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THE SEPARATION OF SUBSTITUTED CARBOHYDRATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Separation of a wide variety of partially and completely substituted carbohydrates was achieved by using two types of gel permeation columns. The relative retention time was dependent on two factors: the relative polarity of the carbohydrate and its molecular weight.

INTRODUCTION

Substituted carbohydrates, in which the hydroxyl groups are replaced with other groups, are widely used in carbohydrate chemistry. Many partially substituted monosaccharides are found in nature, such as the glycosides, nucleosides and nucleotides[1]. Substituted carbohydrates are also used as synthetic intermediates for preparing a wide range of carbohydrate derivatives. Especially useful intermediates are the acetals, ketals and other derivatives which form cyclic intermediates. The formation of partially substituted carbohydrates is also important for determining the structure of complex polysaccharides. The most common procedure involves the complete methylation of the polysaccharide followed by hydrolysis. The position of the linkages between the monomeric units of the polysaccharide corresponds to the position of the free hydroxyl groups of the hydrolyzed monosaccharides[2].

A variety of chromatographic methods for separating substituted carbohydrates is available. Carbohydrates which contain acidic or basic groups can be separated by ion-exchange chromatography. Neutral carbohydrates, which are partially substituted, can be analyzed by gas, column or thin-layer chromatography[3–12]. Recently Grellert and Ballou[13,14] have found that low-molecular-weight exclusion columns (polyacrylamide and dextrans) could be used for separating methylated carbohydrates. They found that the elution volumes were related to the number of methyl groups; the monosaccharides containing four methyl groups came out first, followed by the tri-, di- and monomethylated compounds.

In this study two low-molecular-weight exclusion columns were evaluated. An EM Gel OR-PVA 500 column (vinyl acetate copolymer) and a Poragel 60 Å column (polyacrylamide gel) were tested in order to determine how effective they were in separating a variety of substituted carbohydrates.

EXPERIMENTAL

Apparatus and materials

A Waters Assoc. (Milford, Mass., U.S.A.) Model 202/401 liquid chromatograph equipped with a 1000-p.s.i. pumping system with both ultraviolet (Waters Assoc. Model 202) and differential refractometer detectors (Waters Assoc. Model R-201) was employed. All chromatograms were made at room temperature and at a constant flow-rate. The Poragel 60 Å column (no. 26900) was also obtained from Waters Assoc. The EM Gel OR-PVA 500 (vinyl acetate copolymer) packing material was obtained from EM Labs. (Elmsford, N.Y., U.S.A.). The packing material was initially swelled in methanol for 24 h. After clamping the 2 ft. × $^{1}/_{8}$ in. column in a vertical position, a vacuum was applied at the lower end; then a slurry of the packing material was slowly introduced at the top. Final pressure packing of the column was accomplished by using a high-pressure pump. Furthermore, to prevent foreign matter from being introduced into the ultraviolet or refractometer cells, all newly packed columns were allowed to purge with the appropriate solvent for 3 h before being connected to the inlet lines of the detectors. The conditions for this separation are shown in Table I. The solvents used were the best grade of solvent commercially available (Fisher Pesticide Grade) and were used without further purification.

TABLE I

COLUMN OPERATING PARAMETERS

Parameter	*Poragel 60 Å*	*EM Gel OR-PVA 500*
Length	4 ft.	2 ft.
I.D.	2 mm	2 mm
Solvent	Chloroform	Methanol
Flow-rate	0.51 ml/min	0.27 ml/min
Particle size	37–75 μm	50 μm
Exclusion limit	100–2400	0–300

Sample preparation

The acetates of glucose and galactose were obtained commercially; α-cellobiose, phenyl β-D-glucopyranoside, methyl β-D-glucopyranoside, L-arabinitol and erythritol were acetylated by a known procedure[15]. The benzylidene derivatives of glucose were prepared by the method of Freudenberg *et al.*[16], and the isopropylidene derivatives were prepared using the procedure of Schmidt[17]. The methylated sugars were kindly provided by Dr. E. Zissis of the National Institutes of Health (Bethesda, Md., U.S.A.). The polystyrene and polypropylene glycol standard used for determining the void volume of the columns were purchased from Waters Assoc. The samples (10 mg) were dissolved in the eluting solvent (1 ml) and injected directly into the liquid chromatograph.

RESULTS AND DISCUSSION

A series of substituted carbohydrates were prepared and separated on a polyacrylamide column. The results are shown in Figs. 1 and 2 and summarized in Table II.

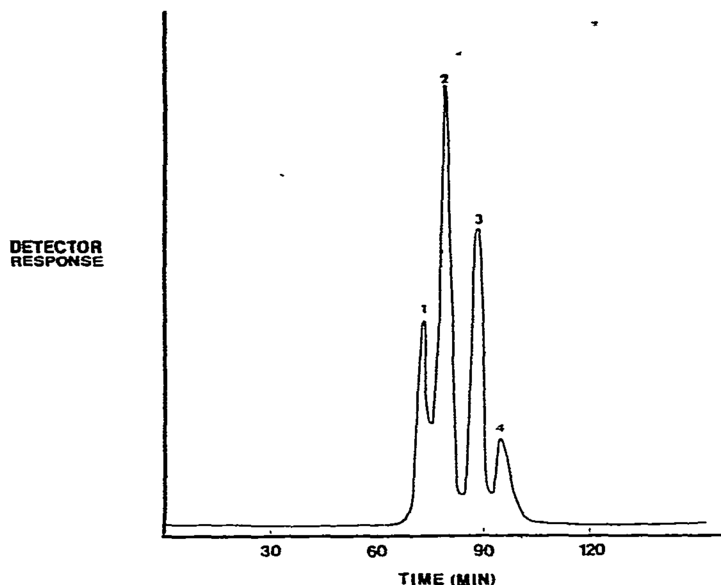

Fig. 1. High-performance liquid chromatography of some substituted carbohydrates on a poly-acrylamide column. 1, α-Cellobiose octaacetate; 2, α-D-glucopyranose pentaacetate; 3, 1,2-O-isopropylidene α-D-glucofuranose; 4, 1,2:5,6-di-O-isopropylidene α-D-glucofuranose.

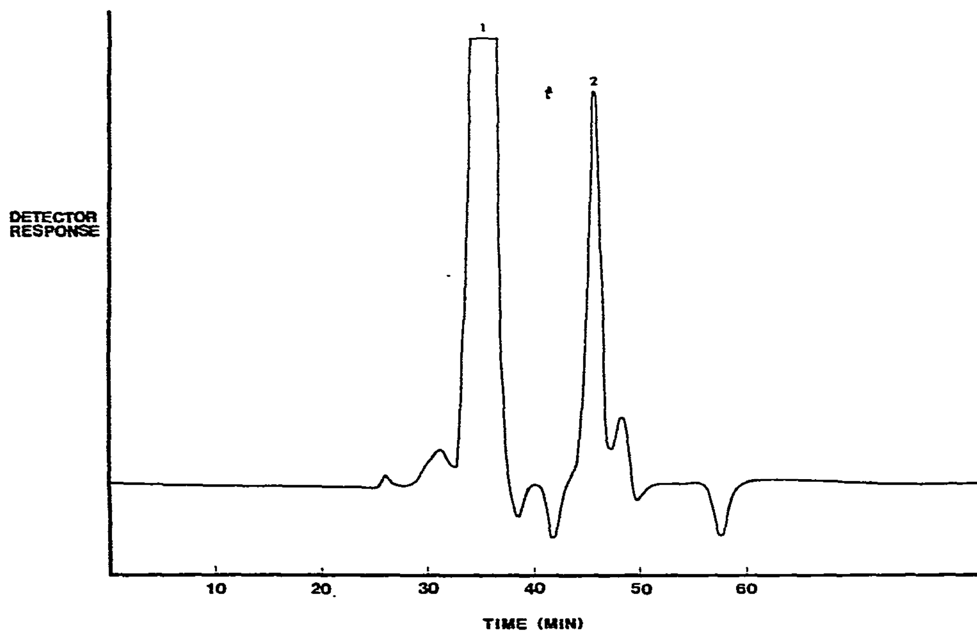

Fig. 2. High-performance liquid chromatography of the products from the reaction of D-glucose with acetone in the presence of zinc chloride. 1, 1,2:5,6-Di-O-isopropylidene α-D-glucofuranose; 2, 1,2-O-isopropylidene α-D-glucofuranose.

TABLE II

SEPARATION OF SUBSTITUTED CARBOHYDRATES ON A PORAGEL 60 Å COLUMN

Retention time values are relative to the retention time of α-D-glucopyranose pentaacetate.

No.	*Compound*	*Molecular weight*	*Relative retention time*
1	α-Cellobiose octaacetate	678.6	0.92
2	Phenyl tetra-O-acetyl β-D-glucopyranoside	424.4	1.03
3	α-D-Glucopyranose pentaacetate	390.3	1.00
4	β-D-Glucopyranose pentaacetate	390.3	0.99
5	α-D-Galactopyranose pentaacetate	390.3	1.01
6	β-D-Galactopyranose pentaacetate	390.3	1.01
7	Methyl tetra-O-acetyl β-D-glucopyranoside	362.3	1.03
8	L-Arabinitol acetate	362.3	1.18
9	Erythritol acetate	290.2	1.07
10	4,6-O-Benzylidene D-glucopyranose	268.3	1.21
11	1,2:5,6-Di-O-isopropylidene α-D-glucofuranose	260.3	1.12
12	2,3,6-Tri-O-methyl-D-glucose	225.2	1.15
13	2,4,6-Tri-O-methyl-D-glucose	225.2	1.15
14	1,2-O-Isopropylidene-α D-glucofuranose	220.2	1.20
15	Polypropylene glycol standard	2020	0.71

One limitation of the Poragel columns is that they cannot be used with aqueous or hydroxylated solvents, but only with organic solvents, such as chloroform or tetrahydrofuran. However, this does not limit their usefulness for substituted carbohydrates, since many are readily soluble in these types of solvents.

α-Cellobiose octaacetate, a disaccharide, was partially separated from the acetylated monosaccharides (Fig. 1). The pentaacetate isomers of galactose and glucose all had approximately the same retention time on this column, while the two acetylated sugar alcohols, L-arabinitol acetate and erythritol acetate, had considerably longer retention times than the acetylated monosaccharides. Partially substituted carbohydrates, which contained some free hydroxyl groups, had retention times which were much longer than those of the completely substituted carbohydrates. In the limited number of compounds studied, it was found that the order of elution was inversely related to the number of free hydroxyl groups. The 1,2:5,6-di-O-isopropylidene α-D-glucofuranose, which contained one free hydroxyl group, had the smallest elution volume. The two methylated carbohydrates, which contained two free hydroxyl groups, came out next, while the monoisopropylidene derivative of D-glucose and the monobenzylidene derivative, which contained three hydroxyl groups, was eluted last. A similar type of elution pattern for methylated sugars has been reported earlier by Grellert and Ballou[13,14] using a polyacrylamide or dextran low-molecular-weight exclusion column.

These types of columns appear to be particularly effective in separating partially substituted carbohydrates. A good example is the separation of the isopropylidene derivatives. The value of this separation method is illustrated graphically in Fig. 2. If D-glucose is reacted with acetone in the presence of zinc chloride, two major products are formed: 1,2:5,6-di-O-isopropylidene α-D-glucofuranose and 1,2-O-isopropylidene α-D-glucofuranose[17]. The two products can be easily separated using an 8-ft. Poragel 60 Å column (Fig. 2).

The second type of column packing material that was investigated was an EM Gel OR-PVA 500, a vinyl acetate copolymer. This material is used in exclusion chromatography for separating compounds with molecular-weight ranges up to 300. The types of separation obtained with a series of carbohydrates are shown in Table III and in Figs. 3 and 4. With the exception of xylose, all of the unsubstituted carbohydrates had about the same retention time. The isomeric methyl D-glucosides have the same retention times while phenyl β-D-glucopyranoside has a longer retention time, even though its molecular weight is much higher than that of the methyl glucosides.

TABLE III

SEPARATION OF SUBSTITUTED CARBOHYDRATES ON A POLYVINYL ACETATE COLUMN

Retention times are relative to D-glucose. The polypropylene glycol standard had an M_n value of 1220.

No.	*Compound*	*Relative retention time*
1	α-Cellobiose octaacetate	1.22
2	Phenyl tetra-O-acetyl β-D-glucopyranoside	2.19
3	α-D-Glucopyranose pentaacetate	2.16
4	β-D-Glucopyranose pentaacetate	2.32
5	α-D-Galactopyranose pentaacetate	2.00
6	β-D-Galactopyranose pentaacetate	2.16
7	Methyl tetra-O-acetyl β-D-glucopyranoside	2.03
8	L-Arabinitol acetate	1.84
9	Erythritol acetate	1.84
10	4,6-O-Benzylidene D-glucopyranose	1.61
11	1,2:5,6-Di-O-isopropylidene α-D-glucofuranose	1.59
12	2,3,6-Tri-O-methyl-D-glucose	1.11
13	2,4,6-Tri-O-methyl-D-glucose	1.10
14	1,2-O-Isopropylidene α-D-glucofuranose	1.23
15	3-O-Methyl D-glucose	1.00
16	D-Mannose	0.97
17	D-Fructose	1.03
18	D-Glucose	1.00
19	D-Xylose	1.10
20	D-Glucitol	1.00
21	D-Arabinitol	1.00
22	Methyl α-D-glucopyranoside	1.00
23	Methyl β-D-glucopyranoside	1.00
24	Phenyl β-D-glucopyranoside	1.22
25	Polypropylene glycol standard	0.69

The acetylated derivatives were retained on the column and, in general, were well separated. Cellobiose octaacetate, a disaccharide, was completely separated from the corresponding acetylated monosaccharide derivatives. The α- and β-isomers of galacto- and gluco-pentaacetates were partially separated on this column (Figs. 3 and 4).

The partially substituted monosaccharides were found to have intermediate retention values on this column. They were eluted after the unsubstituted carbo-

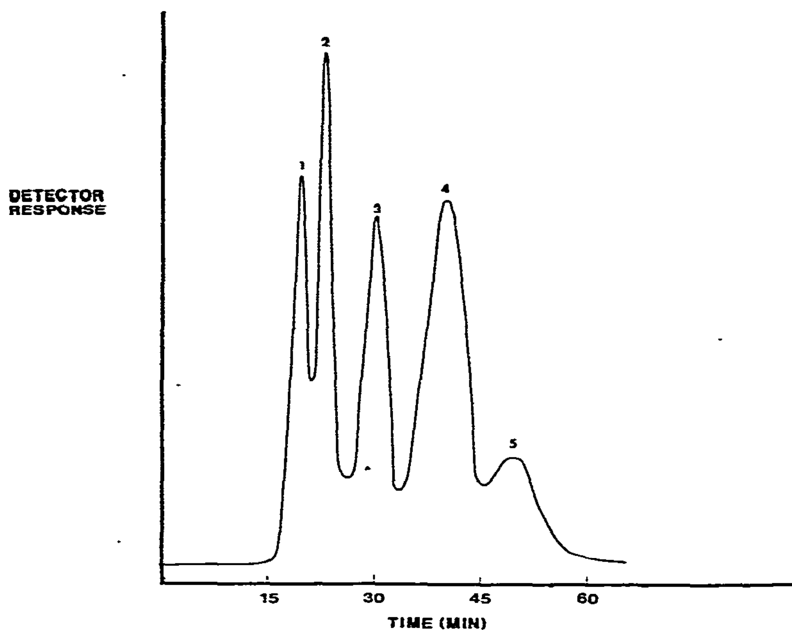

Fig. 3. High-performance liquid chromatography of some partially substituted carbohydrates. 1, 3-O-Methyl D-glucose; 2, 1,2-O-isopropylidene α-D-glucofuranose; 3, 4,6-O-benzylidene D-glucofuranose; 4, α-D-glucopyranose pentaacetate; 5, phenyl β-D-glucopyranose pentaacetate.

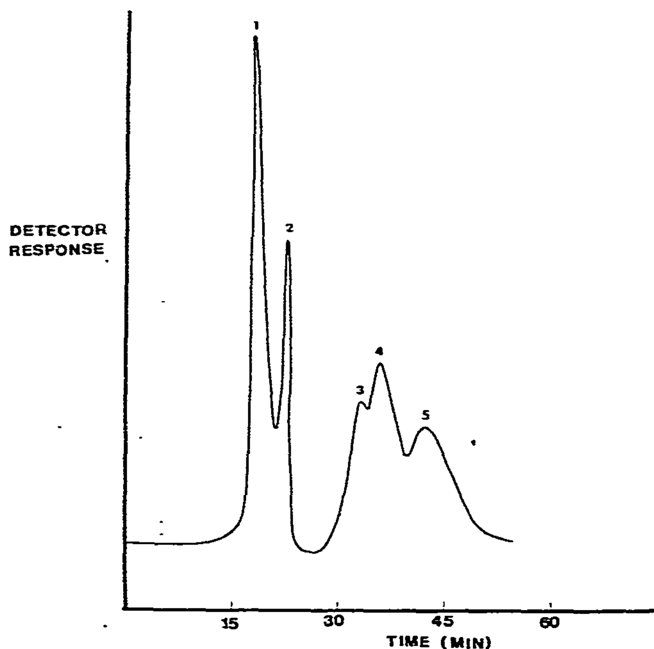

Fig. 4. High-performance liquid chromatography of some substituted carbohydrates. 1, D-Glucose; 2, α-cellobiose octaacetate; 3, erythritol acetate; 4, α-D-galactopyranose pentaacetate; 5, β-D-glucopyranose pentaacetate.

hydrates and before the completely acetylated derivatives. Examples of some of the separations are shown in Figs. 3 and 4.

The relative retention time for the carbohydrates on the polyvinyl acetate column was not dependent on the molecular weight. The separation apparently was due to the relative absorption between the substituted carbohydrates and the column packing. Relatively polar compounds, such as the free sugars, did not interact with the non-polar packing material. However, the partially and completely substituted carbohydrates, which were capable of penetrating into the relatively non-polar gel, interacted very strongly with the packing material.

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