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High-performance liquid chromatography of oligosaccharide alditols and glycopeptides on a graphitized carbon column

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ABSTRACT

The chromatographic behaviour of oligosaccharide alditols and glycopeptides containing neutral and acetamido sugars and sialic acid has been investigated on a HyperCarb porous graphitised carbon column. The alditols were substantially retained and could be eluted in 0–25% acetonitrile–0.05% trifluoroacetic acid in 0.05% aqueous trifluoroacetic acid between 3–30 min for mono- to hexasaccharides. Elution patterns were based on both size, charge and linkage such that isomeric compounds could be separated from each other.

INTRODUCTION

The diversity of closely related carbohydrate structures found in nature provides a unique challenge for the chromatographer. Purification of isomeric oligosaccharides is essential for assigning specific functions and antigenicity [1] and improved methods for high-resolution separation are constantly being sought. A porous graphitised carbon (PGC) stationary phase [2,3] (Shandon Hypercarb) has therefore been investigated as a possible additional column material providing an alternative separation potential to reversed-phase (RP), normal-phase (NP) and anion exchange packings. In order to separate series of related oligosaccharide sequences a combination of RP (normally octadecylsilyl) and NP (amine-bonded) high-performance liquid chromatography (HPLC) has usually been adopted [4,5]. More recently high-pH anion-exchange chromatography has been introduced for isomer separation [5,6]. In the latter methodology neutral reduced oligosaccharides are in general not retained sufficiently for adequate separation and reproducible chromatography and therefore we have been particularly interested in studying the PGC phase for chromatography of isomeric alditols. Koizumi *et al.* [7] have recently published HPLC on PGC columns of monosaccharides and malto-oligosaccharides which show good separation of the α and β anomers. It is therefore more appropriate to use this phase for alditol or glycopeptide separation without the additional complication of anomerisation.

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EXPERIMENTAL

Materials

The oligosaccharides studied are shown in Table I together with the abbreviations used. GlcNAc, CB, CT, SAL, fetuin and trypsin were obtained from Sigma Poole, UK. ^{1}H NMR studies showed that SAL (sialyllactose or N-acetylneuramin-lactose) from bovine colostrum (Sigma batch No. A3001) consisted of approximately 75% of the

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TABLE I

STRUCTURES OF THE OLIGOSACCHARIDES STUDIED AS THEIR ALDITOLS

GalNAc = N-Acetylgalactosamine; GlcNAc = N-acetylglucosamine; Gal = galactose; Glc = glucose; Fuc = fucose; NeuAc = N-acetylneuraminic acid.

	D-GalNAc
	D-GlcNAc
CB	β-D-GlcNAc-(1→4)-D-GlcNAc (chitobiose)
CT	β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-D-GlcNAc (chitotriose)
(G3GN)	β-D-Gal-(1→3)-D-GlcNAc
(G4GN)	β-D-Gal-(1→4)-D-GlcNAc
(G6GN)	β-D-Gal-(1→6)-D-GlcNAc
03	β-D-Gal-(1→4)-β-D-GlcNAc-(1→6)-D-GalNAc
04	β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-D-GalNAc β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-D-GalNAc
N1	β-D-Gal-(1→4)-β-D-GlcNAc-(1 ↘ 6) -D-GalNAc 3) ↗ β-D-Gal-(1
K5(3)	β-D-Gal-(1→4)-β-D-GlcNAc-(1 ↘ 6) -D-GalNAc 3) ↗ β-D-Gal-(1→3/4)-β-D-GlcNAc-(1
LNT	β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal(1→4)-D-Glc
LNNT	β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-β-D-Gal(1→4)-D-Glc
LNFIII	β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-β-D-Gal(1→4)-D-Glc 3 ↑ 1 α-L-Fuc α-L-Fuc 1 ↓ 4
LNFII	β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc
LNFI	α-L-Fuc-(1→2)-β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc
SA3L	α-D-NeuAc-(2→3)-β-D-Gal-(1→4)-D-Glc
SA6L	α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-D-Glc
DSLNT	α-D-NeuAc 2 ↓ 6 α-D-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc

(2→3) isomer (SA3L) and also contained 10% of the (2→6) isomer (SA6L) and 15% of a third unidentified sialylated component, whereas SAL from human milk (Sigma batch No. A2423) was predominantly the (2→6) isomer. The disaccharides G3GN, G4GN and G6GN were obtained by chemical synthesis and kindly supplied by Dr. A. Veyrières (Université Pierre et Marie Curie, Paris, France). The oligosaccharides designated 03, 04, N1 and K5(3) purified from meconium glycoproteins as previously described [8,9] were obtained as their alditols after alkaline–borohydride release of oligosaccharides. The oligosaccharides LNT, LNNT, LNFIII, LNFII, LNFI and DSLNT were from Research Sugars, Long Crendon, Aylesbury, UK. The oligosaccharides obtained from commercial sources and by chemical synthesis were reduced using 20 m*M* sodium borohydride in 0.05 *M* NaOH at 4°C for 18 h, excess borohydride was then destroyed by addition of acetic acid and the sample desalted using Dowex 50-X8 acetate form (3 × 1 cm column) eluted with water.

Apparatus and columns

HPLC analysis was performed using a Gilson system (Anachem, Luton, UK) consisting of two 302 pumps, a 116 UV monitor, 802 manometric module, 802B mixing chamber, Gilson 715 Systems Manager and a Rheodyne 7125 injector. The column was Shandon Hypercarb (Shandon Scientific, Runcorn, UK) 100 × 4.6 mm with an ODS (Hypersil) guard column.

HPLC of oligosaccharide alditols

The flow-rate was 0.75 ml/min using an eluent of acetonitrile–water–0.05% trifluoroacetic acid (TFA) and UV detection at 206 nm. The gradient for neutral oligosaccharide alditols was as follows for eluent A (0.05% aqueous TFA) and B (acetonitrile–0.05% TFA): 0–5 min, 0% B; 5–20 min, 0–15% B; 20–30 min, 15% B; 30–40 min, 15–0% B. For sialylated oligosaccharide alditols the gradient was: 0–5 min, 0% B; 5–40 min, 0–40% B; 40–50 min, 40–0% B.

Glycopeptide mapping

Fetuin was digested with chymotrypsin-free N tosyl-L-phenylalanine-chloromethylketone (TPCK)-treated trypsin (Sigma) as described previously [10] and chromatographed on the PGC column using detection at 210 nm, a flow-rate of 1 ml/min and solvent system as follows for eluent A (0.1% aqueous TFA) and B (acetonitrile/0.1% TFA): 2% B to 82% B in 80 min. Peaks were monitored for hexose using a modification [10] of the method of Dubois *et al.* [11] and the amino acid sequence of peptides in selected peaks were determined using a Model 473A protein sequencer (Applied Biosystems, Warrington, UK) in the pulsed liquid mode. The sample was applied to a polybrene treated filter disc which had been subjected to three pre-wash cycles in 30 μl 0.1% aqueous TFA. Sequencing was carried out for 6–7 cycles of the instrument.

RESULTS

Table II shows the retention times for HPLC of alditols of the oligosaccharides shown in Table I. Monosaccharide acetamido sugar alditols (D-GlcNAc-ol and D-GalNAc-ol) were eluted early

TABLE II

THE RETENTION TIMES OF THE ALDITOLS (-ol) STUDIED USING THE GRADIENT DESCRIBED IN EXPERIMENTAL. RETENTION TIMES (t_R) ARE GIVEN FOR THE ALDITOLS RUN SEPARATELY AND AS A MIXTURE OF THE GROUPS INDICATED BY THE BRACKETS (t_R MIX)

	t_R	t_R mix
GalNAcol	3.64	3–4
GlcNAcol	3.94	3–4
CB-ol	16.97	16.73
CT-ol	18.18	18.15
G3GN-ol	12.75	12.72
G4GN-ol	13.40	13.41
G6GN-ol	15.82	15.75
LNT-ol	20.51	20.42
LNNT-ol	20.98	20.87
LNFII-ol	17.73	17.71
LNFIII-ol	17.65	
LNFI-ol	19.74	
03-ol	18.06	
04-ol	16.90 and 17.25	
N1-ol	18.06	
K5(3)-ol	20.78 (single peak)	
SA3L-ol	22.03	22.03
SA6L-ol	18.50	18.62
DSLNT-ol	27.46	27.56

and could not be separated from each other on the gradient used. There was a good separation of the mono-, di- and trisaccharide alditols of D-GlcNAc (D-GlcNAc-ol, CB-ol and CT-ol) showing a general increased elution time with increased size. The same resolution was achieved when 2 mg of material were loaded and a preparative recovery of >90% was achieved. The presence of a hexose residue as in G3GN-ol, G4GN-ol and G6GN-ol reduced the elution time as compared to CB-ol and the presence of a fucose residue reduced the retention time further, as shown for example by a comparison of the retention times of the linear tetrasaccharide alditols (LNT-ol and LNNT-ol) and their fucosylated analogues (LNF III-ol, LNFII-ol and LNFI-ol).

Within isomeric groups of neutral oligosaccharides the presence of a (1→6) linkage increased the retention time as shown for the disaccharide alditols G3GN-ol, G4GN-ol and G6GN-ol and the trisaccharide alditols 04-ol and 03-ol. More importantly, oligosaccharides having a (1→3) linkage eluted before those having a (1→4) linkage as shown by separation of the two isomers in 04-ol from each other and LNT-ol from LNNT-ol (Fig. 1). However, separation could not be achieved of LNFII-ol and LNFIII-ol which vary in the C-3 and C-4 linkage of D-Gal and L-Fuc to D-GlcNAc, nor of the two isomers present in K5(3)-ol.

The sialic acid-containing oligosaccharide alditols were amongst the longest retained of the alditols tested, but still gave symmetrical peaks with baseline separation, eluting within less than 1 min (Fig. 2). Here the (2→3)-linked trisaccharide alditol was retained longer than the (2→6) which differed from that shown for the neutral oligosaccharide alditols. This represents a facile separation and iden-

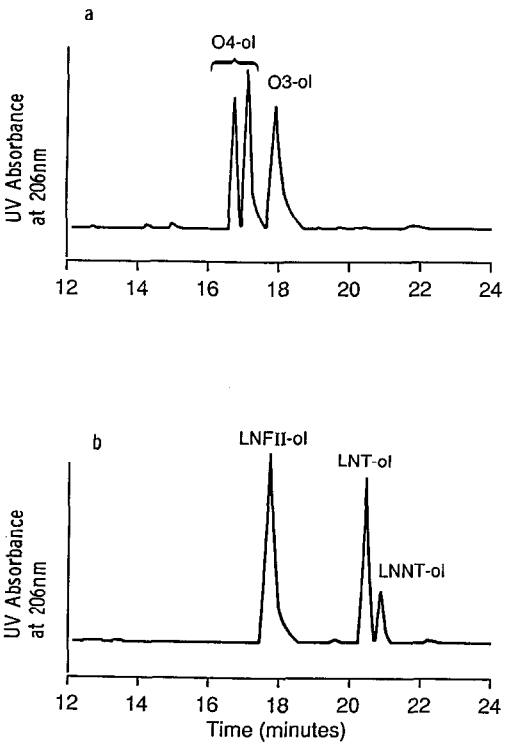

Fig. 1. HPLC on Hypercarb PGC using an acetonitrile–water–0.05% TFA gradient of (a) 1 μg and 3 μg, respectively, of 04-ol (two isomers) and 03-ol, and (b) 10, 5 and 2 μg, respectively, of LNFII-ol, LNT-ol and LNNT-ol.

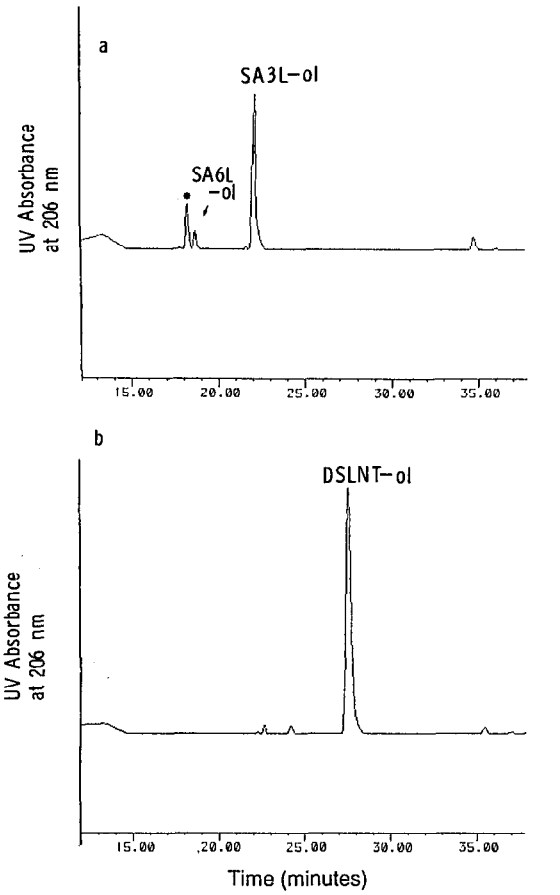

Fig. 2. HPLC on Hypercarb PGC using an acetonitrile–water–0.05% TFA gradient of the alditols obtained from (a) 10 μg of the preparation of SA3L from Sigma, containing 10% SA6L and 15% unknown (*), and (b) DSLNT-ol.

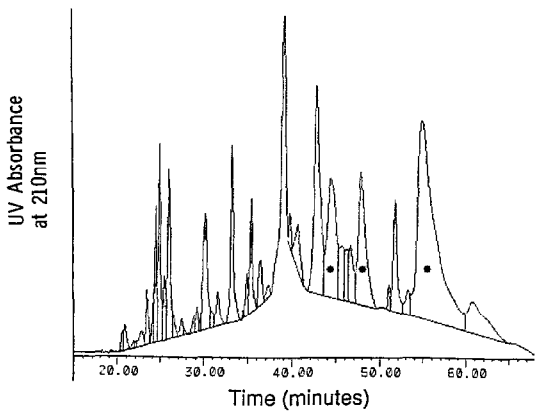

Fig. 3. HPLC on Hypercarb PGC using an acetonitrile–water–0.1% TFA gradient of 200 pmol of a tryptic digest of fetuin; * = peaks containing major glycopeptides.

tification of these oligosaccharides from each other and from an as yet unidentified sialylated component. The chromatographic behaviour of sialylated oligosaccharide sequences has also been studied by analysis of glycopeptides in a trypsin digest of fetuin which has three N-linked and three O-linked sialylated oligosaccharide chains [10]. The conditions chosen for PGC chromatography were the same as those previously used for RP-HPLC on an ODS column [10]. Glycopeptides were identified by hexose assay (Fig. 3) and characterised further by peptide sequence analysis (Table III). The results summarised in Table III show the relative hydrophobicity of the peptides and the diagnostic amino acids detected. The hexose-positive peak eluting at 44–45 min contained the glycopeptide AA 127–168

TABLE III

CHARACTERISATION BY PEPTIDE SEQUENCING OF THE HEXOSE POSITIVE PEAKS IN THE PGC PROFILE OF TRYPSIN DIGESTED FETUIN (GLYCOSYLATION SITES ARE IDENTIFIED BY ASTERISKS)

Elution time (min)		Sequence identification (detected amino acids in bold)[b]	Hydrophilicity/ hydrophobicity index[c]
PGC	ODS[a]		
44	ND[d]	**VWPR**	−357
44	42	**LCPDCPLLAPLNDSR**–[e]	−170
		VVHAVEVALATFNAESNGSYLQLVEISR	−94
48	50	**EPACDDPDTEQAALAAVDYINK**	175
		LCPGR–[e]**IRYFK**	−402
55	54	**R-PTGEVYDIEIDTLETTCHVLDPTPLANCSVR**–[e]	−77
		QQTQHAVEGDCDIHVLK	232
55	62	**AQFVPLPVSVSVEFAVAATDCIAK**	−130
		EVVDPTK-CNLLAEK	29

[a] Data taken from Smith *et al.* [10].
[b] | = disulphide bond.
[c] Calculated for individual non-glycosylated peptides by dividing the sum of the free energy of transfer from solution to surface (ΔF) values for each amino acid [23] by the total number of amino acids present [24]. The most hydrophobic peptides have the lowest negative value.
[d] ND = Not detected.
[e] – = Previously identified [10] non-digested tryptic cleavage site due to close proximity of a glycosylation site or disulphide bond.

having two N-glycosylation sites due to inhibition of hydrolysis of the tryptic cleavage site at arginine 141 by the oligosaccharide chain at asparagine 138. The hydrophobic tetrapeptide VWPR was also detected in this peak. The peak eluting at 48–49 min contained the peptides AA 11–32 and AA 331–335 which are disulphide bonded together. Hexose detection is due to the additional presence of the large glycopeptide AA 228–288 having three O-linked chains, for which peptide sequence data are not obtained [10]. The peak eluting at 55–58 min contained the glycopeptide AA 54–113 having one N-linked chain and two non-digested tryptic cleavage sites. This peak also included a disulphide-linked peptide pair AA 169–103 and AA 194–207 with an undigested tryptic cleavage site adjacent to one of the cystines involved in the disulphide bond.

DISCUSSION

The separation of oligosaccharide alditol isomers differing only by a (1→3) or (1→4) linkage confirms PGC chromatography as a useful adjunct to RP and NP HPLC. The oligosaccharide isomers in 04 for example were not separated in our earlier study [8] although separation was achieved by Lamblin *et al.* [12] who introduced an anion-exchange chromatography step on Durrum AX4 resin (a forerunner of Dionex pellicular anion-exchange resin now being used so successfully in high-pH anion-exchange chromatography to give separation of isomers of non-reduced oligosaccharides [6,13]). In addition, the good baseline and reproducible chromatography together with the high loading that can be achieved without loss of resolution add further to its efficiency. The chromatographic behaviour of alditols on RP (ODS), NP (amine-bonded) and PGC HPLC is different in each case as shown from the following relative t_R data for 03-ol, 04-ol and N1-ol, respectively, RP 1.0/1.2/0.8; NP 1.0/0.9/1.2; PGC 1.0/0.9-0.95/1.0 *i.e.* in RP oligosaccharides having a (1→6) linkage are eluted first and separation is not achieved of the (1→4) and (1→3) isomers [4], whereas in NP the order is reversed [4]. The elution pattern of PGC is similar to NP in that 03-ol and N1-ol elute after the isomers in 04-ol but differs by the fact the separation of the (1→6) containing tri- and tetrasaccharide is not achieved, whereas separation of (1→3) and (1→4) trisaccharide alditols is. A combination of the three phases is probably therefore still required for more complete separation of oligosaccharides of different linkages and sizes. Unfortunately PGC HPLC failed to separate the fucosylated pentasaccharide isomers LNFII-ol and LNFIII-ol which have been successfully resolved only in unreduced form by high-pH anion-exchange [6,13], recycling cation-exchange [14] or RP [4] chromatography.

The separation of non-reduced oligosaccharides varying in their linkage of sialic acid has previously been achieved by HPLC on an amine-bonded NP column using phosphate buffer [5] and more recently by high-pH anion-exchange chromatography [6]. Sialylated oligosaccharide alditols have also been separated by high-pH anion-exchange chromatography [15,16]. The disadvantage of these methods is that they require a high salt concentration which is not as readily removed compared to TFA, thus inhibiting further analysis and making the PGC separation by far the preferred chromatography. For sialylated glycopeptides, similar retention times were found by PGC to HPLC on ODS [10] using the same solvent elution conditions (Table III).

The chromatographic behaviour described herein for oligosaccharide alditols, peptides and glycopeptides and by Koizumi *et al.* [7] for reducing mono- and disaccharides adds to the debate on the mechanism of separation achieved by PGC [17–22]. Compared to the study of Koizumi *et al.* on neutral oligosaccharides we have used 0.05%–0.1% TFA in the eluent in order to widen the scope of the technique to sialylated oligosaccharides and glycopeptides as used by the previous authors [21,22] studying PGC chromatography of other classes of compounds. These authors found the retention of anionic compounds to be dominated by electronic interaction between the solute and delocalised electron clouds on the graphitised carbon whilst cationic compounds were mainly retained by reversed-phase interaction with the hydrophobic carbon surface. Our results agree with a dual chromatographic mechanism based on long retention of glycopeptides having a low negative hydrophilicity/hydrophobicity index (Table III) and others having acidic amino acids and/or sialic acids ionised at pH 2.0 of the eluent. The unique physicochemical properties of PGC provide us with an additional column packing for separation of oligosaccharide alditols, glyco-

peptides and peptides at high sensitivity and preparative efficiency.

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