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Separation of neutral oligosaccharide alditols from human meconium using high-pH anion-exchange chromatography

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ABSTRACT

Neutral reduced oligosaccharides are in general not sufficiently retained to achieve adequate separation and reproducible chromatography using high-pH anion-exchange chromatography. We describe a method to increase the retention using two columns in series. This method has been applied to the separation of oligosaccharides purified from human meconium glycoproteins, obtained as their alditols after alkaline-borohydride release of oligosaccharides. The neutral oligosaccharide alditols were significantly retained upon two CarboPac PA-100 columns, connected in series, and eluted in 80 m*M* sodium hydroxide between 4 and 10 min. Three sialylated alditols studied were substantially retained and could be eluted in a gradient of 0–500 m*M* sodium acetate–80 m*M* sodium hydroxide between 10 and 45 min. The elution patterns were based on their size, charge and linkage, such that oligosaccharide alditol isomers could be separated.

INTRODUCTION

Analytical and preparative separation of oligosaccharides is very important in the investigation of the biological roles of glycoconjugates. Many neutral oligosaccharides, which differ by only one sugar residue or the presence of a $\beta(1\rightarrow6)$ linkage, can be separated on normal-phase HPLC using amino-bonded silica gel and reversed-phase HPLC using alkyl-bonded silica gel [1–7]. However, in general no separation is achieved between $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ positional isomers. Two alternative approaches have been used to achieve isomer separation, either the use of an additional reversed-phase type column based on porous graphitised carbon (PGC) [8,9] or the use of high-pH anion-ex-

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change chromatography coupled with pulsed amperometric detection (PAD). The latter technique has provided (1→3)/(1→4) isomer separation of oligosaccharides with picomole detection [10,11] and has been applied to the analysis of purified oligosaccharides and oligosaccharides derived from both N-linked [10–17] and O-linked chains [18]. Size, charge, composition, linkage position and glycosidic linkage type can all affect retention. However, neutral reduced oligosaccharides are in general not sufficiently retained for adequate separation and reproducible chromatography using this anion-exchange phase [18,19]. A method to increase the alditol retention using two anion-exchange columns in series is described and has been applied to the separation of neutral reduced oligosaccharides derived from human meconium.

EXPERIMENTAL

Materials

The oligosaccharides designated R1, R2, O1, O4, N1, K2(3) and K5(3) were standards of known structure, purified from glycoproteins extracted from human meconium samples of group O neonates of secretor type (*i.e.* having blood-group H activities) as previously described [20,21]. They were obtained as their alditols after alkaline-borohydride degradation in 0.05 *M* sodium hydroxide–1 *M* sodium borohydride, for 16 h at 50°C. Sialyllactose, predominantly the α(2→6)-linked isomer (SA6L) from human milk (Batch No. A2423), and the α(2→3) isomer from bovine colostrum (Batch No. A3001) were both obtained from Sigma Chemical Co. (Poole, Dorset, UK). Chitobiose (CB), GlcNAcβ1→4GlcNAc, was also purchased from Sigma. The synthetic disaccharides G3GN, G4GN and G6GN (Table I) were kind gifts from Dr. A. Veyrières (Université Pierre et Marie Curie, Paris, France) and the synthetic oligosaccharide DSLNT (disialyl-lacto-N-tetraose) was obtained from Research Sugars (Long Crendon, Aylesbury, UK). The commercial sialylated and the synthetic neutral oligosaccharides were reduced to alditols using 20 m*M* sodium borohydride in 50 m*M* sodium hydroxide, at 4°C for 18 h. Following reduction, excess borohydride was degraded by acidification to pH 5, using 50% (v/v) glacial acetic acid in water, and the sample was then deionised using Dowex 50-X8 acetate form (3 × 1 cm column) eluted with 3 column volumes of water. The combined effluent and eluent were lyophilised and the boric acid removed by co-evaporation with several additions of methanol.

HPLC-grade 50% (w/v) sodium hydroxide (*ca.* 12.5 *M*), sodium acetate AnalaR grade, and D(+)melibiose (6-0-α-D-galactopyranosyl-D-glucopyranoside) were purchased from BDH (Poole, Dorset, UK). N-Acetylneuraminic acid and N-glycolylneuraminic acid were purchased from Sigma Chemical Co.

Apparatus and columns

HPLC analysis was performed using a Dionex quarternary advanced gradient pump, coupled with pulsed amperometric detection (Dionex UK, Camberley, Surrey, UK). Both one and two (connected in series) Dionex CarboPac PA-100 (250 × 4 mm I.D.) 8.5 μm anion-exchange columns were used in conjunction with a CarboPac PA-100 (50 × 4 mm I.D.) 8.5 μm guard column. Samples were injected using a 9125 Rheodyne injection valve (Cotati, CA, USA). Chromatograms were analysed using a Spectra-Physics SP4600 integrator (San Jose, CA, USA). All eluents were filtered through 0.2-μm inorganic filters (Anodisc; Whatman, Maidstone, UK) and a Dionex eluent degas module was employed to saturate the eluents with helium gas, to degas and to minimize absorption of CO_2.

HPLC of oligosaccharide alditols

Neutral and sialylated oligosaccharide alditols were dissolved, to a working concentration of 3 μg/ml, in HPLC grade water (Rathburn Chemicals, Walkerburn, Scotland). Samples were filtered through 0.2-μm (pore size) Anotop IC disposable filters (Whatman). Aliquots (50 and 100 μl) of the alditols, containing 320 ng of melibiose (6-0-α-D-galactopyranosyl-D-glucopyranoside) as internal standard, were injected onto the CarboPac columns, equilibrated in 80 m*M* sodium hydroxide. Samples were eluted using a gradient as follows: eluent A (80 m*M*

TABLE I

RETENTION TIMES OF NEUTRAL OLIGOSACCHARIDE ALDITOLS ON ONE ANION-EXCHANGE COLUMN

Oligosaccharide alditols		Alditol t_R (min)	Alditol k' [a]
	GalNAcol	2.32	0.24
R1-ol	Galβ1-3GalNAcol	2.37	0.25
	GlcNAcol	2.45	0.31
R2-ol	GalNAcα1-3GalNAcol	2.54	0.32
G3GN-ol	Galβ1-3GlcNAcol	2.57	0.37
O4-ol	Galβ1-3GlcNAcβ1-3GalNAcol and Galβ1-4GlcNAcβ1-3GalNAcol	2.62 and 4.44	0.37 and 1.30
O1-ol	GlcNAcβ1-6, Galβ1-3 \ GalNAcol	2.90	0.54
CB-ol	GlcNAcβ1-4GlcNAcol	2.59	0.44
G6GN-ol	Galβ1-6GlcNAcol	2.93	0.58
G4GN-ol	Galβ1-4GlcNAcol	3.24	0.69
N1-ol	Galβ1-4GlcNAcβ1-6, Galβ1-3 \ GalNAcol	3.46	0.75
K2(3)-ol	GlcNAcα1-4Galβ1-4GlcNAcβ1-6, Galβ1-3 \ GalNAcol	3.73	1.08
K5(3)-ol	Galβ1-4GlcNAcβ1-6, Galβ1-3/4GlcNAcβ1-3 \ GalNAcol	3.66 and 6.91	0.99 and 2.76

[a] k' = capacity ratio or factor; a measure of solute retention, calculated as retention time of alditol (t_R)-retention time of an unretained solute (individual solvent front, t_0)/t_0. Mean solvent front was 1.85 min (C.V. = 1.40%, n = 13) and the mean retention time of the internal standard (melibiose) was 5.81 min (C.V. = 2.03%, n = 13).

sodium hydroxide) and eluent B (500 m*M* sodium acetate–80 m*M* sodium hydroxide): 0–10 min, 0% B; 10–30 min, 0–20% B; 30–45 min, 20–100% B; 45–50 min, 100% B. The flow-rate was 0.7 ml/min. Detection was by pulsed amperometric detection, using a gold working electrode and a silver/silver chloride reference electrode. The detector output was at 1 μA, with a detection potential (E_1) of +0.05 V, an oxidation potential (E_2) of +0.6 V and a reduction potential (E_3) of −0.6 V. Time constants for each applied potential (on range 2) were t_1 = 720 ms (position 9), t_2 = 300 ms (position 2) and t_3 = 240 ms (position 1) respectively.

RESULTS

Using single Dionex CarboPac column chromatography, the neutral reduced mono- to trisaccharides eluted very rapidly (2 to 3.5 min) (see Table I). The two reduced trisaccharide isomers in sample O4-ol, differing only through

their $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ linked terminal galactose (see Table I), were separated into two separate peaks with retention times of 2.62 and 4.44 min ($k' = 0.37$ and 1.30 respectively). Similarly, the two pentasaccharide isomers of sample K5(3)-ol were also separated with retention times of 3.66 and 6.91 min ($k' = 0.99$ and 2.76). The mean retention time of the column void volume (solvent front) was 1.85 min [coefficient of variation (C.V.) = 1.40%; $n = 13$] and the mean retention time of the internal standard (melibiose) was 5.81 min (C.V. = 2.03%; $n = 13$). In contrast, by using two Dionex CarboPac PA-100 anion-exchange columns connected in series, a significant increase in separation was achieved, with the neutral alditols eluting between 4 and 18 min, and all the alditols studied eluting within 45 min (see Tables II and III). The mean solvent front was at 3.43 min (C.V. = 1.18%; $n = 20$) and melibiose was retained for 13.13 min (C.V. = 1.80%, $n = 20$). High variation in alditol and internal standard elution times could be observed between different chromatographic runs unless the column was regenerated with strong alkaline solution (500 m*M* sodium acetate–80 m*M* sodium hydroxide) and then re-equilibrated in 80 m*M* sodium hydroxide for a period of 25 min, before initiating the next chromatogram. This phenomenon has been previously described with this high-pH anion-exchange phase [18].

Using the dual column method, the two monosaccharide acetamido sugar alditols, D-GalNAcol and D-GlcNAcol, were resolved clear of the solvent front and eluted earlier than the oligosaccharide alditol structures studied; retention times were between 4 and 5 min (see Table II). Linear neutral disaccharide alditols, of the same monosaccharide composition (*e.g.* G3GN-, G6GN- and G4GN-ol) but varying in their linkage position of sugar residues ($\beta1\rightarrow3$, $\beta1\rightarrow6$ and $\beta1\rightarrow4$ respectively), were individually separated between 5 and 7 min ($k' = 0.49$, 0.72 and 0.85 respectively). Three separate peaks were resolved from an admixture of G3GN-, G4GN- and G6GN-ol, with retention times of 5.29, 6.12 and 6.47 min ($k' = 0.54$, 0.78 and 0.89 respectively). Thus, the presence of a $\beta(1\rightarrow3)$ linked galactose reduced the retention time when compared to the $\beta(1\rightarrow6)$ and $\beta(1\rightarrow4)$ isomeric groups (see Fig. 1). The two positional trisaccharide alditol isomers contained in sample O4-ol, differing only through their $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ linked terminal galactose residue, were separated into two peaks with retention times of 5.10 and 9.32 min (see Fig. 2a), with a significant increase in retention ($k' =$ 0.45 and 1.66 respectively) in comparison to the single-column chromatography. Likewise, separation of the two pentasaccharide isomers present in sample K5(3)-ol was also achieved, resolving two peaks with increased retention times of 7.85 and 17.61 min ($k' = 1.24$ and 4.03) (see Fig. 2b). The presence of branched isomerism and the progressive extension of the oligosaccharide alditol side-chains, generally increased retention times on CarboPac PA-100 anion-exchange resin, as demonstrated by the meconium alditols O1-ol (GlcNAcβ1-6{Galβ1-3}GalNAcol), N1-ol (Galβ1-4GlcNAcβ1-6{Galβ1-3}GalNAcol) and K2(3)-ol (GlcNAcα1-4Galβ1-4GlcNAcβ1-6{Galβ1-3}GalNAcol) (see Table II).

In comparison to the neutral alditol structures, the sialylated oligosaccharide alditols studied had by far the longest retention times, being resolved between 25 and 45 min on two anion-exchange columns (see Table III). The $\alpha(2\rightarrow6)$-linked sialyllactitol (SA6L-ol), eluted significantly earlier than the $\alpha(2\rightarrow3)$-linked sialylated isomer, SA3L-ol (see Fig. 3). The di-sialylated oligosaccharide alditol, DSLNT-ol, had the longest retention time on the columns, eluting at 40.34 min ($k' = 10.69$) (Table III). The analysis of an admixture of the three sialylated oligosaccharide alditols, resolved three separate sharp peaks (see Fig. 4). In addition, two acidic monosaccharides, N-acetylneuraminic acid (NeuAc) and N-glycolylneuraminic acid (NeuGc), were also separated from each other by more than 10 min under these chromatographic conditions. NeuGc possessed a much longer elution time (40.03 min, $k' = 10.64$) on the anion-exchange columns than NeuAc (29.63 min, $k' = 7.69$), as has been shown previously [15,18].

DISCUSSION

This study shows that dual column anion-exchange chromatography under high-pH condi-

TABLE II

RETENTION TIMES OF NEUTRAL OLIGOSACCHARIDE ALDITOLS ON TWO ANION-EXCHANGE COLUMNS IN SERIES

Oligosaccharide alditols		Alditol t_R (min)	Alditol k' [a]
	GalNAcol	4.54	0.33
R1-ol	Galβ1-3GalNAcol	4.61	0.35
	GlcNAcol	4.74	0.39
R2-ol	GalNAcα1-3GalNAcol	4.98	0.42
G3GN-ol	Galβ1-3GlcNAcol	5.13	0.49
O4-ol	Galβ1-3GlcNAcβ1-3GalNAcol and Galβ1-4GlcNAcβ1-3GalNAcol	5.10 and 9.32	0.45 and 1.66
O1-ol	GlcNAcβ1-6, Galβ1-3 / GalNAcol	5.48	0.60
CB-ol	GlcNAcβ1-4GlcNAcol	5.75	0.68
G6GN-ol	Galβ1-6GlcNAcol	6.03	0.72
G4GN-ol	Galβ1-4GlcNAcol	6.28	0.85
N1-ol	Galβ1-4GlcNAcβ1-6, Galβ1-3 / GalNAcol	6.45	0.88
K2(3)-ol	GlcNAcα1-4Galβ1-4GlcNAcβ1-6, Galβ1-3 / GalNAcol	8.44	1.48
K5(3)-ol	Galβ1-4GlcNAcβ1-6, Galβ1-3/4GlcNAcβ1-3 / GalNAcol	7.85 and 17.61	1.24 and 4.03

[a] k' = capacity ratio or factor; a measure of solute retention, calculated as (alditol $t_R - t_0)/t_0$. Mean solvent front was 3.43 min (C.V. = 1.18%, $n = 20$) and mean retention time of the internal standard (melibiose) was 13.13 min (C.V. = 1.80%, $n = 20$).

tions is an effective method for the separation of neutral reduced oligosaccharides. The resolution of neutral oligosaccharide alditol isomers differing only by a (1→3), (1→4) or (1→6) linkage, reveals that this method provides an extremely useful separation alternative to alkyl- and amine-bonded phase and PGC-phase column chromatography.

The previous consensus was that neutral alditols in the di- to tetrasaccharide range are poorly retained on the pellicular anion-exchange column, a consequence of the loss of the highly acidic anomeric hydrogen atom seen in the corresponding reducing sugar [22]. Similarly, O'Lloyd and Savage, observed that neutral reduced oligosaccharides derived from various

TABLE III

RETENTION TIMES OF SIALYLATED OLIGOSACCHARIDE ALDITOLS ON TWO ANION-EXCHANGE COLUMNS IN SERIES

Oligosaccharide alditols		Alditol t_R (min)	Alditol k' [a]
SA6L-ol	NeuAcα2-6lactitol	29.80	7.71
SA3L-ol	NeuAcα2-3lactitol	30.50	7.89
DSLNT-ol	NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glcol	40.34	10.69

[a] k' = capacity ratio or factor; a measure of solute retention, calculated as (alditol $t_R - t_0)/t_0$. Mean solvent front was 3.43 min (C.V. = 1.18%, $n = 20$) and mean retention time of the internal standard (melibiose) was 13.13 min (C.V. = 1.80%, $n = 20$).

mucins, also eluted very rapidly (2–3 min) on the column with ineffective fractionation of monosaccharide alditols and little or no separation of larger oligosaccharide chains [18]. In this study, the poor degree of separation of the human meconium derived neutral oligosaccharide alditols achieved using the single anion-exchange column, complemented these previous findings. However, significant retention and separation was achieved using two anion-exchange columns,

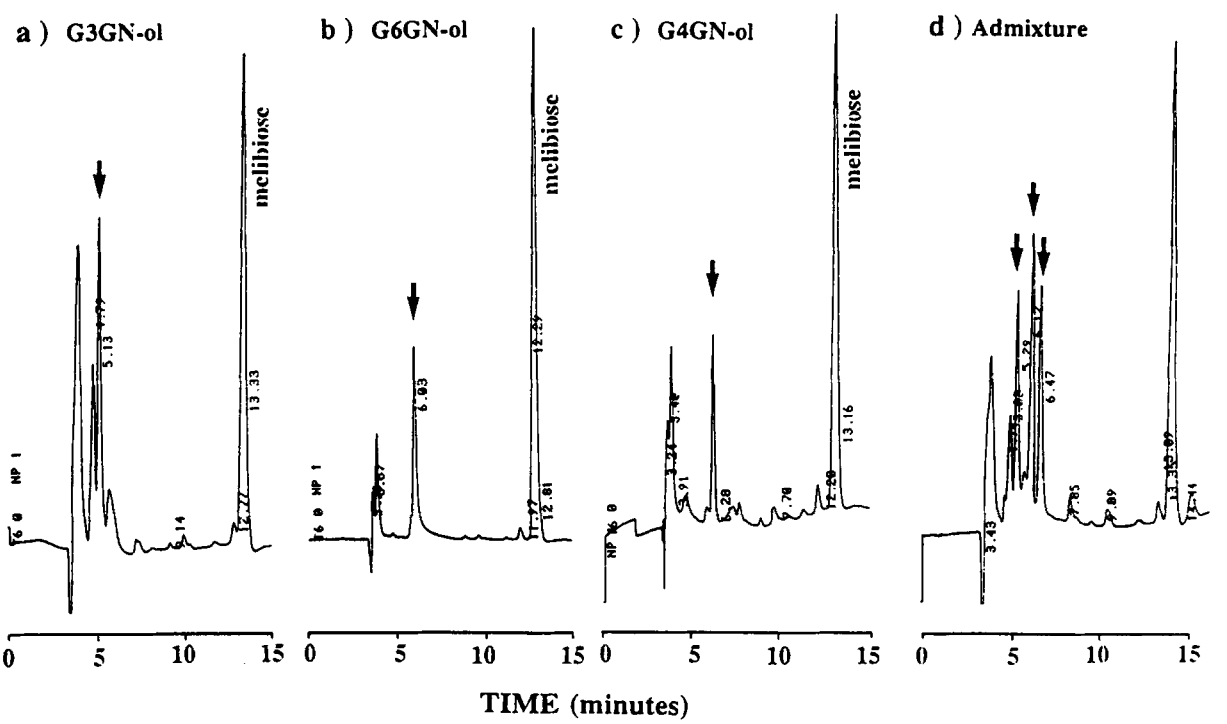

Fig. 1. Chromatographic separation of the (a) β(1→3)-, (b) β(1→6)-, (c) β(1→4)-linked isomer groups of the neutral disaccharide alditol Gal-GlcNAcol, and (d) an admixture of all three isomers (*i.e.* G3GN-, G6GN- and G4GN-ol), using two CarboPac PA-100 high-pH anion-exchange columns. Alditols were eluted in 80 m*M* sodium hydroxide (0–10 min) followed by a gradient of 0–500 m*M* sodium acetate (in 80 m*M* sodium hydroxide) from 10–45 min. A flow-rate of 0.7 ml/min was used and alditols (indicated by arrows) detected with a pulsed amperometic detector (PAD); output was at 1 μA. Internal standard was melibiose (320 ng).

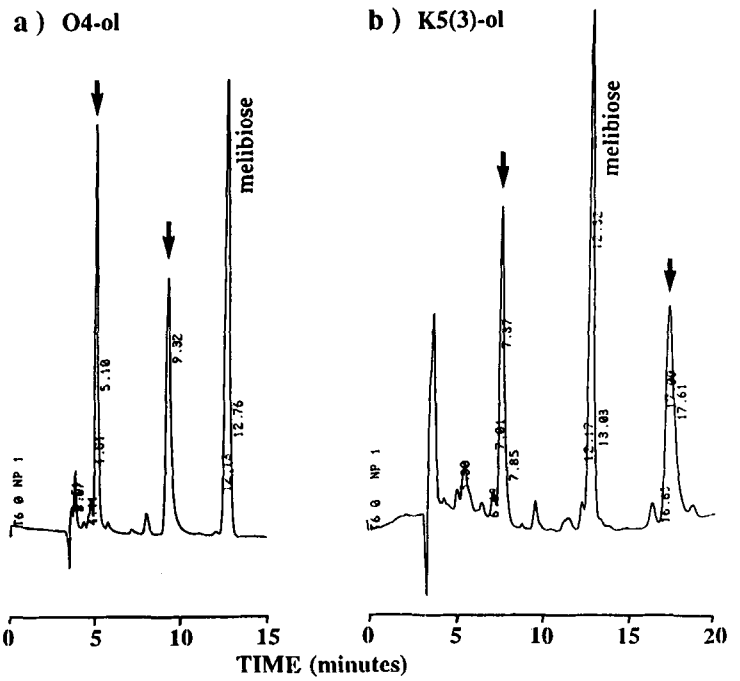

Fig. 2. HPLC separation of neutral oligosaccharide alditol isomers, derived from human meconium, differing by $\beta(1\rightarrow3)$ and $(1\rightarrow4)$ terminal galactose linkages. (a) The two trisaccharide isomers contained in sample O4-ol, and (b) the two pentasaccharide isomers present in K5(3)-ol; as indicated by the arrows. Chromatographic conditions were the same as in Fig. 1. Further structural studies need to be performed to determine the precise order of isomer elution but it seems likely that the $\beta(1\rightarrow3)$ isomer elutes before the $\beta(1\rightarrow4)$ (see Fig. 1).

run in alkaline conditions which obviated the need for post-column addition of strong alkali prior to detection.

Mono acetamido sugar alditols were resolved clear of the solvent front, with GalNAcol eluting fractionally earlier than GlcNAcol; a pattern similar to that previously seen using either PGC-phase [8] or amine-modified reversed-phase column chromatography [6]. The apparent sizes of the monosaccharides are known to be modulated by their linkage site [23–25] and in our study, the separation of oligosaccharide alditol isomers of the same monosaccharide composition but, varying in their linkage position (*e.g.* G3GN-, G4GN- and G6GN-ol), revealed that the presence of the $(1\rightarrow3)$ isomer reduced the retention time. This pattern is similar to that seen using normal-phase [6,7,26,27] and PGC-phase columns [8], where oligosaccharides having a $(1\rightarrow3)$ linkage possess shorter retention times than $(1\rightarrow4)$ and $(1\rightarrow6)$ isomers. In contrast, using reversed-phase chromatography, $(1\rightarrow3)$ and $(1\rightarrow4)$ isomers are inseparable but are retained longer than $(1\rightarrow6)$-linkages [1,2,7]. In previous studies using high-pH anion-exchange chromatography, it was observed that neutral oligosaccharide isomers containing $\beta(1\rightarrow4)$ linkages eluted earlier than $(1\rightarrow3)$ linkages [11,12]. Conversely in this study, for reduced oligosaccharide alditols, the order of elution for neutral oligosaccharide alditols was found to be $(1\rightarrow3) < (1\rightarrow6) \leq (1\rightarrow4)$ isomers, reflecting the different stereochemistry of hydroxyl groups in linear rather than ring form. The exact chemical basis for the retention behaviour of these anionic compounds remains less clear than for their reducing counterparts. However, the distinct separation of $(1\rightarrow4)$ and $(1\rightarrow3)$ oligosaccharide alditol isomers in this study, confirms dual column CarboPac PA-100 anion-exchange chromatography as a useful adjunct to other HPLC techniques.

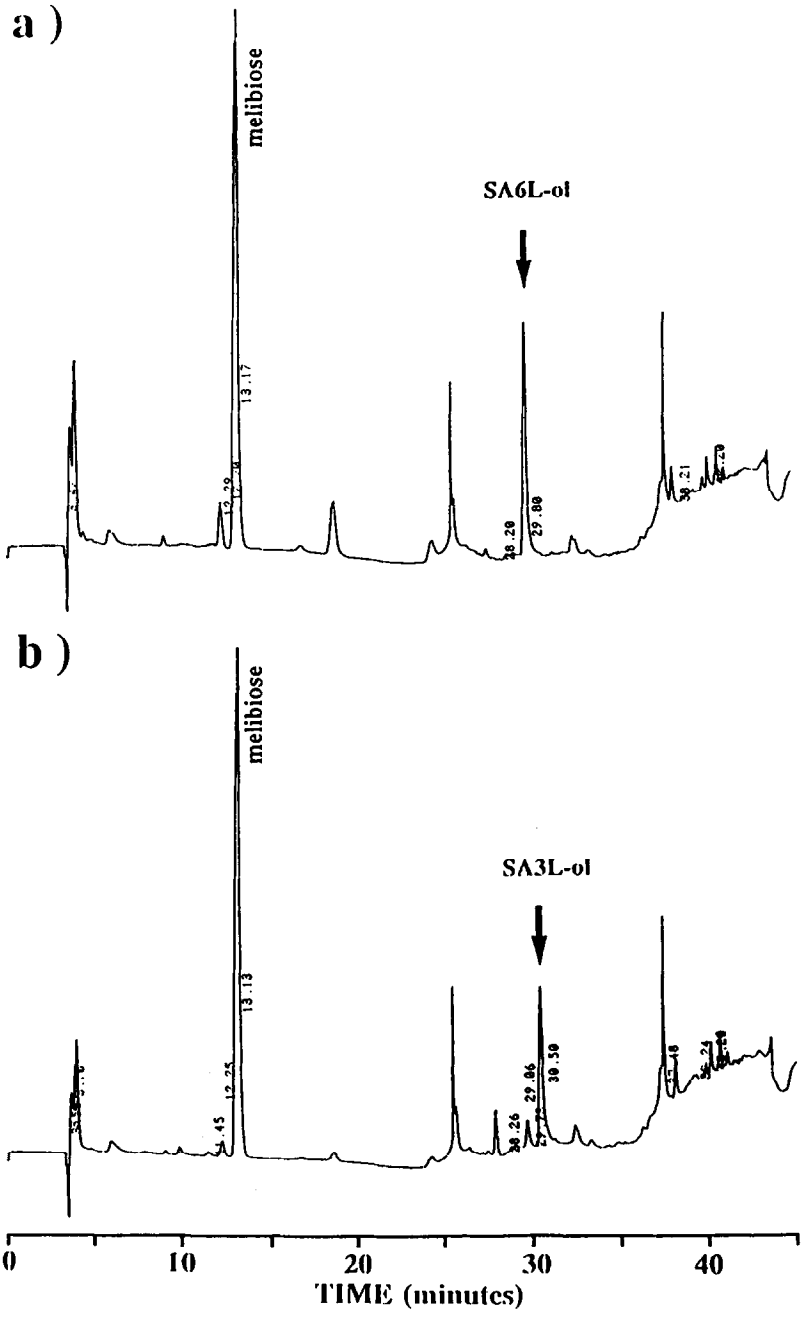

Fig. 3. HPLC separation of the sialyldisaccharide alditols, (a) $\alpha(2\rightarrow6)$ sialyllactitol (SA6L-ol), and (b) $\alpha(2\rightarrow3)$ sialyllactitol (SA3L-ol). Chromatographic conditions as in Fig. 1.

In earlier analyses of oligosaccharides derived from mucins as their alditols [19–21,23,28–30], solute elution was largely based on order of increasing size. Likewise, in the present study, retention times generally increased with increasing oligosaccharide chain length and branching. The two trisaccharide isomers contained in sample O4-ol were extremely well separated using

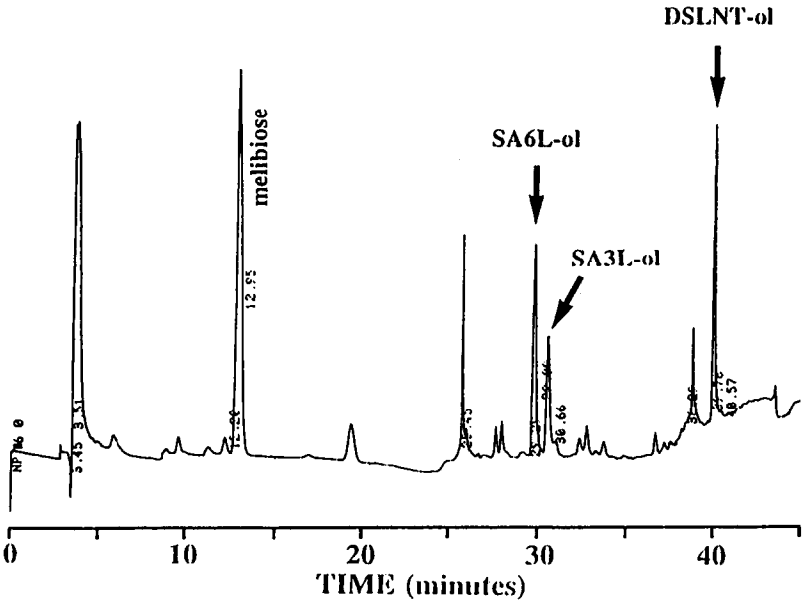

Fig. 4. HPLC separation of an admixture of the three sialic acid-containing oligosaccharide alditols; $\alpha(2\rightarrow6)$ sialyllactitol (SA6L-ol), $\alpha(2\rightarrow3)$ sialyllactitol (SA3L-ol) and DSLNT-ol. Chromatographic conditions as in Fig. 1.

dual column anion-exchange chromatography. The co-migration of oligosaccharides differing only by terminal $\beta(1\rightarrow3)$- or $(1\rightarrow4)$-linked galactose has been previously reported, with the two trisaccharide isomers in O4-ol non-separable using either reversed-phase or normal-phase methodologies [1,2,7,20], unless resolved as their acetylated derivatives [7]. However, some separation of the two trisaccharide isomers has previously been achieved using Durram AX4 anion-exchange resin (a fore-runner of Dionex pellicular anion-exchange resin) [30], or MicroPak AX-5 anion-exchange HPLC [1,2]. Successful separation of the two pentasaccharide isomers present in sample K5(3)-ol, differing in $(1\rightarrow3)$ and $(1\rightarrow4)$ linkage, was also achieved with the dual column anion-exchange method described here. From the neutral disaccharide isomer retention data, it would seem likely that the $\beta1\rightarrow3$-linked trisaccharide alditol elutes before the $\beta1\rightarrow4$ isomer, however further structural analysis would need to be performed to determine the precise elution order.

The separation of acidic reducing sugars varying in their linkage and number of sialic acids has been demonstrated using either amine-bonded normal phase or high-pH anion-exchange chromatography [1,11,15,16]. In contrast to neutral reduced oligosaccharides, sialic acid-containing alditols, derived from sialomucins, have been shown to be strongly retained and well resolved using both PGC [8,9] and high-pH anion-exchange chromatography [18,31]. In the present study, sialic acid-containing reduced oligosaccharides were highly retained on the two high-pH anion-exchange columns, with separation based on both number of and linkage of the sialic acid residues. This is consistent with data from previous studies using either high-pH anion-exchange [18,31] or PGC chromatography [8,9], where substitution with sialic acid in $\alpha(2\rightarrow3)$ linkage lead to increased retention time as compared with substitution in $\alpha(2\rightarrow6)$ linkage to the same residue.

Current opinion suggests that one type of HPLC procedure alone is probably not sufficient to separate and characterise all structural variants which occur. However, the method described in this study is useful both for the isomeric fractionation of acidic sialylated oligosaccharides derived from certain classes of mucins and, more importantly, for mucins con-

taining mainly neutral oligosaccharides. Indeed, this method may even prove beneficial in the resolution of fucosylated oligosaccharide alditols, derived from human mucus glycoproteins, which are poorly retained on various HPLC phases [1,19], except PGC [8]. The successful separation of fucosylated isomers has been achieved as their reducing sugars using high-pH anion-exchange HPLC [11,19]. Thus, the fractionation of N- and O-glycosically bound neutral or sialylated oligosaccharides using dual column anion-exchange HPLC, together with on-line and post-HPLC desalting, would be a good preparative step for structural analysis techniques.

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