. 3 shows elution profiles of typical disaccharides with 4:96 acetonitrile–water at 15°. Except for sophorose and laminaribiose, all reducing disaccharides examined each showed two peaks for their respective anomers. The peak of sophorose split at 30° and became a single peak having a shoulder again at 45°, whereas with 15:85 methanol–water, the peak of sophorose split at 10° and became a single peak at 30°. Although with 4:96 acetonitrile–water, laminaribiose showed a single peak even at 10°, and also by the use of another Hypercarb column, the use of 15:85 methanol–water as the eluent resulted in peak split at temperatures of both 15° and 30°.

The elution order of nine glucodisaccharides depended on the linkage-forms: α,α-(1→1), α-(1→6), α-(1→2), α-(1→4), α-(1→3), β-(1→6), β-(1→2), β-(1→4), and β-(1→3). Retention on Hypercarb is by an adsorption mechanism, and one of the

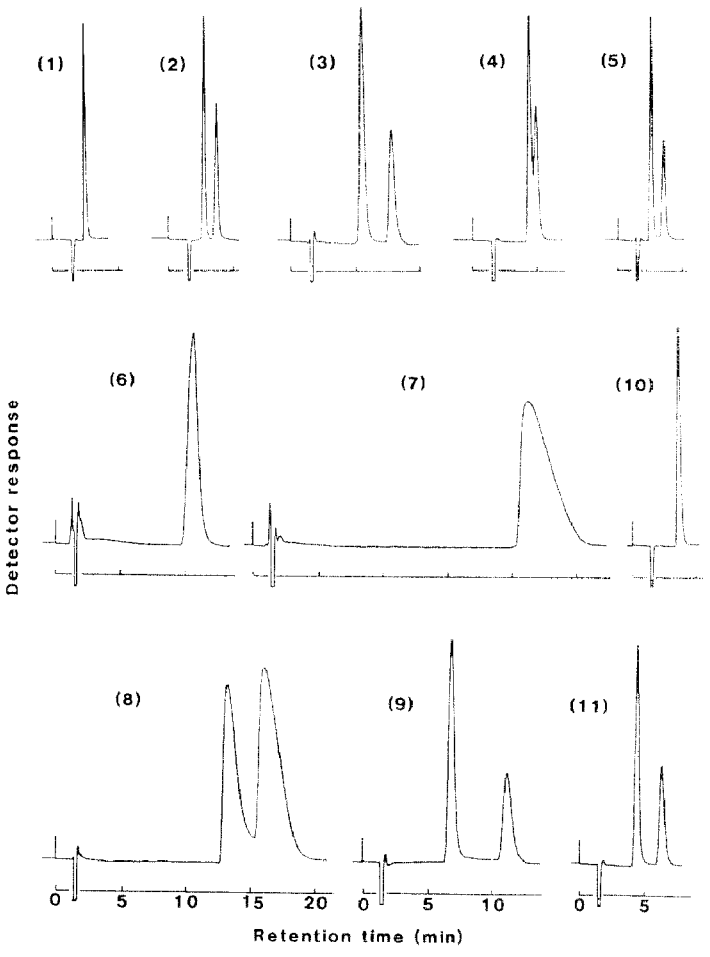

Fig. 3. Chromatograms of typical disaccharides: (1) trehalose, (2) kojibiose, (3) nigerose, (4) maltose, (5) isomaltose, (6) sophorose, (7) laminaribiose, (8) cellobiose, (9) gentiobiose, (10) sucrose, and (11) lactose. Chromatographic conditions: eluent, 4:96 acetonitrile–water; other conditions were as in Fig. 1.

factors to be considered in determining retention is configuration of the molecule. That is, planar molecules are generally more retained than non-planar molecules. The elution order of glucodisaccharides may suggest rankings of relative degrees of planarity of their molecules.

Interestingly, retention of 4-*O*-(β-D-galactopyranosyl)-D-glucose (lactose) resembled that of 4-*O*-(α-D-glucopyranosyl)-D-glucose (maltose) rather than that of 4-*O*-(β-D-glucopyranosyl)-D-glucose (cellobiose); moreover, irrespective of the relatively short T_R, the anomers of lactose were well resolved, and, on the use of 4:96 acetonitrile–water as the eluent, the doublets disappeared at temperatures higher than 75°.

Hypercarb has exceptional chemical and physical stability and can be used throughout the entire pH range. Therefore, the elimination of double peaks of disaccharides was attempted by addition of alkali into the eluent. It was found that mM

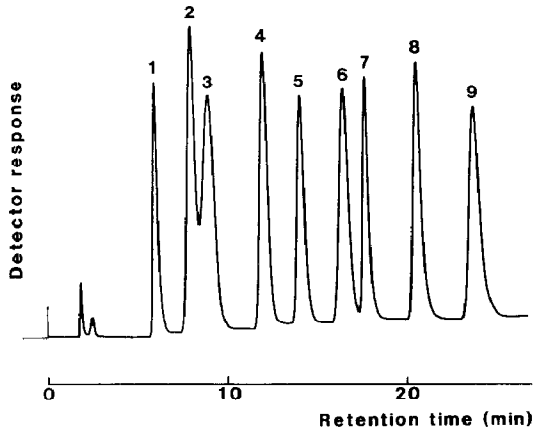

Fig. 4. Separation of glucodisaccharides on a Hypercarb column by gradient elution with mM sodium hydroxide solution containing 1.5–5% acetonitrile. 1 = trehalose; 2 = isomaltose; 3 = kojibiose; 4 = maltose; 5 = nigerose; 6 = gentiobiose; 7 = sophorose; 8 = cellobiose, and 9 = laminaribiose. Chromatographic conditions: eluent A, mM sodium hydroxide solution containing 5% acetonitrile; eluent B, mM sodium hydroxide solution: linear gradient, 0–15 min, 30–100% eluent A; flow rate, 1 mL/min; detector, PAD 2; temperature, ambient.

sodium hydroxide solution containing few per cent of acetonitrile as the eluent is adequate for this purpose. Fig. 4 shows simultaneous separation of nine glucodisaccharides on a Hypercarb column by gradient elution with mM sodium hydroxide solution containing 1.5–5.0% acetonitrile. After the column effluent was made alkaline by passing it between two cation-exchange membranes surrounded by 1.5M sodium hydroxide solution using an anion micromembrane suppressor[9], disaccharides in the effluent were detected with p.a.d.

Cyclomaltaoses and their glucosyl derivatives. — The best separation of cyclomaltaoses (αCD, βCD, and γCD) and that of their 6-*O*-α-D-glucopyranosyl derivatives (G-αCD, G-βCD, and G-γCD) on Hypercarb occurred with water containing a greater percentage of organic modifier than that used for separations on reversed phases, since Hypercarb is more hydrophobic than conventional reversed-phase materials. Acetonitrile, methanol, ethanol, acetone, *N,N*-dimethylformamide, and methyl sulfoxide could be used as the modifier. Table V gives retention data for CDs and G-CDs on a Hypercarb column with acetonitrile modifiers at several temperatures, and Table VI shows similar data with methanol as modifier. The concentration of organic modifier in the eluent and the column temperature both serve to control the retention of CDs and G-CDs.

The retention time tends to decrease with increasing modifier concentration. This retention behavior is, in general, analogous to that observed on reversed phases. However, the elution order of CDs and G-CDs on Hypercarb is quite different from that on reversed phases, as the retention of CDs and G-CDs on Hypercarb were shown to increased with increasing molecular size like that observed with NH_2-bonded phases. Retention times for these compounds on reversed phases are known to increase with decreasing solubility in the eluent[10].

TABLE V

Effects of concentration of acetonitrile and column temperature on retention times of cyclodextrins and glucosyl-cyclodextrins

Conc. of CH_3CN	*Temp. (°)*	*Retention time (min) of*					
		αCD	*βCD*	*γCD*	*G-αCD*	*G-βCD*	*G-γCD*
13%	30	3.5[a]	5.3	9.1	2.5	4.4	9.8
13%	40	3.2[b]	5.4	9.8	2.8	5.1	11.6
13%	50	3.2	5.8	10.8[c]	3.1	6.1	14.3
13%	60	3.4	6.2	12.4[c]	3.5	7.2	17.8[c]
14%	30	2.6[a]	3.7	6.2	2.2	3.2	6.2
14%	40	2.6[b]	4.0	6.6	2.3	3.6	7.2
14%	50	2.7	4.4	7.4[c]	2.5	4.3	9.1
14%	60	2.8	4.8	8.8[c]	2.8	5.2	11.5[c]
15%	30	2.1[b]	2.7	3.9	1.8	2.4	3.8
15%	40	2.1	2.9	4.4	2.0	2.7	4.7
15%	50	2.2	3.3	5.2	2.1	3.2	5.8
15%	60	2.3	3.6	6.0	2.4	3.8	7.2

[a] Peak shows broadening. [b] Peak shows slight broadening. [c] Peak shows slight tailing.

Another interesting fact that is apparent from the data in Tables V and VI is the variation of T_R with temperature. The T_R on C_{18}-bonded silica is greatly affected by temperature and is shown to decrease with increases in column temperature[11]. On the contrary, the T_R on Hypercarb with water containing acetonitrile increased with increases in temperature. Furthermore, even though αCD had the shortest T_R, its peak shape was very broad at lower temperatures and was much improved with rising temperature. In the case of using methanol as the organic modifier, the effect of

TABLE VI

Effects of concentration of methanol in eluent and column temperature on retention times of cyclodextrins and glucosyl-cyclodextrins

Conc. of CH_3OH	*Temp. (°)*	*Retention time (min) of*					
		αCD	*βCD*	*γCD*	*G-αCD*	*G-βCD*	*G-γCD*
50%	30	3.5	6.0	14.5	4.0	7.5	22.0
50%	50	3.0	5.0	12.0	4.0	7.0	20.0
60%	30	2.5	4.0	7.0	3.0	5.0	12.0
60%	40	2.5	4.0	7.0	3.0	5.0	12.0
60%	50	2.5	4.0	7.0	3.0	5.0	12.0
70%	30	2.5	3.5	6.0	2.5	4.0	9.0
70%	40	2.5	3.5	6.0	2.5	4.0	9.5
70%	50	2.5	3.5	6.0	3.0	4.5	10.0

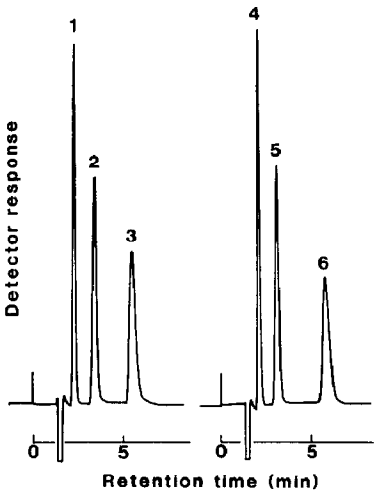

Fig. 5. Elution profiles of CDs and G-CDs: 1 = αCD; 2 = βCD; 3 = γCD; 4 = G-αCD; 5 = G-βCD; and 6 = G-γCD. Chromatographic conditions: eluent, 15:85 acetonitrile–water; temperature, 50°; other conditions as in Fig. 1.

temperature on T_Rs of CDs and G-CDs was much smaller than that observed in the case of acetonitrile, and the effect was essentially nil as compared with the effect of temperature on the C_{18}-bonded silica–methanol–water system. In 50:50 methanol–water the T_Rs of CDs and G-CDs decreased slightly with every 20° increase in temperature, but with 60:40 or 70:30 methanol–water, the temperature had entirely no effect on the T_Rs of CDs. The T_Rs of G-CDs in 70:30 methanol–water increased slightly with raising the temperature from 30° to 50°. In summary, for the mutual separation of three CDs and three G-CDs, the use of the eluents containing higher concentrations of acetonitrile at higher temperature is recommended. Fig. 5 shows elution profiles of CDs and G-CDs on a Hypercarb column with 15:85 acetonitrile–water at 50°.

Separation of positional isomers. — (1) Glucosyl-inositol. A series of oligoglucosyl-inositols were synthesized by the glucosyl-transfer action of the CGTase from *Bacillus ohbensis* with *myo*-inositol as an acceptor and βCD as a donor[12]. In order to elucidate the bonding position of inositol, the glucosyl-inositol fraction was purified by ion-exchange chromatography and successfully crystallized, but positional isomers could not be separated by either recrystallization or by chromatography on either an anion-exchange column or a reversed-phase column. However, the highly steric-sensitive surface of Hypercarb made it possible to separate two positional isomers of glucosyl-inositol (Fig. 6). Although the two isomers were isolated by h.p.l.c. on a Hypercarb column with 0.8–1.0% aq. acetonitrile as the eluent in a high state of purity, and their structures were determined as 4 (or 6)-*O*-(α-D-glucopyranosyl)-*myo*-inositol (**1**) and 5-*O*-(α-D-glucopyranosyl)-*myo*-inositol (**2**) by H–H and C–H COSY n.m.r. spectroscopy, isolation of pure **2** was extremely difficult. In order to exclude a trace contamination of **1** in the fraction of **2**, many repetitive chromatographic separations of the latter fraction were necessary, as the main mechanism of retention on Hypercarb is adsorption.

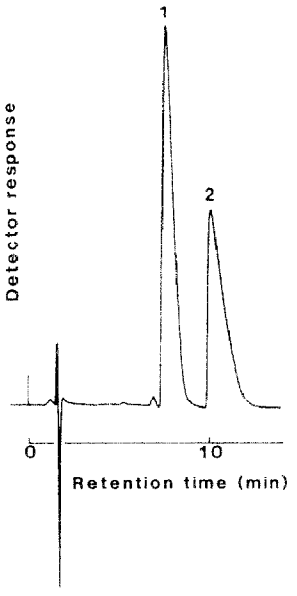

Fig. 6. Separation of positional isomers of glucosyl-inositol: 1 = 4 (or 6)-*O*-(α-D-glucopyranosyl)-*myo*-inositol; 2 = 5-*O*-(α-D-glucopyranosyl)-*myo*-inositol. Chromatographic conditions: eluent, 1:99 acetonitrile–water; temperature, 50°; other conditions as in Fig. 1.

*(2) Dimaltosyl-β*CD. Fig. 7(A) shows a chromatogram of a mixture of dimaltosyl-β-CDs which was prepared from maltose and β-CD through the reverse action of pullulanase. The column used was a C_{18}-bonded silica column, and on this column $6^1,6^2$-di-*O*-(α-maltosyl)-β-CD (**3**) was well resolved from the isomers, $6^1,6^3$-di-*O*-(α-

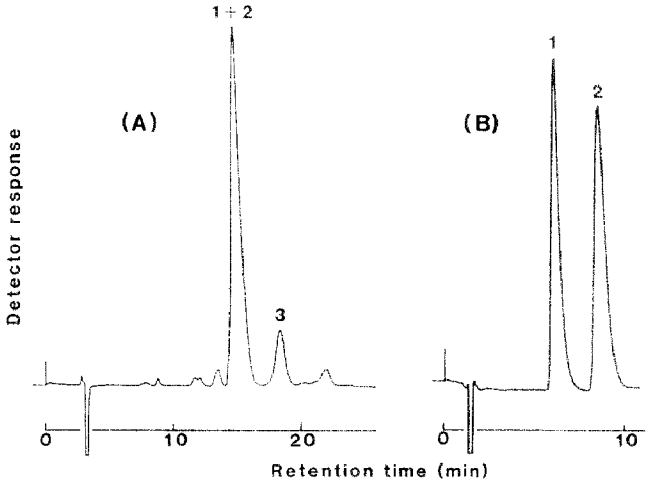

Fig. 7. Separation of positional isomers of dimaltosyl-β-CD on YMC-Pack A-312 (150 × 6 mm i.d.) with 6:94 methanol–water at 35° (A) and on Hypercarb with 17:83 acetonitrile–water at 50° (B). Peaks: 1 = $6^1,6^3$-, 2 = $6^1,6^4$- and 3 = $6^1,6^2$-di-*O*-(α-maltosyl)-β-CD. Flow rate and detector were as in Fig. 1.

maltosyl)-β-CD (**1**) and $6^1,6^4$-di-*O*-(α-maltosyl)-βCD (**2**), whereas the separation of **1** and **2** was impossible. Nevertheless, the resolution of these two isomers (**1** and **2**) could be easily achieved on a Hypercarb column with 17:83 acetonitrile–water at 50° (Fig. 7(B)).

CONCLUSIONS

Chromatographic behavior of carbohydrates on a graphitized carbon column (Hypercarb) is very unique. Although it has been reported that retention on Hypercarb is by an adsorption mechanism, and the graphite structure of the carbon network lends the capability for substrate–solute interactions typically unique to classical reversed phases, namely electron donor–acceptor interactions, the retention mechanism of carbohydrates on this column is rather complicated, *e.g.*, T_κs of mono- and di-saccharides were shown to decrease with rising temperatures like those on reversed phases, whereas those of CDs and branched CDs in eluents containing acetonitrile as an organic modifier were shown to increase with rising temperatures. Furthermore, whereas a small difference of steric sensitivity in two Hypercarb columns from different lots had little effect on the separation of standard samples such as acetone, phenol, *p*-cresol, and 3,5-xylenol, the resolution of certain anomers and positional isomers of carbohydrates was significantly affected by the subtle differences. This phase was surely stable during the course of the study, but by washing the column with dioxane, retention times of branched CDs became very much longer, and the resolution of the positional isomers became much lower. This fact suggests that some organic solvents somewhat alter the surface condition of graphitized carbon, and the small change remarkably affects the chromatographic behavior of some carbohydrates. It is realized through these studies that the exceptional selectivity and resolution power of the Hypercarb column is very effective for carbohydrate mixture separations.

ACKNOWLEDGMENTS

We are grateful to Professor A. Amemura (Fukuyama University) for gifts of sophorose and laminaribiose, and to Professor S. Hizukuri (Kagoshima University) and Dr. T. Nakajima (Tohoku University) for gifts of glucosyl-γCD and kojibiose, respectively.

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