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Reverse Phase HPLC Fractionation of the Oligosaccharide Alditols Isolated from an I-Active Ovarian Cyst Mucin Glycoprotein

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We have used reverse phase high pressure liquid chromatography (HPLC) to separate the reduced oligosaccharides produced by alkaline borohydride degradation of ovarian cyst blood group substances. From a single cyst, six oligosaccharides, ranging from two to seven residues in length, have been isolated by preparative HPLC on C-18 stationary phases using water for elution. The purity of the products and their structures were determined by high field proton NMR spectroscopy in conjunction with exo- and endoglycosidase digestion. All the chains isolated terminated in *N*-acetylgalactosaminitol which was substituted at the 3-postion by galactose and in some cases at the 6-postion by *N*-acetylglucosamine. The largest identified oligosaccharide was a heptasaccharide alditol containing a single α-linked fucose in a Lewis blood group structure (Le^a).

As a result of the similarity of their immunological reactivity to that of red blood cell surfaces, the mucin glycoproteins extracted from ovarian cysts are also known as blood group substances. Recently Kabat [1] has reviewed the structures found in ovarian cyst mucins which were determined by the classical methods of methylation analysis, periodate oxidation and specific enzymic degradation. The structures found in the secreted blood group substances have been assumed to be similar or identical to the carbohydrate sequences of the red cell surface which are the antigens of blood group activity [2-5].

Recent developments in high pressure liquid chromatography (HPLC) have suggested this technique as an alternative to paper chromatography as a method for isolation and analysis of the complex oligosaccharides from glycoproteins. Roussel and coworkers [6]

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have used amino bonded silica in normal phase mode for the isolation of the reduced oligosaccharides from the bronchial mucins of cystic fibrosis patients. We have reported on the use of reverse phase HPLC to separate oligosaccharides of human milk [7] and glycopeptides from ovalbumin [8]. Since reverse phase HPLC is sensitive to differences in linkage stereochemistry and conformation, we propose that it would be useful in separating the complex mixtures of closely related oligosaccharides of ovarian cyst mucins.

The utility of high resolution NMR for structure determination in asparagine-linked glycopeptides has been abundantly demonstrated. This technique also appears to be equally useful in structure determinations in milk oligosaccharides [7] and in mucin oligosaccharides. Vliegenthart and coworkers have reported a number of NMR spectra of reduced oligosaccharides isolated from bronchial mucins [9] and from hog gastric mucin [10]. The method is quite straightforward for cases in which the NMR spectrum of an unknown sample corresponds exactly to that of a previously published oligosaccharide. But it is often possible to identify a structure whose spectrum has not been previously reported by means of comparison with the chemical shifts of similar oligosaccharides. Enzymatic digestions are an especially useful adjunct in such studies allowing the determination of a more complex structure through study of smaller fragments.

Materials and Methods

The HPLC apparatus consisted of a Tracor 990 pump, a Rheodyne 7125 injector and a Schoeffel SF 770 variable wavelength u.v. detector. Alltech Associates 605 RP 5 micron reverse phase columns (250 × 4.6 mm) and Perkin Elmer 3 micron (100 × 4.6 mm) reverse phase columns were eluted with water in all the studies. A precolumn of silica gel was used to improve the life and stability of the column. For preparative separations, fractions were collected using a microfractionator (Gilson). All oligosaccharide alditols were detected by u.v. absorbance due to the amide chromophore at 202 nm.

For ^{1}H-NMR spectroscopic analysis, the samples were dissolved in water, lyophilized and then repeatedly exchanged with $^{2}H_2$ at room temperature followed by lyophilization. The samples were then dissolved in 0.3 ml of high purity $^{2}H_2$ (Merck Sharp and Dohm, USA) in an NMR tube (5 mm) and subjected to NMR spectroscopy at 300 MHz in a Nicolet spectrometer. All the spectra were studied over the temperature range 5 to 75°C in order to avoid interference of the $H^{2}HO$ resonance and to study the effect of temperature on the spectra. The observed chemical shifts are reported relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) using acetone as the internal standard (δ 2.225 ppm downfield to DSS). All spectra were collected in Fourier transform mode.

The glycoprotein was prepared from pseudomucinous fluid of a cystic ovarian carcinoma (Patient blood group A_1, Le^{a-b+}). This material, designated EA in ref. 5, has been characterized previously as I, H and Le^{a} active. For the isolation, following the method of Kabat [11], an equal volume of 0.5 M sodium citrate was added to the ovarian cyst fluid and the mixture centrifuged at 4000 × g for 30 min. The insoluble material was discarded and the supernatant was digested with pepsin (Sigma, St. Louis, MO, USA) (10 mg/l cyst

Table 1. Oligosaccharide structure and notation.

	Compound	Chromato-graphic notation	Structure
1	Disaccharide	A	Galβ1-3 GalNAcOL
2	Core tetrasaccharide	B	Galβ1-3 GalNAcOL 6 Galβ1-4 GlcNAcβ1
3	Core pentasaccharide	Cc	Galβ1-3 GalNAcOL 6 GlcNAcβ1-3 Galβ1-4 GlcNAcβ1
4	Core hexasaccharide	Cd	Galβ1-3 GalNAcOL 6 Galβ1-3 GlcNAcβ1-3 Galβ1-4 GlcNAcβ1
5	Fuco-hexasaccharide	Ca	Galβ1-3 GalNAcOL 6 GlcNAcβ1-3 Galβ1-4 GlcNAcβ1 4 Fucα1
6	Fuco-heptasaccharide	Cb	Galβ1-3 GalNAcOL 6 Galβ1-3 GlcNAcβ1-3 Galβ1-4 GlcNAcβ1 4 Fucα1

fluid) at pH 2.0-2.5 at 37°C. The fluid was again centrifuged to remove insoluble material and the pH adjusted to 5.6 with sodium acetate. Fractional precipitations with 95% ethanol were carried out at 4°C. The precipitates were dissolved in and dialyzed against distilled water and lyophilized. To isolate oligosaccharides from the cyst glycoprotein the procedure of Iyer and Carlson [12] was followed. Accordingly, 500 mg of cyst glycoprotein was dissolved in 50 ml 1.0 M $NaBH_4$ in 0.05 M NaOH and incubated for 16 h at 50°C. The reaction was stopped by neutralizing to pH 7.0 with HCl and concentrating to dryness using a rotary evaporator. Borate was removed by repeated addition and evaporation with about 100 ml of absolute methanol. The dried product was dissolved in distilled water and centrifuged. The clear solution was fractionated on a Bio-Gel P-6 (200-400 mesh, Bio-Rad, Richmond, CA, USA) column of 2.0 × 100 cm, using water for elution.

Each gel fraction was assayed for neutral sugar by the phenol-sulfuric acid test [13] and studied by HPLC. The major peaks on the Bio-Gel chromatogram were freeze dried and studied by HPLC using water as an eluant. Fractions corresponding to desired peaks were collected and their purity and structure determined by rechromatography and high field ^{1}H NMR.

Glycosidase digestions were used to assist in the oligosaccharide structure determinations (for structures and compound notation see Table 1). 2 mg of a heptasaccharide alditol (Cb) was dissolved in 1 ml of 0.05 M sodium acetate buffer (pH 5.0) and 200 μl (1 unit) of α-fucosidase (Sigma) from bovine kidney was added. The reaction mixture was kept at 37°C for 16 h. Finally the reaction mixture was assayed by reverse phase HPLC using the Perkin Elmer 3μ (100 × 4.6 mm) column and water as an eluant. The hexasaccharide alditol (Cd) was also run as an authentic sample. The reaction product of fucosidase digestion of Cb (fucoheptasaccharide alditol) was identified by ^{1}H NMR spectroscopy.

For the hydrolysis of hexasaccharide alditol by endo-β-galactosidase, the incubation mixture contained 10 nmol of the substrate and 50 units of the enzyme in 100 μl of 0.01M sodium acetate buffer, pH 5.8. After incubating at 37°C for 17 h, the reaction mixture was evaporated to dryness and analyzed by TLC using *n*-butanol/acetic acid/water, 2/1/1 by vol, as solvent. Oligosaccharides were made visible by spraying the plate with diphenylamine reagent [14]. Endo-β-galactosidase was isolated from *Escherichia freundii* as described previously [15].

Results and Discussion

A typical elution curve for the ovarian cyst oligosaccharide alditols on the P-6 column (Fig. 1) shows peaks A, B and C, the major components which contribute 53% of the total

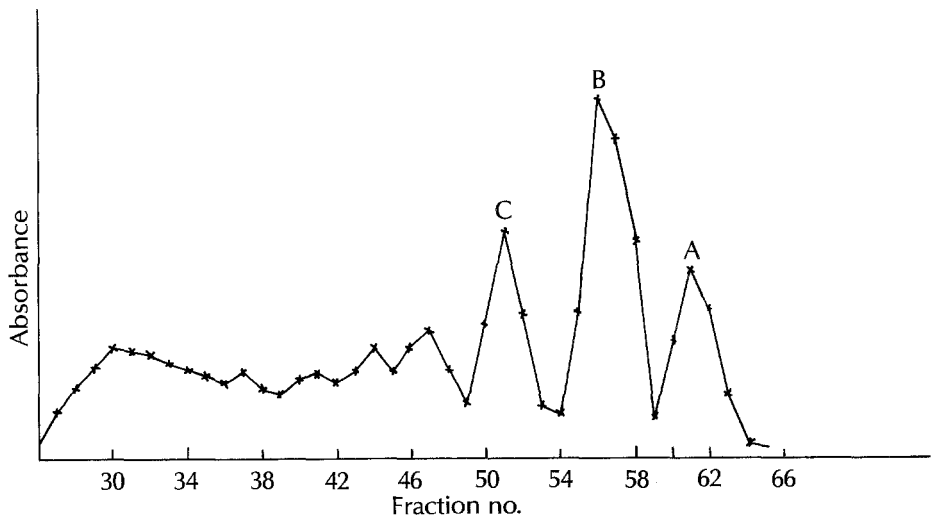

Figure 1. Bio-Gel P-6 chromatogram of the products of borohydride degradation of the ovarian cyst glycoprotein. Peaks A (disaccharide), B (tetrasaccharide) and C were selected for further analysis. Detection by phenol-sulfuric acid test at 490 nm.

Table 2. HPLC retention time of ovarian cyst oligosaccharide alditols.

	Compound	Peak after Bio-Gel P-6 (Fig. 1)	Retention time (min.)	
			Column 1[a]	Column 2[a]
1	Disaccharide	A	3.1	2.6
2	Core tetrasaccharide	B	3.8	3.0
3	Core pentasaccharide	C	6.1	4.6
4	Core hexasaccharide	C	6.9	5.1
5	Fuco-hexasaccharide	C	5.1	3.9
6	Fuco-heptasaccharide	C	5.4	4.1

[a] Chromatographic conditions. Column 1: Alltech 5μC_{18} column (25 cm × 4.6 mm), flow 0.5 ml/min. Column 2: Perkin Elmer 3μC_{18} column (10 cm × 4.6 mm), flow 1 ml/min.

carbohydrate isolated from the glycoprotein as determined by the phenol-sulfuric acid assay [13].

Analytical HPLC of fraction A shows only one major peak in u.v. detection (Table 2). After preparative HPLC, the rechromatography of this compound showed a single peak. The ^{1}H chemical shifts of the structural reporter group protons of this compound given in Table 3 are identical with those of the disaccharide alditol Galβ1-3GalNAcOL as reported earlier by Van Halbeek *et al.* [9]. Hence the major compound of fraction A is a disaccharide alditol, Galβ1-3GalNAcOL, whose HPLC retention time is given in Table 2.

Fraction B is the major carbohydrate constituent in this ovarian cyst glycoprotein, contributing the 25% of the total glycoprotein carbohydrate detected by phenol-sulfuric acid [13] in the P-6 column fractionation. HPLC of fraction B shows a single major peak with other trace components. 300 MHz ^{1}H-NMR (discussed below) and ^{13}C NMR spectroscopy [16] show this to be a pure sample of a branched tetrasaccharide (Table 1).

Fraction C contributes 14% of the total carbohydrate present in the P-6 chromatogram. HPLC of gel fraction C (Fig. 2) shows four major components, the retention times of which are given in Table 2. The fractions Ca, Cb, Cc and Cd were collected by preparative HPLC. Their rechromatography shows that fractions Cb, Cc and Cd are more than 90% pure while fraction Ca is about 60% pure. These estimates of purity were confirmed by 300 MHz ^{1}H NMR studies (discussed below). The structures of these oligosaccharides were determined by 300 MHz ^{1}H NMR spectroscopy and by enzymic treatment as discussed later.

The ^{1}H chemical shifts of the structural-reporter group protons of compound B (major) show three β anomeric protons and an alditol expected for a reduced tetrasaccharide (Table 4). The resonances of the protons of the *N*-acetylgalactosaminitol residue have been assigned by Vliegenthart and coworkers [9, 10] and can be recognized in our spectrum. The chemical shifts of H-2 (δ 4.393 ppm) and H-5 (δ 4.283 ppm) of *N*-acetylgalactosaminitol are characteristic of substitution at the C-3 position by β-galactose and at C-6 by

Table 3. 300 MHz ^{1}H NMR resonances of reduced oligosaccharides from ovarian cyst.

Residue	Reporter group	Disaccharide (A)	Core hexasaccharide (Cd) 24°C	Core hexasaccharide (Cd) 75°C	Core pentasaccharide (Cc) 24°C	Core pentasaccharide (Cc) 75°C	Fuco-heptasaccharide (Cb) 24°C	Fuco-heptasaccharide (Cb) 75°C	Fuco-hexasaccharide (Ca) 75°C
β-^{3}Gal	H-1	4.483	4.463	4.477	4.470	4.478	4.470	4.475	4.510
β-^{4}Gal	H-1		4.441	4.450	4.450	4.455	4.484	4.502	4.477
	H-4		4.154	4.144	4.130	4.125	4.154	4.141	4.150
	H-2	4.402	4.396	*	4.401	*	4.395	*	*
GalNAc-OL	H-5	4.203	4.283	4.250	4.271	4.245	4.285	4.244	4.240
	NAcOL	2.067	2.066	2.064	2.067	2.063	2.066	2.064	2.064
β-^{6}GlcNAc	H-1		4.560	4.578	4.555	4.575	4.560	4.574	4.575
	NAc		2.066	2.058	2.058	2.055	2.066	2.058	2.056
β-^{3}GlcNAc	H-1		4.727	4.782	4.703	4.771	4.70	4.771	4.761
	NAc		2.029	2.032	2.032	2.038	2.036	2.038	2.044
	H-1						5.030	5.025	5.027
α-^{4}Fuc	H-5						*	4.782	4.788
	H-6						1.183	1.188	1.189

* Values are obscured by H^{2}HO resonance.

β-*N*-acetylglucosamine [9]. The chemical shift of H-1 and the amide methyl protons of the β-^{6}GlcNAc linked to *N*-acetylgalactosaminitol at C-6 are 4.560 and 2.064 ppm respectively [9]. Two β-Gal H-1 shifts (by integration) are found at 4.465 ppm, one attached to the C-3 of *N*-acetylgalactosaminitol while the other is attached to the C-4 position of ^{6}GlcNAc. The linkage of Galβ1-4-^{6}GlcNAcβ is also indicated by the ^{13}C NMR spectrum by the downfield shift of the C-4 of β-*N*-acetylglucosamine [16]. These facts indicate that the major component of peak B is a tetrasaccharide alditol, whose structure is given in Table 1. The same compound has been isolated from cystic fibrosis mucin and its ^{1}H NMR spectrum is identical to ours [9].

In the course of changing the probe temperature in order to shift the residual H^{2}HO resonances relative to that of carbon bound protons, we have observed a marked temperature dependence for many of the resonance of the tetrasaccharide alditol. The data in Table 4 show that the chemical shifts of H-2 and H-5 of *N*-acetylgalactosaminitol move upfield with increasing temperature while those of H-1 of β-galactose, β-*N*-acetylgalactosamine and H-4 of *N*-acetylgalactosaminitol move downfield. At higher temperature, the shape of the H-1 resonance of β-^{6}GlcNAc changes, becoming broad, due to the virtual coupling as reported by Brisson and Carver [17] for glycopeptides. This observation suggests that the chemical shifts of H-2 and H-3 of *N*-acetylglucosamine become similar at high temperature.

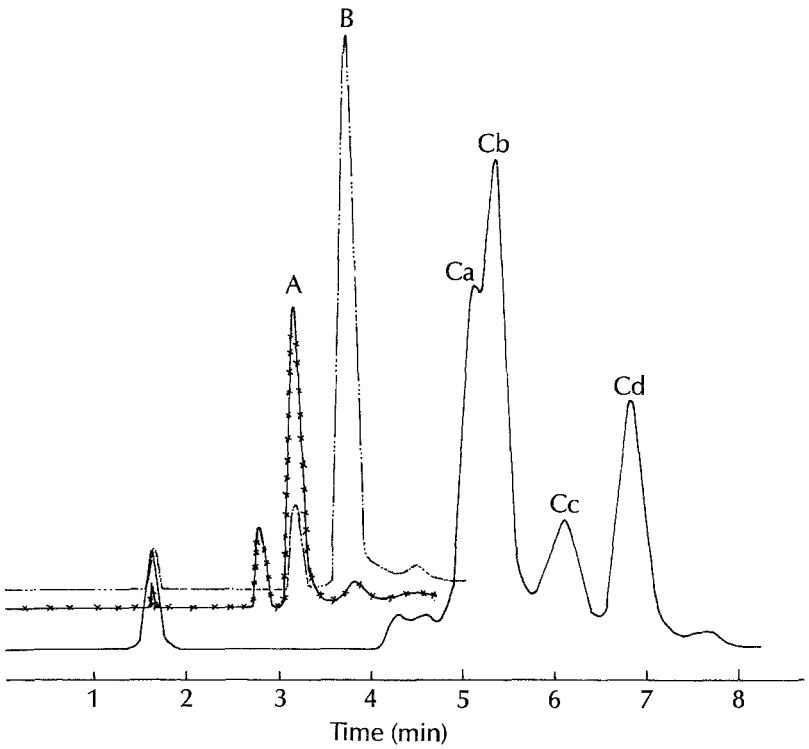

Figure 2. Reverse phase HPLC of the Bio-Gel P-6 fractions eluted with water. U.v. (202 nm) detection. Fraction A (-✳-), fraction B (- - -) and fraction C subfractions Ca, Cb, Cc and Cd (—).

The chemical shifts of the structural reporter group protons of component Cd isolated by preparative HPLC from gel fraction C is given in Table 3. We have recorded the spectrum at two different temperatures because of the temperature dependence of the chemical shifts of the anomeric resonances and of the H^2HO resonance. The integration of the anomeric region (Fig. 3) implies that the compound Cd is a hexasaccharide containing five β-anomeric proton resonances and one alditol. The characteristic resonances of GalNAcOL H-2 (at 4.396 ppm) and H-5 (at 4.283 ppm) indicate that the *N*-acetylgalactosaminitol is substituted at C-3 by β-galactose and at C-6 by β-*N*-acetylglucosamine [9]. The resonance at 4.560 is characteristic of the anomeric proton of β-^{6}GlcNAc linked to *N*-acetylgalactosaminitol. The resonance at 4.727 ppm is assigned to H-1 of β-^{3}GlcNAc linked to β-^{4}Gal. The *N*-acetyl region shows resonances at 2.066 due to *N*-acetylgalactosaminitol and β-^{6}GlcNAc (compare the tetrasaccharide) and the resonance at 2.029 ppm is due to β-^{3}GlcNAc. The integration of the region between 4.420 to 4.500 ppm implies that there are three β-galactose residues. It is difficult to assign the resonances to the three specific β-galactose residues, but it is reasonable to assume that the resonances of two β-^{3}Gal non-reducing terminal residues are at the same chemical shift (4.463 ppm) while β-^{4}Gal is at 4.441 ppm. The assignment of β-^{4}Gal is based on comparison to the resonances of the pentasaccharide discussed below. The presence of a narrow doublet (J = 3.2 Hz) at 4.154 ppm is characteristic of a Gal H-4 which is substituted at the C-3 position

Table 4. Effect of temperature on the chemical shifts of core tetrasaccharide (B).

Residue	Reporter group	5°C	25°C	45°C	60°C	75°C
β-^{6}GlcNAc	H-1	4.553	4.560	4.565	4.577	4.582
β-^{3}Gal β-^{4}Gal	H-1	4.465	4.465	4.470	4.477	4.480
GalNAcOL	H-2	4.411	4.393	4.380	4.375	4.367
	H-3	4.067	4.059	4.055	4.057	4.057
	H-4	3.448	3.468	3.492	3.500	3.510
	H-5	4.299	4.283	4.267	4.257	4.248
^{6}GlcNAc	Me	2.069	2.064	2.063	2.063	2.063
	H-6	4.010	4.000	4.000	4.000	4.000

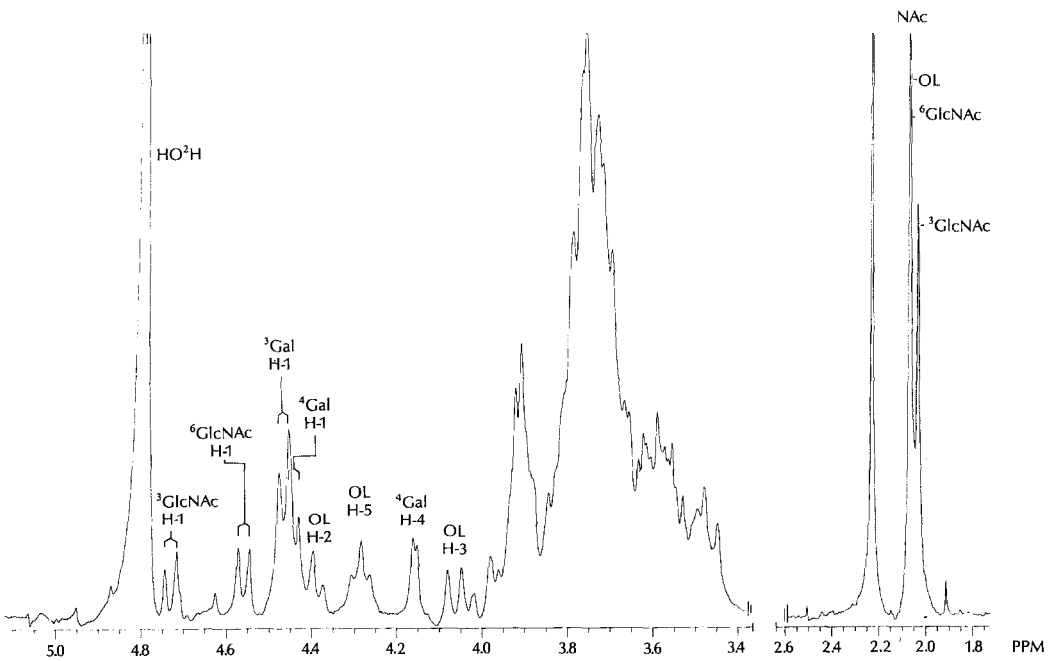

Figure 3. Proton NMR spectrum (300 MHz) of the core hexasaccharide alditol Cd at 24°C.

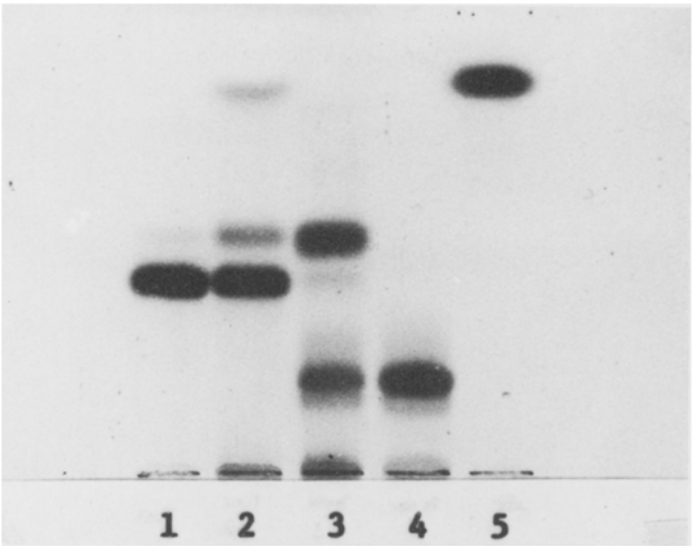

Figure 4. TLC of oligosaccharides and their endo-β-galactosidase digestion products. Lane 1, lacto-*N*-tetraose. Lane 2, lacto-*N*-tetraose plus endo-β-galactosidase. Lane 3, hexasaccharide alditol Cd plus endo-β-galactosidase. Lane 4, hexasaccharide alditol Cd. Lane 5, glucose.

[7]. Comparison of this spectrum with that of the tetrasaccharide shows that this compound is an extension of the tetrasaccharide formed by addition of the disaccharide Galβ1-3GlcNAcβ1-3 to β-^{4}Gal or β-^{3}Gal. ^{13}C NMR studies of this compound show that the extension is at C-3 of one of the β-galactosyl residues [16]. The above data indicate that this hexasaccharide is an extension of the core tetrasaccharide (B) by the addition of a β-^{3}GlcNAc to one of the galactoses (either ^{3}Gal or ^{4}Gal), and that this β-^{3}GlcNAc must be further substituted in either the 3- or the 4-position by a third residue of galactose. Our discussion (below) of the structure of a fucosylated derivative (Cb) of this hexasaccharide clearly indicates that it is the former (Type I) structure which is present.

Whether the disaccharide Galβ1-3GlcNAcβ1-3 is attached to the 3- or 4-linked galactose can be determined by endo-β-galactosidase digestion. This enzyme is expected to cleave only the latter structure, and not the former in which the only internal galactose linkage is β1-3. TLC data (Fig. 4) show that one product of endo-β-galactosidase digestion of Cd has the same migration as the major product of digestion of lacto-*N*-tetraose, for which we expect a trisaccharide product, Galβ1-3GlcNAcβ1-3Gal. The other product from Cd gives a faint band with a TLC mobility similar to that of lacto-*N*-tetraose. Since this is expected to be a reduced branched trisaccharide with only one galactose residue, it does not stain well with diphenylamine. These data, as well as the ^{13}C NMR spectrum [16], indicate that the compound is a hexasaccharide alditol as shown in Table 1. The ^{1}H NMR spectrum shows that the compound is more than 95% pure. Although contamination by the tetrasaccharide could not be identified by ^{1}H NMR, the HPLC shows it to be free of the di- and tetrasaccharides.

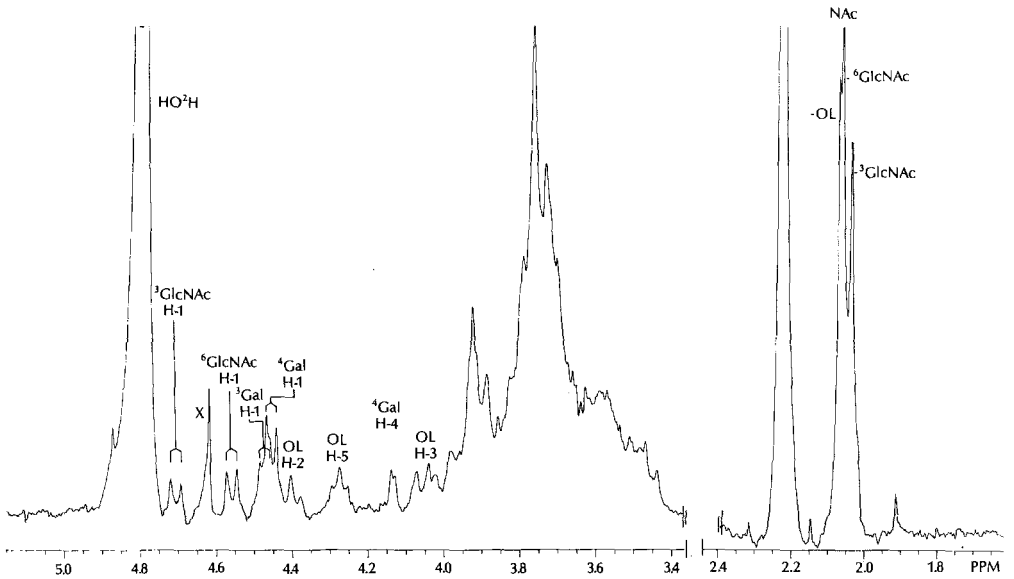

Figure 5. Proton NMR spectrum (300 MHz) of the core pentasaccharide alditol Cc at 24°C. X represents a spinning side band.

The chemical shifts of the structural reporter group protons of component Cc isolated by preparative HPLC from fraction C of the P-6 column are given in Table 3. The integration of the anomeric region in Fig. 5 implies that this compound has four β-anomeric resonances and an alditol. The characteristic resonances in the 24°C spectrum of GalNAcOL H-2 (δ 4.401 ppm) and H-5 (4.271 ppm) show that the *N*-acetylgalactosaminitol is substituted at C-3 by β-galactose and at C-6 by β-*N*-acetylglucosamine. The doublet at 4.555 is assigned to H-1 of β-^{6}GlcNAc linked to *N*-acetylgalactosaminitol and that at 4.703 is assigned to H-1 of β-^{3}GlcNAc linked to β-^{4}Gal. The amide methyl resonances of these two *N*-acetylglucosamine residues are at 2.058 (^{6}GlcNAc) and 2.032 ppm (^{3}GlcNAc) respectively. The integration of the β-galactose anomeric proton region shows the presence of two residues, one linked to the 3-position of *N*-acetylgalactosaminitol at 4.470 ppm and the other at 4.450 ppm assigned to β-^{4}Gal. This is a tentative assignment based on analogies to the core hexa- and tetrasaccharides. The resonance assigned to H-4 of galactose at 4.130 ppm shows that one galactose is substituted at the C-3 position. A comparison of this spectrum (Fig. 5) with the core hexasaccharide alditol spectrum shows that this compound has one β-galactose less than the hexasaccharide alditol. Although our data do not exclude the structure GlcNAcβ1-3Galβ1-3[Galβ1-4GlcNAcβ1-6]GalNAcOL, analogy to the hexasaccharide (Cd) suggests the structure for Cc given in Table 1. It is impossible to detect impurities due to tetrasaccharide and hexasaccharide alditols in this compound by ^{1}H NMR but the HPLC shows that these contaminants could only be present below the 5% level.

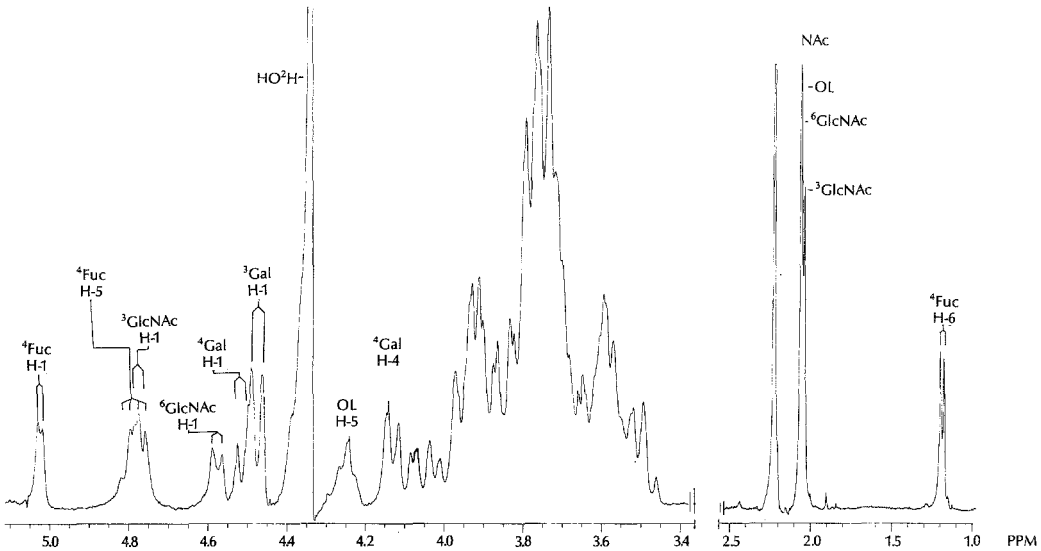

Figure 6. Proton NMR spectrum (300 MHz) of the fuco-heptasaccharide alditol Cb at 77°C.

The 300 MHz NMR spectrum (77°C) of component Cb isolated by preparative HPLC from fraction C of the P-6 column is given in Fig. 6 and the chemical shifts of the structural reporter group protons are given in Table 3. An integration of the anomeric region shows one α-fucose, five β-anomeric protons and one *N*-acetylgalactosaminitol which implies a fuco-heptasaccharide alditol, while an integration of the methyl region shows three amide methyl groups (two *N*-acetylglucosamine and one *N*-acetylgalactosaminitol). The resonances at 5.025, 4.782 and 1.188 ppm have been assigned in the 75°C spectrum to H-1, H-5 and H-6 of α-^{4}Fuc, respectively, indicating that α-^{4}Fuc is linked to ^{3}GlcNAc [7]. The chemical shift of H-5 of α-^{4}Fuc is determined by the spin decoupling difference spectroscopy (SDDS) experiment [18]. The characteristic resonances at 4.395 and 4.285 ppm in the 24°C spectrum assigned to H-2 and H-5 of *N*-acetylgalactosaminitol respectively, imply that *N*-acetylgalactosaminitol is substituted by β-galactose at C-3 and β-*N*-acetylglucosamine at C-6. At 75°C, these resonances are shifted upfield and the H-2 resonance is obscured by the H^2HO signal (Fig. 6). The resonances at 4.574 and 2.058 ppm are assigned to H-1 and the amide methyl of β-^{6}GlcNAc respectively, while those of β-^{3}GlcNAc are found at 4.771 and 2.038 ppm. The integration of the β-galactose anomeric region indicates three β-galactosyl residues. The anomeric proton resonance of the β-^{3}Gal (non-reducing terminal) is found at 4.475 ppm, a chemical shift similar to that in the hexasaccharide (Cd) while the anomeric proton resonance of β-^{4}Gal appears at 4.502 ppm. A narrow doublet at 4.141 is assigned to H-4 of ^{4}Gal. A comparison of this spectrum with the hexasaccharide spectrum (Fig. 3) suggests that the compound Cb has the core

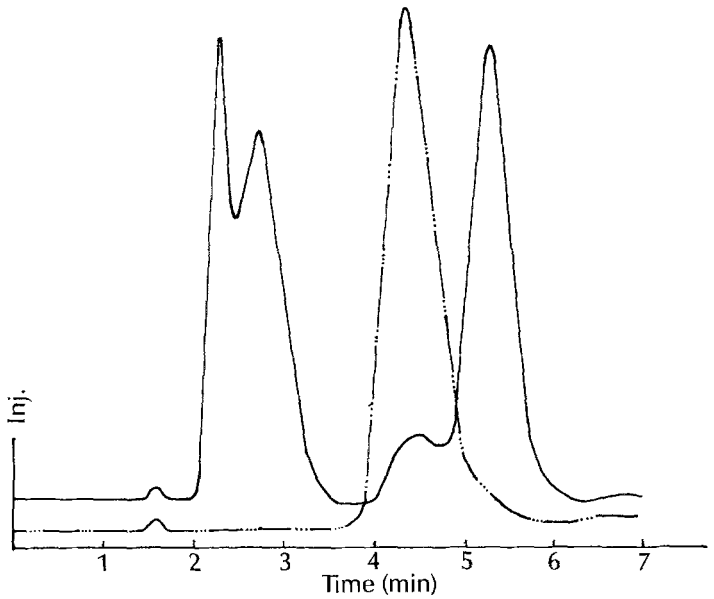

Figure 7. Reverse phase HPLC of the fuco-heptasaccharide Cb (- - -) and of the products of α- fucosidase digestion (—). The retention time of the major product corresponds to that of the hexasaccharide Cd, whose identity was confirmed by NMR.

hexasaccharide alditol structure (Cd) with the addition of one α-^{4}Fuc residue as may be shown by treatment of Cb by α-fucosidase. The chromatography of the α-fucosidase digestion products of Cb given in Fig. 7 indicates that more than 90% of the heptasaccharide alditol (Cb) is converted to the hexasaccharide alditol (Cd). The ^{1}H NMR spectrum of the digestion product of Cb is identical to that of the hexasaccharide alditol (Cd), confirming that the heptasaccharide (Cb) has the hexasaccharide (Cd) core structure. It is hard to detect impurities due to tetrasaccharide and the fuco-hexasaccharide (discussed below) by ^{1}H NMR spectroscopy but the HPLC shows that the contamination is below the 5% level.

Analytical HPLC of the component Ca shows that it is approximately 60% pure and it is contaminated with heptasaccharide alditol. The ^{1}H chemical shifts of compound Ca are given in Table 3. The ^{1}H NMR spectrum (not given) is very similar to that of the heptasaccharide Cb. The basic differences between these two are in the β-galactose anomeric region. The integration of the 4.45 to 4.52 ppm region of Ca and Cb shows that compound Cb has three β-Gal H-1 protons while Ca has between two and three B-Gal H-1 protons, which implies that Ca is contaminated with Cb. By a comparison of this spectrum and the heptasaccharide spectrum, one may conclude that the compound Ca has the fuco-hexasaccharide structure shown in Table 1.

Conclusion

The effect of temperature on the ^{1}H chemical shifts of the tetrasaccharide alditol (B) is quite remarkable, especially for the *N*-acetylgalactosaminitol resonances and to a lesser extent for the anomeric protons. This temperature dependence suggests that the *N*-acetylgalactosaminitol is flexible and that the conformation of the tetrasaccharide alditol (B) is temperature dependent.

The EA ovarian cyst oligosaccharides have recently been studied by Tanaka *et al.* [5]. They have reported disaccharide (A), the major core tetrasaccharide (B), the fuco-heptasaccharide (Cb), a fuco-pentasaccharide with a Fucα1-2 substitution on the ^{4}Gal of tetrasaccharide (B) and a decasaccharide. We did not detect the latter two oligosaccharides, but we have isolated three additional oligosaccharides, i.e. Ca, Cc and Cd. The differences could be explained by variation in sample composition obtained from this very large multi-loculated cyst.

In previous work we have shown that reverse phase HPLC can be applied to the fractionation of oligosaccharides and glycopeptides containing *N*-acetyl amino sugars [7, 8]. In this publication we demonstrate that the method can be applied to fractionation of ovarian cyst oligosaccharide alditols. Since oligosaccharide-alditols give only one peak rather than the two reducing terminal anomeric peaks found for oligosaccharides, the chromatogram is simplified and shows increased resolution. The retention time of the fuco-heptasaccharide (Cb) is lower than that of the hexasaccharide (Cd) and pentasaccharide (Cc) illustrating the fact that the reverse phase separations are based not only on molecular size but also on stereochemical and conformational features of the carbohydrate chain.

The 300 MHz ^{1}H NMR spectroscopic analysis of the six oligosaccharide alditols obtained from this ovarian cyst glycoprotein shows that their typical structural features can readily be elucidated by ^{1}H NMR. The presence of *N*-acetylgalactosaminitol and fucose is obvious from their typical sets of structural reporter groups (H-2, H-5, NAc, and H-1, H-5, Me, respectively). The β-*N*-acetylglucosamine is identified by H-1 and *N*-acetyl signals. The chemical shifts of H-2 and H-5 of *N*-acetylgalactosaminitol provide the nature of the substitution at C-3 and C-6 of this residue. Comparison of the NMR spectra of the core hexasaccharide Cd (Fig. 3) with that of the fuco-heptasaccharide Cb (Fig. 6) reveals a resonance at 4.075 ppm which is unique to the latter. Since the two oligosaccharides differ by a single fucose residue and no signal in this chemical shift region can be assigned to fucose, the resonance is proposed to be a "structural reporter" characteristic of Le^a structures. This signal can be assigned to H-3 of *N*-acetylglucosamine substituted by ^{4}Fuc and ^{3}Gal by analogy to the milk oligosaccharides lacto-*N*-fucopentaose III [7] and a difucosylated derivative of lacto-*N*-hexaose (Dua VK, Goso K, Dube VE, Bush CA, manuscript in preparation).

It seems clear that reverse phase HPLC and high field NMR are extremely useful methods for isolation and structural determination of oligosaccharide alditols from ovarian cyst glycoproteins as well as their enzymatic digestion products. Current work in our laboratory suggests that is should be possible, using a combination of reverse phase and normal phase HPLC, to isolate and quantitatively analyze all of the oligosaccharide

products from alkaline borohydride degradation, making available a complete catalog of all the oligosaccharide structures in a cyst mucin [19].

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References

1 Kabat EA (1982) Am J Clin Pathol 78:281-92.
2 Watkins WM (1972), in Glycoproteins, 2nd edition, ed. Gottschalk A, Elsevier, New York, p 830-91 (p 886).
3 Rovis L, Anderson B, Kabat EA, Gruezo F, Liao J (1973) Biochemistry 12:5340-54.
4 Rovis L, Anderson B, Kabat EA, Gruezo F, Liao J (1973) Biochemistry 12:1955-61.
5 Tanaka M, Dube V, Anderson B (1984) Biochim Biophys Acta 798:283-90.
6 Lamblin G, Boersma A, Lhermitte M, Roussel P, Van Halbeek H, Mutsaers JHGM, Vliegenthart JFG (1983) in Proc 7th Int Symp Glycoconjugates, eds. Chester MA, Heinegård D, Lundblad A, Svensson S, Secretariat, Lund, p 565.
7 Dua VK, Bush CA (1983) Anal Biochem 133:1-8.
8 Dua VK, Bush CA (1984) Anal Biochem 137:33-40.
9 Van Halbeek H, Dorland L, Vliegenthart JFG, Hull WE, Lamblin G, Lhermitte M, Boersma A, Roussel P (1982) Eur J Biochem 127:7-20.
10 Van Halbeek H, Dorland L, Vliegenthart JFG, Kochetkov NK, Arbatsky NP, Derevitskaya VA (1982) Eur J Biochem 127:21-29.
11 Kabat EA (1956) Blood Group Substances, Their Chemistry and Immunochemistry, Academic Press, New York, p 125-29.
12 Iyer RN, Carlson DM (1971) Arch Biochem Biophys 142:101-5.
13 Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Anal Chem 2:350-56.
14 Harris G, McWilliam JC (1954) Chem Ind 249.
15 Nakagawa H, Yamada T, Chien J, Gardas A, Kitamikado M, Li SC, Li Y-T (1980) J Biol Chem 255:5955-59.
16 Dua VK, Panitch M, Rohr TE, Bush CA (1984) Anal Biochem, in press.
17 Brisson JR, Carver JP (1982) J Biol Chem 257:11207-9.
18 Gibbons WA, Beyer CF, Dadok J, Speicher RF, Wyssbrod HR (1975) Biochemistry 14: 420-29.
19 Dua VK, Dube VE, Bush CA (1984) Biochim Biophys Acta,in press.