medulla resulted in an increase in extracellular adrenaline levels in the posterior hypothalamus and a simultaneous increase in mean arterial pressure. Extracellular adrenaline increased to 56% of pre-stimulation control values when a corresponding blood pressure rise of 47.7 mmHg (+65%) was observed. Neither adrenaline or mean arterial pressure increased when the stimulating electrodes were outside the C₁ region (Fig. 2). No change was seen in hypothalamic extracellular levels of noradrenaline, dopamine, DOPAC and 5-HIAA during the stimulation period; however, there was a significant increase in extracellular noradrenaline levels in the post-stimulation perfusate sample. This delayed increase in noradrenaline may be the result of a reflex response to the increase in blood pressure and not due to the electrical stimulus spreading to the A₁ region. The increase in adrenaline levels in the hypothalamus after stimulation of the C₁ region gives support to the evidence for an adrenergic pressor pathway from the rostral ventrolateral medulla to the hypothalamus. The lack of correlation between an increase in extracellular levels of noradrenaline, dopamine, DOPAC and 5-HIAA and mean arterial pressure after C₁ stimulation suggests that the increase in mean arterial pressure during electrical stimulation of the C₁ region relates to a specific increase in adrenaline levels. However, the C₁ region also innervates the spinal cord (Ross et al., 1981). An adrenergic pathway descends from the C₁ region to the intermediolateral column of the spinal cord. Stimulation of the C₁ region may also increase adrenaline levels in the intermediolateral column causing a corresponding increase in mean arterial pressure. Further studies are necessary to determine whether the pressor responses are mediated through an adrenergic pathway to the hypothalamus or by a direct pathway to the spinal cord.

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The combined use of h.p.l.c. on reverse-phase and amine-bonded silica column supports in oligosaccharide purification

Reverse-phase chromatography of oligomers of glucose (Cheetham et al., 1981) and of N-acetylglucosamine (Blumberg et al., 1982) with ODS column packings gave a separation with an increased retention time for oligomers with higher \( M_r \) values. However, when oligosaccharides containing both neutral and acetylated oligosaccharide alditols by using silica, silica bonded with octadecyl (ODS) or with aminopropyl (APS) groups and an anion-exchange resin.

The application of high-performance liquid chromatography to the purification of oligosaccharides containing neutral and acetamido sugars

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Oligosaccharides which occur in body fluids, such as milk and urine, or as the carbohydrate chains of secreted and cell-surface glycoproteins are usually found as complex mixtures of closely related molecules. Their purification involves the separation, not only of molecules of different size and composition, but also of isomers which vary in linkage position or anomeric configuration. Our studies on the structural characterization and assignment of the antigenicities of cell-surface carbohydrates has been made possible by the finding that the oligosaccharides of interest are also present on secreted glycoproteins such as the mucins of human meconium (Wood et al., 1979; Gooi et al., 1981, 1983a, b, Hounsell et al., 1985) and in milk (Gooi et al., 1981; Hounsell et al., 1981a; Gooi et al., 1985), which can be obtained in relatively large amounts. However, because of the heterogeneity of the carbohydrate structures, efficient chromatographic methods are required for purification before structural and antigenic elucidation.

H.p.l.c. has proved to be of great value in oligosaccharide purification and analysis because of the wide range of adsorbents and solvent systems available, the relatively short separation time and the high yield, which allow several different chromatographic separations to be carried out. More than one chromatographic system has been shown to be necessary for purification of oligosaccharide isomers (Hounsell et al., 1981a, b, 1984a, 1985) and thus we have investigated the h.p.l.c. separation of native and acetylated oligosaccharide alditols by using silica, silica bonded with octadecyl (ODS) or with aminopropyl (APS) groups and an anion-exchange resin.

Several different chromatographic systems have been used to resolve the oligosaccharides. These include: adsorption chromatography on diverse stationary phases (ODS, amino-bonded, octadeyl); gel filtration; paper electrophoresis; conventional ion-exchange chromatography on DEAE-cellulose; and reverse-phase liquid chromatography on reversed-phase packings (ODS) or reversed-phase bonded silica particles.

The application of high-performance liquid chromatography to the purification of oligosaccharides containing neutral and acetamido sugars

Abbreviations used: ODS, silica bonded with octadecyl groups; APS, aminopropyl bonded with aminopropyl groups; h.p.t.l.c., high-performance thin-layer chromatography.

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1979; Hounsell et al., 1984a). Within this size separation, isomers having a 1-6 branch had a significantly longer retention time (Bergh et al., 1983; Hounsell et al., 1984a).

Because of the differing chromatographic behaviour of oligosaccharides on reverse-phase and amino-bonded columns, a combination of the two systems can often separate oligosaccharides which are not resolved in one system alone (Hounsell et al., 1984a). This is illustrated by the retention times given in Table 1 for h.p.l.c. of the mono- to tetra-saccharide fractions obtained by Biogel P4 gel filtration chromatography of oligosaccharides released from human meconium glycoproteins (Hounsell et al., 1985) by mild alkali/borohydride degradation (Iyer & Carlson, 1971; Hounsell et al., 1984b) and isolated by dialysis and ion-exchange chromatography (Dowex 50X8 and 1X2, hydrogen and acetate form respectively). Preparative chromatography was carried out isocratically using water and acetonitrile/water (65:35, v/v) with the ODS and APS columns, respectively. These solvent systems gave satisfactory column retention and, in the latter case, was the minimum percentage of water for adequate solubility of the oligosaccharides for preparative separations. The longer retention of the larger oligosaccharides in this series (to be reported elsewhere) made it possible to use gradient elution and a wider range of water/acetonitrile eluents. However, it was still found that separation of some isomers could only be achieved by a combination of h.p.l.c. on both APS and ODS columns.

As exemplified by h.p.l.c. of the two isomers Ga\(\beta\)1-3GlcNAc\(\beta\)1-3GalNAc-ol and Ga\(\beta\)1-4GlcNAc\(\beta\)1-3GalNAc-ol (Table 1) there were certain oligosaccharides which could not be separated by h.p.l.c., on either APS or ODS columns. The co-migration of oligosaccharides differing only by a 1-3 or 1-4 linkage to GlcNAc has been reported previously (Hounsell et al., 1984a). We have therefore investigated two further h.p.l.c. systems, discussed below, for separation of oligosaccharide alditols.

**H.p.l.c. of derivatized oligosaccharides using ODS and APS column supports**

The previous study (Hounsell et al., 1984a) has shown that reduced oligosaccharide isomers not separated by h.p.l.c. on ODS or APS could be resolved as their acetylated derivatives by h.p.l.c. on silica using 4% propan-2-ol in dichloromethane/heptane (55:45, v/v). The isomers separated in this system were as follows. Ga\(\beta\)1-3GlcNAc\(\beta\)1-3Gal-ol from Ga\(\beta\)1-4GlcNAc\(\beta\)1-3Gal-ol, and Ga\(\beta\)1-3GlcNAc\(\beta\)1-6Gal-ol from Ga\(\beta\)1-4GlcNAc\(\beta\)1-6Gal-ol. As the last two isomers (with 1-6 linkage) can be separated from the first two (with 1-3 linkage) on APS (Hounsell et al., 1984a), the two systems together provide separation of all four isomers. These oligosaccharides, obtained by chemical synthesis and kindly provided by Dr. A. Veyrères (Université d’Otal, France; Augé et al., 1979), have structures closely related to those identified in mucins (Table 1; Hounsell & Feizi, 1982) and therefore h.p.l.c. of acetylated derivatives should be of value for separation of oligosaccharides obtained from this source.

Acetylation, high-performance thin-layer chromatography (h.p.t.l.c.) and h.p.l.c. have also been used to purify the following isomers from a fraction of human milk (kindly provided by Dr. E. Prieels, University of Brussels).

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>ODS Retention time (min)</th>
<th>APS Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalNAc-ol</td>
<td>3.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Ga(\beta)1-3GalNAc-ol</td>
<td>3.6</td>
<td>10.6</td>
</tr>
<tr>
<td>Ga(\beta)1-4GlcNAc-ol</td>
<td>4.4</td>
<td>10.6</td>
</tr>
<tr>
<td>GlcNAc(\beta)1-3GalNAc-ol</td>
<td>4.9</td>
<td>10.1</td>
</tr>
<tr>
<td>Ga(\beta)1-3GlcNAc(\beta)1-3GalNAc-ol</td>
<td>5.9</td>
<td>12.9</td>
</tr>
<tr>
<td>Ga(\beta)1-4GlcNAc(\beta)1-3GalNAc-ol</td>
<td>5.9</td>
<td>12.9</td>
</tr>
<tr>
<td>Ga(\beta)1-4GlcNAc(\beta)1-6GalNAc-ol</td>
<td>5.1</td>
<td>13.8</td>
</tr>
<tr>
<td>GlcNAc(\beta)1-6GalNAc-ol</td>
<td>4.0</td>
<td>13.8</td>
</tr>
<tr>
<td>Ga(\beta)1-3GalNAc(\beta)1-4Glc-ol</td>
<td>4.2</td>
<td>16.0</td>
</tr>
<tr>
<td>Ga(\beta)1-4GlcNAc(\beta)1-4Glc-ol</td>
<td>4.2</td>
<td>16.0</td>
</tr>
</tbody>
</table>

H.p.l.c. on an ODS column (Fig. 1; first chromatogram) gave two major peaks and several minor components. The major component eluting at 9.8 min was shown by n.m.r. analysis to contain non-fucosylated material whereas that eluting at 12.3 min contained fucosylated molecules. On acetylation and preparative separation by h.p.t.l.c., several products designated B–G were collected as shown in Fig. 2. Each was re-purified by h.p.t.l.c. giving B1, B2, C1, D1, D2, E1, E2, F1, F2 and G in sufficient quantities for further analysis (Fig. 2). These were de-O-acetylated and chromatographed on the APS column (Fig. 1). Each component isolated by h.p.t.l.c. gave two or more components on h.p.l.c. and retention times suggested some overlap between bands. C1 was shown by n.m.r. analysis to be LNFP 11-01, and n.m.r. analysis showed that each contained both LNT-ol and LNFP II-ol. A combination of chromatographic techniques was, therefore, necessary to separate these two oligosaccharides. E2 and F2 were indistinguishable by h.p.l.c., and n.m.r. analysis showed that they were LNNT-ol. In summary, acetylation and t.l.c. resolved LNFP III-ol from LNFP II-ol and LNT-ol from LNNT-ol, whilst h.p.l.c. on

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*Table 1. H.p.l.c. of mono- to tetra-saccharides obtained from a glycoprotein preparation of human meconium after mild acid hydrolysis (to remove fucose and sialic acid residues), mild alkali/borohydride degradation, ion-exchange chromatography (Dowex 50X8 and 1X2 resins in the H\(^+\) and OAc\(^-\) forms, respectively) and Biogel P4 gel filtration*

The ODS- and APS-Hypersil column packings were from Shandon Southern Products, Runcorn, U.K. The ODS column was eluted with water and analytical APS chromatography was carried out with gradient elution from 75:25 (v/v) acetonitrile/water to 65:35 (v/v) at 10 min and 35:65 (v/v) at 20 min. Detection was at 208 nm. The boxes represent complete separations.
The first chromatogram shows the original profile of the tri- to hexa-saccharide alditol fraction. Chromatograms B1, B2, C1, E1, D1, D2/E1 and E2/F2 were of bands obtained by h.p.t.l.c. of reduced and acetylated oligosaccharides. H.p.l.c. was performed on a column (0.4 cm x 30 cm) of APS-Hypersil (Shandon Southern Products, Runcorn, U.K.) using isocratic elution with acetonitrile/water (65:35, v/v).

APS was necessary to resolve LNFP II-ol from LNT-ol and several minor components. Interestingly, band G was shown by n.m.r. analysis after de-acetylation to contain pure LNFP II-ol; it therefore represents a partially acetylated product of LNFP II-ol which must be the more difficult of the oligosaccharides in the mixture to acetylate.

Application of ion-exchange h.p.l.c. to the separation of neutral oligosaccharides

H.p.l.c. or h.p.t.l.c. of acetylated derivatives is one possible method for the separation of oligosaccharides obtained from natural sources and differing only by 1–3 or 1–4 linkage to GlcNAc. However, acetylation, separation and de-O-acetylation is inconvenient for preparative work and therefore other methods have been sought. From the studies of Roussel and colleagues (Lamblin et al., 1980; Van Halbeek et al., 1982) it was suggested that such isomers are resolved by ion-exchange chromatography using classical column sizes and flow rates and the anion-exchange resin DAX4 (Durrum) eluted with borate buffer,
because they reported the two isomers Gaℓβ1-3GlcNAcβ1-3GalαN-ac-ol and Gaℓβ1-4GlcNAcβ1-3GalαN-ac-ol in pure form from a preparation of human bronchial mucins which had initially been purified in this way rather than by Bio-Gel P4 gel filtration. Several studies have been reported using classical ion-exchange chromatography in mono- and oligo-saccharide analysis. In general (Jandera & Churacek, 1974; Goulding, 1975; Ladisch et al., 1978; Scobell & Brobst, 1981), it was found that for cation-exchange resins eluted with water and anion-exchangers eluted with water or borate buffers, but not aqueous ethanol, the retention times for oligosaccharides increases with decreasing number of hydroxyl groups. A separation is therefore achieved mainly by size exclusion. These ion-exchange systems adapted to h.p.l.c. may therefore offer an alternative to classical size exclusion chromatography and, as shown by the study reported above (Van Halbeek et al., 1982), may allow further separation of isomers. Interestingly, Bio-Gel P4 chromatography also gave a partial separation of the two trisaccharide isomers referred to above from human meconium glycoproteins. In general (Jandera et al., 1982), may 0.1 could not be achieved using several different acetonitrile/water mixtures. However, a separation of LNT-ol and LNNT-ol and the synthesis and assumes importance in the genetic variant

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**Some recent high-performance liquid chromatography assays of steroid hormones and their conjugates**

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Abbreviations used: 16-OHDHAS, 16-hydroxydehydro-epi-androsterone sulphate; E,3G, oestriol-3-glucosiduronate; E,16G, oestriodiol-16-glucosiduronate; E,17G, oestriodiol-17-glucosiduronate; E,3S, oestriol-3-sulphate; DIAS, dehydro-epi-androsterone sulphate.

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During late pregnancy oestriol is produced in relatively large amounts by biosynthetic reactions involving both the foetal adrenal gland and the placenta; the measurement of oestriol excretion in maternal urine or of its concentration in maternal plasma is commonly used for the monitoring of foetal wellbeing after the 30th week of pregnancy. 16-Hydroxydehydro-epi-androsterone sulphate (16-OHDHAS) is the immediate precursor in this biosynthesis and assumes importance in the genetic variant

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