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QUANTITATIVE MICROANALYSIS OF OLIGOSACCHARIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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ABSTRACT

The rapid separation and quantitative determination of per-*O*-benzoyl oligosaccharides were obtained using high performance, liquid chromatography. Oligosaccharides were completely *O*-benzoylated without concomitant *N*-benzoylation of acetamidodeoxyhexoses. Benzoylation prior to analysis allowed a quantitative determination of picomolar amounts because the absorbance at 230 nm of these derivatives is directly proportional to the number of benzoyl groups present. Separation by normal and reversed-phase chromatography was demonstrated, and the best resolution was obtained on an Ultrasphere octyl column. Excellent separations of oligosaccharides containing up to 10 sugar residues present in mannosidosis urine and of malto-oligosaccharides containing up to 15 sugar residues present in Karo syrup were achieved within an analysis time of 30 min. Anomers of maltose, maltotriose, and maltotetraose were separated; for this reason, reduction of complex samples prior to analysis is advisable. The effect of linkage configuration on retention time was tested with reduced, α-linked di- and tri-glucopyranosides. The presence of an acetamidodeoxyhexose residue in an oligosaccharide significantly reduced its retention time, whereas branching had the opposite effect. A linear response was obtained for the injection of 1–600 pmol of raffinose, and the detection limit was 0.5 pmol. Derivatization and analysis of raffinose was shown to yield reproducible results within the range 0.01–1 μmol, and, with special precautions to minimize losses, as little as 100 pmol could be analyzed successfully.

INTRODUCTION

The separation of complex oligosaccharides on the basis of size has traditionally required lengthy gel filtration with destructive chemical assay or radioactive monitoring of a portion of the column effluent[1,2]. More recently, high-performance liquid chromatography (h.p.l.c.) has provided an alternative method for separation of

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oligosaccharides, which promises faster analysis times and increased resolution[3-5]. However, the refractive-index monitor frequently employed is insensitive and precludes the use of gradient elution, thus placing serious constraints on the size range of oligosaccharides that can be conveniently resolved by h.p.l.c.

Perbenzoylation of glycoconjugates prior to h.p.l.c. analysis permits the use of gradient elution for the rapid separation of components of different size and allows their nondestructive analysis by a sensitive u.v. detector. Previous studies from this laboratory have demonstrated the usefulness of this procedure for the microanalysis of glycosphingolipids[6,7] and monosialogangliosides[8]. This report describes the preparation and rapid h.p.l.c. analysis of picomolar amounts of perbenzoylated oligosaccharides. Preliminary reports of this work have appeared[9-11].

RESULTS AND DISCUSSION

Reversed-phase h.p.l.c. analysis of perbenzoylated oligosaccharides. — Perbenzoylation of oligosaccharides prior to analysis serves two important functions: (a) it produces derivatives having improved chromatographic properties on reversed-phase columns as compared to the native oligosaccharides; and (b) it imparts a

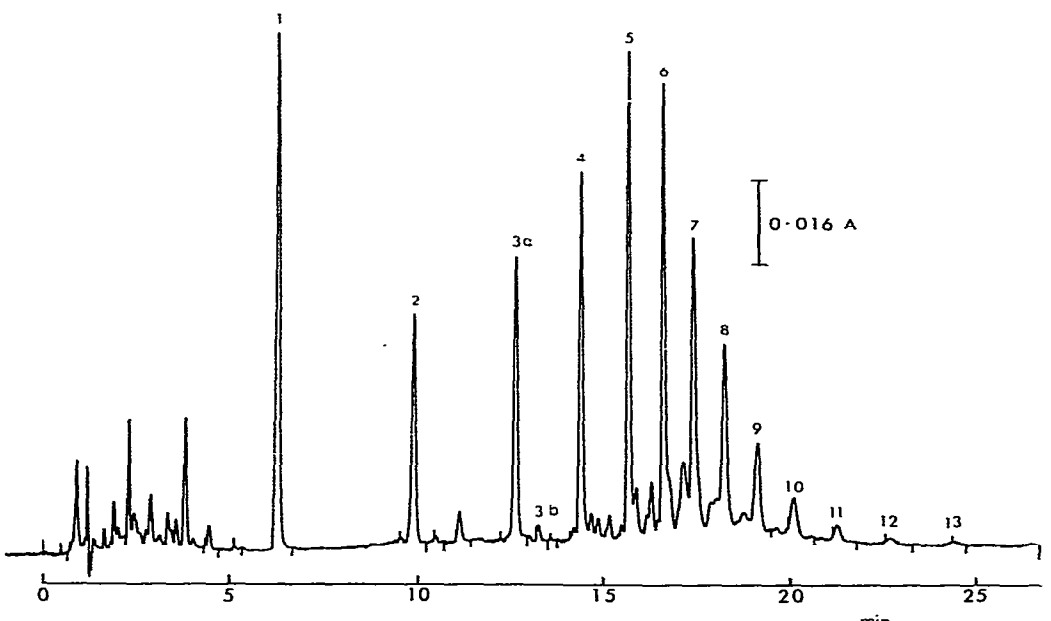

Fig. 1. Separation of D-glucose and malto-oligosaccharides from a fraction of Karo syrup enriched in higher-mol.-wt. oligomers by ethanol precipitation, which was reduced and then benzoylated with benzoic anhydride. Ordinate: u.v. absorbance. Column, Ultrasphere octyl; elution conditions, a 15-min linear gradient of 80% (v/v) acetonitrile in water to absolute acetonitrile at 2 mL/min; u.v. detection at 230 nm; chart speed, 1 cm/min. The number beside each peak indicates its d.p. Peak 3(b) is isomaltotriose. Peaks eluted prior to D-glucose (1) are caused by traces of benzoylating reagents.

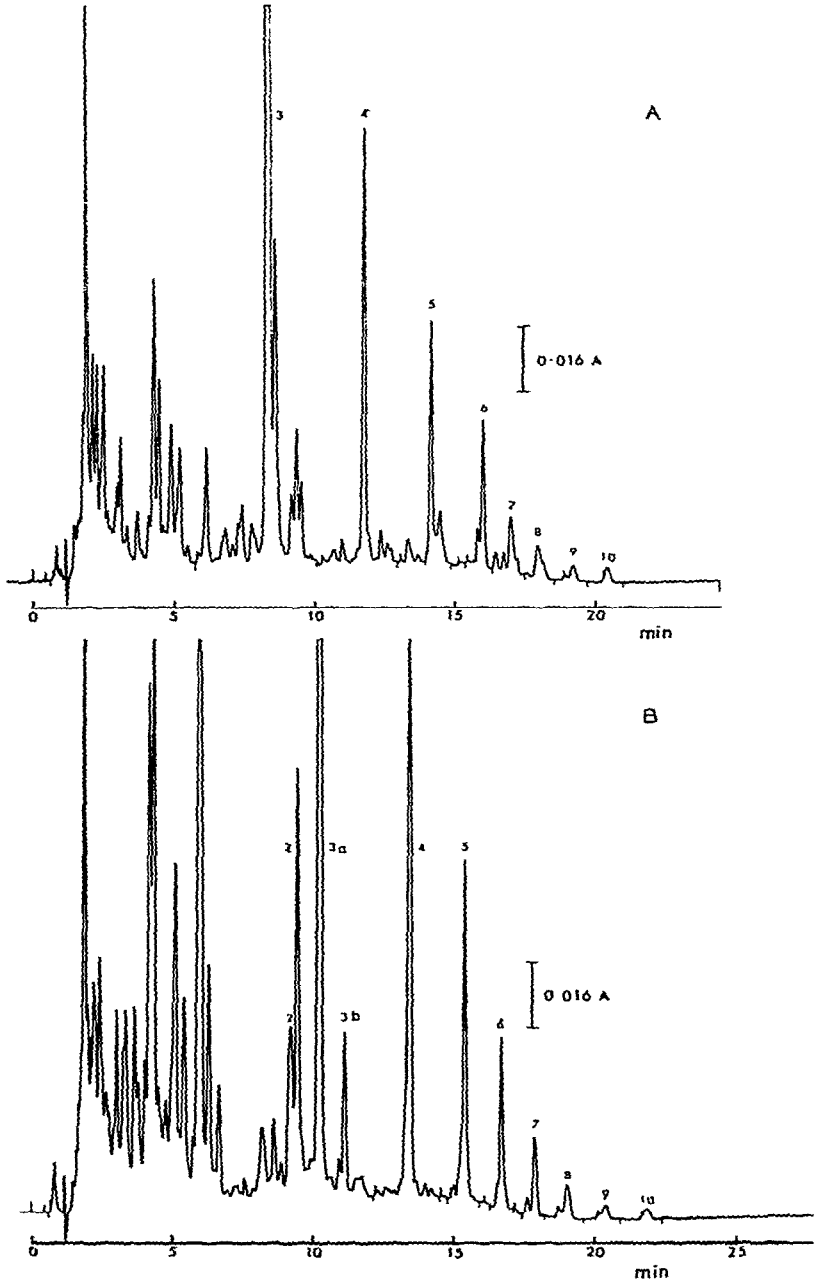

Fig. 2. Separation of oligosaccharides from mannosidosis urine. (a) Separation of nonreduced oligosaccharides, and (b) separation of reduced oligosaccharides. The urine was desalted and treated as described in the Experimental section and then benzoylated with benzoic anhydride. Ordinate: u.v. absorbance. Analysis conditions are given in the legend to Fig. 1. The number beside each peak indicates its d.p. Unmarked early peaks are due to monosaccharides and other components in urine that react with benzoic anhydride.

large extinction coefficient, at 230 nm, that facilitates the u.v. detection and quantitative determination of picomolar amounts of individual oligosaccharides. This is illustrated by the resolution of oligomers present in Karo syrup by reversed-phase h.p.l.c. (Fig. 1). Injection of <1 nmol of total benzoylated oligosaccharides was sufficient for the separation and quantitative determination of reduced malto-oligosaccharides ranging in size from D-glucose (263 pmol) to a degree of polymerization (d.p.) of 13 (<1 pmol) with an analysis time of only 25 min. Trace amounts of oligosaccharides having d.p. 14 and 15 could be detected by overloading the column with sample. The elution of oligosaccharides was in the order of increasing molecular size, which corresponded to the incremental increase in absorption due to the presence of three additional benzoyl groups per additional hexose unit. Characteristic oligosaccharides from mannosidosis urine having d.p. 3 ($Man_2GlcNAc$; 774 pmol) to d.p. 10 ($Man_9GlcNAc$; 3 pmol) were readily analyzed within 22 min, as shown in Fig. 2.

Benzoylation conditions. — Complete benzoylation of glycoconjugates can be achieved by treatment with either benzoyl chloride in pyridine[6], or benzoic anhydride

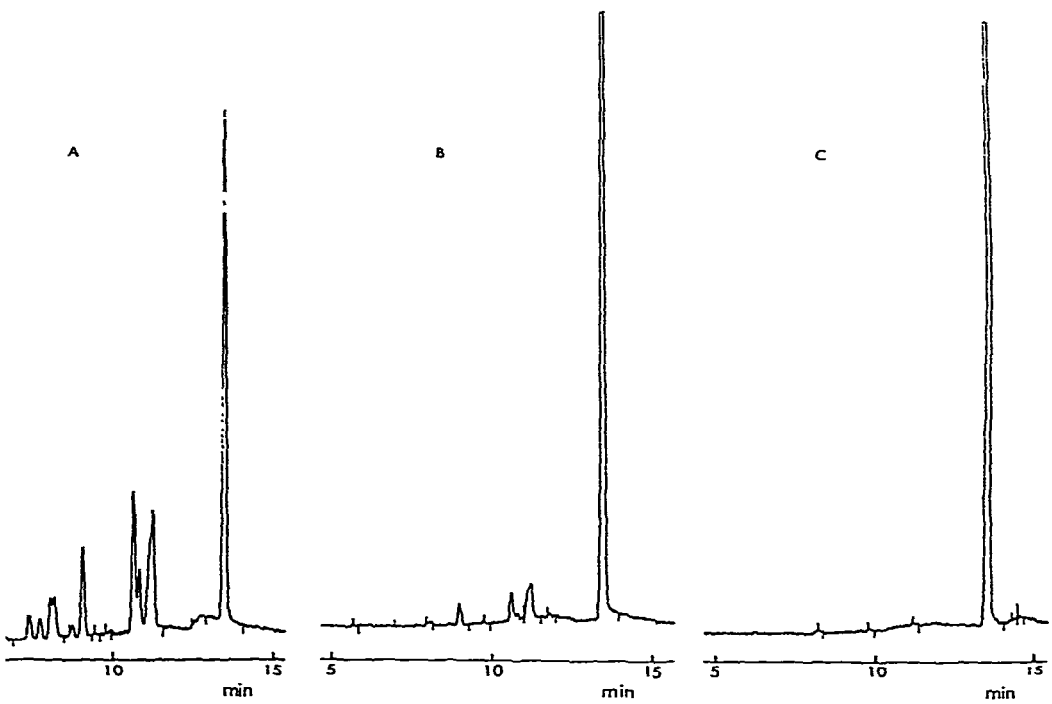

Fig. 3. H.p.l.c. analysis of under-benzoylated raffinose: A, 2-min benzoylation; B, 5-min benzoylation; and C, 120-min benzoylation. All samples were benzoylated with benzoic anhydride, and the reaction was stopped at the indicated time by the addition of water (4.5 mL). The volume injected corresponded to 500 pmol of raffinose in each case. Peaks of under-benzoylated compounds are more polar and, therefore, were eluted earlier than the completely benzoylated raffinose. Ordinate: u.v. absorbance. Analysis conditions are given in the legend to Fig. 1.

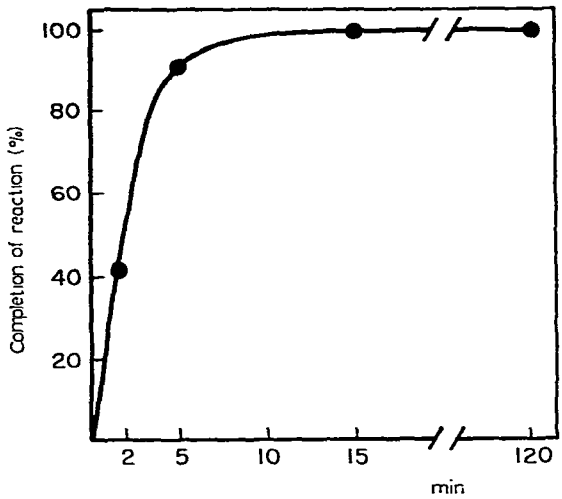

Fig. 4. Time course of benzoylation. A standard of raffinose was benzoylated at 37° with benzoic anhydride for various times and then subjected to h.p.l.c. analysis. The area of the fully benzoylated raffinose peak is expressed as a percentage of the total area from all the raffinose peaks.

in pyridine with 4-dimethylaminopyridine as acylation catalyst[12]. Benzoylation of malto-oligosaccharides with benzoyl chloride in pyridine for 16 h at 37° was found to give complete benzoylation. These conditions were originally developed for the analysis of neutral glycosphingolipids[6]. However, acetamidodeoxyhexoses undergo *N*-benzoylation in addition to *O*-benzoylation on derivatization with benzoyl chloride, whereas benzoic anhydride yields solely the *O*-benzoyl derivatives[12]. Quantitative *N*-debenzoylation cannot be achieved because a mixture of benzamido- and acetamidodeoxyhexoses is obtained on mild alkaline hydrolysis. For this reason, derivatization with benzoic anhydride is well-suited for both analytical and preparative h.p.l.c. of oligosaccharides containing acetamidodeoxyhexoses, such as those obtained from mannosidosis urine.

It is crucial that benzoylation be complete because incomplete benzoylation gives rise to additional peaks on h.p.l.c. Benzoylation of raffinose (11 free hydroxyl groups) with benzoic anhydride for only 2 min yielded ~60% underbenzoylated products, which are more polar and are therefore less strongly retained on a reversed-phase column (Fig. 3A). After a 5-min benzoylation, ~10% of incompletely benzoylated products were obtained (Fig. 3B), but these disappeared with longer reaction times (Fig. 3C). The time course of perbenzoylation of raffinose with benzoic anhydride at 37° is shown in Fig. 4. It can be seen that, under these conditions, benzoylation proceeds rapidly and is complete in 15 min. In order to ensure complete benzoylation of long-chain oligosaccharides having a d.p. up to 15 (~48 hydroxyl groups), we standardized the reaction time at 4 h. This was adequate for quantitative benzoylation of all oligosaccharides tested.

Excess benzoylating reagents must be removed prior to h.p.l.c., otherwise

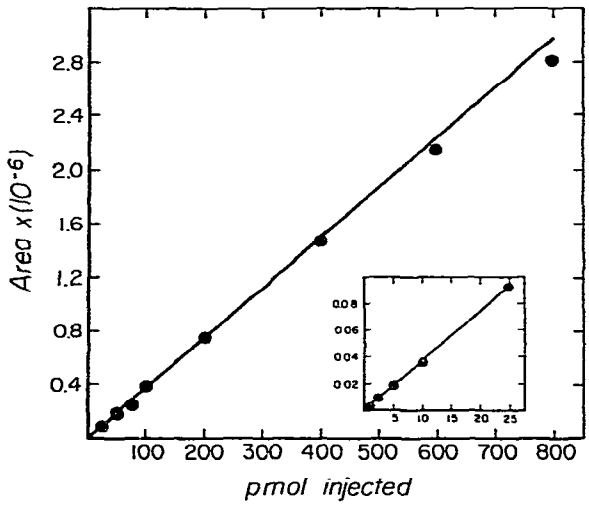

Fig. 5. Linearity of the u.v. absorbance response at 230 nm to the amount of raffinose injected onto an Ultrasphere octyl column. The eluted raffinose was determined by measuring the u.v. absorption at 230 nm with a Schoeffel SF 770 detector (set at maximum sensitivity) coupled to an Autolabs System I computing integrator. The deviation from linearity at the top of the range can be corrected by attenuating the signal at the detector.

peaks eluted early are lost in a huge background absorbance. Solvent extraction has been successful in eliminating the excess of reagents after benzoylation of glycosphingolipids[6]; however, when this technique was applied to benzoylated oligosaccharides, recoveries were variable. For this reason, we examined the usefulness of partition chromatography on a C18 Sep-pak cartridge for routine sample-preparation. Following benzoylation, the sample was diluted with distilled water and applied to the Sep-pak as described in the Experimental section. Excess reagents were eluted with aqueous pyridine, and benzoylated oligosaccharides subsequently with acetonitrile.

Recovery and sensitivity. — The linearity of the detector response was investigated by injection of progressive dilutions of a benzoylated-raffinose standard. As shown in Fig. 5, the response was linear over the range of 1–600 pmol with the u.v. detector set at maximum sensitivity (range 0.01 at 230 nm). Linearity above this value could be restored by attenuating the signal at the detector.

By adding small amounts of [^{3}H]raffinose to samples prior to benzoylation, we have established that the recovery of radioactivity in the acetonitrile eluent from the Sep-pak column is 81% ($\pm$5.4) and is constant over the entire range from 100 pmol to 100 nmol, thus making the procedure eminently suitable for processing very small amounts of samples. More than 90% of the injected radioactivity was recovered in the raffinose peak after h.p.l.c. For the combined derivatization and analysis of amounts of raffinose ranging from 10 nmol to 1 μmol, the overall response was >90% of maximum with excellent reproducibility. For the successful analysis of material in amounts <10 nmol special precautions must be taken to minimize losses.

TABLE I

UNIFORMITY OF RESPONSE FOR DERIVATIZATION AND ANALYSIS OF VARIOUS AMOUNTS OF RAFFINOSE[a]

Amount benzoylated (nmol)	*Amount injected[b] (pmol)*	*Mean response per nmol (Area × 10⁻⁶)[c]*
0.1	10	3.38 ±1.22 (9)
0.3	30	3.02 ±0.90 (6)
1	100	2.69 ±0.78 (4)
3	200	3.42 ±1.17 (3)
10–1000[d]	200	4.15 ±0.14 (8)

[a]All tubes were silanized to minimize losses. The samples of raffinose (0.1–1 nmol) were benzoylated with 100 μL of benzoic anhydride reagent; the remainder were benzoylated with 500 μL. Duplicate injections of samples containing >1 nmol differed by <2%; duplicate injections of 100- and 300-pmol samples differed by <7%. Based on recovery of counts from raffinose samples to which a small amount of [³H]raffinose had been added, recovery from the column of Sep-pak was 81% (±5.4) and was uniform over the range from 100 pmol to 100 nmol. [b]Volume injected, 20 μL. [c]±Standard deviation, with number of samples given in parentheses. [d]Two samples each of 10, 30, 100, and 1000 nmol.

These include silanization of glassware to reduce adsorptive losses, and micro-benzoylation in 100 μL of reagent. As shown in Table I, >70% of maximum response could be obtained for the analysis of as little as 100 pmol of raffinose, although not surprisingly the variation between samples increased significantly.

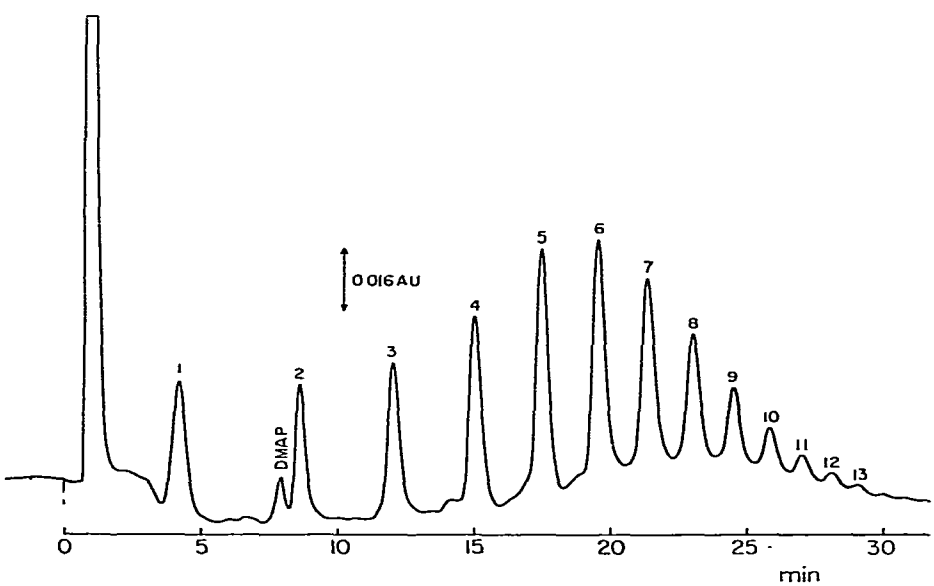

Fig. 6. Adsorption chromatography of mono- and oligo-saccharides of Karo syrup on a Zipax column. Reduced Karo syrup was derivatized with benzoic anhydride. The number by each peak indicates its d.p.; DMAP: 4-dimethylaminopyridine. Ordinate: u.v. absorbance. The column was irrigated for 26 min at a flow rate of 2 mL/min with a linear gradient of 2.5 to 47.5% (v/v) of 1,4-dioxane in hexane; chart speed, 1 cm/min.

It has been shown that the molar extinction of per-*O*-benzoylated glycoconjugates is independent of the structure of the compound studied and is strictly additive as a function of the degree of substitution[6,13]. The absorbance increment for each additional benzoyl group is ~12 600. Because of the complexity of the Karo syrup and mannosidosis urine samples, it was not possible to use an internal standard. Quantitative determination was best achieved by determining the response, per benzoyl group, of a known amount of a pure oligosaccharide standard that had been derivatized at the same time as the experimental sample. For this purpose, a non-reducing trisaccharide such as raffinose is ideal. The detection limit (twice the noise level) for raffinose is 0.5 pmol. Thus, for oligosaccharides of d.p. >9, a realistic detection limit is 0.2 pmol. This increase in sensitivity with increasing d.p. is a most desireable feature for trace analysis, as the converse is true for many detection procedures, for example, those that depend on derivatization of the reducing, terminal sugar residue.

Chromatographic conditions. — (*a*) *Adsorption chromatography.* Zipax is a pellicular silica gel, which has proved useful for the separation of benzoylated glycosphingolipids and monosialogangliosides on the basis of their sugar content[6,8]; so it was a logical starting point for our studies on oligosaccharides. Reduced, benzoylated oligosaccharides from Karo syrup of d.p. 1–13 were separated on Zipax with a linear gradient of 1,4-dioxane in hexane at a flow rate of 2 mL/min. (Fig. 6). The order of elution was the same as that observed with Ultrasphere C-8. A comparison of Figs. 1 and 6 clearly reveals the superiority of the reversed-phase separation. A Lichrosphere

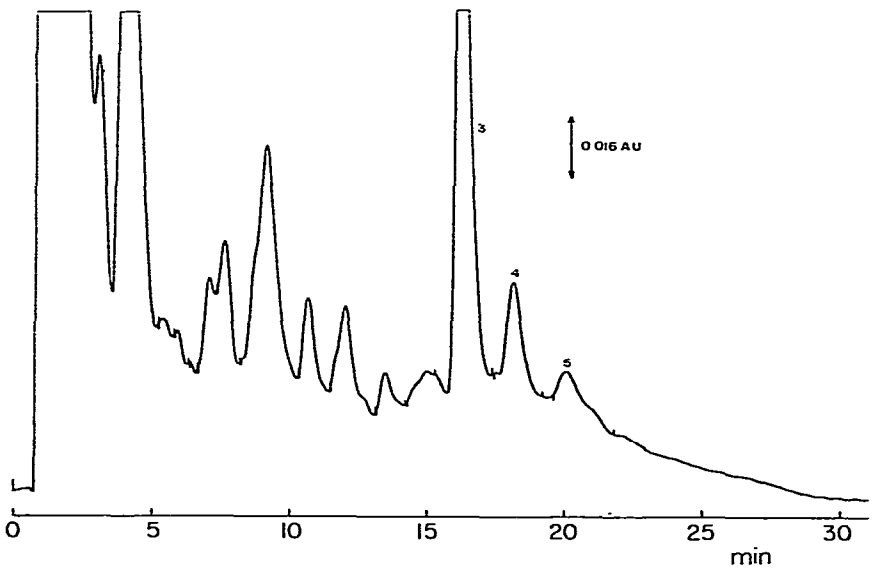

Fig. 7. Reduced, neutral oligosaccharides isolated from mannosidosis urine were derivatized with benzoic anhydride and analyzed on a column of Zipax. Ordinate: u.v. absorbance. For chromatographic conditions, see the legend to Fig. 6. The number of each peak indicates its d.p.

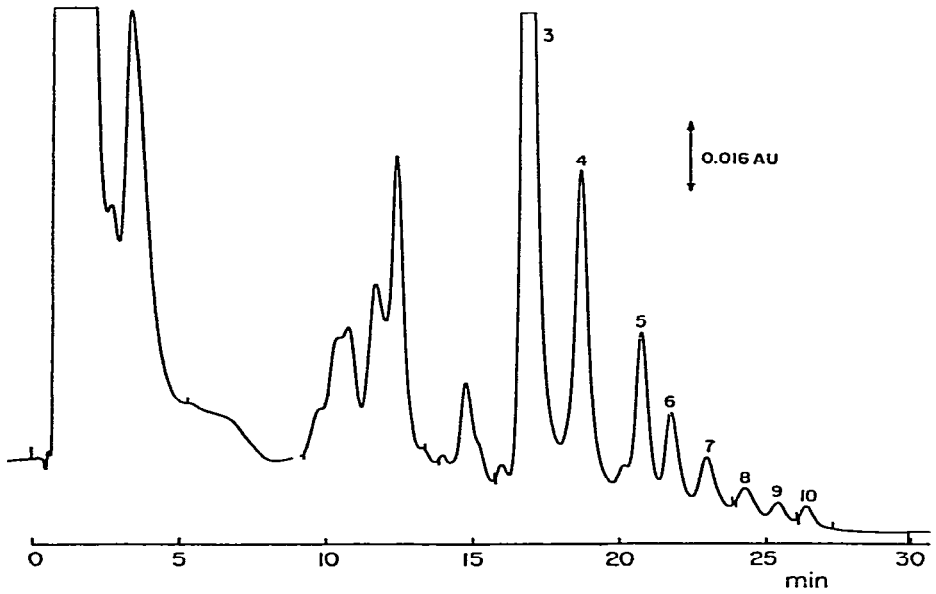

Fig. 8. Reduced, neutral oligosaccharides isolated from mannosidosis urine were derivatized with benzoyl chloride and analyzed on a column of Zipax. Ordinate: u.v. absorbance. For chromatographic conditions, see the legend to Fig. 6. The number by each peak indicates its d.p.

Si-4000 column was tried on the premise that the smaller particle-size (10 μ) and larger pore-size would provide a higher chromatographic efficiency than the pellicular Zipax. This was not found to be the case: the two profiles were virtually superimposable, with the exception of the earlier elution of sorbitol on Si-4000.

The h.p.l.c. analysis on Zipax of reduced mannosidosis oligosaccharides after derivatization with benzoic anhydride was disappointing (Fig. 7). Only the smaller, unbranched oligosaccharides[14,15] of d.p. 3–5 were eluted under these conditions, the larger oligosaccharides being retained on the column. Attempts to elute these by increasing the concentration of 1,4-dioxane in the eluting solvent were unsuccessful. However, all the oligosaccharides known to be present could be eluted after derivatization with benzoyl chloride (Fig. 8). Evidently, the presence of the additional *N*-benzoyl group is essential for the elution from silica gel of branched chain oligosaccharides of d.p. >5. Presumably, the acetamido group interacts strongly with the silica gel unless it is diacylated.

(*b*) *Reversed-phase chromatography.* A column of octadecylsilane (Lichrosorb RP-18) was tried initially for the separation of benzoylated malto-oligosaccharides. It proved to be too retentive, since oligosaccharides of d.p. >6 could not be eluted, even with absolute acetonitrile. The choice of stronger eluotropic solvents was severely limited by the requirement for u.v. transparency at 230 nm. However, with a column of octylsilane (Ultrasphere octyl), we were able to resolve, within 30 min, all the oligosaccharides present in Karo syrup, by means of a 15-min linear gradient of acetonitrile in water at a flow rate of 2 mL/min (Fig. 1). The excellent resolution of

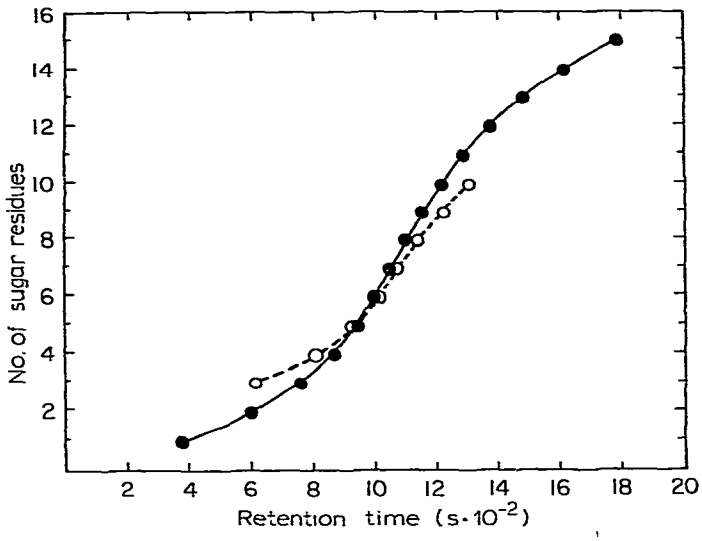

Fig. 9. Plot of retention time on an Ultrasphere octyl column *vs.* number of sugar residues. Oligosaccharides from Karo syrup of d.p. 1–15 (—●—) and from mannosidosis urine of d.p. 3–10 (--○--) were benzoylated with benzoic anhydride and analyzed as described in the legend to Fig. 1. The shape of the curve for oligosaccharides from mannosidosis urine is affected by two opposing factors: (*a*) decreased polarity associated with an acetamido group, and (*b*) increased binding affinity due to branching.

the Ultrasphere octyl column may be attributed partly to the good mass-transfer inherent in partition chromatography, and partly to the spherical nature and small size (5 μ) of the packing. This column gave a much higher resolution than that of a column of Lichrosorb RP-8 packed with irregular 10-μ particles that we first utilized. The resolving power of the Ultrasphere octyl column was sufficient to be able to resolve minor components that had escaped attention previously.

A plot of the number of sugar residues *vs.* retention time for reduced mannosidosis oligosaccharides and malto-oligosaccharides (Fig. 9) indicates that different homologous series of oligosaccharides give rise to different curves. This can probably be attributed to an increased interaction with the support due to branching, as the malto-oligosaccharides are linear, whereas the more-highly-retained mannosidosis oligosaccharides of d.p. >5 are branched[16]. The earlier elution of the lower mol.-wt. mannosidosis oligosaccharides is due to the increased polarity associated with the acetamido group.

The h.p.l.c. analysis of both reduced and nonreduced oligosaccharides from mannosidosis urine after benzoic anhydride derivatization is shown in Fig. 2. It is apparent that reduction and substitution with a single additional *O*-benzoyl group substantially increased the retention time of each of the characteristic mannosidosis oligosaccharides. Addition of an *N*-benzoyl group by derivatization with benzoyl chloride further increased the retention time (data not shown), and this can be utilized to deduce the presence of an acetamidodeoxyhexose in an unknown oligosaccharide.

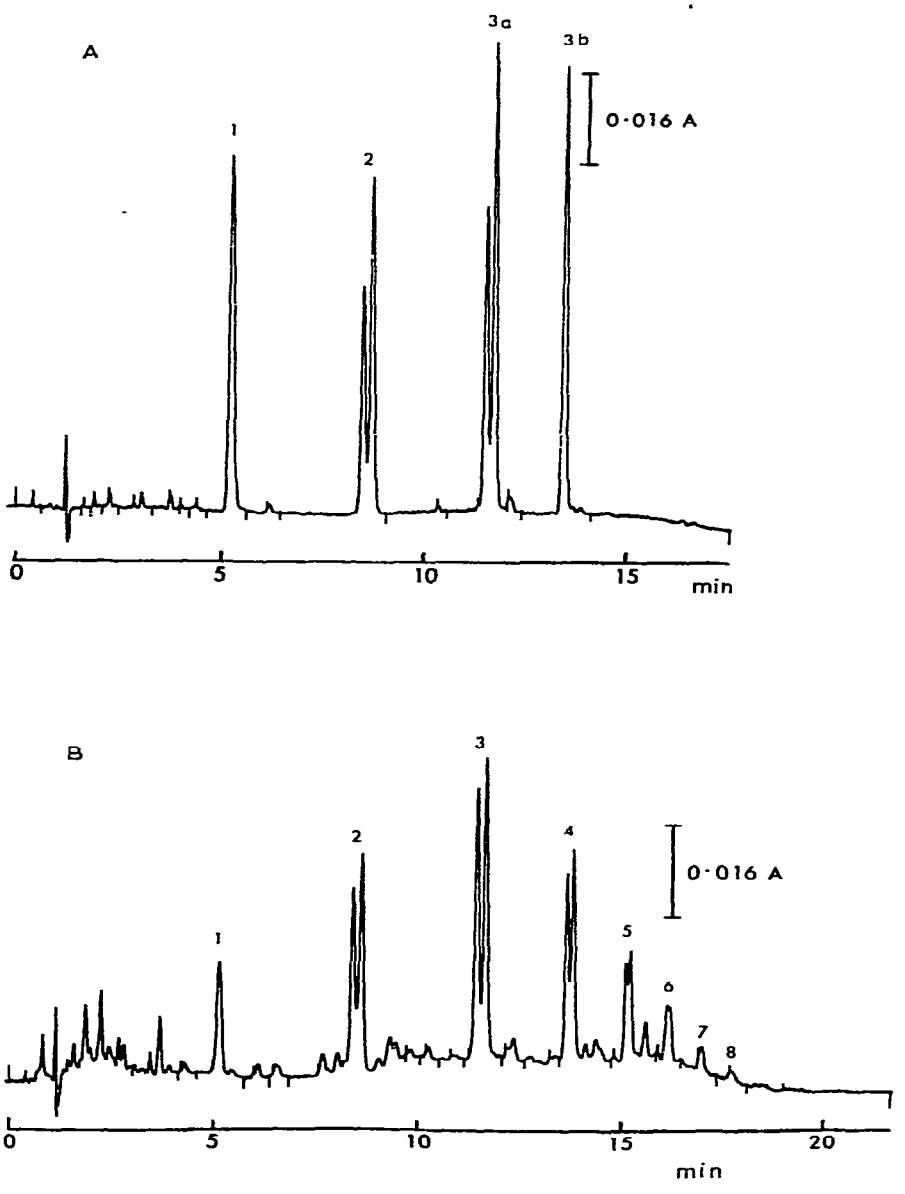

Fig. 10. Separation of benzoylated anomers by reversed-phase chromatography on an Ultrasphere octyl column. A, Separation of nonreduced standards: (1) D-glucose; (2) maltose; (3a) maltotriose; and (3b) raffinose. B, Separation of nonreduced malto-oligosaccharides from Karo syrup. The number by each peak or pair of peaks indicates the d.p. Anomers of maltose, maltotriose, and maltotetraose were separated by 13, 12 and 9 s, respectively. A 15-min gradient of 80–100% (v/v) acetonitrile in water was used at a flow rate of 2 mL/min. Chart speed, 1 cm/min. Ordinate: u.v. absorbance.

Separation of anomers. — The high resolution of reduced malto-oligosaccharides on Ultrasphere C-8 encouraged us to examine the separation of anomers. The separation of standards D-glucose, maltose, maltotriose, and raffinose is shown in Fig. 10 (top), and a similar profile is given by Karo syrup (Fig. 10, bottom). D-Glucose, anomalously, gave only a single peak, as did raffinose, as was to be expected for a nonreducing sugar. However, anomers of maltose, maltotriose, and maltotetraose were separated by 13, 12, and 9 s, respectively. For this reason, reduction of complex samples is advisable.

Because of interest in the isolation of native mannosidosis oligosaccharides for use as possible sugar acceptors in *in vitro* enzyme assays, or as a convenient starting point for organic synthesis of potential intermediates in glycoprotein biosynthesis[17], the ability to separate mannosidosis oligosaccharides without prior reduction is important. Fortuitously, it appears from Fig. 2a that one anomer of these oligosaccharides predominates after benzoic anhydride derivatization[10], which should make isolation a simpler task. The parent oligosaccharide can be regenerated by treatment of the benzoylated derivative with mild alkali[12].

Separation of positional isomers. — The separation of reduced maltotriose and isomaltotriose (Fig. 11) established the ability of the Ultrasphere octyl column to separate certain isomers that differ in their position of linkage. This ability was further tested with reduced D-glucopyranosyl disaccharides (Table II). In the α-series, kojibiose, nigerose, maltose–isomaltose, and trehalose are separable, but there is insufficient resolution to separate maltose from isomaltose. Kojibiose, cellobiose, and gentiobiose are inseparable. The degree of separation of kojibiose, nigerose, and maltose is illustrated in Fig. 12.

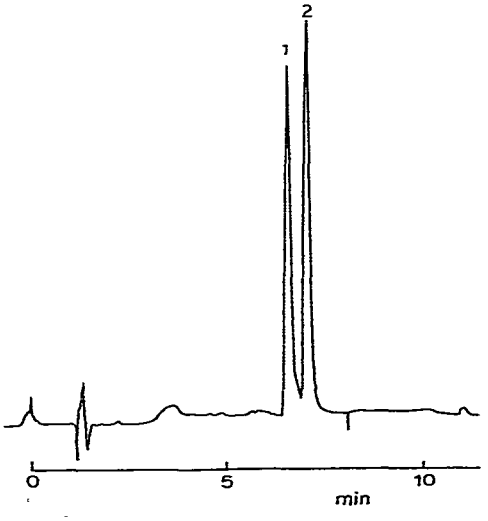

Fig. 11. Separation of reduced, benzoylated maltotriose and isomaltotriose on an Ultrasphere octyl column: (1) maltotriose and (2) isomaltotriose. Ordinate: u.v. absorbance. An 8 min gradient of 90–100% (v/v) acetonitrile in water was used at a flow rate of 2 mL/min. Chart speed, 1 cm/min.

TABLE II

H.P.L.C. OF REDUCED D-GLUCOPYRANOSYL DISACCHARIDES[a]

Disaccharide	*Linkage*	*Retention time (s)*
Trehalose	α,α-(1,1)	523
Kojibiose	α-(1→2)	574
Nigerose	α-(1→3)	699
Maltose	α-(1→4)	612
Isomaltose	α-(1→6)	595
Cellobiose	β-(1→4)	574
Gentiobiose	β-(1→6)	573

[a]The sugars were separated isocratically on a column of Ultrasphere octyl in 85% (v/v) acetonitrile in water at 2 mL/min.

Analysis of malto-oligosaccharides. — Quantitative data for the analysis of Karo-syrup components, illustrated in Fig. 1, are given in Table III. The total analysis required less than 1 nmol of benzoylated oligosaccharides. D-Glucose (263 pmol) comprises approximately one third of the total, maltose–maltohexose account for another half, and decreasing amounts of higher mol.-wt. oligosaccharides make up the remainder. Approximately 1 pmol of oligosaccharide having d.p. 12 was detected, whereas the oligosaccharide having d.p. 13, although present, did not give a peak that was integrated. The increase in sensitivity with size, already mentioned, is offset slightly by the isocratic elution, in absolute acetonitrile at the end of the gradient, of the high mol.-wt. oligosaccharides, and as a result the peaks are less sharp and integration is more difficult. Minor components seen between the major oligo-

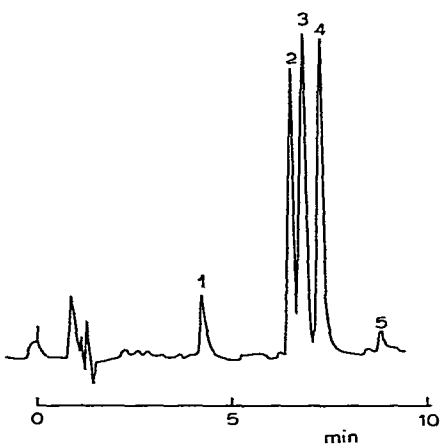

Fig. 12. Separation of reduced, benzoylated disaccharides on an Ultrasphere octyl column: (1) D-glucose; (2) kojibiose; (3) maltose; (4) nigerose; and (5) maltotriose. Ordinate: u.v. absorbance. An 8-min gradient of 85–100% (v/v) acetonitrile in water was used at a flow rate of 2 mL/min. Chart speed, 1 cm/min.

TABLE III

QUANTITATIVE DETERMINATION OF REDUCED MONO- AND OLIGO-SACCHARIDES OF KARO SYRUP[a]

D.p. of saccharide (peak)	*Retention time (s)*	*Number of benzoyl groups*	*Area ($\times 10^{-6}$)*	*Normalized area ($\times 10^{-6}$)*	*Amount (pmol)*	*Molar ratio (percent)*
1	377	6	0.616	1.129	263	35.5
2	598	9	0.280	0.342	80	10.8
3(a)	762	12	0.337	0.309	72	9.7
3(b)	798	12	0.018	0.017	4	0.5
4	868	15	0.412	0.302	70	9.4
5	944	18	0.504	0.308	72	9.7
6	999	21	0.571	0.299	70	9.4
7	1047	24	0.449	0.244	57	7.7
8	1096	27	0.315	0.128	30	4.0
9	1149	30	0.158	0.058	14	1.9
10	1209	33	0.066	0.022	5	0.7
11	1280	36	0.035	0.011	3	0.4
12	1364	39	0.019	0.005	1	0.2
13	1466	42	[b]		<1	

[a]These data were obtained from the analysis of Karo syrup shown in Fig. 1. The areas were normalized to 11 benzoyl groups, and the amount of each oligosaccharide was calculated from the response of a standard of raffinose (11 benzoyl groups; area, 4.3×10^3 per pmol). [b]No response of integrator.

saccharides may be isomalto oligosaccharides. Maltose and isomaltose could not be separated (see Table II), but maltotriose was well-separated from isomaltotriose (Fig. 11). We cannot completely exclude the possibility that these extra peaks are due to incomplete benzoylation, but the following evidence is against this interpretation: (a) benzoylation for both 1 and 16 h gave identical profiles; and (b) oligosaccharides of d.p. 10–15 gave symmetrical peaks with no shoulders on the trailing edge, as would be expected for underbenzoylation of oligosaccharides of this size.

Analysis of mannosidosis-urine oligosaccharides. — Patients with mannosidosis excrete a homologous series of oligosaccharides (see Fig. 13). Examination of the elution profile of reduced oligosaccharides (Fig. 2b) reveals that the oligosaccharide having d.p. 10 appears as a symmetrical peak, but those having d.p. 4–9 exhibit shoulders on the trailing edge, suggesting the presence of structural isomers that are partially separated. This is consistent with recent reports on the structure of the branched oligosaccharides in mannosidosis urine[16,25]; from acetolysis data, it was deduced that there were two isomers, each having d.p. 4, 5, and 6, and three isomers, each having d.p. 7, 8, and 9. The oligosaccharide having d.p. 10, however, was shown to be a single component.

Quantitative analysis of 10 μL of reduced oligosaccharides from mannosidosis urine, as shown in Fig. 2b, is given in Table IV. $Man_2GlcNAc$ (774 pmol) accounted for two-thirds of the total; proportions of the higher homologs decreased linearly down to only 3 pmol of $Man_9GlcNAc$. The relative abundances are in reasonable

TABLE IV

QUANTITATIVE DETERMINATION OF REDUCED OLIGOSACCHARIDES FROM MANNOSIDOSIS URINE[a]

Oligosaccharide fraction[b]	*D.p. of oligosaccharide (peak)*	*Retention time (s)*	*Number of benzoyl groups*	*Area ($\times 10^{-6}$)*	*Normalized area ($\times 10^{-6}$)*	*Amount (pmol)*	*Molar ratio (percent)*	
							This analysis	*Ref. 16*
$Man_2GlcNAc$	3(a)	617	11	3.329	3.329	774	65.1	66.5
GalNAc(Fuc)Gal	3(b)	670	10	0.275	0.303	70		
$Man_3GlcNAc$	4	808	14	1.313	1.032	240	20.2	16.4
$Man_4GlcNAc$	5	926	17	0.641	0.415	97	8.2	5.2
$Man_5GlcNAc$	6	1005	20	0.312	0.172	40	3.4	5.1
$Man_6GlcNAc$	7	1075	23	0.180	0.086	20	1.7	3.8
$Man_7GlcNAc$	8	1143	26	0.108	0.046	11	0.9	1.9
$Man_8GlcNAc$	9	1224	29	0.047	0.018	4	0.4	0.7
$Man_9GlcNAc$	10	1312	32	0.032	0.011	3	0.2	0.4

[a]The urine from a 31 year-old mannosidosis patient (blood group A, secretor) was desalted and derivatized with benzoic anhydride. The h.p.l.c. profile is given in Fig. 2B. The amount injected was equivalent to 10 μL of urine. The areas were normalized to 11 benzoyl groups, and the amount of each oligosaccharide was calculated from the response of a standard of raffinose (11 benzoyl groups; area, 4.3×10^3 per pmol). [b]The structures of these oligosaccharide fractions are given in Fig. 13.

Trisaccharide

αMan→3βMan→4GlcNAc

Tetrasaccharides

Major αMan→2αMan→3βMan→4GlcNAc

Minor αMan→3βMan→4GlcNAc
6
↑
αMan

Pentasaccharides

Major αMan→2αMan→2αMan→3βMan→4GlcNAc

Minor αMan→3βMan→4GlcNAc
6
↑
αMan→3αMan

Heptasaccharides

αMan→3βMan→4GlcNAc
6
↑
αMan→3αMan
6
↑
αMan→2αMan

αMan→3βMan→4GlcNAc
6
↑
αMan→2αMan→3αMan
6
↑
αMan

αMan→2αMan→3βMan→4GlcNAc
6
↑
αMan→2αMan→3αMan
6
↑
αMan

Nonasaccharides

αMan→2αMan→3βMan→4GlcNAc
6
↑
αMan→2αMan→3αMan
6
↑
αMan→2αMan

αMan→2αMan→2αMan→3βMan→4GlcNAc
6
↑
αMan→2αMan→3αMan
6
↑
αMan

αMan→2αMan→2αMan→3βMan→4GlcNAc
6
↑
αMan→3αMan
6
↑
αMan→2αMan

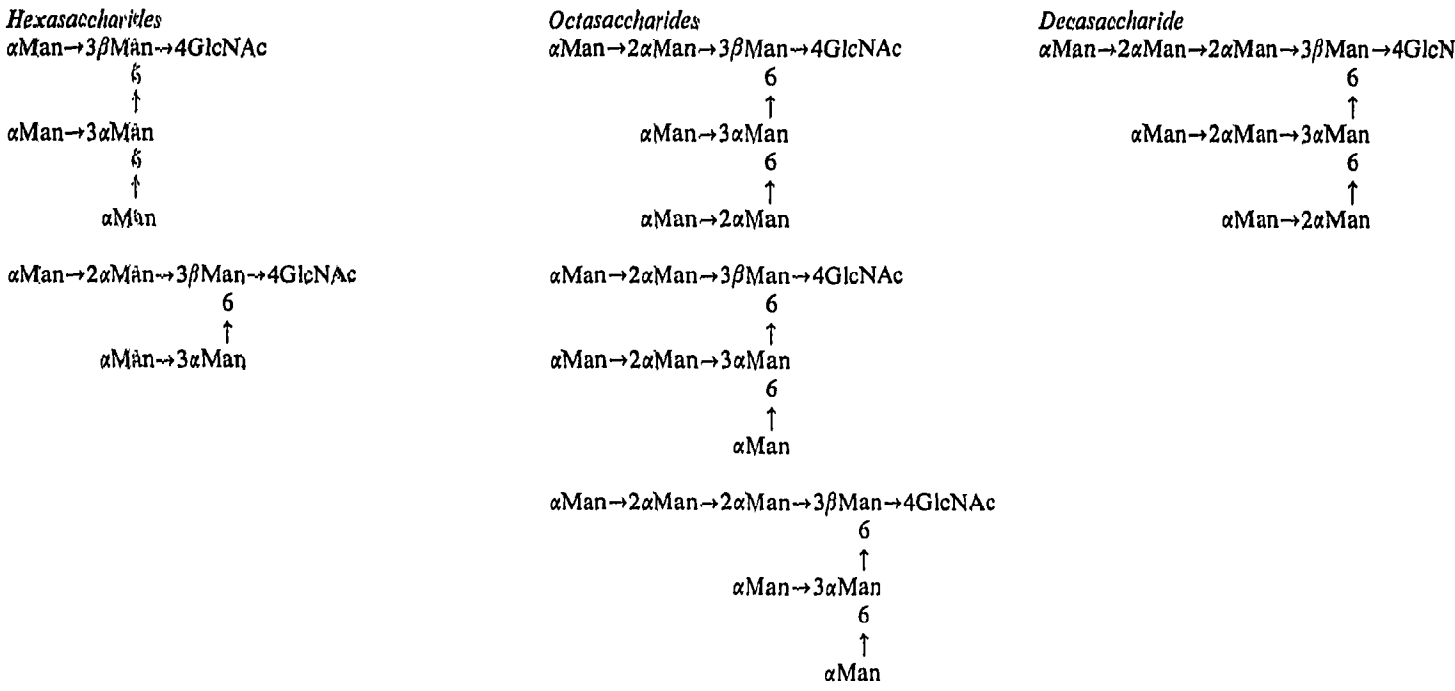

Fig. 13. Structures of oligosaccharides isolated from mannosidosis urine[16,25].

agreement with recent results obtained by borotritide reduction and conventional fractionation of urine from another mannosidosis patient[16]. We cannot say whether the observed differences are due to biochemical variation between the patients or to the different methodologies used.

CONCLUSIONS

Because of the rapid separation, extreme sensitivity, and ease of quantitative determination, the method described herein has considerable potential for the micro-analysis of oligosaccharides from biological sources. Since the detection is non-destructive, individual components can easily be collected for determination of radio-activity or for further analysis. Clearly, this degree of sensitivity is not really necessary for analysis of the large amounts of oligosaccharides found in urines from patients with glycoprotein or glycolipid storage diseases, although it is certainly convenient. Nevertheless, h.p.l.c. of perbenzoylated oligosaccharides from mannosidosis urine facilitates the quantitative determination of minor components that are undetectable by h.p.l.c. of the original oligosaccharides[18]. This analytical procedure has enabled us to determine quantitatively the low level of $Man_2GlcNAc$ (0.1–0.4 nmol/mL) found in the serum of a patient suffering from mannosidosis[19] and could be used to monitor the level of oligosaccharides in the serum of patients having other storage diseases.

The potential clinical uses include the analysis of cerebrospinal and amniotic fluids, and the examination of the stored material in cultured skin-fibroblasts and tissue biopsies from patients suffering from disorders of carbohydrate metabolism. The procedure may be applied to the rapid isolation of intermediates involved in glycoprotein processing and their acetolysis products. It could also be used to study changes in the level of "high-mannose" glycoproteins with the state of growth or differentiation of cells[20,21].

Accounts of the reversed-phase separation of permethylated[22] and peracetylated[4] oligosaccharides on C-18 columns have appeared. However, until reliable h.p.l.c.–mass-spectrometer interfaces become more generally available, perbenzoylation is clearly the method of choice where the highest-possible sensitivity is desired.

EXPERIMENTAL

Preparation of derivatives. — (*a*) *With benzoic anhydride.* The samples containing <1 μmol of total sugars in a screw-cap tube (13 × 100 mm) were dried *in vacuo* in the presence of phosphorus pentaoxide and benzoylated for up to 4 h at 37° with pyridine (0.5 mL) containing 10% of benzoic anhydride (Sigma Chemical Co., St. Louis, MO 63178) and 5% 4-dimethylaminopyridine (Sigma) as catalyst. The samples containing 1 nmol or less of total sugars were benzoylated, in silanized tubes, with 100 μL of reagent. The excess reagents were removed by a modification of the method of Daniel[23]. Following benzoylation, each sample was diluted with water

(4.5 mL), shaken in a Vortex, and applied to a column of C-18 Sep-pak (Waters Associates, Milford, MA 01757). The eluate was collected in the original tube and reapplied twice to the Sep-pak to ensure maximal binding. The column of Sep-pak was then washed with 10% (v/v) aqueous pyridine (10 mL), followed by distilled water (5 mL). The benzoylated oligosaccharides were eluted with acetonitrile (15 mL) into a 50-mL, round-bottomed flask and evaporated by rotary evaporation. Each column of Sep-pak was re-used up to five times.

(*b*) *With benzoyl chloride.* The oligosaccharides were benzoylated with 10% (v/v) benzoyl chloride (Eastman Kodak Co., Rochester, NY 14650) in pyridine (0.5 mL) for 16 h at 37°. The benzoylated products were separated from the excess of reagents by solvent partition as previously described[23].

High-performance, liquid chromatography. — (*a*) *By reversed phase.* Benzoylated samples were dissolved in a specific volume of acetonitrile and aliquots (20 μl) were injected onto a prepacked column (4.6 mm i.d. × 25 cm) of Ultrasphere octyl by means of an Altex 905–42 injector (Altex Scientific Inc., Berkeley, CA 94710) having a 20-μL injection loop. A linear gradient of acetonitrile in water was established with an Altex model 420 flow controller and two model 110 A pumps. The output at 230 nm from a variable–wavelength u.v. detector (Model SF770; Schoeffel Instruments Div., Kratos Inc., Westwood, NJ 07675) was connected in series to an Autolabs system 1 computing-integrator (Spectra-Physics Inc., Autolab Div., Santa Clara, CA 95051) and a strip-chart recorder. A gradient from 80% (v/v) acetonitrile–water to pure acetonitrile flowing for 15 min at a rate of 2 mL/min was used routinely at a sensitivity of 0.16 absorption units full scale. The packed columns and Burdick and Jackson h.p.l.c.-grade solvents were obtained from Rainin Instruments Co. Inc., (Woburn, MA 01801).

(*b*) *By adsorption.* The samples for adsorption chromatography were dissolved in carbon tetrachloride and injected onto a column (2.1 mm i.d. × 50 cm) packed with either Zipax pellicular (DuPont Instruments, Wilmington, DE 19898) or Si-4000 (E. Merck, D-61 Darmstadt, Germany) silica gel. A linear gradient of 1,4-dioxane in hexane at a flow rate of 2 mL/min was generated with a Waters model 660 solvent programmer and model 6000 pumps. The residual adsorption at 230 nm due to 1,4-dioxane was eliminated by passing the solvent gradient through a pre-column, high-pressure reference cell as previously described[7].

Sources of oligosaccharides. — Commercially available di- and tri-saccharides were obtained from Sigma Chemical Co. Kojibiose and nigerose octaacetate were provided by Drs. K. Matsuda and I. Goldstein, respectively; nigerose was *O*-deacetylated by barium methoxide in methanol. Except where indicated, all oligosaccharides were reduced with sodium borohydride prior to benzoylation. Light Karo corn syrup was precipitated with aqueous ethanol (85%, v/v) to obtain a fraction that was enriched in higher mol.-wt. oligomers. The ethanol–precipitation step was repeated, and a portion of the precipitate was reduced with sodium borohydride. This preparation of Karo syrup contained measureable amounts of malto-oligosaccharides having d.p. 1–13, plus traces of higher-mol.-wt. oligomers. The urine from a patient with

mannosidosis was desalted with a mixed-bed resin, further purified by adsorption chromatography on charcoal–Celite, and then subjected to a second ion-exchange chromatography to obtain a neutral oligosaccharide fraction as previously described[24]. The charcoal–Celite step was shown to be an essential part of the procedure, as very low yields of benzoylated oligosaccharides were obtained when it was omitted, probably owing to consumption of the reagent by nonsaccharide components of the urine.

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REFERENCES

1 F. SCHMIDT AND B. S. ENEVOLDSEN, *Carbohydr. Res.*, 61 (1978) 197–209.
2 M. NATOWICZ AND J. U. BAENZIGER, *Anal. Biochem.*, 105 (1980) 159–164.
3 M. D'AMBOISE, N. NOËL, AND T. HANAI, *Carbohydr. Res.*, 79 (1980) 1–10.
4 G. B. WELLS AND R. L. LESTER, *Anal. Biochem.*, 97 (1979) 184–190.
5 E. K. GUM, JR. AND R. D. BROWN, JR., *Anal. Biochem.*, 82 (1977) 372–375.
6 M. D. ULLMAN AND R. H. MCCLUER, *J. Lipid Res.*, 18 (1977) 371–378.
7 M. D. ULLMAN AND R. H. MCCLUER, *J. Lipid Res.*, 19 (1978) 910–913.
8 E. G. BREMER, S. K. GROSS, AND R. H. MCCLUER, *J. Lipid Res.*, 20 (1979) 1028–1035.
9 P. F. DANIEL, D. F. DEFEUDIS, I. T. LOTT, AND R. H. MCCLUER, *Proc. Int. Symp. Glycoconjugates, 5th*, (1979) 120–121.
10 P. F. DANIEL, I. T. LOTT, AND R. H. MCCLUER, in G. L. HAWK (Ed.), *Biological/Biomedical Applications of Liquid Chromatography III*, Marcel Dekker, New York, 1981.
11 P. F. DANIEL, *Trans. Am. Soc. Neurochem.*, 11 (1980) 250.
12 S. K. GROSS AND R. H. MCCLUER, *Anal. Biochem.*, 102 (1980) 429–433.
13 F. NACHTMANN, *Z. Anal. Chem.*, 282 (1976) 209–213.
14 N. E. NORDEN, A. LUNDBLAD, S. SVENSSON, P.-A. OCKERMAN, AND S. AUTIO, *J. Biol. Chem.*, 248 (1973) 6210–6215.
15 N. E. NORDEN, A. LUNDBLAD, S. SVENSSON, AND S. AUTIO, *Biochemistry*, 13 (1974) 871–874.
16 K. YAMASHITA, Y. TACHIBANA, K. MIHARA, S. OKADA, H. YABUUCHI, AND A. KOBATA, *J. Biol. Chem.*, 255 (1980) 5126–5133.
17 C. D. WARREN, R. W. JEANLOZ, AND G. STRECKER, *Carbohydr. Res.*, 92 (1981) 85–101.
18 N. M. K. NG YING KIN AND L. S. WOLFE, *Anal. Biochem.*, 102 (1980) 213–219.
19 I. T. LOTT AND P. F. DANIEL, *Neurology*, in press.
20 T. MURAMATSU, N. KOIDE, C. CECCARINI, AND P. H. ATKINSON, *J. Biol. Chem.*, 251 (1976) 4673–4679.
21 N. KOIDE, T. MURAMATSU, AND A. KOBATA, *J. Biochem. (Tokyo)*, 85 (1979) 149–155.
22 B. S. VALENT, A. G. DARVILL, M. MCNEIL, B. K. ROBERTSEN, AND P. ALBERSHEIM, *Carbohydr. Res.*, 79 (1980) 165–192.
23 P. F. DANIEL, *J. Chromatogr.*, 176 (1979) 260–263.
24 G. STRECKER AND A. LEMAIRE-POITAU, *J. Chromatogr.*, 143 (1977) 553–569.
25 F. MATSUURA, H. A. NUNEZ, G. A. GRABOWSKI, AND C. C. SWEELEY, *Arch. Biochem. Biophys.*, 207 (1981) 337–352.