Carbohydrate Research, 188 (1989) 1–7
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SEPARATION OF FUCOSYLATED OLIGOSACCHARIDES USING HIGH-pH ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED-AMPEROMETRIC DETECTION*

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(Received August 18th, 1988; accepted for publication February 4th, 1989)

ABSTRACT

Fucosylated oligosaccharides of the βGal(1→4)GlcNAc-, βGal(1→4)Glc-, and βGal(1→3)GlcNAc-series were chromatographed on a high-performance anion-exchange pellicular resin under alkaline conditions (pH ≃13). Fucosylation of either lactose, lactosamine (Type II chains), or lacto-*N*-biose (Type I chains) oligosaccharides markedly decreased the retention time (10–38 min) of the non-fucosylated form. The magnitude of the reduction was related to whether fucose replaced Gal [αFuc(1→3)→GlcNAc], whether fucose was α-(1→2)-linked to Gal at the end of a chain, or whether fucose was linked in a subterminal position [α(1→3) or α(1→4)] to Gal or GlcNAc. The results suggest that the decreases in retention times of fucosylated oligosaccharides (10–38 min) is not attributable to the absence of a 6-OH in Fuc but instead to steric and substitution effects which affect the interaction of the most readily ionizable groups of Fuc (2-OH), Gal (2-OH), and GlcNAc (3-OH) with the stationary phase. We show that high-pH anion-exchange chromatography can effectively separate 1→2, 1→3, and 1→4 fucose positional isomers in a single chromatographic step.

INTRODUCTION

With the recognition of a potential role of fucosylated glycoconjugates in development[1], differentiation[2], and malignant transformation[3], there is a growing interest in the isolation and characterization of these compounds from normal and mutated cell lines[4]. We have recently shown, using high-pH anion-exchange (h.p.a.e.) chromatography under alkaline conditions, that αFuc(1→3)GlcNAc con-

*Contribution No. 1413 from the McCollum-Pratt Institute. This investigation was supported by National Institutes of Health Grant DK31376. Presented at the XIVth International Carbohydrate Symposium, Stockholm, Sweden, August 14–19, 1988.

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0008-6215/89/$ 03.50

taining oligosaccharides* are widely separated from their non-fucosylated counterparts[5]. In this study, we further investigated the chromatographic selectivity of this method for fucosylated oligosaccharides. We found that both lactosamine- and lacto-*N*-biose-type oligosaccharides having fucose linked subterminal to GlcNAc [either $\alpha(1\rightarrow3)$ or $\alpha(1\rightarrow4)$] were much more weakly retained (10–38 min) than their non-fucosylated forms, whereas a terminal linkage of αFuc$(1\rightarrow2)$ to Gal had considerably less effect.

RESULTS AND DISCUSSION

We have proposed that the retention times of oligosaccharide oxyanions on pellicular anion-exchange resins can be correlated with both the relative acidities of hydroxyl groups (extrapolated from monosaccharide studies[6]) and accessibility of oxyanions to the stationary phase[5]. The anomeric hydroxyl group of reducing sugars is the most acidic (p*K*a $\simeq$12) and the other ring hydroxyl groups have a hierarchy of acidity of 2-OH $\gg$ 6-OH > 3-OH > 4-OH, with p*K*a values[6] up to 14. In 2-acetamido-2-deoxypyranosides it has been indirectly shown (by retention on strong anion-exchange resins) that the 3-OH group has a comparable p*K*a to the 2-OH group of unsubstituted pyranosides because of its proximity to the acetamido group[7]. Therefore, during anion-exchange chromatography of these oligosaccharides at pH $\simeq$13, the bulk of the retention time can be attributed to the effectiveness of the interaction of the anomeric oxyanions of the reducing sugars, the 2-OH groups of the unsubstituted pyranoses, and the 3-OH groups of the 2-acetamido-2-deoxypyranosides to the positively charged pellicular resin.

Fig. 1 and Table I show the chromatographic data of a series of fucosylated and related non-fucosylated compounds. Lactose (**10**) was retained 5 min longer than lactosamine (**1**). If the p*K*a of the 2-OH group of pyranosides and the 3-OH group of 2-acetamido-2-deoxypyranosides are comparable[7], then apparently the 4-substitution of βGal hinders access of the 3-OH group of GlcNAc or 2-OH of Gal. The trisaccharide αFuc$(1\rightarrow3)\beta$GlcNAc$(1\rightarrow2)$Man (**2**) had a 9 min and 2 min shorter retention-time than either lactosamine or GlcNAc (retention time of 9.0 min), respectively. Molecular modeling, using the hard-sphere exoanomeric approach[8], showed that the acetamido group in compound **2** approximates the 2-OH group of fucose (Rao *et al.*, unpublished results). Therefore, the much shorter retention time of compound **2** relative to **1** and **3** was explained by the combined effect of removal of the 3-OH group of GlcNAc by substitution and blocking of the 2-OH group of Fuc with the acetamido group of GlcNAc. The result was a trisaccharide having approximately the same retention-time as a reducing monosaccharide (6–10 min under these gradient conditions). A similar trend was noted by comparing the retention times of the di-branched lactosamine-type structures, compounds **7** and **8**.

*All designated sugars are the D enantiomers, except for fucose, which is the L enantiomer.

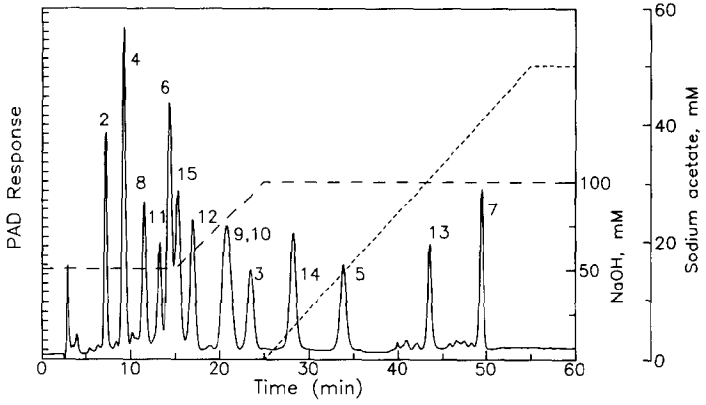

Fig. 1. H.p.a.e. chromatography of neutral and fucosylated oligosaccharides on CarboPak PA-1. An aliquot (50 μL) containing 750 pmol each of compounds 2–15 was injected onto a CarboPak PA-1 column at 0.7 mL/min, and eluted with a gradient of NaOH (dashed line) and NaOAc (dotted line) as described under "Materials and Methods". Detection was by pulsed amperometry, 1000 nA full scale. The numbers above each peak correspond to those in Table I.

We next investigated the retention time effects of subterminal Fuc substitutions on Type I [βGal(1→3)GlcNAc-] and II [βGal(1→4)GlcNAc-] chains. Addition of αFuc(1→3) to the GlcNAc of a Type II chain resulted in a 19–28 min decrease in retention times as compared to non-fucosylated counterparts (compare compounds **5** and **6**; compounds **7** and **9**). If all readily ionizable hydroxyl groups were equally available to the stationary phase, compound **9** should have a retention time comparable to compound **7**, as substitution of the 3-OH groups to the two branch GlcNAc groups should be compensated for by the addition of the two Fuc 2-OH groups. We attribute the 28-min shorter retention of **9** to both removal of 3-OH groups through Fuc substitution and steric hindrance of the Fuc 2-position oxyanions by the acetamido groups of the GlcNAc residues. The result was a nonasaccharide having the same retention time as lactose.

The h.p.a.e. procedure effectively separates βGal(1→4)GlcNAc from βGal(1→3)GlcNAc chains[5]. For example, Lacto-*N*-tetraose (**13**) and Lacto-*N*-neotetraose (**5**) were separated by 10 min. The greater acidity of the 3-OH group of GlcNAc relative to its 4-OH predicts that compound **5** should be eluted later than **13**. Conformational analysis[8,9] of these two types of linkages showed that the disaccharide unit, βGal(1→3)GlcNAc, has a distinct hydrophilic face produced by both monosaccharides. In contrast, for the βGal(1→4)GlcNAc sequence, the hydroxyl groups of Gal and GlcNAc are in distinct planes and do not form a planar "sheet" of hydrophilic residues. We speculated[5] that this distinct conformational difference allowed the βGal(1→3)GlcNAc-containing structures to interact more strongly with the positively charged resin, even though 3-OH is substituted. Presumably, in certain conformations, the less well ionized hydroxyl groups contribute to column retention time. Substitution to the βGal(1→3)GlcNAc unit

TABLE I

RETENTION TIMES OF FUCOSYLATED OLIGOSACCHARIDES AND RELATED COMPOUNDS USING h.p.a.e.–p.a.d.

Number	*Oligosaccharide structure*	*Retention times (min)*[a]
1	βGal(1→4)GlcNAc	16.2
2	αFuc(1→3)βGlcNAc(1→2)Man	7.1
3	βGal(1→4)βGlcNAc(1→6)Man	23.4
4	βGal(1→4)βGlcNAc(1→2)Man ↗ αFuc(1→3)	9.3
5	βGal(1→4)βGlcNAc(1→3)βGal(1→4)Glc	33.8
6	βGal(1→4)βGlcNAc(1→3)βGal(1→4)Glc ↗ αFuc(1→3)	14.4
7	βGal(1→4)βGlcNAc(1→2)αMan(1→6) ↘ Man ↗ βGal(1→4)βGlcNAc(1→2)αMan(1→3)	49.4
8	αFuc(1→3)βGlcNAc(1→2)αMan(1→6) ↘ Man ↗ αFuc(1→3)βGlcNAc(1→2)αMan(1→3)	11.5
9	αFuc(1→3) ↘ βGal(1→4)βGlcNAc(1→2)αMan)1→6) ↘ Man ↗ βGal(1→4)βGlcNAc(1→2)αMan(1→3) ↗ αFuc(1→3)	21.0
10	βGal(1→4)Glc	21.0
11	βGal(1→4)Glc ↗ αFuc(1→3)	13.2
12	αFuc(1→2)βGal(1→4)Glc ↗ αFuc(1→3)	17.0
13	βGal(1→3)βGlcNAc(1→3)βGal(1→4)Glc	43.6
14	αFuc(1→2)βGal(1→3)βGlcNAc(1→3)βGal(1→4)Glc	28.2
15	βGal(1→3)βGlcNAc(1→3)βGal(1→4)Glc ↗ αFuc(1→4)	15.3

[a]Retention times were determined by using the gradient described under "Materials and Methods".

with α-(1→4)-linked fucose (**15**) resulted in an almost 30-min decrease in retention time of the Lacto-*N*-tetraose structure (**13**). Terminal αFuc(1→2) linkage to Gal had a less dramatic effect on the retention time of a Lacto-*N*-tetraose chain with a 15-min decrease in retention time.

Substitution of lactose chains also resulted in decreased retention times. Addition of αFuc(1→3) to lactose (**11**) decreased its retention time by ≃7 min. Either the 3-OH group of Glc is substantially charged or the 3-linked Fuc sterically blocks other charged groups (namely the 2-OH group of Glc). Addition of another αFuc(1→2) to the terminal Gal (**12**) gave a 4-min increase in retention time, implying some access of 2-OH of a terminal Fuc to the stationary phase.

Fucosylated oligosaccharides have been separated using reverse-phase h.p.l.c.[10,11]. In these studies, linear tetrasaccharides fucosylated at the subterminal GlcNAc residue were less strongly retained (≃1–2 min) than their non-fucosylated counterparts, and a conformational argument was invoked to explain the earlier elution of the theoretically more hydrophobic fucosylated oligosaccharides[10]. One difference in reverse-phase and h.p.a.e. elution order was that fucosylated compound **14** was reported to be more retained (≃2 min)[10] than compound **13**, whereas we found that compound **14** eluted ≃15 min earlier than compound **13**.

In summary, h.p.a.e. can widely separate commonly encountered fucosylated oligosaccharides from their non-fucosylated counterparts. The bulk of the interaction is apparently a result of conformational features of the oligosaccharides, which determine the efficiency of interaction of the most readily ionizable groups of the oligosaccharides (the reducing anomeric hydroxyl group, the 2-OH groups of unsubstituted pyranoses, and the 3-OH group of 2-acetamido-2-deoxypyranosides) with the pellicular anion-exchange resin. Although this study has emphasized the effects of fucose, substitution by other sugars should have similar effects. Less-dramatic differences in retention times (but equally as important for desired separations) may be a result of participation of oxyanions at different ring positions, particularly from the 6-OH group. The potential effect of substitution on the p*K*a values of neighboring hydroxyl groups was not considered. However, the overall result is enhanced chromatographic selectivity, apparently based on the spatial arrangement of oxyanions.

MATERIALS AND METHODS

Materials. — Compounds **1** and **10** were from Sigma Chemical Co. (the number corresponds to the designation of oligosaccharides in Table I). Compounds **5, 6, 11–12,** and **14–15** were purchased from BioCarb, Lund, Sweden. The other compounds used in this study were donated by the following individuals: **2, 4, 8,** and **9** from Dr. Hans Lönn, University of Stockholm, Department of Organic Chemistry; **3** and **7** from Dr. J. Lönngren, University of Stockholm, Department of Organic Chemistry; and **13** from Dr. V. Ginsburg, National Institutes of Health.

Sodium hydroxide solution (50%, w/w) was purchased from Fisher Scientific (Rockville, MD). Sodium acetate was from J. T. Baker (Philipsburg N.J.). Nylon membranes were from Schleicher and Schuell (Keene, NH).

Chromatographic apparatus. — The system used for h.p.a.e.–p.a.d. consisted of a Dionex GPM pump and model p.a.d. 2 pulsed amperometric detector. The

Dionex Eluant Degas Module was employed to sparge and pressurize the eluants with helium. For the present separations, eluant *A* was mM NaOH, eluant *B* was 100mM NaOH, and eluant *C* was 100mM NaOH containing 0.2M NaOAc. These solutions were prepared by suitable dilution of 50% NaOH solution with glass-distilled water. Eluants containing NaOAc were filtered through 0.2-μm nylon membranes before use. Sample injection was *via* a Spectra-Physics SP8780 autosampler equipped with a 200-μL sample loop. The Rheodyne injection valve on the autosampler was equipped with a Tefzel rotor seal to withstand the alkalinity of the eluants. Oligosaccharides were separated on a column (4 × 250 mm) of Dionex CarboPac PA-1 pellicular anion-exchange resin using a flow rate of 0.7 mL/min at ambient temperature, equipped with an Dionex AG-6 guard column. The gradient program used to elute oligosaccharides in the present study was as follows. The sample was introduced into a system equilibrated at 50% eluant *A*, 50% eluant *B*. This proportion was maintained for 15 min, at which time the proportion of eluant *B* was increased linearly to 100% at 25 min. A linear gradient to 75% eluant *B*, 25% eluant *C* at 55 min was then executed. Isocratic elution at the latter condition was maintained for 10 min, with return to initial conditions at 70 min. Time between injections was 85 min. The NaOH (300mM) was added to the post-column effluent *via* a mixing tee at a flow rate of 0.7 mL/min, using a Dionex DQP-1 Pump.

Pulsed-amperometric detection was used with a gold working electrode and triple-pulse amperometry[12]. The following pulse potentials and durations were used for detection of oligosaccharides: $E_1 = 0.05$ V ($t_1 = 360$ ms); $E_2 = 0.80$ V ($t_2 = 120$ ms); and $E_3 = 0.60$ V ($t_1 = 420$ ms). The response time of the p.a.d. 2 was set to 3 s. Chromatographic data were collected and plotted using either a Spectra-Physics model SP4270 integrator or Waters model 840 software.

ACKNOWLEDGMENTS

Samples prepared by Drs. H. Lönn, J. Lönngren, and V. Ginsburg were kindly provided by Professor Y. C. Lee. The discussions of oligosaccharide conformations with Dr. B. N. Narasinga Rao is gratefully acknowledge. The Dionex Carbohydrate Analyzer was purchased with National Institutes of Health Research Grant DK09970 and experiments were performed in the laboratory of Professor Y. C. Lee.

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