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Hydrophilic-interaction chromatography of complex carbohydrates

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Abstract

Complex carbohydrates can frequently be separated using hydrophilic-interaction chromatography (HILIC). The mechanism was investigated using small oligosaccharides and a new column, PolyGLYCOPLEX. Some carbohydrates exhibited anomer separation, which made it possible to determine the orientation of the reducing end relative to the stationary phase. Amide sugars were consistently good contact regions. Relative to amide sugars, sialic acids and neutral hexoses were better contact regions at lower levels of organic solvents than at higher levels. HILIC readily resolved carbohydrates differing in residue composition and position of linkage.

Complex carbohydrate mixtures could be resolved using volatile mobile phases. This was evaluated with native glycans and with glycans derivatized with 2-aminopyridine or a nitrobenzene derivative. Both asialo- and sialylated glycans could be resolved using the same set of conditions. With derivatized carbohydrates, detection was possible at the picomole level by UV detection or on-line electrospray mass spectrometry. Selectivity compared favorably with that of other modes of HPLC. HILIC is promising for a variety of analytical and preparative applications.

1. Introduction

Many proteins and lipids of higher organisms are glycosylated. Recently, interest in the free carbohydrate chains has mounted with the discovery that they can be involved in very specific interactions with receptor proteins (e.g., lectins) and intact cells. This can control cell adhesion, which is involved in such phenomena as inflammation or microbial activity [1–3].

The carbohydrate moieties of mammalian proteins and lipids can be complex. Typically, they are composed of the following sugars: the amide sugars N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc); the neutral hexoses glucose (Glc) (rare in mammalian glycoproteins), galactose (Gal), mannose (Man) and fucose (Fuc); and sialic acids (of which over 30 are known from natural sources [1]). The method of linkage can vary in three respects: (1) linkage via the α- or β-anomer; (2) position of linkage ($1\rightarrow6$; $2\rightarrow3$; $1\rightarrow4$; etc.); (3) branching

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(i.e., linkage of more than one sugar to a residue). All of these factors are important in determining the biological activity of a complex carbohydrate. Chromatographic methods for this class of solutes should be sensitive to these differences.

Various methods have been used for chromatography of complex carbohydrates. Some of the more widely used methods are as follows:

Anion-exchange chromatography (conventional) [4]. This works only with carbohydrates containing acidic residues (e.g., sialic acid or phosphate groups).

Reversed-phase (RP) HPLC. Usually this involves attachment of a hydrophobic chromophore or fluorophore to the reducing end by reductive amination; derivatives of 2-aminopyridine (PA) are increasingly being used for this purpose [5,6]. The eluting carbohydrates are easy to detect using conventional HPLC detectors, and can be analyzed by mass spectrometry (MS). Recently, RP-HPLC of underivatized carbohydrates has been performed with a Hypercarb S column [7].

Size-exclusion chromatography. This mode is useful in combination with other modes [8].

High-performance anion-exchange chromatography (HPAEC). This subset of anion exchange was developed by Hardy and Townsend [9,10]. The mobile phases contain 0.1 *M* NaOH; sugar hydroxyl groups ionize at the high pH and adsorb to anion-exchange resins, from which they can be eluted with a salt gradient (e.g., sodium acetate). HPAEC has excellent selectivity for linkage and composition variations in complex carbohydrates. Upon elution, carbohydrates can be detected at the picomole level with pulsed amperometric detection (PAD). The salts in the mobile phase are sometimes inconvenient when other detection methods are used, or in preparative applications.

Hydrophilic-interaction chromatography (HILIC). This features a "normal-phase" combination of a hydrophilic stationary phase and a hydrophobic (mostly organic) mobile phase [11]. A partition mechanism is probably involved [11–14] (rather than "hydrogen bonding", as stated in some reports). Elution is in order of least to most polar. Amino-silica columns, widely used for analysis of neutral sugars, have also been evaluated with complex carbohydrates [15,16]. Elution of sialylated oligosaccharides from these columns requires a significant level of salt in the mobile phase [17], reflecting the superimposition of electrostatic attraction on the hydrophilic interaction. Neutral materials used in this mode include TSKgel Amide-80, which has been used in two-dimensional HPLC of PA-derivatized carbohydrates along with RP-HPLC [3,18], and GlycoPAK N, which has been used with native glycans [19].

HILIC is a rational mode to develop for carbohydrates, which are polar solutes. Earlier, a neutral material, poly(2-hydroxyethyl aspartamide)-silica, was developed for general HILIC of polar solutes [11]. This material works well with glycopeptides and homologous oligomers of carbohydrates, but retention of complex carbohydrates is inadequate. Accordingly, a new material, PolyGLYCOPLEX, has been developed for such applications. This material was used here to study the utility of HILIC for topical carbohydrate separations, and to explore the mechanisms governing selectivity in this mode.

2. Materials and methods

2.1. Reagents

The complex carbohydrate library from native bovine fetuin was a gift of Dr. Rao Thotakura (NIH, Bethesda, MD, USA). It was obtained via hydrazinolysis with a GlycoPrep 1000 system from Oxford GlycoSystems (Abingdon, UK).

Biantennary oligosaccharides (Figs. 7 and 8) were released enzymatically from human apotransferrin (Sigma, St. Louis, MO, USA) using PNGase F. Reductive amination with 2-aminopyridine was performed using borane–dimethylamine complex, as per the improved method of Hase et al. [20]. The products were purified using column chromatography and characterized using LDMS, electrospray MS, and 300 MHz ^{1}H NMR.

The GPI anchor glycans were isolated from

purified VSG (variant surface glycoprotein) of *Trypanosoma brucei brucei* by aqueous HF dephosphorylation, then re-N-acetylation using [^{14}C]acetic anhydride (Amersham, UK) [21,22]. The resulting neutral glycans are labelled in the GlcNAc residue proximal to the terminal inositol residue (cf. Fig. 9).

Xyloglucans were obtained from tamarind seeds (*Tamarindus indica*) using a published method [23], and from pea root (*Pisum sativum*) cell walls by extraction with an *endo*-β-1,4-glucanase [24]. The reducing ends were derivatized with a *p*-nitrobenzene derivative; the procedure is described elsewhere [25,26].

The sialic acids were from Sialomed (Baltimore, MD, USA).

All other carbohydrates were purchased from Sigma.

2.2. HPLC columns and apparatus

PolyGLYCOPLEX columns were prepared by PolyLC (Columbia, MD, USA). The neutral, hydrophilic material is derived from silica with a covalent coating of polysuccinimide, using a procedure similar to that detailed in previous papers [11,27]. The amount of residual charge is quite low, but not absent. HPAEC was performed with HPLC systems, PAD detectors, and CarboPac PA1 columns from Dionex (Sunnyvale, CA, USA). RP-HPLC (Fig. 14) was performed with a Spherisorb 5 ODS1 column, 250 × 4.6 mm (Phase Separations, Norwalk, CT, USA). Radioactivity detection was performed on-line with a Raytest Ramona detector equipped with a 200-μl X-cell solid scintillator flow cell (Raytest Instruments, Sheffield, UK).

2.3. Apparatus for MS and narrow-bore and microbore HPLC

In Fig. 8, the HPLC system was Model 172 from Applied Biosystems Division of Perkin-Elmer (Foster City, CA, USA). Microbore LC–MS (Figs. 11–13) was performed with HPLC system Model 140B from Applied Biosystems with a 5-μl sample loop. Post-column addition of 0.1% acetic acid at 8 μl/min was accomplished through an Upchurch low-dead-volume tee (Upchurch Scientific, Oak Harbor, WA, USA) connected by fused-silica tubing (50 μm I.D.) to a Harvard Apparatus (South Natick, MA, USA) Model 22 syringe pump. The combined effluent was again split using an Upchurch low-dead-volume tee. A length of fused silica (50 μm I.D.) from one outlet went directly to the tip of the ion spray needle of the mass spectrometer. The other outlet of the tee was connected to the UV detector by a length of 100 μm I.D. fused-silica tubing. The split ratio of 1:9 (MS:UV detector) was accomplished by adjusting the lengths of the fused-silica tubing. UV detection at 275 nm was accomplished with a Applied Biosystems Model 785A detector with a 2.4-μl flow cell.

MS detection was accomplished with a Sciex API-III triple quadrupole mass spectrometer fitted with an ion spray source (PE Sciex, Canada). The mass analyzing quadrupole (Q1) was scanned over a m/z range of 1214–1542, at a scan rate of 2.85 s/scan in steps of 0.5 u. The orifice potential was set at 50 V. The mass spectrometer was calibrated with a mixture of polypropylene glycols 425, 1000 and 2000 ($3.3 \cdot 10^{-5}$, $1 \cdot 10^{-4}$ and $2 \cdot 10^{-4}$ *M*, respectively), dissolved in water–methanol–formic acid (50:50:0.1), with 1 m*M* NH_4OAc. Q1 was adjusted for unit mass resolution (approx. 50% peak valley). All data were recorded by Sciex data acquisition software (Tune 2.1.2) on a Macintosh IIfx computer.

3. Results

3.1. HILIC of small carbohydrates

Initially, HILIC was performed with small oligosaccharides. These small solutes might be expected to have fewer interactions with the stationary phase than larger oligosaccharides, facilitating the interpretation of the data. While uncharged oligosaccharides were retained when the mobile phase contained only MeCN and water, sialic acids and small sialylated oligosaccharides (e.g., sialyllactose) eluted in the void volume (data not shown), owing to ion-exclusion

effects. This phenomenon has been noted before [28]. Accordingly, 10 m*M* triethylamine phosphate (TEAP) [11] was included in the mobile phases for the initial studies.

Figs. 1 and 2 show that sialic acids and small oligosaccharides can be resolved by HILIC on the PolyGLYCOPLEX column. The resolution of the sialic acids is at least as good as any currently in the literature. The position of linkage affects retention as much as residue composition; an oligosaccharide with a (1→6) or (2→6) link is better retained than one with a (1→4) or (2→3) link. This confirms earlier observations with HILIC columns [15,17]. From Figs. 1 and 2, it is evident that either amide sugar or sialic acid residues promote retention of a carbohydrate through hydrophilic interactions. Upon comparing peaks F and G in Fig. 1, one could conclude that a neutral hexose at the reducing end promotes retention more than an amide sugar, again in accordance with previous observations [15,16]. Oligosaccharides with an amide sugar at the reducing end (Fig. 1, peak F, and Fig. 2), and GlcNAc itself, are resolved into separate peaks for the α- and β-anomers, linked by a continuum of the intermediate forms. This separation of anomers also accounts for the tailing peaks for Neu5Ac and Neu5Gc; Neu5Ac2en, which does not form anomers, elutes as a symmetrical peak. This phenomenon has been noted before when neutral stationary phases were used for HILIC

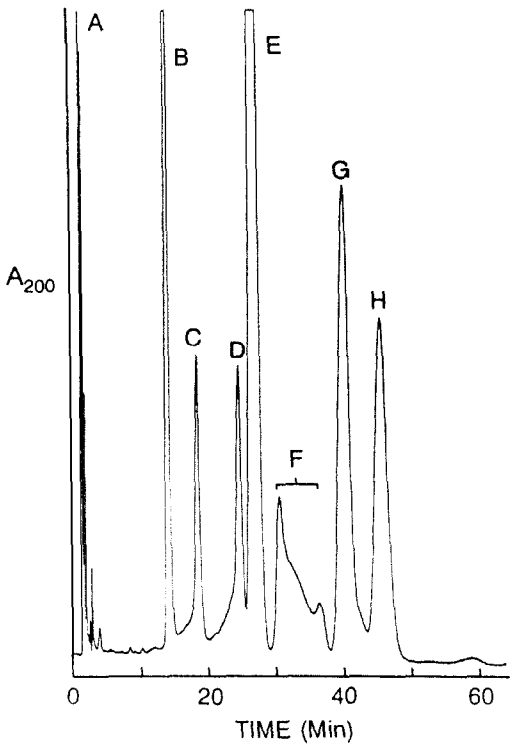

Fig. 1. HILIC of small carbohydrates. Column: PolyGLYCOPLEX, 200 × 4.6 mm (5 μm). Mobile phase (isocratic): 10 m*M* TEAP, pH 4.4, in MeCN–water (80:20, v/v). Flow-rate: 1.0 ml/min. Detection: A_{200} = 0.05 AUFS. Peaks: A = monosaccharides; B = 2,3-didehydro-2,6-anhydro-N-acetylneuraminic acid (Neu5Ac2en); C = N-acetylneuraminic acid (Neu5Ac); D = N-glycolylneuraminic acid (Neu5Gc); E = sialyl(2→3)lactose; F = sialyl(2→6)-N-acetyllactosamine; G = sialyl(2→6)lactose; H = disialyllactose.

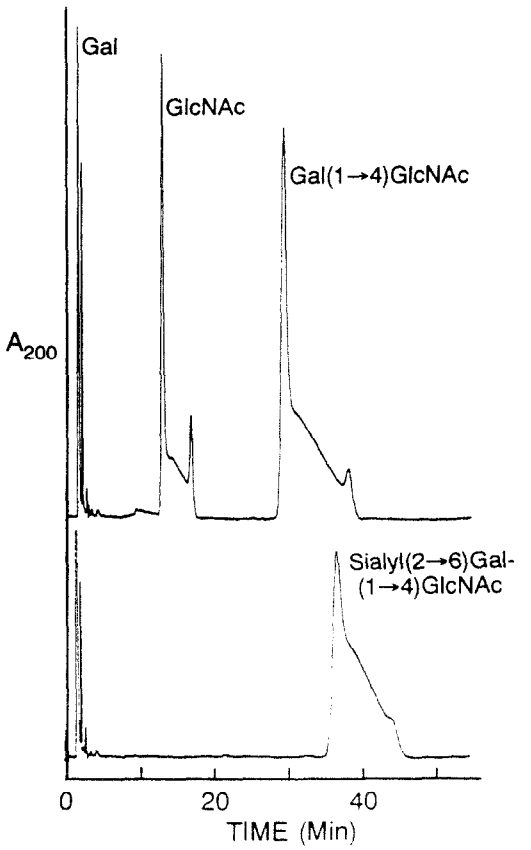

Fig. 2. HILIC of GlcNAC-containing carbohydrates. Conditions as in Fig. 1.

[19,29]; mutarotation is slow on the time scale of HPLC with these mobile phases. Mutarotation is accelerated by high pH. Thus, addition of 0.1% organic amine to the mobile phase can collapse the anomer doublets when a neutral column is used [29]. Similarly, anomer separation is normally not observed in HILIC with amino-silica columns, owing to the basicity of this stationary phase (although even these columns will resolve anomers if the mobile phase is sufficiently acidic [30]).

In some chromatographic separations, a solute can be oriented in such a manner that a particular "contact region" [31] is favored for interaction with the stationary phase surface. This phenomenon is not limited to partition modes. If a carbohydrate displays anomer separation, the implication is that the reducing end is a good contact region. Lack of an anomer effect implies that the reducing end is not the preferred contact region. Anomer separation can thus serve as a reporter function for solute orientation during chromatography. Fig. 3 is a schematic of such oriented contact; orientation is determined by the position of the amide sugar, which is the preferred contact region. This hypothesis on orientation is falsifiable. It was tested by inverting the sequence of amidated sugar and neutral hexose. GlcNAc(1→6)Gal does exhibit a symmetrical peak in Fig. 4, which would seem to confirm the hypothesis. However, at lower levels of acetonitrile (MeCN) in the mobile phase (inset), a small amount of anomer separation becomes evident with this compound too. This

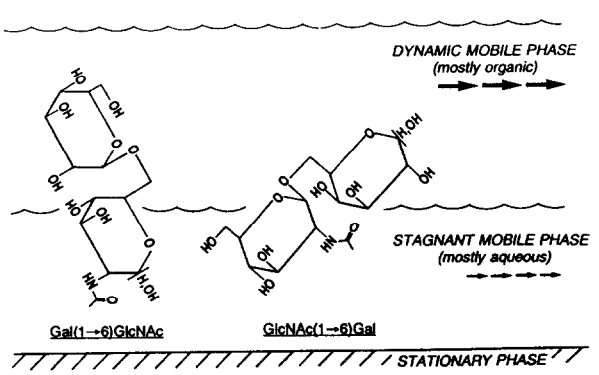

Fig. 3. Contact orientation and anomer effects in HILIC.

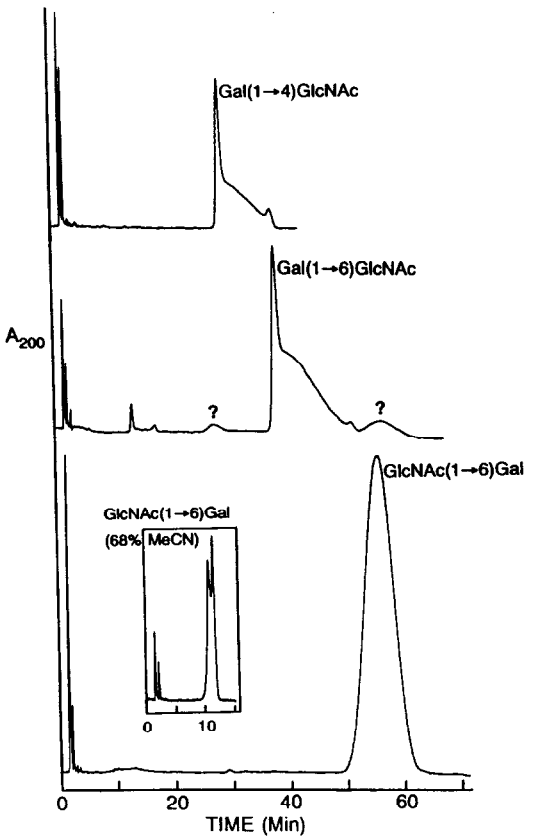

Fig. 4. Effect of sequence on anomer separation. Conditions as in Fig. 1, except for the inset, where MeCN–water ratio is 68:22 (v/v).

implies that the selectivity of the column changes somewhat with MeCN concentration; at lower levels of MeCN, neutral sugars serve as contact regions to a limited extent. At higher levels, amidated sugars dominate the chromatography.

Fig. 5 presents this shift in retention and selectivity for a number of carbohydrates. At relatively low levels of MeCN, sialic acids are very good contact regions. As the MeCN concentration increases, their role as contact regions diminishes relative to other sugars. Thus, the best separation of disialyllactose from the other carbohydrates is observed at the lowest level of MeCN in this study. Additive effects are evident in some cases [Gal(1→4)GlcNAc vs. GlcNAc] but not in others [sialyl(2→3)lactose vs. Gal(1→4)GlcNAc]. Fig. 2 confirms that the

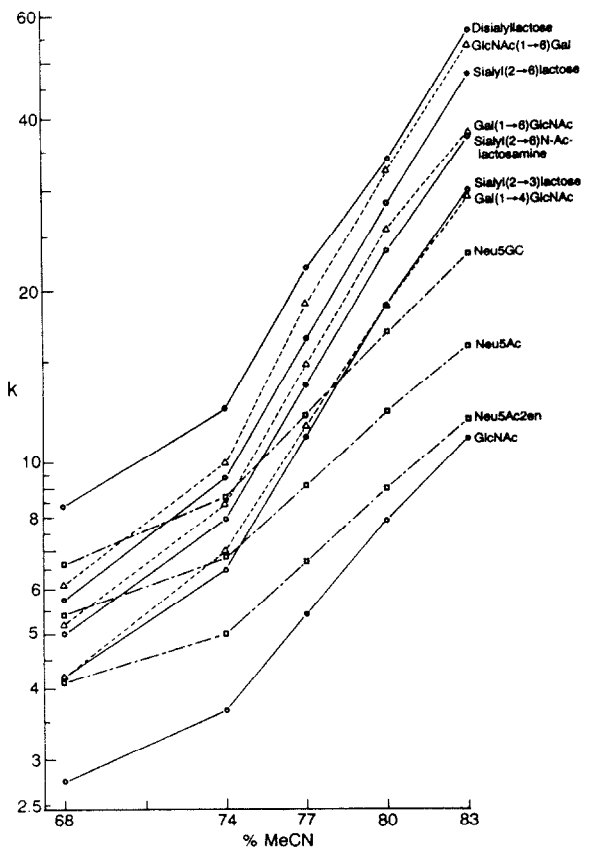

Fig. 5. Retention and selectivity vs. %MeCN. Conditions as in Fig. 1. The retention factor was determined from the formula $k = (t_R - t_0)/t_0$, where t_0 is the elution time for toluene, a non-retained solute, at a given flow-rate (*Note*: this replaces the expression for capacity factor, as per the new IUPAC nomenclature for chromatography).

addition of a sialyl residue has little effect on the orientation of contact as well as on the degree of retention; the amidated sugar is clearly the preferred contact region.

It seems counterintuitive that replacement of a residue which is a poor contact region with a better one (e.g., GlcNAc for Glc) results in a shorter retention time. The results probably reflect the partition nature of the HILIC mode. Retention is proportional to the degree to which a solute partitions into the stagnant aqueous layer on the surface of the stationary phase. With an amide sugar at the reducing end, a small oligosaccharide contacts the surface in an orientation which leads to less partitioning into the aqueous layer than if the orientation were inverted. The two phenomena —orientation and extent of partition— are independent to some extent. It should be noted that trisaccharides where the middle residue can be either GlcNAc or a neutral hexose do not differ much in retention in HILIC, compared with the consequences of such substitution at the reducing end [15]. A substitution in the middle of the oligosaccharide probably does not have much effect on its orientation or the degree of partitioning.

3.2. HILIC of underivatized complex carbohydrates

Fig. 6 shows the resolution of the underivatized glycan library cleaved from native bovine fetuin via hydrazinolysis. This library reportedly contains glycans which were N- and O-linked [6]. The most abundant single species is an O-linked disialylated tetrasaccharide [6]. This is consistent with the retention times in Fig. 6. Reasonable retention was obtained even though the mobile phase did not contain an electrolyte; evidently it is not necessary with larger oligosaccharides. These generally contain amide sugars, which

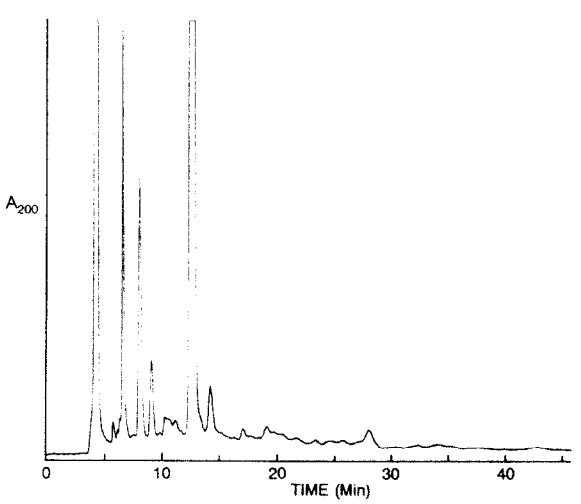

Fig. 6. HILIC of the complex carbohydrate library from native bovine fetuin. Sample: 6 μg (cf. Materials and methods section). Mobile phase: MeCN–water (80:20, v/v) (no TEAP). Other conditions as in Fig. 1.

would be good alternatives to sialic acids as contact regions. Since elution was isocratic, it was possible to detect the glycans at 200 nm. The lack of anomer separation probably reflects the presence of good contact regions in addition to the reducing end. The degree of anomer separation is in fact influenced by the composition of larger glycans [19].

3.3. HILIC of derivatized complex carbohydrates

This class was evaluated with biantennary N-linked oligosaccharides from apotransferrin. The PA derivatives were prepared as described in the Materials and methods section. Fig. 7 shows their isocratic separation by HILIC. N-Linked glycans are generally larger than the O-linked glycans prominent in Fig. 6, and less MeCN is necessary to obtain adequate retention. Retention and resolution are better with 65% MeCN (Fig. 7-II) than with 60% (Fig. 7-I). The last major peak (eluting at 18 min in Fig. 7-II) is the structure indicated. Mass spectral data (not shown) indicate that the peak eluting at 13 min in Fig. 7-II is the indicated glycan minus a terminal Gal residue. Davies et al. [7] have noted the presence of (Gal-minus) forms in commercial asialofetuin. The monosialyl form of this glycan was also examined. Judging from the data in Figs. 7-I and -II, such a sample could contain the following four components: (1) the monosialylated form with the structure indicated; (2) the monosialylated form minus a terminal Gal residue; (3 and 4) the asialo forms (± a Gal residue). Fig. 7-III indicates that all four of these forms are present, with the monosialyl forms (± a Gal) resolved at 60%. Clearly sialylation increased retention in comparison with the asialo forms. Separation of the monosialyl forms was worse at higher levels of MeCN (Fig. 7-IV), in contrast to the effect on the asialo forms (Fig. 7-II). Evidently the effectiveness of sialic acid as a contact region decreased relative to the other residues as the level of MeCN increased. This is consistent with the retention factors in Fig. 5.

Fig. 8 shows analysis of the same samples by

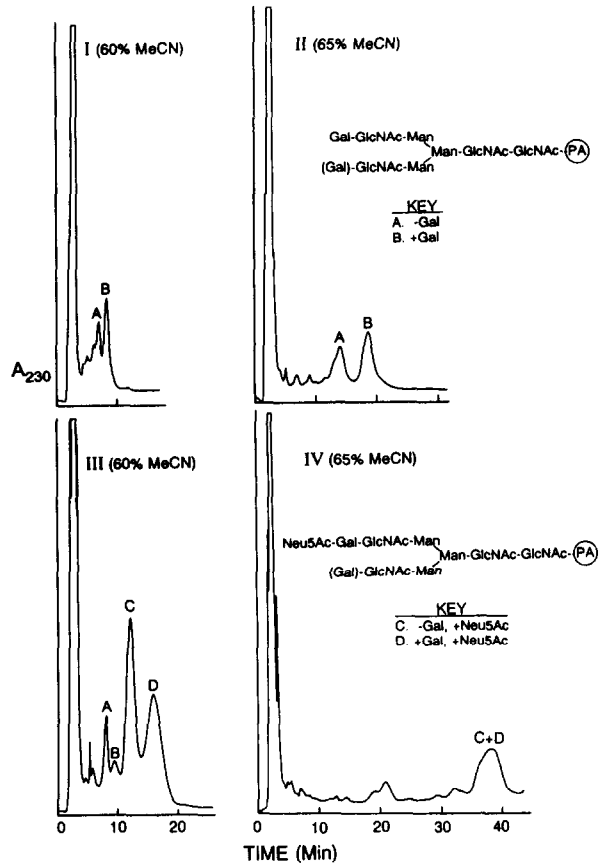

Fig. 7. HILIC of biantennary oligosaccharides from apotransferrin (PA derivatives). Column: PolyGLYCOPLEX, 200 × 2.1 mm (5 μm). Mobile phase (isocratic): MeCN–water (60:40, v/v) (I and III) or (65:35, v/v) (II and IV). Flow-rate: 0.25 ml/min.

HPAEC. The two major asialo forms are resolved to baseline, with sharp peaks. However, the two major monosialyl forms coincide under these conditions (Fig. 8, bottom). The presence of the sialyl group appears to dominate the interaction with the stationary phase in this mode; removal of the Gal does not affect retention.

3.4. HILIC of a GPI anchor glycan

Some proteins are attached to membranes through a glycan which is attached, via inositol, to a phospholipid. Such a "GPI anchor" glycan was obtained from the coat of a trypanosome as

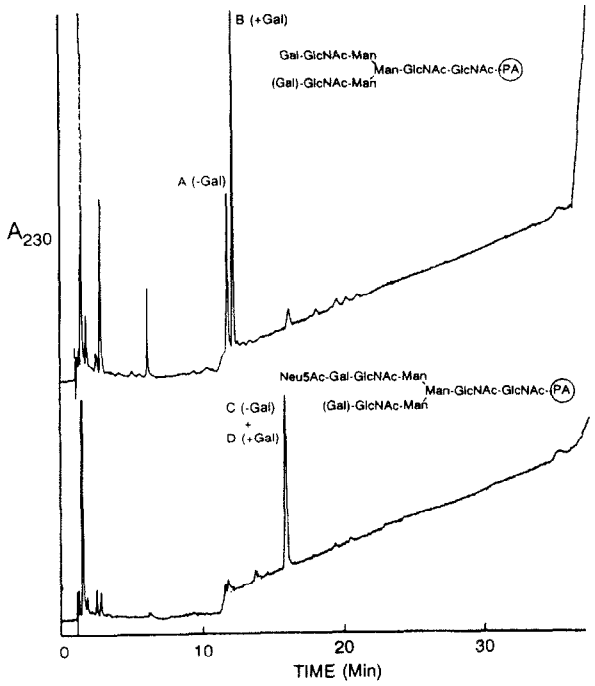

Fig. 8. HPAEC of biantennary oligosaccharides (same sample as Fig. 7). Column: CarboPac PA1. Flow-rate: 1.0 ml/min. Mobile phases: (eluent A) 100 m*M* NaOH; (eluent B) 100 m*M* NaOH + 500 m*M* sodium acetate. Gradient: 0–2 min, 1% B; 2–27 min, 1–50% B; 27–45 min, 50–100% B.

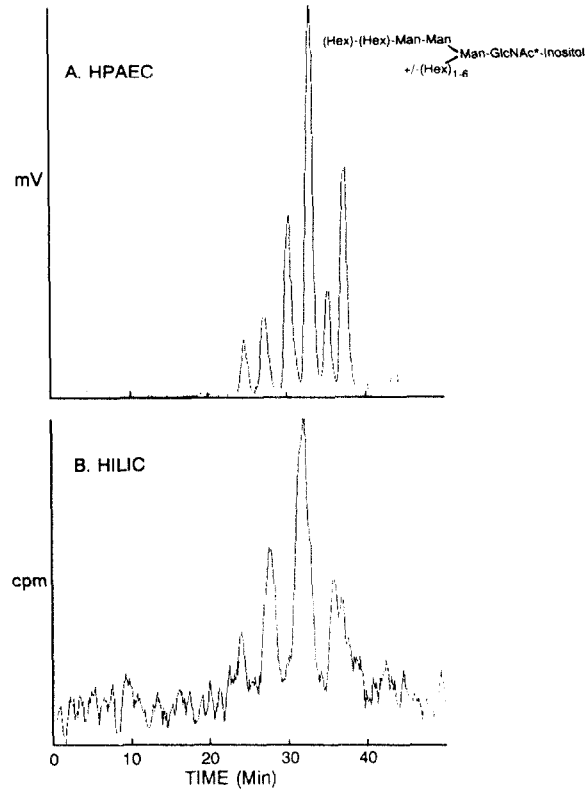

Fig. 9. GPI-anchor glycans (see Materials and methods section). The sample contained approx. 10 000 cpm, labelled at the GlcNAc residue as described in the Materials and methods section. (A) HPAEC; column: CarboPac PA1; mobile phases: (A) 150 m*M* NaOH, (B) 150 m*M* NaOH + 0.25 *M* sodium acetate; gradient: 5–20% B over 50 min; flow-rate: 0.6 ml/min; detection: PAD. (B) HILIC; column: PolyGLYCOPLEX, 200 × 4.6 mm (5 μm); mobile phase (isocratic): MeCN–water (65:35, v/v); flow-rate: 0.6 ml/min; detection: radioactivity detector (on-line).

described in the Materials and methods section. Fig. 9 shows the analysis of the product by HPAEC with detection by PAD (top) and HILIC with on-line radioactivity detection (bottom). The composition of the termini is quite variable, and is described elsewhere [32]. Both modes afford somewhat similar selectivity, although the efficiency of HPAEC is significantly better. The poor signal-to-noise ratio in the HILIC run reflects the small quantity of sample analyzed. Recovery of applied counts from this system is in excess of 95%.

3.5. Applications with MS

Fig. 10 shows a mixture of xyloglucans resolved by HILIC; the reducing termini have been derivatized with a *p*-nitrobenzene (PNB) derivative. The volatility of HILIC mobile phases permits convenient MS identification of eluted solutes. This was investigated with the xyloglucan mixture. The effluent was split between an absorbance detector and electrospray MS. Since the PNB-oligosaccharides were not reliably protonated upon elution (in contrast to results obtained with PA derivatives), a small amount of acetic acid was added post-column to insure that the derivatives were sufficiently charged for analysis by electrospray MS.

In Fig. 11 (left), it appears that resolution was significantly worse with a microbore column than with the regular analytical column used for Fig. 10. From comparison with the mass spectrum

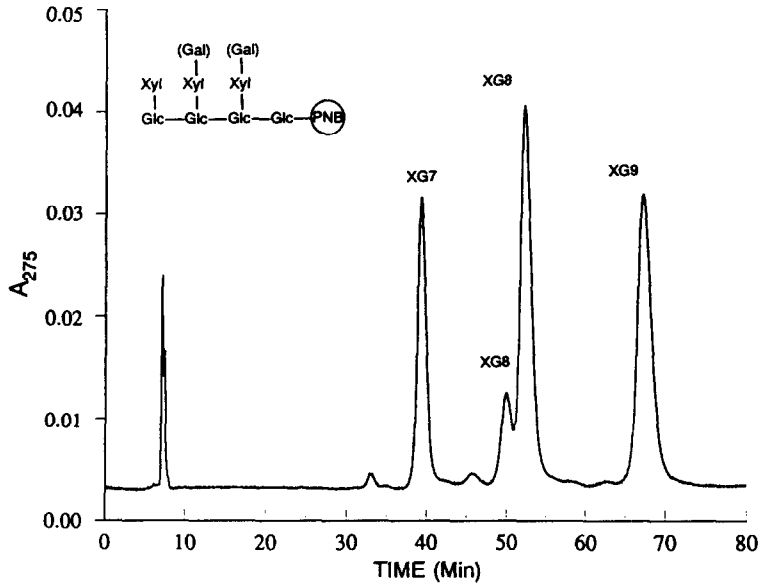

Fig. 10. HILIC of xyloglucans (PNB derivatives). XG7, XG8 and XG9 contain 0, 1 and 2 Gal residues, respectively. The two peaks labelled XG8 represent isomers with Gal in alternative positions [23]. Column: PolyGLYCOPLEX, 200 × 9.4 mm (5 μm). Mobile phase (isocratic): MeCN–water (70:30, v/v). Flow-rate: 1.0 ml/min.

(Fig. 11, right), it is evident that this loss of resolution reflects extracolumn bandspreading from connections and a UV detector not optimized for microbore work.

The mass spectrum in Fig. 11 was obtained using the total ion current (TIC) mode; the MS functioned as a general HPLC detector. The window of ion current can also be narrowed to

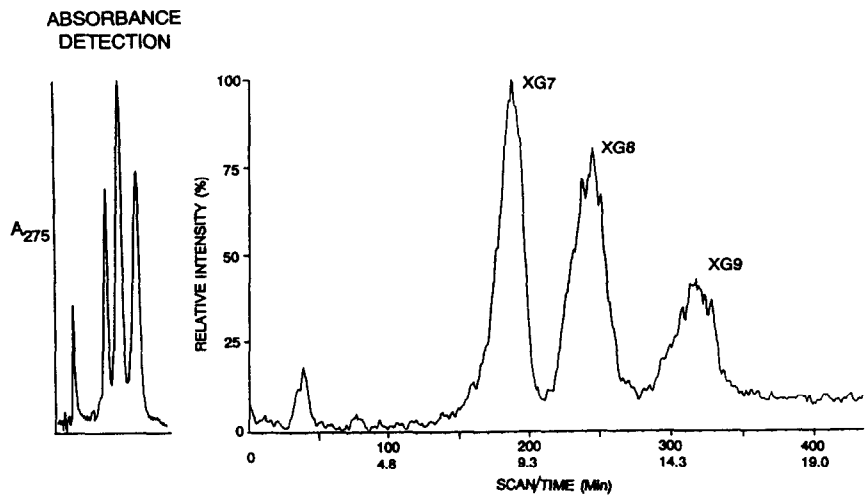

Fig. 11. Microbore HILIC–on-line MS of PNB-xyloglucans. Column: PolyGLYCOPLEX, 150 × 1.0 mm. Mobile phase as in Fig. 10. Flow-rate: 50 μl/min. Sample: 150 ng/1 μl (injected using a Rheodyne reverse-flow valve; see Materials and methods). Detection: (UV) $A_{275} = 0.025$ AUFS; MS, see Materials and methods. With these conditions, the positional isomers of PNB-XG8 were not resolved.

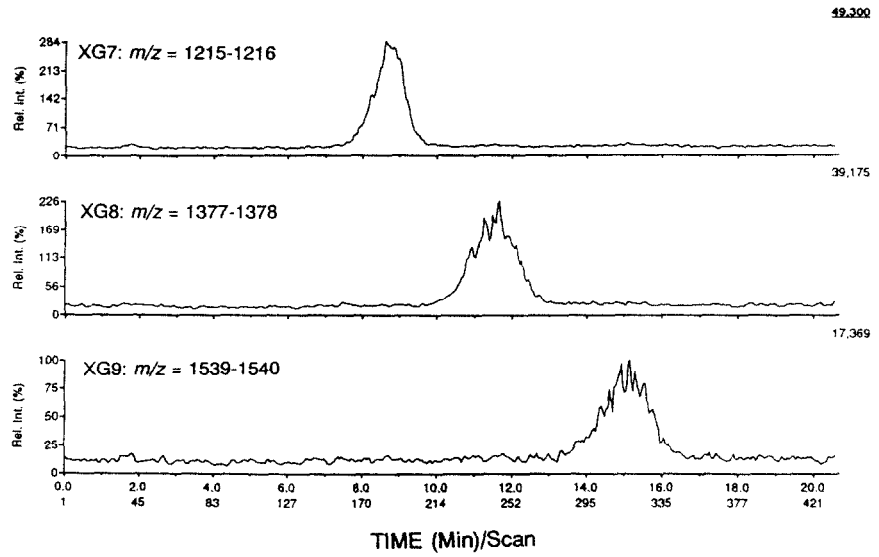

Fig. 12. Microbore HILIC-MS of PNB-xyloglucans: computer-generated selected ion chromatograms of Fig. 11.

the point that only a single species is detected, as shown in Fig. 12. This demonstrates the feasibility of determining the presence and molecular mass of a particular complex carbohydrate even if not completely resolved from others in the eluent. A similar study has been performed with PA derivatives on a RP-HPLC column [33].

The above data were obtained with approximately 50 pmol each of the PNB-xyloglucans. Optimization of the extracolumn equipment and the chromatographic conditions should permit analysis of carbohydrate derivatives in the low picomole range.

3.6. Selectivity of HILIC vs. RP-HPLC

RP-HPLC can be used with volatile mobile phases, which makes it convenient to use for direct MS of complex carbohydrate derivatives. Fig. 13 compares the performance of HILIC and RP-HPLC with PNB derivatives of complex carbohydrates from pea seedlings. The selectivity of the two modes is somewhat complementary, but overall the selectivity of HILIC is superior to that of RP-HPLC in this case. It should be noted that while Davies et al. [7] were able to resolve underivatized complex carbohydrates on a Hypercarb S column in the RP-HPLC mode, the column was unable to distinguish between (Gal-plus) and (Gal-minus) forms (cf. Fig. 7), and monosialyl forms were poorly resolved from asialo forms.

4. Discussion

4.1. HILIC with PolyGLYCOPLEX and other columns

HILIC with the PolyGLYCOPLEX column is promising with all of the categories of complex carbohydrates examined. Isocratic elution with 65 or 70% MeCN may suffice to resolve most complex carbohydrates in an acceptable time frame. Retention of smaller carbohydrates may require 80% MeCN. With particularly complex samples, it may be necessary to use a gradient from 80–65% MeCN. There may be solubility problems with larger underivatized glycans. However, PolyGLYCOPLEX seems to afford a given degree of retention at lower levels of MeCN than is necessary with other neutral HILIC materials. For example, if one corrects for differences in column volume, with the same glycan and mobile phase, retention time is about

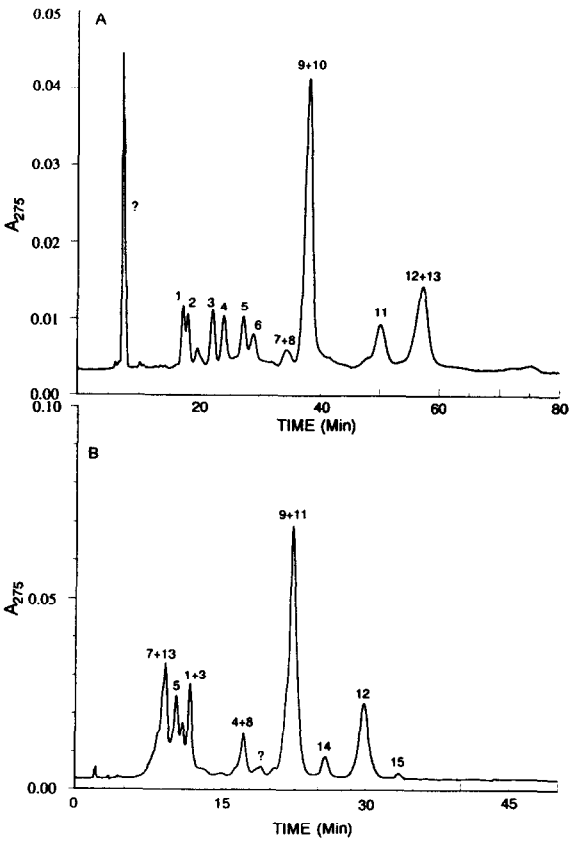

Fig. 13. HILIC and RP-HPLC of carbohydrates (PNB derivatives) from extract of pea root (*P. sativum*). (A) HILIC; column and conditions as in Fig. 10. (B) RP-HPLC; column: see Materials and methods; elution: 9–12% MeCN (aqueous) in a linear gradient over 50 min; flow-rate: 1.0 ml /min. Mobile phases were sparged with helium prior to use. Components were determined by off-line electrospray MS of collected fractions. Electrospray MS is unable to identify the specific pentose and hexose residues and their sequences, or to identify branched structures. Peaks (P = pentose; H = hexose; F = fucose): 1 = $(P)_4$; 2 = $(H)_3$; 3 = $(P)_5$; 4 = $(H)_3(P)_2$; 5 = $(H)_4$; 6 = $(P)_6$; 7 = $(H)_5$; 8 = $(H)_4(P)_2$; 9 = $(H)_4(P)_3$; 10 = $(P)_7$; 11 = $(H)_5(P)_3$; 12 = $(H)_5(P)_3F$; 13 = $(H)_6$; 14 = $(H)_6(P)_3F$; 15 = $(H)_6(P)_3(F)_2$.

four times longer on PolyGLYCOPLEX than on GlycoPAK N (e.g., Fig. 7 vs. Ref. [19], Fig. 2).

Sialic acids, amide sugars, and neutral hexoses all contribute significantly to retention when incorporated into an oligosaccharide which contains at least one good contact residue. The degree to which each residue affects retention depends on whether or not it is at the reducing end and on the level of organic solvent in the mobile phase. Relative to amide sugars, sialic acids and neutral hexoses contribute more to retention at lower levels of organic solvent than at higher levels. This permits selectivity to be manipulated. In order to generate detailed rules relating retention to composition, it will be necessary to examine the retention times of a large set of complex carbohydrates, using several sets of elution conditions (e.g., low vs. high organic; presence or absence of electrolytes).

Amino-silica and basic polymeric materials have two disadvantages relative to PolyGLYCOPLEX. First, they are inconvenient to use with sialylated oligosaccharides because of the extra electrostatic attraction; it necessitates increasing salt gradients. Second, some of the more common groups for derivatizing complex carbohydrates are positively charged at low pH. They are effectively run on amino-silica columns only at pH ranges high enough to uncharge the derivative (e.g., pH 7.3 with PA derivatives). Neutral HILIC columns do not have this disadvantage, although this is not apparent to all users; the pH 7.3 mobile phases have been uncritically carried over in some published work with such columns.

4.2. HILIC vs. other modes

HPAEC

Both methods have good selectivity for residue composition, number, and linkage position. HPAEC is decidedly more efficient. PAD is convenient with HPAEC. However, PAD can also be used with HILIC, through post-column addition of sodium or lithium hydroxide solution [34–36]. The non-volatile nature of HPAEC mobile phases is inconvenient for sample collection and MS analysis. The latter is possible through the use of in-line ion-suppressor membranes to remove cations post-column [37,38]; the procedure currently requires larger glycans to be run in the negative ion mode. HILIC seems to be more straightforward for direct MS analysis, since no post-column treatment is necessary.

RP-HPLC

In RP-HPLC, the only really good contact region is the chromophore or fluorophore at the reducing end. This may account for the lesser selectivity of RP-HPLC compared with HILIC.

The selectivity of the two modes is sufficiently complementary that it is reasonable to continue to use them in a two-dimensional sequence [3,18].

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