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Separation of mucin oligosaccharide-alditols by high performance liquid chromatography on alkylamine-bonded silica columns. Effects of structural parameters

André Klein, Christophe Carnoy, Jean-Marc Lo-Guidice, Geneviève Lamblin and Phillippe Roussel

Unité des Protéines, I.N.S.E.R.M. U16, Place de Verdun, F-59045 Lille (France)

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ABSTRACT

Forty five oligosaccharide-alditols, purified after reductive β-elimination of human bronchial mucins, were analyzed by HPLC on alkylamine-bonded silica column (Lichrosorb-NH_2). The comparison of their structural features and retention times permitted the extension of some previous findings. The chromatographic behavior of the oligosaccharide depends more on the accessibility of oligosaccharide hydroxyl groups than on the sugar composition. The use of this type of fractionation is very efficient for low-molecular-mass oligosaccharide-alditols but needs to be completed by a second chromatographic step for higher molecular-mass oligosaccharide-alditols.

INTRODUCTION

Human bronchial mucins are constituted of 60 to 80% of carbohydrates[1]. These carbohydrate chains are *O*-glycosyl-linked to serine or threonine and can be released as oligosaccharide-alditols by alkaline borohydride treatment[2]. The mucin oligosaccharides are extremely polydisperse, with regard to size diversity, numbers of isomers, and variable acidic characters due to the presence of sialyl and sulfate residues. Techniques such as gel filtration or paper chromatography are not efficient enough to resolve the heterogeneity of these oligosaccharides. During the last decade, HPLC techniques have been developed to isolate bronchial mucin oligosaccharides in sufficient amount and purity to allow their primary structure determination by NMR and mass spectrometry. The fractionation is based on the use of silica gel derivatized with aminopropyl groups[3,4], or on the sequential utilization of two different columns, one containing silica gel derivatized with

Correspondence to: Professor P. Roussel, Unité des Protéines, I.N.S.E.R.M. U16, Place de Verdun, F-59045 Lille, France.

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aminopropyl groups (normal-phase chromatography) and the other with octadecyl groups (reverse–phase chromatography)[5,6].

Silica derivatized with primary amine groups has been successfully used for the separation of numerous mono-, oligo-, and poly-saccharides[7–9]. The separation is mainly due to hydrogen bonding between the carbohydrate hydroxyl groups and the matrix[10]. The effects of some structural parameters have been described by Blanken et al.[11]. In the present study, we have compared the retention times and the structural features of 45 neutral oligosaccharide-alditols, thus extending the previous observations of Blanken et al.[11].

EXPERIMENTAL

Preparation of oligosaccharide-alditols and HPLC. — Forty five oligosaccharides (**1–45**) were obtained by alkaline-reductive degradation of mucin glycopeptides isolated from the sputum of a secretor patient with blood group O ($Le^{a^-b^+}$)[5,6] and of a nonsecretor patient ($Le^{a^+b^-}$)[12].

HPLC was performed with a Varian (Walton-on-Thames, UK), model 5000, equipped with a Lichrosorb-NH_2 column (Merck, Darmstadt, FRG) (4 × 250 mm, particle size 5 μm) at room temperature, and with a linear gradient of acetonitrile–water (17:3, up to 3:2), for 60 min at a flow rate of 1 mL/min. Oligosaccharide peaks were detected by absorption at 206 nm. Two sets of experiments were performed. Since there was a decrease in column efficiency due to the matrix breakdown by hydrolysis of the bonded phase, the retention times are expressed relative to that of 2-acetamido-2-deoxy-D-galactitol taken as 1.0.

RESULTS

Structures, retention times (RT), and relative retention times (RRT) of the oligosaccharides are reported in Table I.

The effect of a given structural element was calculated from the difference of the relative retention times of the oligosaccharide-alditols possessing or not this structural element (ΔRRT). The differences were plotted as a function of the number of sugar units, and the linear regression and the correlation factor were calculated and are reported in Table II.

Effects of a terminal, nonreducing β-D-galactopyranosyl group. — The differences in relative retention times (ΔRRT) of oligosaccharide-alditols couples **1–2**, **4–5**, **4–7**, **12–14**, **13–17**, **23–24**, **23–26**, **30–31**, **30–45**, **31–35**, **31–41**, and **32–36** were plotted against the number of units of the oligosaccharide-alditols. They gave a linear plot with an intercept of 1.198 that corresponds to the retardation effect of an additional D-galactose unit, and a slope of −0.124 that corresponds to the effect of the water gradient on the retention times[10,11,13] (Fig. 1 and Table II).

TABLE I

Retention times (RT) and relative retention times (RRT) of human bronchial mucin oligosaccharide-alditols [a]

Oligosaccharide	RT (min)	RRT
1 Gan (standard unit)	11.0, 11.2	1.00
2 G → 3Gan	23.2, 23.3	2.10
3 F → 2G → 3Gan	25.3	2.30
4 Gn → 3G → 3Gan	31.5	2.81
5 G → 3Gn → 3G → 3Gan	40.3	3.60
6 Gn → 6(Gn → 3)G → 3Gan	40.0	3.57
7 G → 4Gn → 3G → 3Gan	40.6, 41.3	3.69
8 G → 4(F → 3)Gn → 3Gan	45.5, 46.6	4.15
9 F → 2G → 4(F → 3)Gn → 3G → 3Gan	50.4	4.58
10 F → 4(G → 3)Gn → 3G → 3Gan	46.6	4.16
11 G → 4(F → 3)Gn → 3(Gn → 6)G → 3Gan	52.8	4.71
12 Gn → 6(G → 3)Gan	34.1, 34.5	3.09
13 F → 2G → 3(Gn → 6)Gan	36.5	3.32
14 G → 4Gn → 6(G → 3)Gan	42.9, 43.8	3.91
15 G → 4(F → 3)Gn → 6(G → 3)Gan	48.5, 49	4.39
16 F → 2G → 4Gn → 6(G → 3)Gan	47.6	4.33
17 G → 4Gn → 6(F → 2G → 3)Gan	44.4	4.04
18 F → 2G → 4Gn → 6(F → 2G → 3)Gan	48.5	4.41
19 G → 4(F → 3)Gn → 6(F → 2G → 3)Gan	48.5	4.41
20 F → 2G → 4(F → 3)Gn → 6(F → 2G → 3)Gan	53.5	4.86
21 G → 4Gn → 3G → 3(G → 4Gn → 6)Gan	55.7	5.06
22 G → 4(F → 3)Gn → 3G → 3(G → 4Gn → 6)Gan	58.8	5.35
23 Gn → 3Gan	20.2, 20.5	1.83
24 G → 3Gn → 3Gan	30.0, 29.8	2.70
25 F → 2G → 3Gn → 3Gan	38.2	3.47
26 G → 4Gn → 3Gan	31.2, 31.5	2.83
27 F → 2G → 4Gn → 3Gan	36.5	3.32
28 G → 4(F → 3)Gn → 3Gan	38.3	3.42
29 F → 2G → 4(F → 3)Gn → 3Gan	42.9	3.90
30 Gn → 3(Gn → 6)Gan	31.5	2.81
31 G → 4Gn → 6(Gn → 3)Gan	40.6, 41.3	3.69
32 F → 2G → 4Gn → 6(Gn → 3)Gan	44.7	4.06
33 G → 4(F → 3)Gn → 6(Gn → 3)Gan	46.6	4.16
34 F → 2G → 4(F → 3)Gn → 6(Gn → 3)Gan	50.4	4.58
35 G → 4Gn → 6(G → 4Gn → 3)Gan	47.6, 48.5	4.33
36 F → 2G → 4Gn → 6(G → 4Gn → 3)Gan	51.2	4.65
37 F → 2G → 4Gn → 3(G → 4Gn → 6)Gan	51.2	4.65
38 F → 2G → 4Gn → 6(F → 2G → 4Gn → 3)Gan	55.1	5.01
39 F → 2G → 4(F → 3)Gn → 3(F → 2G → 4Gn → 6)Gan	58.8	5.36
40 F → 2G → 4(F → 3)Gn → 6(G → 4Gn → 3)Gan	56.5	5.14
41 G → 4Gn → 6(G → 3Gn → 3)Gan	48.5	4.41
42 F → 2G → 3Gn → 3(G → 4Gn → 6)Gan	52.6	4.78
43 F → 2G → 4Gn → 6(F → 2G → 3Gn → 3)Gan	56.5	5.14
44 F → 2G → 4(F → 3)Gn → 6(F → 2G → 3Gn → 3)Gan	60.6	5.51
45 G → 3Gn → 3(Gn → 6)Gan	40.3	3.60

[a] Two values of RT are given when the compound was isolated from mucins of two different patients[5,12]. Abbreviated structures: G, β-D-Gal*p*-(1 →; Gn, β-D-Glc*p*NAc-(1 →; F, α-L-Fuc*p*-(1 →; and Gan, D-GalNAcol

TABLE II

Correlation between the retardation effect and the number of sugar units when a nonreducing, terminal group is added to an oligosaccharide-alditol [a]

Group added	Effect on retention time	
	ΔRRT	R
β-D-Gal*p*	1.205–0.126*n*	0.95
β-D-Glc*p*NAc	1.027–0.760*n*	0.52
α-L-Fuc*p*-(1 → 2)- to:		
β-D-Gal*p*-(1 → 3)-D-GalNAcol	0.354–0.059*n*	0.89
β-D-Gal*p*-(1 → 4)-[H or α-L-Fuc*p*-(1 → 3)]-D-GlcNAc	0.518–0.024*n*	0.47
β-D-Gal*p*-(1 → 4)-D-GlcNAc	0.565–0.041*n*	0.74
β-D-Gal*p*-(1 → 4)-[α-L-Fuc*p*-(1 → 3)]-D-GlcNAc	0.520–0.015*n*	0.46
α-L-Fuc*p*-(1 → 3)- to:		
H or α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 4)-D-GlcNAc	0.707–0.050*n*	0.72
β-D-Gal*p*-(1 → 4)-D-GlcNAc	0.871–0.099*n*	0.99
α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 4)-D-GlcNAc	0.887–0.073*n*	0.97
α-L-Fuc*p*-(1 → 2)- and α-L-Fuc*p*-(1 → 3)- to:		
β-D-Gal*p*-(1 → 4)-D-GlcNAc	1.346–0.106*n*	0.98

[a] ΔRRT is the difference of relative retention times; *n* the number of sugar units; and R is the correlation factor of the linear regression.

Effect of a terminal, nonreducing 2-acetamido-2-deoxy-β-D-glucopyranosyl group. — The effect of a terminal, nonreducing 2-acetamido-2-deoxy-β-D-glucopyranosyl group was deduced from the comparison of the ΔRRT of the couples **1–23**, **2–4**, **2–12**, **3–13**, **4–6**, **8–11**, **23–30**, and **24–45**. The linear plot deduced from the differences of relative retention times has an intercept of 1.027 and a slope of −0.07 with a correlation factor of 0.52 (Table II), indicating that the population of coupled oligosaccharides studied is not homogeneous; a distinction has to be made

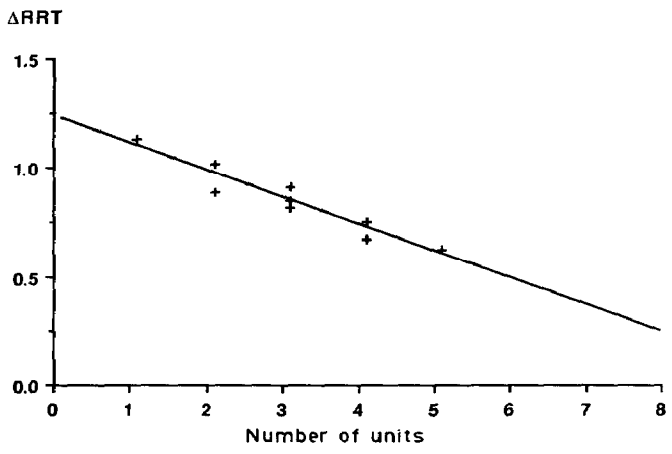

Fig. 1. Correlation between the retardation effect and the number of sugar units when a terminal, nonreducing β-D-galactopyranosyl group is added to an oligosaccharide-alditol. ΔRRT = 1.205 − 0.126*n*, R = 0.95.

as to whether the β-D-Glc*p*NAc group is linked (1 → 3) or (1 → 6). For (1 → 3)-linked β-D-Glc*p*NAc groups (oligosaccharide-alditols pairs **1–23** and **2–4**) the ΔRRT are 0.84 and 0.73, respectively. The couples **2–12**, **3–13**, **23–30**, and **24–45** indicated the effect of a β-D-Glc*p*NAc group linked (1 → 6) to GalNAcol; the retardation effect (ΔRRT) with values ranging from 0.9 to 1 is higher than that of the (1 → 3)-linked analogs previously described by Blanken et al.[11]. Contrary to previous suggestions, a β-D-Glc*p*NAc group (1 → 6)-linked to a D-galactose unit does not seem to induce a large effect: the ΔRRT of the pairs **4–6** and **8–11** are 0.76 and 0.56, respectively[11].

*Effect of a nonreducing, terminal α-L-fucopyranosyl group (1 → 2)-linked to β-D-Gal*p*-(1 → 3)-D-GalNAcol.* — The linear plot of the ΔRRT of couples **2–3**, **12–13**, **14–17**, **15–19**, and **16–18** has an intercept of 0.354 and a slope of −0.059, indicating a weak retardation effect of the α-L-fucopyranosyl group, as previously observed[11] (Table II). The addition of an α-L-fucopyranosyl group (pair **15–19**) does not change the retention time.

Effect of a nonreducing, terminal α-L-fucopyranosyl group (1 → 2)-linked to a (1 → 4)-linked β-D-galactopyranosyl residue. — The effect of an α-L-fucosyl group (1 → 2)-linked to a (1 → 4)-linked β-D-galactosyl residue was deduced from the plot obtained with the ΔRRT of oligosaccharide-alditols pairs **8–9**, **14–16**, **17–18**, **19–20**, **26–27**, **28–29**, **31–32**, **33–34**, **35–36**, **35–37**, **37–38**, **42–43**, and **43–44**. The intercept has a value of 0.518, indicating a retardation effect higher than in the previous group. The value of the slope is −0.024 and that of the correlation factor 0.47 (Table II). Two subgroups could be distinguished. (*a*) The first group corresponds to the retardation effect induced by the addition of an α-L-fucosyl group (1 → 2)-linked to a Type 2 disaccharide [β-D-Gal*p*-(1 → 4)-D-GlcNAc], to give an H-Type 2 determinant [α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 4)-D-GlcNAc]. This could be measured by comparing the couples **14–16**, **17–18**, **26–27**, **31–32**, **35–36**, **35–37**, **37–38** and **42–43**. The linear plot has an intercept of 0.565, a slope of −0.04, and a correlation factor of 0.74. (*b*) The second group corresponds to the addition of an α-L-fucosyl group (1 → 2)-linked to an X determinant {β-D-Gal*p*-(1 → 4)-[α-L-Fuc*p*-(1 → 3)]-β-D-GlcNAc} to give a Y determinant {α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 4)-[α-L-Fuc*p*(1 → 3)]-D-GlcNAc}. It induces a retardation effect that can be deduced from the comparison of the ΔRRT of couples, **8–9**, **19–20**, **28–29**, and **33–34**. The equation of the linear plot has values of 0.52 as intercept and −0.015 for the slope with a correlation factor of 0.85 (Table II).

Effect of a nonreducing, terminal α-L-fucopyranosyl group (1 → 3)-linked to a 2-acetamido-2-deoxy-D-glucose residue. — The ΔRRT of oligosaccharide-alditols pairs, **7–8**, **14–15**, **17–19**, **18–20**, **21–22**, **26–28**, **27–29**, **31–33**, **32–34**, **36–40**, **38–39**, and **43–44**, allowed us to draw a linear plot with a correlation factor of 0.72 (Table II). The values of the intercept and of the slope are 0.707 and −0.050, respectively. Two subgroups were observed. (*a*) One corresponds to the addition of an α-L-fucosyl group (1 → 3)-linked to a Type 2 disaccharide to give an X determinant {β-D-Gal*p*-(1 → 4)-[α-L-Fuc*p*-(1 → 3)]-D-GlcNAc}. The ΔRRT values

were obtained from the comparison of couples, **7–8**, **14–15**, **17–19**, **21–22**, **26–28**, and **31–33**. The values of the intercept and of the slope of the linear plot are 0.871 and −0.099, respectively, with a correlation factor of 0.99 (Table II). (*b*) The effect of adding an α-L-fucosyl group (1 → 3)-linked to an H Type 2 determinant {α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 4)-D-GlcNAc} to give a Y determinant {α-L-Fuc*p*-(1 → 2)β-D-Gal*p*-(1 → 4)-[α-L-Fuc*p*-(1 → 3)]-D-GlcNAc} could be observed from the ΔRRT of the couples, **18–20**, **27–29**, **32–34**, **36–40**, **38–39**, and **43–44**. The values of the intercept and of the slope are 0.887 and −0.773, respectively, with a correlation factor of 0.97 (Table II).

Effect of two nonreducing, terminal α-L-fucopyranosyl groups (1 → 2)- and (1 → 3)-linked in a Y determinant. — The ΔRRT obtained from the couples, **7–9**, **17–20**, **26–29**, **31–34**, **35–40**, **36–39**, and **42–44**, indicated the effects of adding two α-L-fucosyl groups to a Type 2 determinant. The value of the intercept and of the slope are 1.346 and −0.106, respectively, with a correlation factor of 0.97 (Table II).

DISCUSSION

The sugar composition of an oligosaccharide influences the retention on an alkylamine-bonded column, for example, the retardation effect induced by a D-galactose unit is superior to that induced by 2-acetamido-2-deoxy-D-glucose and L-fucose units. Blanken et al.[11] and Green and Baenziger[14] described an increase in retention time induced by a (1 → 6) link or a (2 → 6)-linked α-sialyl group, as compared to a (1 → 3) link and a (2 → 3)-linked α-sialyl groups, respectively. They explained this phenomenon by the flexibility of the linkages, allowing an increased interaction of hydroxyl groups with the matrix. In the present series of oligosaccharides, this phenomenon was also observed when a β-DGlc*p*NAc residue was linked (1 → 6) to a D-GalNAcol residue. In two cases, couples **4–6** and **10–11**, where a β-D-Glc*p*NAc residue is linked (1 → 6) to a D-galactose unit, the ΔRRT were lower than expected (0.76 and 0.55). However, the number of observed cases is too low to draw any definitive conclusion.

The effects of an additional α-L-Fuc*p* group could be classified in order of importance. The retardation effect induced by this group is lower in the structure α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 3)-D-GalNAcol than in the H Type 2 determinant. The highest retardation effect is due to an α-L-fucosyl group (1 → 3)-linked to a D-GlcNAc unit in a Type 2 disaccharide.

These effects may be compared with those observed on a octadecylsilane-bonded column (C_{18} column) where oligosaccharides possessing an α-L-Fuc*p* group (1 → 3)-linked to a D-GlcNAc unit (in an X or a Y determinant) are not retained, whereas oligosaccharides with a (1 → 2)-linked α-L-Fuc*p* group in an H determinant or linked to a β-D-Gal*p*-(1 → 3)-D-GalNAcol structure are strongly retained[6]. The retardation effect on a Lichrosorb-NH_2 column is due to the accessibility of hydroxyl groups[10,11,15], whereas on C_{18} columns, the elution order depends on the

hydrophobicity of the oligosaccharide which is mainly due to the methyl or acetamido groups of the sugar units[16] and their accessibility[17].

The influence of an α-L-fucosyl group (1 → 3)-linked to a D-GlcNAc unit in an X or a Y determinant on the retention time of an oligosaccharide can be explained by the more hydrophilic character of this type of oligosaccharide having hydroxyl groups in a more external position and the L-fucose methyl group in a more inside position, than is the case of an oligosaccharide having a (1 → 2)-linked α-L-Fuc*p* group in an H determinant, as already mentioned by Hindsgaul et al.[18].

During analysis of the different linear plots, it became obvious that the more precise is the structural element studied in a series of oligosaccharides, the better is the correlation factor of its linear plot. The chromatographic behavior of carbohydrates on an alkylamine-bonded column was first explained by the total number and the location of hydroxyl groups[10,14]. Blanken et al.[11] pointed out the influence of the (1 → 6) linkage. In the present study, the behavior of mucin oligosaccharide-alditols seems to depend overall on their conformation.

For the fractionation of mucin-oligosaccharide alditols, Lichrosorb-NH_2 is a very efficient medium, but not sufficient when the number of sugar residues becomes large. In the case of human respiratory mucins, where the heterogeneity is preponderant, the separation on Lichrosorb-NH_2 had to be complemented by a second step on an octadecylsilane-bonded silica column[20].

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