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LIQUID ION-EXCHANGE CHROMATOGRAPHY UNDER PRESSURE OF MILK OLIGOSACCHARIDES USING A PULSED AMPEROMETRIC DETECTOR

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ABSTRACT

Several oligosaccharides from human milk were separated completely on a Dionex AS6 ion-exchange column under mild alkaline conditions, such that no degradation of oligosaccharides was detectable after incubation for 6 h at room temperature in the eluent buffer. In general, both the presence of fucosyl groups and branching within oligosaccharide chains tend to reduce retention times for oligosaccharides in this system. Thus, both lacto-*N*-fucopentaose II {β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc} and lacto-*N*-fucopentaose III {β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc} were eluted before lacto-*N*-fucopentaose I [α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc], and all three fucopentaoses were eluted earlier than lacto-*N*-tetraose [β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc]. 2′-Fucosyllactose [α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-D-Glc] and 3-fucosyllactose {α-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc} were separated completely under a wide range of conditions. Introduction of substituents at either the O-3 or O-4 of 2-acetamido-2-deoxy-D-glucose produced large differences in retention: lacto-*N*-tetraose and lacto-*N*-neotetraose [β-D-Gal-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc] could be separated by more than 8 min. The l.c. ion-exchange chromatographic system described represents a useful addition to existing methods for separating oligosaccharides derived from glycoproteins and glycolipids.

INTRODUCTION

Human milk is a rich source of oligosaccharides, many of which are identical to the sugar chains found in glycoproteins or glycolipids. Thus, milk oligosaccharides are widely used in studies of the specificities of glycosyltransferases, glycosidases, lectins, and antibodies involved in biosynthesis and recognition of secreted or membrane-bound glycoconjugates[1–8]. Oligosaccharides from human milk have been isolated by gel filtration, ion exchange, and paper chromato-

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graphy[9,10]. Several methods that utilize liquid chromatography under pressure (l.c.) have proved faster and more convenient than paper chromatography for separation of underivatized milk oligosaccharides, but these methods have produced few improvements in chromatographic resolution of sugar chains having similar structures[11–14]. Early in the 1960's, Austin *et al.*[15] used a hydroxide form of a strong base anion-exchange resin to separate isomers of glycosides with water as a solvent. Later, Rocklin and Pohl[16] demonstrated that carbohydrates can be separated with ion-exchange columns under alkaline conditions and can be detected by pulsed amperometry. The present work describes an improved l.c. separation of human milk oligosaccharides using the Dionex ion-exchange column AS6 and a pulsed amperometric detector (PAD). The results indicate that this chromatographic system offers superior separation characteristics for many oligosaccharides when compared with methods previously available, as well as simple and highly sensitive detection of complex sugars.

RESULTS

Fig. 1 shows a typical chromatographic profile for the major milk oligosaccharides containing two to six simple sugars (see Scheme 1 for abbreviations, structures, and numbering of oligosaccharides). Individual oligosaccharides migrated

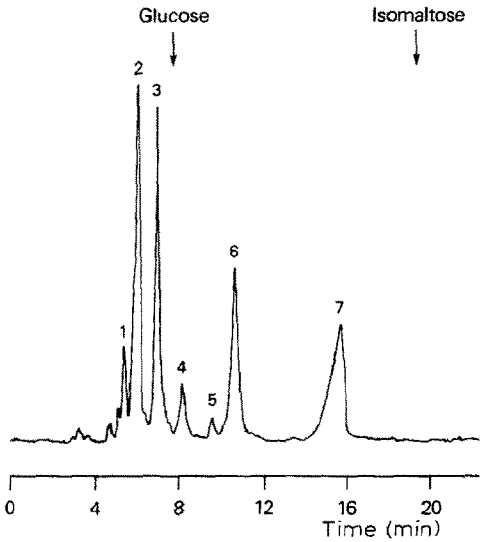

Fig. 1. L.c. separation of a mixture of neutral oligosaccharides isolated by gel filtation of milk from a Le(a−b+) donor (see ref. 9). A sample containing a total of 50 μg material was injected in distilled water (50 μL) and eluted with 30mM NaOH plus 25mM sodium acetate at 0.5 mL/min. The column eluate was mixed with 0.3M NaOH added at 0.5 mL/min before passage through a pulsed amperometric detector: Peak 1, LND I (**11**); peak 2, LNF II (**9**), plus LNF III (**10**); peak 3, 3FL (**5**) plus LNnT (**7**); peak 4, LNF I (**8**); peak 5, Lac (**3**); peak 6, 2'FL (**4**); and peak 7, LNT (**6**).

α-D-Glc*p*-(1→6)-D-Glc
Isomaltose (1M2) (**1**)

D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Glc
Isomaltotriose (IM3) (**2**)

β-D-Gal*p*-(1→4)-D-Glc
Lactose (Lac) (**3**)

α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-D-Glc
2′-Fucosyllactose (2′FL) (**4**)

β-D-Gal*p*-(1→4)-D-Glc
3
↑
1
α-L-Fuc*p*
3-Fucosyllactose (3FL) (**5**)

β-D-Gal*p*-(1→3)-β-D-Glc*p*-NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
Lacto-*N*-tetraose (LNT) (**6**)

β-D-Gal*p*-(1→4)-β-D-Glc*p*-NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
Lacto-*N*-neotetraose (LNnT) (**7**)

α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*-NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
Lacto-*N*-fucopentaose I (LNF I) (**8**)

β-D-Gal*p*-(1→3)-β-D-Glc*p*-NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
4
↑
1
α-L-Fuc*p*
Lacto-*N*-fucopentaose II (LNF II) (**9**)

β-D-Gal*p*-(1→4)-β-D-Glc*p*-NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
3
↑
1
α-L-Fuc*p*
Lacto-*N*-fucopentaose III (LNF III) (**10**)

α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*-NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
4
↑
1
α-L-Fuc*p*
Lacto-*N*-difucohexaose I (LND I) (**11**)

α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc
DimannosylGlcNAc ($Man_2GlcNAc$) (**12**)

α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc
Oligosaccharide from ceramide trihexoside (CTH) (**13**)

β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc
6
↑
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*
Oligosaccharide from asialotransferrin (**14**)

Scheme 1. Structures of oligosaccharides.

TABLE I

RETENTION VALUES OF SOME MONO- AND OLIGO-SACCHARIDES

Sugar	*Retention time*[a] *(min)*	*k'*	*Glucose unit*	*Glucose unit on Bio-Gel P-4*[b]
Glucose	7.69	1.56	1.00	1.00
Isomaltose (**1**)	19.28	5.43	2.00	2.00
Isomaltotriose (**2**)	43.58	13.5	3.00	3.00
LND I (**11**)	5.18	0.73	0.465	
LNF II (**9**), alditol	5.91	0.970	0.620	
LNF III (**10**)	5.92	0.973	0.623	6.3
LNF II (**9**)	6.00	1.00	0.640	6.23
LNnT (**7**)	6.80	1.27	0.810	5.64
3FL (**5**)	6.92	1.31	0.836	3.33
LNF I (**8**)	8.20	1.73	1.11	6.64
Lac (**3**)	9.62	2.20	1.17	2.38
2'FL (**4**)	10.63	2.56	1.25	3.36
LNT (**6**)	15.58	4.19	1.68	5.64

[a]The l.c. conditions were the same as in the legend to Fig. 1. The dead volume was 3.00 min (*k'* 0).
[b]Data from ref. 24. Sugars were in their alditol form.

as single peaks. Splitting of reducing oligosaccharides into α and β anomers, as occurs in most reported l.c. systems[11–13], was not observed. Relative retentions for several human milk oligosaccharides on AS6 medium are shown in Table I. Separation of oligosaccharides in this system is affected by several parameters. Retention values tend to increase with addition of simple sugars to form larger structures[16], as demonstrated by separation of members of the isomaltosyl series containing more than 40 glucosyl units (Fig. 2). This trend also is evident for some milk oligosaccharides; for example, retention increased in the order lactose (Lac, **3**) < 2'-fucosyllactose (2'FL, **4**) < lacto-*N*-tetraose (LNT, **6**). However, retention may be profoundly influenced by at least three other factors.

Firstly, linear oligosaccharides have larger retention values than branched oligosaccharides of similar structure and identical composition. For example, lacto-*N*-fucopentaoses II and III [LNF II (**9**) and LNF III (**10**)] were eluted earlier than their linear isomer, lacto-*N*-fucopentaose I (LNF I, **8**). Likewise, 3- (3FL, **5**) was eluted well before 2'-fucosyllactose (2'FL, **4**).

Secondly, oligosaccharides that contain L-fucose have much lower retention values than oligosaccharides of similar structure and composition that lack L-fucose. For example, the pentasaccharides LNF II (**9**), LNF III (**10**), and LNF I (**8**) all were eluted earlier than the tetrasaccharide LNT (**6**), despite their larger size. Similarly, 3FL (**5**) was eluted earlier than Lac (**3**). The same effect was observed for lacto-*N*-difucohexaose I (LND I, **11**), which has the same structure as LNF II (**9**) except for an additional L-fucosyl group and was eluted earlier than the pentasaccharides.

Finally, linkage positions within oligosaccharide chains may exert strong

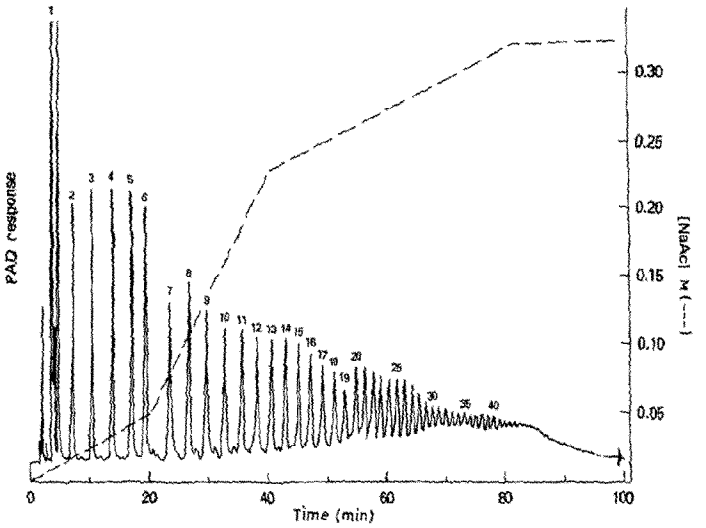

Fig. 2. L.c. separation of isomaltosyl oligodextrins derived from partial acid hydrolysis of Dextran D9206. A sample (2.5 mg) of hydrolysis products dissolved in distilled water (50 μL) was injected and eluted at 1.0 mL/min with 0.15M NaOH, with sodium acetate added in the gradient shown.

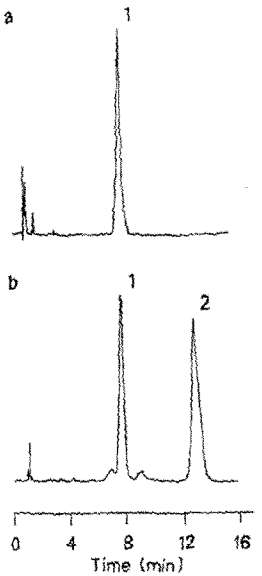

Fig. 3. L.c. separation of LNT (6) and LNnT (7): (a) A sample containing a total of 50 μg authentic LNT (6) was injected in distilled water (50 μL) and eluted with 0.15M NaOH at 0.8 mL/min. (b) A sample containing 100 μg of a mixture of LNT (6) plus LNnT (7) was analyzed as described in (a): Peak 1, LNT (6); peak 2, LNnT (7).

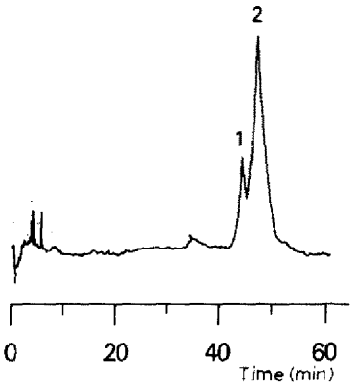

Fig. 4. L.c. separation of LNF II (**9**) and LNF III (**10**). A sample containing 5 μg of material was injected in distilled water (50 μL) and eluted with 30mM NaOH at 0.5 mL/min: Peak 1, LNF III (**10**); peak 2, LNF II (**9**).

influence on retention. For example, as shown in Fig. 3, two tetrasaccharides, lacto-*N*-neotetraose (LNnT, **7**) and lacto-*N*-tetraose (LNT, **6**), that differ only in that nonreducing terminal galactosyl groups are linked to O-4 and O-3, respectively, were separated with a resolution factor as high as 4.3.

Separation of oligosaccharides having identical composition and branching pattern sometimes could be achieved by relatively minor changes in pH or ionic strength (or both) of the eluting buffer. Under conditions shown in Fig. 1, LNF II (**9**) and LNF III (**10**) migrated with almost identical retention values. At lower ionic strength, these isomers were partially resolved (Fig. 4).

The stability of selected milk oligosaccharides at room temperature (25°)

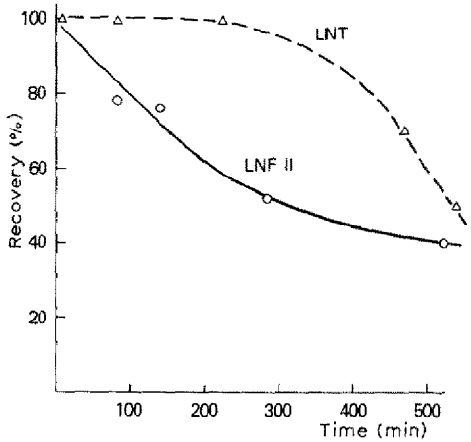

Fig. 5. Recoveries of LNF II (**9**) and LNT (**6**) after incubation in 0.15M NaOH at 25°. A total of 50 μg of each oligosaccharide was dissolved in 0.15M NaOH (500 μL) and incubated with constant stirring at room temperature. Samples (50 μL) were withdrawn from the mixture at the times indicated and analyzed immediated under the conditions described in the legend to Fig. 1: (—○—○—) LNF II (**9**), (--△--△--) LNT (**6**).

TABLE II

MOLECULAR RATIOS OF MONOSACCHARIDES DETERMINED BY L.C. AFTER HYDROLYSIS OF OLIGOSACCHARIDES AND GLYCOCONJUGATES[a]

Oligosaccharide or glycoconjugate	*Molecular ratio*				
	Fuc	*GlcN*	*Gal*	*Glc*	*Man*
3FL (**5**)	1.00		1.02	0.98	
LNF II (**9**)	1.00	1.06	2.10		
LNT (**6**)		0.94	2.02	1.00	
$Man_2GlcNAc$ (**12**)		1.00			1.89
CTH (sugar chain) (**13**)			2.00	0.82	
Asialotransferrin (sugar chain) (**14**)		4.00	1.80		2.70

[a]Conditions for hydrolysis and l.c. analysis are given in the text and in legend to Fig. 6. Data are from Figs. 6 and 7.

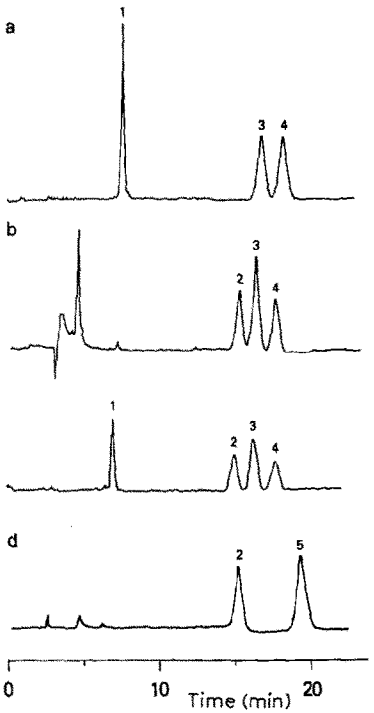

Fig. 6. L.c. analysis of the monosaccharide compositions of oligosaccharide hydrolyzates. Each hydrolyzate, prepared as described in the Experimental section, was dissolved in distilled water, and a sample (50 μL) containing ~100 pmol was injected and eluted with 17.5mM NaOH at 0.8 mL/min: (a) 3FL (**5**); (b) LNT (**6**); (c) LNF II (**9**); and (d) $Man_2GlcNAc$ (**12**). Peak 1, L-fucose; peak 2, 2-amino-2-deoxy-D-glucose; peak 3, D-galactose; peak 4, D-glucose; and peak 5, D-mannose.

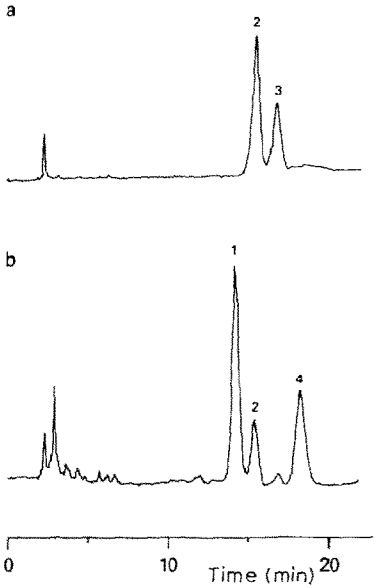

Fig. 7. L.c. analysis of the monosaccharide compositions of trihexaosyl ceramide (a) and asialo-transferrin (b). Each hydrolyzates, prepared as described in the Experimental section, was dissolved in distilled water, and a sample (50 μL) was injected and eluted with 17.5mM NaOH at 0.8 mL/min: Peak 1, 2-amino-2-D-deoxyglucose; peak 2, D-galactose; peak 3, D-glucose; and peak 4, D-mannose.

under the alkaline conditions used for chromatography on the AS6 column was determined by analyzing aliquots of purified oligosaccharides dissolved in 0.15 or 0.03M NaOH after various periods of incubation. Most milk oligosaccharides undergo at least 50% alkaline degradation in 0.15M NaOH at room temperature after 500 min, but the rates of degradation may vary considerably, as illustrated for LNT (**6**) and LNF II (**9**) in Fig. 5. On the other hand, no significant degradation of LNT (**6**), LNF II (**9**), 3FL (**5**), or 2'FL (**4**) was observed after 500 min in 0.03M NaOH.

The same column used for separation of oligosaccharides also can be used for analysis of monosaccharide composition of oligosaccharides, either as free compounds or as sugar chains covalently linked to glycosphingolipids or glycoproteins (Table II). The complex sugars are hydrolyzed in 5M trifluoroacetic acid for 4 h at 100°, and after evaporation of the acid, the products of hydrolysis are dissolved in distilled water and injected underivatized onto the AS6 column. As shown in Figs. 6 and 7 and in Table II, the molar ratios of monosaccharides derived from authentic standards are generally within 10% of the theoretical values. Hexoses, deoxyhexoses, and amino sugars give essentially identical molar responses. No interfering products from ceramides or amino acids were detected with PAD. As little as 10 pmol of each monosaccharide could be detected with this system.

DISCUSSION

The growing number of investigations into the structures and functions of oligosaccharides released by chemical[17,18] or enzymic[19,20] methods from glycoproteins or glycolipids would benefit from improved methodologies for separating and detecting complex sugars. The present method may be especially useful as a complement to gel filtration chromatography, which separates oligosaccharides mostly according to molecular weight (see Table I).

The physical basis for separation in the present system, while not completely understood, appears to be dependent mainly on the overall acidity of oligosaccharide molecules. As p*Ka* values of carbohydrates tend to fall into the range of 12–14, these molecules are likely to undergo ionization in the presence of >0.01M sodium hydroxide. According to Rendleman[21], the relatively high acidity of reducing sugars is due to the high lability of the hydrogen atom of the hemiacetal (anomeric) hydroxyl group, a condition resulting from inductive effects of the ring oxygen atom. A higher retention value for LNF II (**9**) than for its alditol derivative (Table I) may be thus explained. As seen from the data in this paper and in a previous report[16], a major benefit of the AS6 ion-exchange system is that peaks containing reducing sugars are not split into α and β anomers. Thus, the effects of anomerity *per se* on overall acidity seem to be relatively minor. Inductive effects of the ring oxygen atom also have been invoked to explain the observation that, for methyl glycosides, OH-2 of the sugar ring is the most acidic group. The remaining ring hydroxyl groups decrease in acidity in the following order[22]: OH-2 $\gg$ OH-6 > OH-3 > OH-4.

Whatever the origins for variability in pK values of ring hydroxyl groups, their effects are dramatic when structurally diverse oligosaccharides are chromatographed on ion-exchange media in water containing > 0.01M sodium hydroxide. Chromatography of the isomaltosyl series of oligodextrins in this system gave a separation profile similar to that obtained in other systems that separate oligosaccharides according to molecular weight[10] or the total number of ring hydroxyl groups[23]. Resolution of successive oligomers containing more than 40 glucosyl residues probably reflects a stepwise proportional increase in overall acidity with each added set of electrostatistically identical hydroxyl groups in a homologous series. In contrast, chromatography of various isomeric milk oligosaccharides having identical molecular weights and sugar compositions [*e.g.*, LNT (**6**) and LNnT (**7**); LNF I (**8**) and LNF II (**9**); and 2′FL (**4**) and 3FL (**5**)] showed wide variations in relative retention values (Table I). The p*Ka* values are not known for all the ring hydroxyl groups of oligosaccharides, but, as noted above, it appears that they may vary enough that overall acidity of the molecules could be profoundly reduced by, for example, removal of a hydrogen atom at a highly acidic hydroxyl group and replacement at the same site with a glycosidically linked sugar ring that itself contains no highly acidic ring hydroxyl group. Introduction of a deoxysugar such as fucose, which lacks OH-6, might exaggerate this effect. Combined inductive

effects created by introducing ring substituents, such as of the acetamido group at C-2 of GlcNAc and glycosidic oxygen atoms at various positions on sugar rings, probably exert large differential effects on the acidities of the proximate unsubstituted ring hydroxyl groups. While these effects are likely to form the basis for the large differences in retention values for LNT (**6**), LNnT (**7**), and their fucosylated derivatives **8** and **9**, speculation as to the roles of individual hydroxyl groups is premature. The use of this l.c. system to systematically explore effects of sugar ring substituents on overall acidity of oligosaccharides might prove beneficial.

The order of elution of milk oligosaccharides on some reverse-phase l.c. (r.p.l.c.) columns[11–13] is similar to that observed on AS6, although separation on the latter is much superior. It is widely accepted that separation on r.p.l.c. is based on relative hydrophobicities, but unexpectedly early elution of fucose-containing and branched carbohydrate chains is not consistent with this interpretation. In other r.p.l.c. systems, introduction of fucosyl residues tend to retard elution of oligosaccharides[24]. In light of the present data, it would be interesting to explore r.p.l.c. of oligosaccharides more thoroughly to determine whether overall acidity of the molecules may sometimes play a role in their interaction with reverse-phase columns.

Although the preparative capacity of the AS6 analytical column has not been explored intensively yet, a scaled-up system might offer a distinct advantage over other l.c. systems, such as normal phase silica where amine-containing solvents are used or primary amino-bonded silicas, both of which have the potential for unwanted Schiff base formation[22].

EXPERIMENTAL

Oligosaccharides. — Oligosaccharides from human milk were purified according to the procedure described by Kobata and assoc.[9,10]. Partial acid hydrolysis of Dextran D9206, average M_r of 9400 (Sigma Chemical Co.), was carried out in 0.1M HCl for 2 h at 100°. Isomaltose and isomaltotriose were isolated from the partial hydrolyzate by gel filtration on Bio-Gel P-4 using water containing 0.02% of sodium azide as eluent[25]. Asialotransferrin was prepared by desialylation of transferrin[26] (Sigma). Ceramide trihexoside (**13**), purified from sheep erythrocyte stroma, was kindly provided by Dr. Carl Alving. α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc (**12**) from urine of human patients with mannosidosis was a gift from Dr. Arne Lundblad. All samples were dissolved in de-ionized water prior to injection on l.c.

Chromatography. — Separations were carried out on a Dionex Series 4000i HPLC system with a Dionex Ion Pac Column HPLC AS6 (Dionex Corp.) in series with a Guard Column HPIC AG6. The system is equipped with a pulsed amperometric detector containing a gold electrode to which potentials of E_1 0.1, E_2 0.6, and E_3 −0.8 *V* were applied for durations times T_1 300, T_2 120, and T_3 300 ms. A Dionex pump model DQP-1 was used for post-column addition of 0.3M NaOH. A

Li-Chroma-Damp III pulse dampener and 45 m of 0.175-mm i.d. plastic tube were placed in series between the pump and detector to minimize hydraulic pulse. A 50% (w/w) solution of NaOH (Fisher Sci. Co.) and sodium acetate (Mallinkrodt Science Prods. Div.) was used to formulate eluents in water purified by passage through a Milli Q purification system (Millipore Corp.). All sovents were degassed under reduced pressure and stored in closed, pressurized vessels with He sparging.

Trifluoroacetic acid hydrolysis. — Samples (0.1 μg) of oligosaccharides were hydrolyzed with 5M trifluoroacetic acid (200 μL) for 4 h at 100°. Hydrolyzates were dried under a stream of dry N_2 in a heating block maintained at 37° (Pierce Chem. Co.). Samples were redissolved in de-ionized water and analyzed.

Preparative l.c. — To recover oligosaccharides for preparative purposes, fractions (1 mL) were collected into tubes containing the same volume of 0.15M acetic acid, and pooled fractions were desalted either by gel filtration through Bio-Gel P-4, or by mixed-bed, ion-exchange on AG 50W-X4 (H^+) plus AG 1-X80 (OH^-) resins.

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