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SEPARATION OF SUBSTITUTED CARBOHYDRATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY. II*

G. D. McGINNIS and P. FANG

Forest Products Utilization Laboratory, Mississippi State University, Mississippi State, Miss. 39762 (U.S.A.)

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SUMMARY

A high-performance liquid chromatographic technique is described for the separation of a variety of partially and completely substituted carbohydrates using a microparticulate silica gel column. The solvents were varied depending on the relative polarity of the carbohydrate. The compounds studied were unsubstituted glycosides, isopropylidene and benzylidene derivatives, partially methylated carbohydrates, and a series of completely acetylated carbohydrates. The results of this study indicate that high-performance liquid chromatography can be used for qualitative and quantitative analyses of a wide variety of substituted carbohydrates.

INTRODUCTION

In the last five years high-performance liquid chromatography (HPLC) has developed into a versatile and sensitive method for quantitative separation of organic compounds. Application of this technique to carbohydrates has generally been limited to the unsubstituted monosaccharides[2–4], nucleosides[5] and nucleotides[6,7]. In this study we have studied the separation of a wide variety of partially and completely substituted carbohydrates using HPLC. The compounds studied included monosubstituted carbohydrates, a series of di-, tri- and tetra-substituted carbohydrates, as well as some completely substituted derivatives. All the compounds were separated using a column packed with microparticulate silica gel. The solvents were varied depending on the relative polarity of the carbohydrate.

EXPERIMENTAL

Apparatus and material

A Waters liquid chromatograph (Model 202/401), equipped with a 5000 p.s.i. pumping system (Milton Roy Model No. 396) and with both ultraviolet and differential refractometer detectors was employed. All chromatograms were made at room

* For part I, see ref. 1.

temperature and monitored using the refractive index detector. The column contained 10-μm silica gel (Partisil 10, No. D-010) and was purchased prepacked from Whatman, Clifton, N.J., U.S.A. It was a stainless-steel column. All solvents were the best commercial grade available and were dried just prior to use over a column of anhydrous silica gel (12–42 mesh). The conditions for these separations were as follows: sample size, 10 μl; flow-rate, 1.2 ml/min; temperature, ambient; sample concentration, 1.2 mg/ml; detector, differential refractometer; length, 25.0 mm; I.D., 4.6 mm and particle size, $\pm$ 10 μm.

Sample preparation

The unsubstituted monosaccharides, glycosides, and the acetylated compounds were obtained commercially. Samples of some of the less common glycosides and methylated sugars were kindly provided by Dr. N. K. Richtmyer and Dr. E. Zissis. The glycosidation reaction of mannose was done following the procedure of Cadotte *et al.*[8]. The benzylidine and isopropylidene derivatives of D-glucose and D-mannose were prepared using known procedures[10]. The di-O-isopropylidene derivatives of D-fructose were obtained using the procedure of Ohle and Koller[12]. β-Cellobiose octaacetate was anomerized in the presences of zinc chloride and acetic anhydride at 80° for 15 min[8]. The glycosidation reaction of tetra-O-acetyl α-D-glucopyranosyl bromide was done following the method of Koenigs and Knorr[13].

RESULTS AND DISCUSSION

A series of monosubstituted carbohydrates was separated on a silica gel column using the eluting solvent acetonitrile–water (9:1). The results are shown in Table I and Fig. 1. In general, it was found that the glycosides were well separated on this column. Some anomeric glycosides, such as the methyl α- and β-D-mannopyranosides, were completely separated on the column, while the anomeric phenyl and methyl D-glucopyranosides and the methyl α- and β-D-xylopyranosides were partially separated on this column.

The value of this technique for following glycosidation reactions is illustrated in Fig. 2. When D-mannose is reacted with methanol in the presence of an acidic ion-exchange resin, a mixture of glycosides is formed. The concentration of the individual furanosides and pyranosides is related to the reaction time[8]. With short reaction times the products consist of both the anomeric pyranosides and furanosides. At longer reaction times, the products after 30 min and 18 h reaction time is shown in Fig. 2. At short reaction times four peaks were obtained using HPLC. The first peak consisted of a mixture of two products which were not separated on this column. Evidence, based on gas chromatography retention time studies, indicated that this peak contained a mixture of furanosides. The second peak consisted of methyl α-D-mannopyranoside, and the third component was methyl β-D-mannopyranoside. A trace of D-mannose (Peak 4) was also present after 30 min. The two major products after 18 h reaction time consisted of a mixture of methyl α- and β-D-mannopyranosides (Fig. 2).

Unsubstituted monosaccharides could also be separated on this column using the same eluting solvent that was used for the glycosides (Table II). It was found that the retention time of the monosaccharide was considerably longer than the corre-

TABLE I

RETENTION TIME OF SOME MONOSUBSTITUTED CARBOHYDRATES

For conditions, see under Experimental.

Compound	*Retention time (min)*
Methyl α-D-xylopyranoside	12.4
Methyl β-D-xylopyranoside	11.8
Methyl α-L-arabinopyranoside	14.2
Methyl β-D-ribopyranoside	11.5
Methyl β-D-glucopyranoside	17.7
Methyl α-D-glucopyranoside	15.9
Phenyl α-D-glucopyranoside	10.0
Phenyl β-D-glucopyranoside	8.8
Methyl α-D-mannopyranoside	15.3
Methyl β-D-mannopyranoside	18.3
Methyl α-D-galactopyranoside	20.1
Methyl β-D-galactopyranoside	19.5
Methyl α-D-altropyranoside	15.3
Methyl α-D-gulopyranoside hydrate	18.3
3-O-Methyl D-glucose	15.9

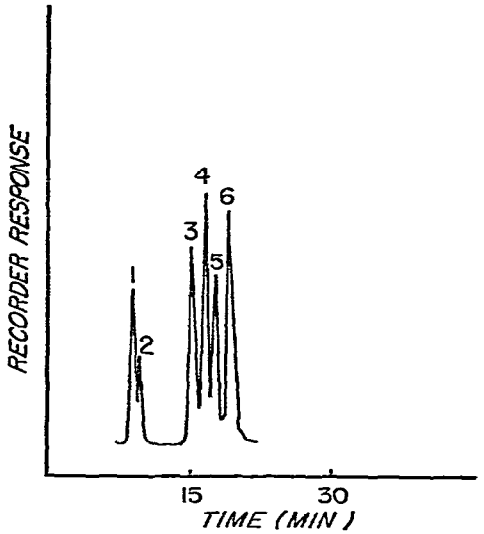

Fig. 1. Separation of a mixture of glycosides. Peaks: 1 = phenyl β-D-glucopyranoside; 2 = phenyl α-D-glucopyranoside; 3 = methyl α-D-mannopyranoside; 4 = methyl α-D-glucopyranoside; 5 = methyl β-D-glucopyranoside; 6 = methyl α-D-galactopyranoside.

sponding methyl glycosides. D-Glucose and D-xylose gave fairly sharp peaks; the other monosaccharides, however, gave broad peaks, apparently due to some mutarotation during passage through the column.

The next series of compounds that was separated on this column was a series of di-, tri- and tetra-substituted carbohydrates (Figs. 3–5). The 2,3,6-tri-O-methyl

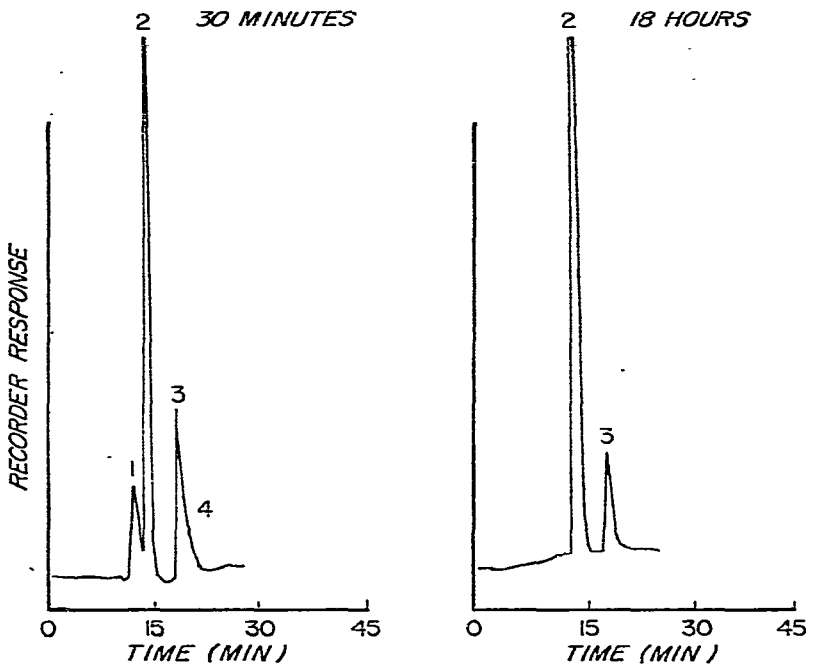

Fig. 2. The reaction products from D-mannose and methanol in the presences of an acidic ion-exchange resin after 30 min and 18 h. Peaks: 1 = unknown; 2 = methyl α-D-mannopyranoside; 3 = methyl β-D-mannopyranoside; 4 = D-mannose.

TABLE II

RETENTION TIME OF SOME MONOSACCHARIDES

Compound	*Retention time (min)*
D-Xylose	14.2
L-Arabinose	18.4
D-Ribose	14.7
D-Glucose	22.2
D-Mannose	22.4
D-Galactose	25.4
D-Fructose	21.3

D-glucose could be partially separated from 2,4,6-tri-O-methyl D-glucose using acetonitrile–water (18:1) as the eluting solvent. The same solvent could also be used for separating 4,6-O-benzylidene D-glucopyranose from 1,2-O-isopropylidene α-D-glucopyranose.

For the separation of the diisopropylidene derivatives of glucose and mannose, it was necessary to use a less polar eluting solvent consisting of ethyl acetate–hexane (3:1). With this solvent the 2,3:5,6-di-O-isopropylidene D-mannofuranose could be completely separated from 1,2:5,6-di-O-isopropylidene α-D-glucofuranose (Fig. 4).

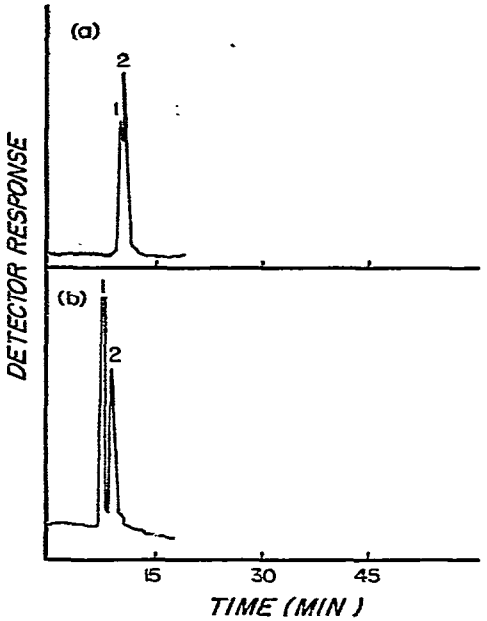

Fig. 3. The separation of some partially substituted carbohydrates. (a) Peaks: 1 = 2,3,6-tri-O-methyl D-glucose; 2 = 2,4,6-tri-O-methyl D-glucose. (b) Peaks: 1 = 4,6-O-benzylidene D-glucopyranose; 2 = 1,2-O-isopropylidene α-D-glucopyranose.

When D-fructose is reacted with acetone in the presence of an acidic catalyst, two products are formed: the 1,2:4:5-di-O-isopropylidene β-D-fructopyranose and 2,3:4,5-di-O-isopropylidene β-D-fructopyranose[7]. These two products can be partially separated on the silica column using ethyl acetate–hexane (3:1) as the eluting solvent (Fig. 5).

The next group of carbohydrates that was studied was a series of completely substituted carbohydrates. Retention time for these compounds, using equal volumes of ethyl acetate and hexane as the eluting solvent, is given in Table III. In general, it was found that a wide variety of the acetylated derivatives could be separated on

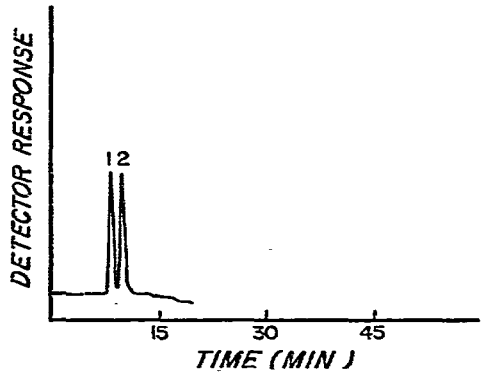

Fig. 4. The separation of the di-O-isopropylidene derivatives of D-glucose and D-mannose. Peaks: 1 = 2,3:5,6-di-O-isopropylidene D-mannofuranose; 2 = 1,2:5,6-di-O-isopropylidene α-D-glucofuranose.

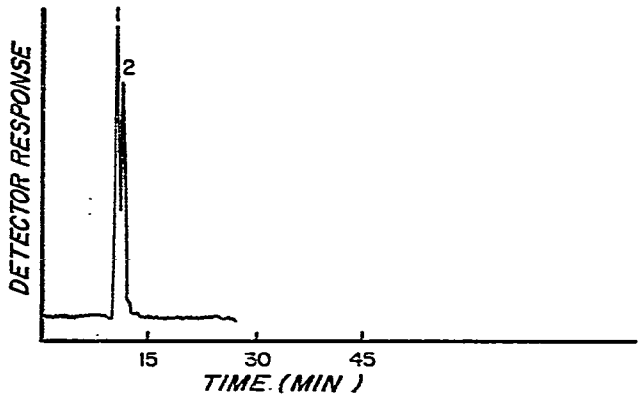

Fig. 5. The separation of the di-O-isopropylidene derivatives of D-fructose. Peaks: 1 = 1,2:4,5-di-O-isopropylidene β-D-fructopyranose; 2 = 2,3:4,5-di-O-isopropylidene β-D-fructopyranose.

TABLE III

RETENTION TIME OF SOME ACETYLATED CARBOHYDRATES

Compound	*Retention time (min)*
α-Cellobiose octaacetate	38.0
β-Cellobiose octaacetate	36.1
α-D-Glucopyranose pentaacetate	17.2
β-D-Glucopyranose pentaacetate	15.9
α-D-Galactopyranose pentaacetate	15.9
β-D-Galactopyranose pentaacetate	16.0
L-Arabinitol pentaacetate	14.0
Erythritol tetraacetate	12.6
Tetra-O-acetyl-α-D-glucopyranosyl bromide	11.5
Tetra-O-acetyl-α-D-galactopyranosyl bromide	11.1
Methyl α-D-glucopyranoside tetraacetate	16.3
Methyl β-D-glucopyranoside tetraacetate	17.3
Phenyl α-D-glucopyranoside tetraacetate	11.7
Phenyl β-D-glucopyranoside tetraacetate	13.0
Phenyl α-D-galactopyranoside tetraacetate	11.8

this column. The chromatograms of the anomeric phenyl and methyl D-glucopyranosides are shown in Fig. 6.

Some examples of the use of this analytical technique for following reactions of acetylated carbohydrates are shown in Figs. 7 and 8. When -cellobiose octaacetate is heated in the presence of zinc chloride and acetic anhydride, it undergoes equilibration between the α- and β-forms. At equilibrium the major component (80%) is α-celliobiose octaacetate[8]. HPLC can be used to monitor the reaction (Fig. 7). The top chromatogram is β-cellobiose octaacetate (starting material), the middle curve is the reaction mixture containing both isomers, and the bottom curve is the purified α-cellobiose octaacetate.

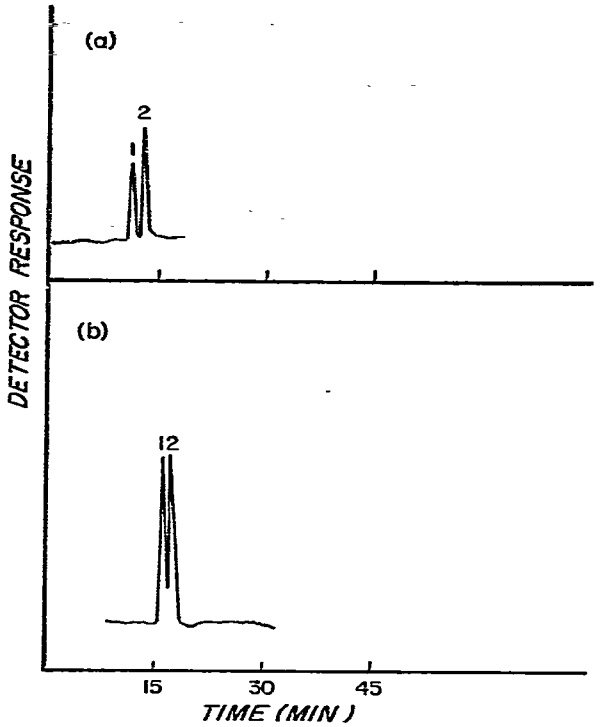

Fig. 6. Separation of the anomeric phenyl and methyl D-glucopyranosides. (a) Peaks: 1 = phenyl tetra-O-acetyl-α-D-glucopuranoside; 2 = phenyl tetra-O-acetyl-β-D-glucopyranoside. (b) Peaks: 1 = methyl tetra-O-acetyl-α-D-glucopyranoside; 2 = methyl tetra-O-acetyl-β-D-glucopyranoside.

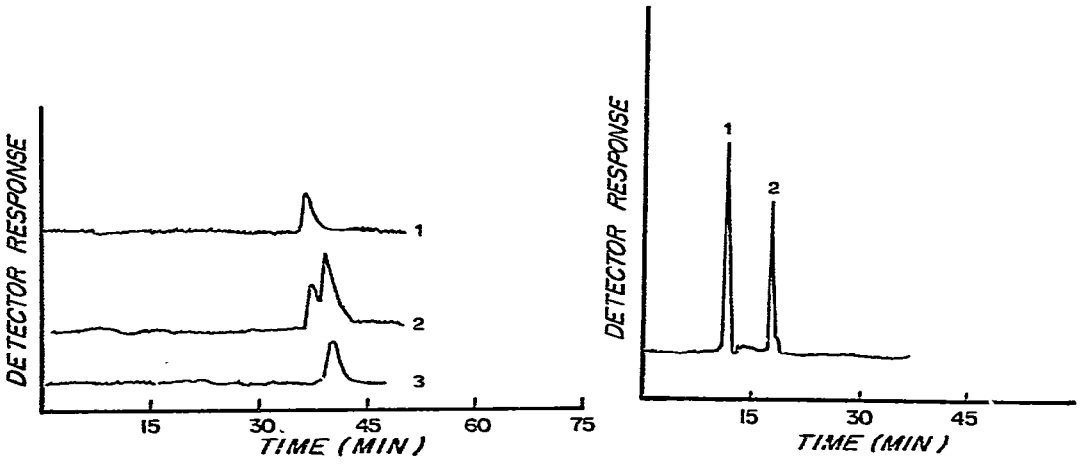

Fig. 7. Products from the anomerization of β-cellobiose octaacetate. 1 = β-Cellobiose octaacetate; 2 = reaction mixture; 3 = α-cellobiose octaacetate.

Fig. 8. Chromatogram of tetra-O-acetyl α-D-glucopyranosyl bromide (1) and methyl tetra-O-acetyl glucopyranoside (2).

HPLC can also be used to monitor the Koenigs-Knorr glycosidation reactions. In Fig. 8 is the chromatogram of the tetra-O-acetyl-α-D-glucopyranosyl bromide (starting material) and the corresponding acetylated methyl glycosides (final product), formed by the reaction of acetobromo sugar, methanol and silver carbonate.

The results of this study indicate that HPLC with a microparticulate silica gel column can be used to separate a wide range of substituted carbohydrates. By appropriate selection of the solvent, separation can be achieved from the very polar nonsubstituted monosaccharides to the relatively less polar acetylated derivatives.

HPLC has many advantages over the other types of separation techniques for carbohydrates. Carbohydrates can be analyzed directly without preparation of a derivative. The technique is relatively fast (5–15 min) and can be used for both qualitative and quantitative analyses. Thermally labile carbohydrates which cannot be analyzed by gas chromatography can be analyzed by this method.

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