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EVALUATION OF THE LIQUID CHROMATOGRAPHIC SEPARATION OF MONOSACCHARIDES, DISACCHARIDES, TRISACCHARIDES, TETRASACCHARIDES, DEOXYSACCHARIDES AND SUGAR ALCOHOLS WITH STABLE CYCLODEXTRIN BONDED PHASE COLUMNS

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SUMMARY

Both α- and β-cyclodextrin (CD) bonded phase columns were evaluated for their ability to separate carbohydrates and related molecules. Chromatographic data on approximately 50 solutes are reported. Mobile phases consisting of acetonitrile–water or acetone–water produce the best separations. Separations could be run in isocratic or gradient modes. The α-CD column produced slightly more efficient separations than the β-CD column. Both cyclodextrin columns seemed to be more efficient and selective than alkylamine and ion-exchange columns. The stability and reproducibility of the CD columns were excellent and showed little deterioration after several thousand injections. The retention of saccharides on CD bonded phases seems to be related to the number of available hydroxy groups per solute and to the size of the solute. Analysis times and detection sensitivity are discussed.

INTRODUCTION

The separation, identification and quantitation of carbohydrates by liquid chromatography (LC) have received an increasing amount of attention in recent years. Earlier paper and thin-layer chromatographic methods tended to be relatively time consuming and often afforded less resolution than needed for complex mixtures[1–6]. Gas chromatographic methods gave sample resolution and sensitivity but the analytes required prior derivatization to make them volatile[7–10]. Many of the earlier LC methods also used carbohydrate derivatives rather than native compounds[11–16]. The most recent reports involve the direct LC analysis of underivatized carbohydrates[17–19]. Often these separations require specific types of columns such as alkylamine bonded phases, polyol derivatized phases or various ion-exchange media in which specific counter ions are added to affect the separation. Each of these stationary phases have characteristics that are useful for carbohydrate separations. However, there can also be shortcomings, particularly in terms of column stability, lifetimes and reproducibility. An alternative approach is to use an underivatized stationary phase and add a diamine modifier to the mobile phase[20]. Both comprehensive[21,22] and critical[23,24] reviews on the LC separation of carbohydrates have been published.

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Cyclodextrin (CD) bonded phase LC columns developed in our laboratory are used extensively for the separation of optical, geometric and structural isomers[25–31]. Also, they are used increasingly in routine reversed-phase and normal-phase LC separations[32]. CD bonded phase media is relatively stable, particularly in eluents such as those used to separate carbohydrates on alkylamine, diol and polyol stationary phases (i.e., water–organic mixtures containing high percentages of the organic modifier). As the CD bonded phase contains no amine functionality, Schiff base formation is avoided, which should make quantitative results more reliable. In this work, two different CD bonded phase columns (α- and β-CD) were evaluated for the separation of a large variety of saccharides. The effect of different organic modifiers was examined and column efficiency, stability and reproducibility were evaluated. Also considered were the effect of isocratic *versus* gradient separation, detection methods and anomer formation.

EXPERIMENTAL

All the carbohydrates were obtained from Sigma (St. Louis, MO, U.S.A.) except heptose, which was obtained from Aldrich (Milwaukee, WI, (U.S.A.) and α-, β- and γ-CD, which were obtained from Ensuiko Sugar Refining (Japan). All high-performance liquid chromatographic (HPLC) grade solvents were obtained from Fisher Scientific (St. Louis, MO, U.S.A.). All chemicals were used without further purification.

CD bonded phase columns (α-CD or Cyclobond III and β-CD or Cyclobond I) were obtained from Advanced Separation Technologies (Whippany, NJ, U.S.A.). The 250 × 4.6 mm I.D. stainless-steel columns were packed with 5 μm diameter stationary phase support.

A Shimadzu LC-6A liquid chromatograph was used for all separations. A C-R3A or C-R2AX Chromatopac was used to record the chromatograms and for data reduction. Either a Simadzu variable-wavelength detector or a Waters R401 differential refractometer was used to detect the eluted species. Molar absorptivities were measured by use of a Hitachi U-2000 spectrophotometer. All separations were done at room temperature (22°C).

RESULTS AND DISCUSSION

General behavior

The effect of three different organic modifiers (methanol, acetonitrile and acetone) on the separation of a variety of saccharides and related solutes on CD bonded phases was examined. In general the best separations occurred with water–organic solvents containing a high percentage of organic modifier (65–90%). Methanol consistently produced the poorest separations and was not considered to be a viable modifier for most carbohydrate separations on these columns. When methanol was used, the retention times often were too short and similar for many of the solutes. However, excellent chromatochrams could be obtained when using acetonitrile or acetone as modifier. Fig. 1 shows a typical isocratic separation of seventeen mono-, di- and trisaccharides on an α-CD column with an acetonitrile modifier. Fig. 2 shows a gradient separation of nineteen saccharides on a β-CD column, also with an

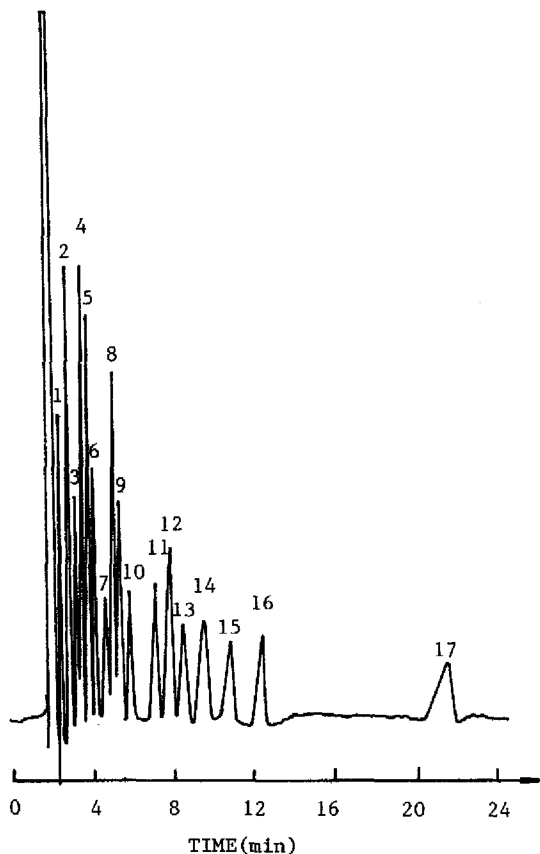

Fig. 1. Isocratic separation of mono-, di- and trisaccharides on a 25-cm α-CD bonded phase column. Mobile phase, acetonitrile–water (78:22, v/v); flow-rate, 1.5 ml/min; refractive index detection. Approximately 40 μg of each sample were injected. Peaks: 1 = phenyl-β-D-glucopyranoside; 2 = 1-deoxy-1-nitro-D-sorbitol; 3 = D-erythrose; 4 = 2-deoxy-D-ribose; 5 = ribose; 6 = lyxose; 7 = tagatose; 8 = xylose; 9 = glucose; 10 = galactitol; 11 = sucrose; 12 = cellobiose; 13 = lactose; 14 = melibiose; 15 = melezitose; 16 = raffinose; 17 = stachyose.

acetonitrile modifier. Gradient separations were very straightforward on CD bonded phases. Column re-equilibration was rapid (2–3 column volumes of mobile phase) and it offered traditional advantages of peak sharpening and reduction of retention for late-eluting solutes. The addition of salts or buffers to the mobile phase seemed to offer no particular advantage with respect to separation efficiency or selectivity. This is very different from what was observed for the separation of ionizable enantiomers on these columns[30]. Consequently, no additional mobile phase additives were used for any of the carbohydrate separations reported here.

Table I gives retention data for a variety of different saccharides and related compounds on the β-CD column and Table II for the α-CD column. Results for both acetonitrile and acetone modifiers are shown. The relative retention order of the saccharides is similar for both α- and β-CD columns, although there are a few

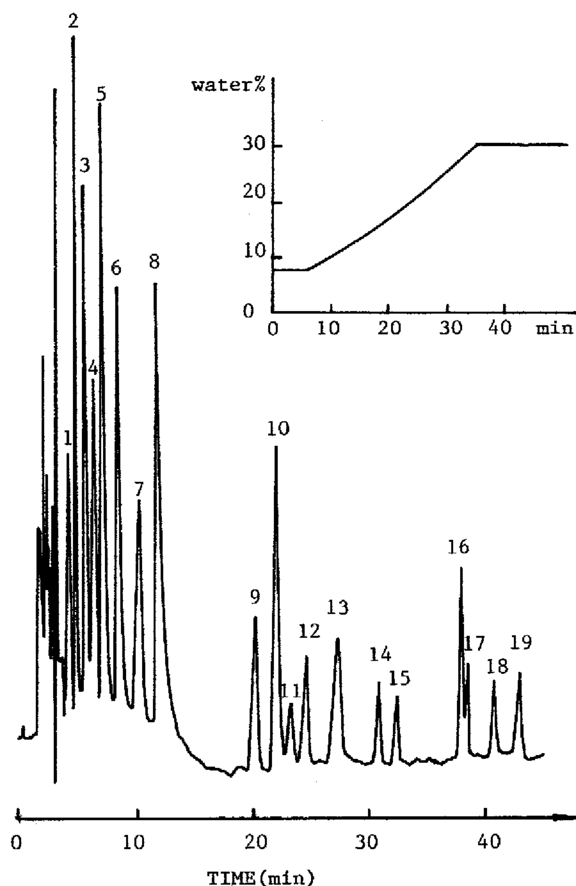

Fig. 2. Gradient separation of nineteen carbohydrates on a 25-cm β-CD bonded phase column. An acetonitrile–water mobile phase was used and the change in composition is shown in the insert. The flow-rate was 1.0 ml/min and UV detection (195 nm) was used. Peaks: 1 = phenyl-β-D-glucopyranoside; 2 = 2-deoxy-D-ribose (8 μg); 3 = ribose (8 μg); 4 = xylose (24 μg); 5 = talose (24 μg); 6 = sorbose (24 μg); 7 = glucose (24 μg); 8 = sorbitol (24 μg); 9 = sucrose (31 μg); 10 = turanose (31 μg); 11 = maltose (31 μg); 12 = lactose (31 μg); 13 = melibiose (31 μg); 14 = melezitose (31 μg); 15 = maltotriose (31 μg); 16 = stachyose (31 μg); 17 = α-cyclodextrin (58 μg); 18 = β-cyclodextrin (68 μg); 19 = γ-cyclodextrin (78 μg).

differences. In general, the monosaccharides elute before diaccharides, which elute before trisaccharides. By controlling the mobile phase composition one can separate a wide range of different carbohydrates (as in Fig. 1 and 2) or one can alter the conditions to obtain maximum separation of related compounds of similar size (Fig. 3). Another interesting fact that is apparent from the data in Tables I and II is that there is a consistant elution order for families of deoxy-sugars, sugars and sugar alcohols. The deoxy-sugar tends to elute before the related sugar and they both elute before the analogous sugar alcohol. In general, the greater the number of available hydroxy groups in a compound, the greater is the retention. Also, some disaccharide pairs that are reportedly difficult to separate (*e.g.*, maltose and lactose) are easily resolved on both α- and β-CD columns.

TABLE I

CHROMATOGRAPHIC RETENTION DATA FOR SACCHARIDES SEPARATED ON A β-CYCLODEXTRIN COLUMN WITH TWO DIFFERENT MOBILE PHASES

Compound	*Mobile phase acetonitrile–water (85:15, v/v)*		*Mobile phase acetone–water (90:10, v/v)*	
	t_r *(min)*	k'	t_r *(min)*	k'
Erythrose	3.12	0.48	4.23	0.41
2-Deoxy-D-ribose	3.45	0.64	–	–
Glycerine	3.47	0.65	–	–
Glyceraldehyde	3.57	0.71	4.56	0.53
Rhamnose	3.63	0.73	–	–
Ribose	3.74	0.78	4.60	0.53
6-Deoxy-D-glucose	3.83	0.82	–	–
Lyxose	4.04	0.93	4.84	0.61
2-Deoxy-D-glucose	4.05	0.93	–	–
Erythritol	4.07	0.94	–	–
Threitol	4.10	0.95	–	–
Xylose	4.12	0.96	4.97	0.66
6-Deoxy-D-galactose	4.13	0.97	–	–
2-Deoxy-D-galactose	4.17	0.99	–	–
Talose	4.23	1.02	4.82	0.61
Arabinose	4.30	1.05	5.15	0.72
Tagatose	4.54	1.16	5.18	0.73
Fructose	4.73	1.25	5.43	0.81
Ribitol	4.73	1.25	–	–
Arabitol	4.80	1.29	–	–
Sorbose	4.81	1.29	5.44	0.81
Xylitol	4.88	1.33	–	–
Allose	4.91	1.34	–	–
Mannose	4.91	1.34	–	–
Glucose	5.36	1.55	5.87	0.96
Galactose	5.57	1.65	6.17	1.06
Sorbitol	5.77	1.75	–	–
Mannitol	5.80	1.76	–	–
Galactitol	5.87	1.80	–	–
D-*gluco*-D-*gulo*-Heptose	6.17	1.94	6.13	1.04
Sucrose	7.79	2.71	6.99	1.33
Turanose	8.51	3.05	7.38	1.46
myo-Inositol	8.62	3.10	–	–
Cellobiose	9.02	3.30	8.23	1.60
Maltose	9.09	3.33	7.87	1.62
Maltitol	10.00	3.76	–	–
Lactulose	–	–	8.66	1.74
Lactose	10.16	3.84	8.85	1.95
Gentiobiose	10.75	4.12	9.50	2.00
Melibiose	11.56	4.51	9.67	2.23
Melezitose	14.18	5.76	10.13	2.38
Maltotriose	16.44	6.83	11.61	2.67
Raffinose	17.20	7.19	12.29	2.88
Stachyose	39.0	17.57	26.0	7.23

TABLE II

CHROMATOGRAPHIC RETENTION DATA FOR SACCHARIDES SEPARATED ON AN α-CYCLODEXTRIN COLUMN WITH TWO DIFFERENT MOBILE PHASES

Compound	*Mobile phase acetonitrile–water (80:20, v/v)*		*Mobile phase acetone–water (85:15, v/v)*	
	t_r *(min)*	*k'*	t_r *(min)*	*k'*
Erythrose	5.82	0.74	4.53	0.24
Rhamnose	–	–	4.81	0.32
Glyceraldehyde	–	–	4.83	0.33
2-Deoxy-D-ribose	6.23	0.86	4.86	0.34
Glycerine	6.47	0.94	5.09	0.40
Ribose	6.74	1.01	4.98	0.37
6-Deoxy-D-glucose	6.79	1.04	5.08	0.40
3-O-Methyl-D-glucose	7.05	1.11	–	–
2-Deoxy-D-glucose	7.19	1.15	5.19	0.43
Lyxose	7.24	1.17	5.21	0.43
Xylose	7.32	1.20	5.33	0.46
2-Deoxy-D-galactose	–	–	5.40	0.48
Erythritol	7.47	1.23	5.54	0.50
Talose	7.51	1.27	5.13	0.41
6-Deoxy-D-galactose	–	–	5.71	0.57
Arabinose	7.87	1.35	5.73	0.57
Tagatose	8.01	1.40	5.51	0.51
Ribitol	8.30	1.48	5.72	0.57
Fructose	8.32	1.50	5.84	0.60
Sorbose	8.38	1.52	5.62	0.53
Arabitol	8.43	1.53	5.91	0.62
Mannose	8.47	1.54	5.79	0.59
Allose	–	–	6.00	0.65
Xylitol	8.48	1.54	6.01	0.65
Glucose	9.04	1.74	6.20	0.70
Galactose	9.45	1.83	–	–
D-*gluco*-D-*gulo*-Heptose	–	–	6.38	0.75
Sorbitol	9.56	1.86	6.43	0.77
Mannitol	9.62	1.88	–	–
Galactinol	9.74	2.01	6.56	0.80
Sucrose	11.31	2.40	6.63	0.82
Turanose	11.61	2.48	6.98	0.92
Cellobiose	12.27	2.64	7.85	1.16
Maltose	12.33	2.70	7.30	1.01
myo-Inositol	13.04	2.91	9.70	1.67
Maltitol	13.23	2.97	7.03	0.93
Lactose	13.23	2.97	8.22	1.26
Melibiose	14.40	3.30	8.83	1.43
Melezitose	15.62	3.68	8.53	1.34
Maltotriose	–	–	8.80	1.42
Raffinose	17.58	4.26	9.25	1.54
α-Cyclodextrin	–	–	12.97	2.56
Stachyose	27.87	7.34	13.88	2.81
β-Cyclodextrin	–	–	15.6	3.29
γ-Cyclodextrin	–	–	18.93	4.20

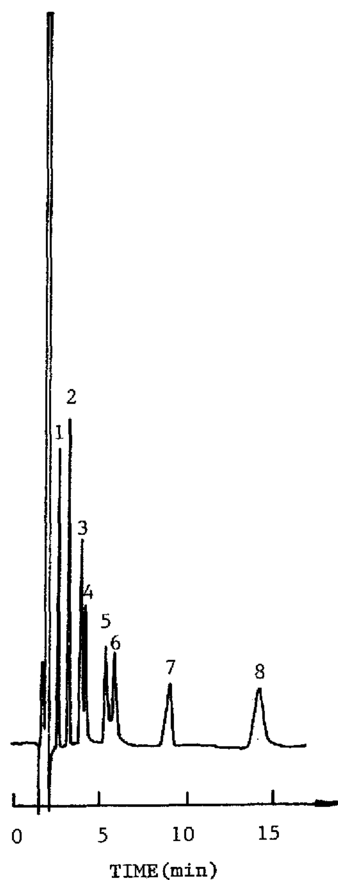

Fig. 3. Chromatogram showing the isocratic separation of eight sugar alcohols on a 25-cm β-CD column. Mobile phase, acetonitrile–methanol–water (95:5:5, v/v); flow-rate, 2.0 ml/min; refractive index detection. Approximately 80 μg of each solute were injected. Peaks: 1 = glycerine; 2 = erythritol; 3 = ribitol; 4 = arabitol; 5 = sorbitol; 6 = galactitol; 7 = *myo*-inosytol; 8 = maltitol.

The concentration of organic modifier in the mobile phase controls the retention and selectivity of the saccharide on cyclodextrin columns. The retention tends to increase with increasing modifier concentration (see Fig. 4 and 5). This retention behavior is analogous to that reported for sugars on alkylamine, polyol and diol columns[24]. At equivalent mobile phase compositions, α-CD columns generally show a greater retention than β-CD columns (Figs. 4 and 5).

Efficiency

Van Deemter plots for several saccharides are shown in Figs. 6 and 7 for the α- and β-CD column, respectively. The optimum efficiency (*i.e.*, the smallest height equivalent to a theoretical plate, *H*) varied from solute to solute. The α-CD column seemed to produce more efficient separations than the β-CD column. Both columns contained an identical 5-μm spherical silica support. The efficiencies at optimum

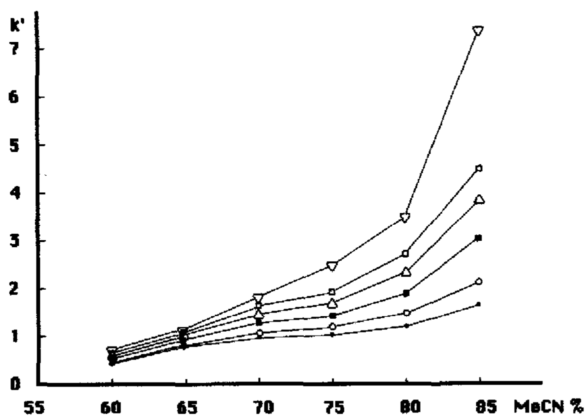

Fig. 4. Plot showing the effect of acetonitrile (MeCN) concentration (%, v/v) in the mobile phase on the capacity factors of several carbohydrates eluted from a 25-cm α-CD column. Solutes: ● = ribose; ○ = lyxose; ■ = fructose; △ = glucose; □ = sorbitol; ▽ = sucrose.

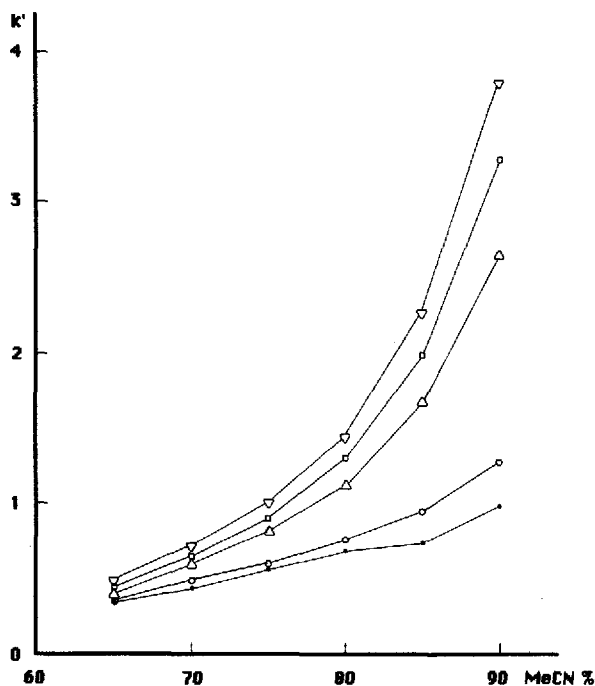

Fig. 5. Plot showing the effect of acetonitrile (MeCN) concentration (%, v/v) in the mobile phase on the capacity factors of several carbohydrates eluted from a 25-cm β-CD column. Solutes: ● = fructose; ○ = glucose; △ = sucrose; □ = maltose; △ = lactose.

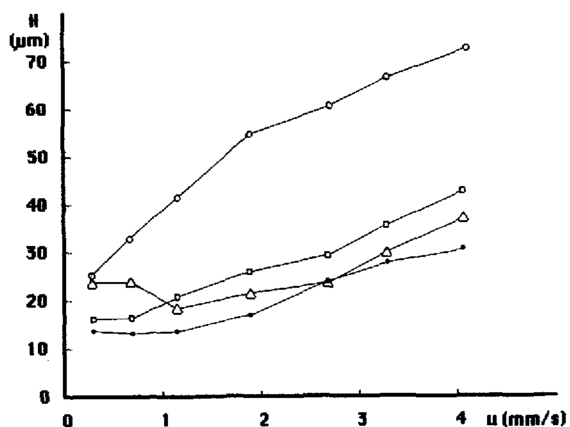

Fig. 6. Van Deemter plots of flow-rate *versus* height equivalent to a theoretical plate (H) for ● = ribose; △ = sorbitol; □ = sucrose and ○ = glucose. A 25-cm α-CD column was used with acetonitrile–water (78:22, v/v) as mobile phase.

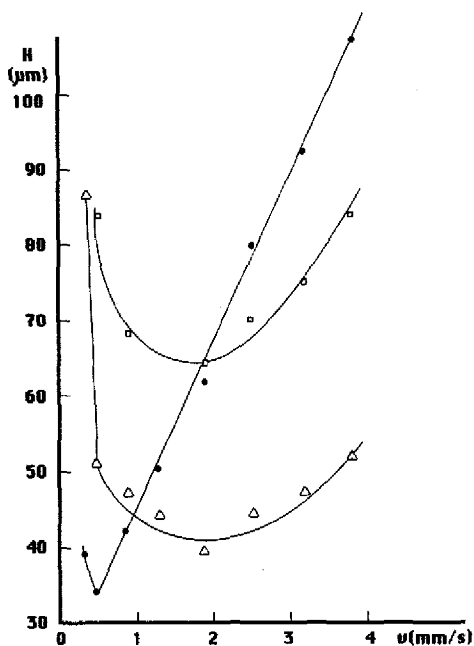

Fig. 7. Van Deemter plots of flow-rate *versus* height equivalent to a theoretical plate (H) for ● = fructose; △ = sucrose and □ = lactose. A 25-cm β-CD column was used with acetonitrile–water (85:15, v/v) as mobile phase.

flow-rates ranged from *ca.* 4000 to 8000 plates per 25 cm column for β-CD and from *ca.* 6000 to 19000 plates per column for α-CD. These efficiencies are higher than those reported for other columns used to separate carbohydrates[24]. There are a number of possible reasons for the higher efficiency. First, the mobile phase can be adjusted easily to suppress or enhance the separation of most anomers. This, coupled with small temperature modifications, can eliminate most band broadening due to the partial separations of anomers. Anomeric separations are very sensitive to mobile phase composition. In general, mobile phases containing higher percentages of organic modifiers tend to enhance the separation of anomers and *vice versa.* An extensive study of the separation of sugar anomers has been completed recently[33]. Retention of saccharides on cyclodextrin bonded phases is probably due to adsorption via hydrogen bonding and dipolar interactions but not to inclusion complex formation. The CD cavity is probably occupied by the organic modifier, which is present in very high concentrations. Further, the large, bulky CDs tend sterically to prohibit solute molecules from reaching the silica gel surface where other retention mechanisms may be important (*e.g.*, binding to free silanols). Thus the exchange kinetics of saccharides between stationary and mobile phases may be more rapid for CD bonded phases under the conditions outlined above. Another possible factor that could contribute to efficiency is that the base packing material (silica gel) and packing method for the CD bonded phases might be better than that of other columns used for carbohydrate analysis. As CD columns are used extensively for difficult enantiomeric separations, both the silica gel and packing technology had to be optimized and continuously checked. However, the manufacturing and packing procedures used by the companies that produce different LC columns are often unknown and it is therefore difficult to know the significance of these factors or compare them.

Stability and reproducibility of CD columns

One of the advantages of α- and β-CD columns for the analysis of carbohydrates is their high stability and reproducibility. As no amine functionality exists on these bonded phases, the formation of Schiff bases is avoided. Consequently, quantitative results may be more accurate[24]. Also, self-hydrolysis is not a significant problem as it is for alkylamine bonded phases. As an example, a β-CD column approximately 1 year old that had received over 3000 standard injections was compared with a new column. The chromatograms are shown in Fig. 8. It is apparent that the older β-CD column is nearly as selective and efficient as the new column. Two aspects of these results should be emphasized. First, the separations were done with pure sales. If samples are injected that contain impurities which are irreversibly adsorbed on the column, then the retention behavior must change with time. This is true for any column. Second, CD bonded phases are particularly stable in water–organic solvents containing $>50\%$ modifier. Many times when separating compounds other than carbohydrates predominantly aqueous mobile phases are used. In these instances, it remains advisable to place a presaturator containing silica gel prior to the injection loop. Again, this is advisable for any silica gel-based column.

Analysis time

The time of analysis can be controlled by adjusting the mobile phase composition, the flow-rate and, to a lesser extent, the temperature. As indicated in

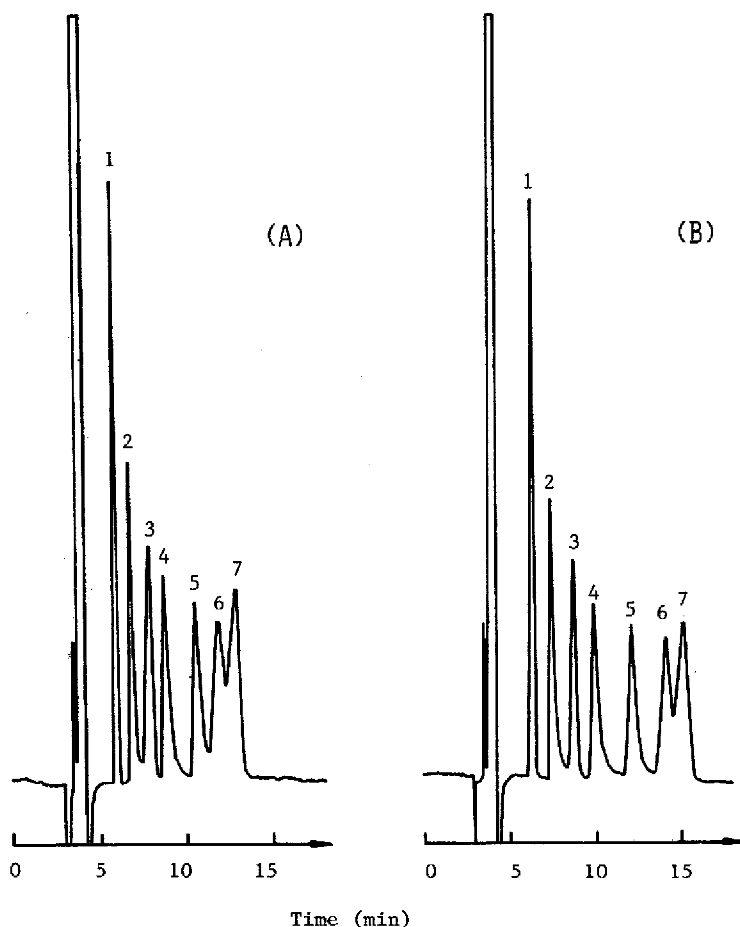

Fig. 8. Comparison of the separations obtained on (A) an old β-CD column (25 cm × 4.6 mm I.D., *ca.* 1 year old and with > 3000 injections) and (B) an analogous new β-CD column. In both instances the mobile phase was acetonitrile–water (95:5, v/v) and the flow-rate was 1.0 ml/min. Peaks: 1 = 2-deoxy-D-ribose; 2 = ribose; 3 = xylose; 4 = talose; 5 = sorbose; 6 = mannose; 7 = glucose.

Figs. 4 and 5, the retention of saccharides decrease as the water content of the mobile phase increases (*i.e.*, the opposite of traditional reversed-phase LC). This allows one to decrease the analysis time, often at the expense of resolution. The optimum efficiency occurs at normal LC flow-rates between 0.5 and 2.0 ml/min (Fig. 6 and 7). Most of the chromatograms shown in this work were generated using a flow-rate of 1.5 ml/min; however, flow-rates of 3.0 and 4.0 ml/min have been used for many saccharide separations. In general, the analysis times on CD bonded phase columns under conditions of optimum efficiency are comparable to those reported for octadecylated silica gel and are less than those reported for amino phases and ion-exchange phases[24].

Detection

It was noted previously that when using an acetonitrile–water mobile phase,

TABLE III

MOLAR ABSORPTIVITIES OF SACCHARIDES AT WAVELENGTHS BETWEEN 190 AND 215 nm

Aqueous solutions were used in all determinations.

Compound	*Wavelength (nm)*					
	190	*195*	*200*	*205*	*210*	*215*
Ribose	149	53	16	6	4	3
Lyxose	114	39	12	4	2	2
Glucose	45	11	3	1	1	1
Fructose	144	57	20	9	4	3
Sorbitol	95	31	9	2	1	1
Sucrose	139	45	13	5	2	1
Lactose	73	17	3	1	0	0
Raffinose	173	42	8	2	0	1
α-Cyclodextrin	64	15	7	4	3	3
β-Cyclodextrin	82	30	14	12	11	11
γ-Cyclodextrin	121	34	17	12	10	9

many saccharides can be detected via absorption (of wavelengths $\leqslant$200 nm)[34]. The molar absorptivities of several saccharides (at wavelengths between 190 and 215 nm) are given in Table III. At lower wavelengths, UV detection of saccharides is sometimes (but not always) more sensitive than refractive index detection. The relative sensitivity

TABLE IV

COMPARISON OF THE SENSITIVITIES OF ULTRAVIOLET AND REFRACTIVE INDEX DETECTION OF SACCHARIDES

Data were calculated from chromatograms generated with a β-CD column. The flow-rate was 1.5 ml/min and the mobile phase composition was acetonitrile–water (85:15, v/v), except where indicated otherwise.

Compound	*Minimum detectable amount (μg)*	
	UV (195 nm) *(0.01)*	*RI* *(8×)*
2-Deoxy-D-ribose	0.084	0.88
Ribose	0.094	1.2
Xylose	0.43	2.2
Fructose	0.13	2.2
Glucose	0.47	2.0
Sorbitol	0.23	1.7
Sucrose	0.91	3.1
Turanose	0.50	2.3
Maltose	2.6	4.7
Lactose	2.1	4.0
Melibiose	1.9	5.2
Melezitose	3.0	5.2
Maltotriose	5.3	8.7
α-Cyclodextrin*	16.2	2.0
β-Cyclodextrin*	18.9	2.6
γ-Cyclodextrin*	23.6	2.9

* Mobile phase: acetonitrile–water (70:30, v/v).

depends on the nature of the saccharide (*i.e.*, molar absorptivity) and its size. The minimum detectable amounts of sixteen saccharides for the variable-wavelength UV and refractive index (RI) detectors used in this study are given in Table IV. In general, UV detection can be up to ten times more sensitive for the smaller monosaccharides on a weight basis. UV and RI detection are of approximately equal sensitivity for trisaccharides but RI detection is more sensitive for larger carbohydrates such as CDs (Table IV).

CONCLUSIONS

Both α- and β-CD bonded phase columns appear to separate a variety of carbohydrates. The smaller saccharides usually elute before the larger polysaccharides. It appears that retention is closely related to the number of available hydroxy group per molecule. This may explain why deoxy-sugars are retained less than the analogous sugars and that neither are retained as much as the corresponding sugar alcohols. The efficiency and stability of CD columns appear to be superior to those of alkylamine bonded phases. The analysis times on CD bonded phases are comparable to those on reversed-phase columns and often shorter than those on amino and ion-exchange stationary phases. The choice of UV *versus* RI detection is dependent on the mobile phase used and the molecular weight of the carbohydrates.

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