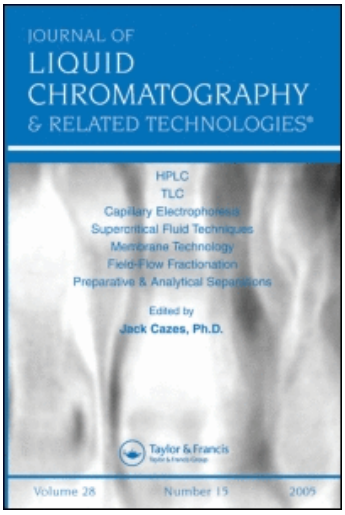


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# SEPARATION OF OLIGOSACCHARIDE ISOMERS CONTAINING ACETAMIDO AND NEUTRAL SUGARS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatography (HPLC) has been investigated for the separation of the following reduced oligosaccharides containing neutral and acetamido sugars;
Galβ1-3GlcNAc-ol and Galβ1-4GlcNAc-ol,
Galβ1-3GlcNAcβ1-6Gal-ol,
Galβ1-4GlcNAcβ1-6Gal-ol,
Galβ1-4GlcNAcβ1-3Gal-ol,
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-ol (LNT-ol),
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-ol (LNNT-ol),
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-ol,
Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc-ol (LNFII-ol),
Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc-ol (LNFIII-ol).
These alditols were studied as standards for the separation of mixtures of reduced oligosaccharides obtained from glycoproteins.

A combination of several HPLC systems using normal and reverse phase column packings was required for separation of the isomers as follows. A novel chromatography system using acetylated alditols eluted from silica (Hypersil) with dichloromethane/hexane/isopropanol as mobile phase separated the disaccharides and the first trisaccharide from the next two. These last two trisac-



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charides could in turn be separated as non-acetylated alditols chromatographed on Hypersil eluted with aqueous acetonitrile containing 0.05% tetraethylenepentamine (TEPA) or on silica chemically bonded with aminopropyl groups (APS-Hypersil). The pentasaccharides were resolved as acetylated alditols chromatographed on reverse phase, octodecyl silica (ODS-Hypersil).

Isomeric separation of the tetrasaccharides was not achieved. However LNT-ol could be obtained essentially free of LNNT-ol by isolation of its di-N-acetylated product. The third tetrasaccharide studied was readily separated from the other tetrasaccharides and the pentasaccharide isomers on normal or reverse phase chromatography because of its greater acetamido/neutral sugar ratio. In general the varying ratios of acetamido/neutral sugars and their different glycosidic linkages conferred distinct but predictable chromatographic properties to the alditols on silica and reverse phase chromatography.

## INTRODUCTION

An important requirement in the analysis of the detailed structure and function of the carbohydrate chains of glycoconjugates is the purification of oligosaccharide isomers. Multiple oligosaccharide isomers are present in secretions such as milk and are released from certain glycoproteins, for example those of mucin-type. Such oligosaccharide structures, although having very similar chemical properties, are, for example, recognised as distinct antigens by anti-carbohydrate antibodies (1).

High-performance liquid chromatography (HPLC) offers several advantages over paper, thin-layer (TLC) and column chromatographies in the speed and ease of preparative separation. Furthermore, many different types of HPLC column packings and a wide range of mobile phases are available. This flexibility can be exploited for isomer separation.

Although there have been several reports of the analytical separation of oligosaccharides by HPLC (2-9) only a limited number of isomers has been purified and in general the separation of reduced oligosaccharides has not been systematically investigated. We have compared several HPLC systems having different stationary and mobile phases and radioactivity or UV detection for the chromatography of oligosaccharide alditols and their acetylated derivatives. Di- to penta-saccharides which contain both acetamido and neutral sugars and vary only in the positions of the

glycosidic linkages were separated and a protocol was formulated for the purification of oligosaccharides of various sizes and ratios of acetamido and neutral sugars which would be obtained from glycoproteins.

## EXPERIMENTAL

### Materials and Reagents

The oligosaccharides Galβ1-3GlcNAc, Galβ1-4GlcNAc, Galβ1-3GlcNAcβ1-6Gal, Galβ1-4GlcNAcβ1-3Gal, Galβ1-4GlcNAcβ1-6Gal, and Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc obtained by chemical synthesis (10,11) were provided by Professor S. David and Drs. C. Augé and A. Veyières, Orsay, France. The oligosaccharides Galβ1-3GlcNAcβ1-3Galβ1-4Glc, Galβ1-4GlcNAcβ1-3Galβ1-4Glc, Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc and Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc obtained from human milk were provided by Dr. W.M. Watkins of this Institute. Chitotriitol was prepared by the partial acid hydrolysis of chitin. Tritiated sodium borohydride (10 Ci/mmole) was obtained from Amersham International (Amersham, U.K.). Sodium borohydride, ammonium acetate, tetraethylenepentamine (TEPA), isopropanol, hexane and dichloromethane were AnalaR grade from BDH Chem. Ltd. (Poole, U.K.). Acetonitrile was 'S' grade from Rathburn Chem. Ltd. (Walkerburn, U.K.).

### Derivatisation of Oligosaccharides

Oligosaccharides were reduced in 0.1 M borate buffer pH 9 containing 0.1 M sodium borohydride. After standing for 16 hr at 4°C the samples were adjusted to pH 4.5 with glacial acetic acid and evaporated with methanol (4 x 5 ml). Radioactively labelled oligosaccharides were obtained by prior treatment for 2 hr at 4°C with tritiated sodium borohydride (1mCi/100 nmole oligosaccharide) Samples for acetylation were dried overnight over phosphorus pentoxide and acetylated for 3 h at 100°C in 1:1 acetic anhydride/pyridine followed by evaporation and chloroform/water extraction. Non-acetylated samples were applied in water to a Bond Elut SCX cation exchange column (Analytichem Int.Inc., Harbor City, CA, USA) primed with methanol. The eluate and 3 x 0.5 ml water washings were collected and lyophilised before being dissolved in water for HPLC.

HPLC Apparatus

A Varian Associates (Walnut Creek, CA., U.S.A.) model 5000 liquid chromatograph was used with either a Varian UV-50 variable wavelength detector operating at 190-210 nm or a Berthold LB 503 HPLC radioactivity monitor employing a solid scintillant cell (130 µl) which would readily detect $10^5$ cpm.

HPLC Columns and Mobile Phases

Hypersil (5µm spherical silica), APS-Hypersil (silica chemically bonded with aminopropyl groups) and ODS-Hypersil (octadecylsilica) were obtained from Shandon Southern Products (Runcorn, U.K.). The columns, 250x5mm, were eluted at a flow rate of 1 ml/min. The mobile phases for the APS-Hypersil and ODS-Hypersil were varying proportions of acetonitrile in either water adjusted to pH2.9 with hydrochloric acid, 15mM phosphate buffer pH 5.2 (ref 7) or 0.5 M ammonium acetate (pH 7.0). The mobile phases for the Hypersil column were either acetonitrile-water each containing 0.05% tetraethylenepentamine (TEPA; for oligosaccharide alditols) or isopropanol in 70:30 dichloromethane/hexane (for acetylated oligosaccharide alditols).



## RESULTS AND DISCUSSION

HPLC of Oligosaccharide Alditols using Silica Eluted with Organic Solvents and Silica Modified with TEPA or Chemically Bonded with Aminopropyl Groups.

Previous studies using TLC have shown that native oligosaccharide alditol isomers not separable on silica could be resolved after acetylation (12-15). Figure 1 shows the equivalent separation by HPLC of acetylated di- and tri-saccharide alditols on a Hypersil column eluted isocratically with 5% isopropanol in 70:30 dichloromethane/hexane. The separation of Galβ1-3GlcNAc-ol from Galβ1-4GlcNAc-ol and of Galβ1-3GlcNAcβ1-6Gal-ol from Galβ1-4GlcNAcβ1-6Gal-ol and Galβ1-4GlcNAcβ1-3Gal-ol was achieved. The acetylated oligosaccharides containing the Galβ1-3GlcNAc linkage had a shorter retention time than those having a Galβ1-4GlcNAc linkage. These last two trisaccharides having a Galβ1-4GlcNAc linkage could not be separated as acetylated deriva-

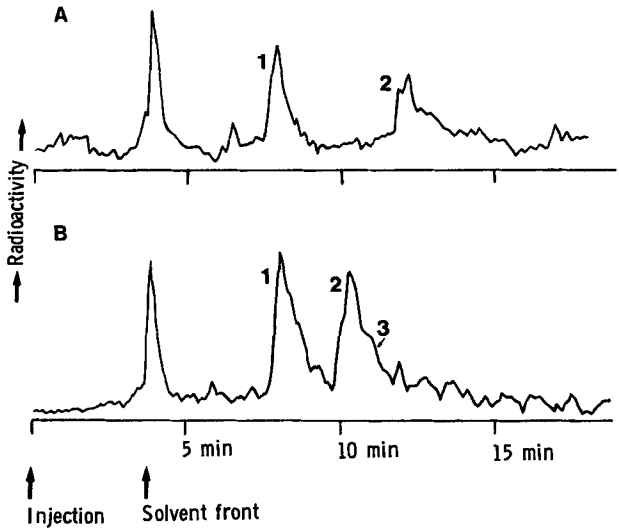


FIGURE 1 HPLC of acetylated di- and tri-saccharide alditol isomers. Column, Hypersil (250x5mm); eluent, 5% isopropanol in dichloromethane-hexane (70:30 v/v); flow rate, 1 ml/min; detector, radioactivity monitor.
A1 Galβ1-3GlcNAc-ol; A2 Galβ1-4GlcNAc-ol;
B1 Galβ1-3GlcNAcβ1-6Gal-ol;
B2 Galβ1-4GlcNAcβ1-6Gal-ol;
B3 Galβ1-4GlcNAcβ1-3Gal-ol.



tives on Hypersil using any of the conditions tested (20 min gradient of 4-14% isopropanol or isocratic elution with 2,3,4 and 5% isopropanol in 70:30 dichloromethane/hexane). However, as shown in Figure 2, they could be separated as non-acetylated derivatives on the Hypersil column eluted with aqueous acetonitrile containing TEPA. Thus a combination of HPLC systems using acetylated and non-acetylated derivatives was capable of purifying all three trisaccharide alditols.

The capacity factors (k') given in Table 1 show the differing retention behaviours of the acetylated and non-acetylated derivatives on silica chromatography. The non-acetylated alditols behaved similarly on the APS-Hypersil column and on the silica column modified with TEPA. In each case the trisaccharides having

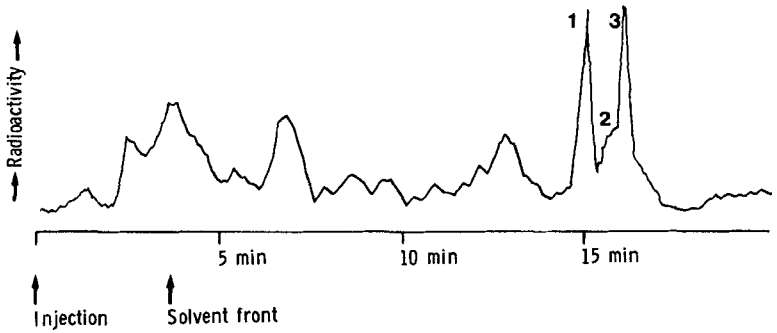


FIGURE 2 HPLC of tri-saccharide alditol isomers. Column, Hypersil (250x5 mm); eluent, 20 min linear gradient elution (75-55%) acetonitrile in water, both solvents containing 0.05% TEPA; flow rate, 1ml/min; detector, radioactivity monitor.
1, Galβ1-4GlcNAcβ1-3Gal-ol;
2, Galβ1-3GlcNAcβ1-6Gal-ol;
3, Galβ1-3GlcNAcβ1-6Gal-ol.



a GlcNAcβ1-6Gal-ol linkage had a longer retention time than that having a GlcNAcβ1-3Gal-ol linkage. A similar finding has been documented for non-reduced oligosaccharides chromatographed on silica columns bonded with amine groups (7,16). Besides the differing behaviour conferred by their linkages, oligosaccharides with a greater acetamido/neutral sugar ratio eluted slower as their acetylated derivatives and faster when underivatised. For example the fucosylated pentasaccharides were not retained on chromatography of their acetylated derivatives due to their low acetamido/neutral sugar ratio. Thus the order of elution of the oligosaccharides is not necessarily related to their size and an initial molecular sizing step is recommended prior to HPLC of mixtures of oligosaccharides.

Chromatography of acetylated LNT-ol gave a second peak with a k' of 0.77 using the Hypersil column and elution with 4-14% isopropanol in 70:30 dichloromethane/hexane (results not shown). This was presumed to be di-N-acetylated LNT-ol previously reported (3) to be formed on O-acetylation. It was partially converted on rechromatography to the peak eluting at k' 2.19 (Table 1) which

TABLE 1

Capacity Factors (k') of Oligosaccharide Alditols Chromatographed on Silica (Hypersil) and Silica Bonded With Aminopropyl (APS-Hypersil) or Octadecylsilyl (ODS-Hypersil) Groups.

| Oligosaccharide Alditols | k' | | | | | | |
|---|---|---|---|---|---|---|---|
| | Hypersil | | APS-Hypersil | ODS-Hypersil | | | |
| | Acetylated | Non-acetylated | | Acetylated | Non-acetylated | | |
| | 4-14% (20 min) $CH_3CH(OH)CH_3$ in $CH_2Cl_2$/ hexane 70:30 | 75-55% (20 min) $CH_3CN$ in $H_2O$/TEPA pH 9.9 | 75-55% (20 min) $CH_3CN$ in $H_2O$/HCl pH 2.9 | 30-60% (20 min) $CH_3CN$ in $NH_4OAc$ pH 7 | 100% $H_2O$ pH 2.9 (HCl) | 100% 15 mM phosphate pH 5.2 | 100% 0.5M $NH_4OAc$ pH 7 |
| GlcNAc-ol | 2.42 | 1.78 | 1.24 | 1.75 | 0.94 | 0.25 | 0.00 |
| Galβ1-3GlcNAc-ol | 1.73 | 2.16 | 2.09 | 3.31 | 1.31 | NT | NT |
| Galβ1-4GlcNAc-ol | 2.16 | 2.03 | 2.01 | 3.34 | 1.35 | NT | 0.00 |
| Galβ1-3GlcNAcβ1-6Gal-ol | 2.17 | 3.57 | 2.84 | 5.08 | 0.97 | 0.27 | NT |
| Galβ1-4GlcNAcβ1-6Gal-ol | 2.30 | 3.68 | 2.98 | 5.12 | 1.10 | 0.37 | NT |
| Galβ1-4GlcNAcβ1-3Gal-ol | 2.35 | 3.41 | 2.53 | 4.97 | 1.00 | 0.35 | NT |
| Galβ1-3GlcNAcβ1-3Galβ1-4Glc-ol | 2.19 | 4.31 | 4.34 | 5.77 | 0.97 | 0.40 | 0.00 |
| Galβ1-4GlcNAcβ1-3Galβ1-4Glc-ol | 2.22 | 4.30 | 4.32 | 5.92 | 0.93 | 0.36 | NT |
| Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-ol | NT | NT | 2.42 | 4.30 | 1.27 | NT | NT |
| Galβ1-3(Fucα1,4)GlcNAcβ1-3Galβ1-4Glc-ol | 0.00 | 5.00* | 5.07* | 6.73 | 1.09 | NT | NT |
| Galβ1-4(Fucα1,3)GlcNAcβ1-3Galβ1-4Glc-ol | 0.00 | 5.00* | 5.07* | 7.20 | 1.00 | NT | NT |

$k' = V_1 - V_o \div V_o$ where $V_1$ = elution volume of solute and $V_o$ = elution volume of non-retained peak;

NT = not tested; ⊓⊔ = separation of isomers. *Chromatographed as mixtures only and the isomers were not resolved.

was characterised as fully O-acetylated mono-N-acetylated LNT-ol by direct probe electron impact mass spectrometry (E.F. Hounsell and A.M. Lawson, unpublished results). LNNT-ol and the mono-, di- and tri-saccharides used in the present study did not appear to undergo di-N-acetylation as deduced by their chromatography as a single peak and mass spectrometry. Therefore, although none of the HPLC systems tested achieved an isomeric separation of the tetrasaccharides, LNT-ol could be separated from its isomer LNNT-ol by harvesting the di-N-acetylated product of LNT-ol.

Separation of the tetra- and penta-saccharide isomers and the two trisaccharides having the 1-6Gal-ol linkage could not be achieved on chromatography of their non-acetylated derivatives on either the Hypersil or APS-Hypersil column although several conditions were explored; 20 min gradients of 75-55%, 70-30%, 60-40% acetonitrile and isocratic elution with 65%, 70% and 75% acetonitrile in water (with both solvents containing 0.05% TEPA for chromatography on the Hypersil column). A comparison of the two types of column showed that the in situ loading of silica with TEPA resulted in a much more stable and reliable system compared to aminopropyl silica which has a shorter column life and may vary from batch to batch.



## HPLC of Oligosaccharide Alditols on ODS-Hypersil

As shown in Table 1 the retention behaviours of the acetylated derivatives on an ODS-Hypersil column eluted with a gradient of acetonitrile in ammonium acetate resembled those of non-acetylated alditols on Hypersil and APS-Hypersil, but differed from acetylated derivatives chromatographed on Hypersil. However, complete separation of the trisaccharide isomers Galβ1-4GlcNAcβ1-6Gal-ol and Galβ1-4GlcNAcβ1-3Gal-ol could not be achieved on the ODS-Hypersil column although several conditions were explored (20 min gradients of 30-60% and 40-60% acetonitrile and isocratic elution with 50%, 45% and 40% acetonitrile in 0.5 M ammonium acetate).

Fig. 3A shows an example of the separation of acetylated oligosaccharide alditols that can be achieved by ODS-Hypersil.

The two isomers of lacto-N-fucopentaose (LNF), Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc-ol (LNFII) and Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-ol (LNFIII), in a preparation of LNFII obtained from human milk were completely resolved. Rechromatography of the peak eluting at 27 min gave the original peak and a peak which co-chromatographed with per-O-acetylated LNFII. After de-O-acetylation the two peaks were indistinguishable. These characteristics indicated that the peak was the di-N-acetylated product of LNFII.

Fig. 3B shows the chromatography of per-O-acetylated tetrasaccharide Galβ1-3GlcNAcβ1-3Galβ1-4Glc-ol (LNT) and its di-N-acetylated product discussed above. Also shown in Fig. 3 is the chromatography of the acetylated tetrasaccharide Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-ol and an unidentified peak also presumed to be its di-N-acetylated product.

Non-acetylated alditols were only retained on the ODS-Hypersil column when 100% aqueous eluent at pH 2.9 or pH 5.2 was used (Table I) and no separation of isomers was achieved. When ammonium acetate at pH 7 was used as the eluent none of the oligosaccharides tested were retained. The increased elution time at pH 3 of the disaccharides and the tetrasaccharide having a 1:1 acetamido/neutral sugar ratio (Table 1) and the further increased elution time of chitotriitol (GlcNAcβ1-4GlcNAcβ1-4GlcNAc-ol, k' 1.88; results not shown) suggest that reverse phase chromatography of non-acetylated alditols may be useful for the separation of larger oligosaccharide isomers containing a high acetamido/neutral sugar ratio, for example those obtained by base/borohydride degradation of mucin- type glycoproteins having a reduced-end N-acetylgalactosaminitol.

Recovery of Oligosaccharide Alditols by Preparative HPLC

Reduced oligosaccharides are used for preparative separation by HPLC for two main reasons. Firstly, HPLC usually results in the separation of the α and β anomers at the reducing end of oligosaccharides and therefore two peaks have to be collected and characterised for each oligosaccharide. This property has been

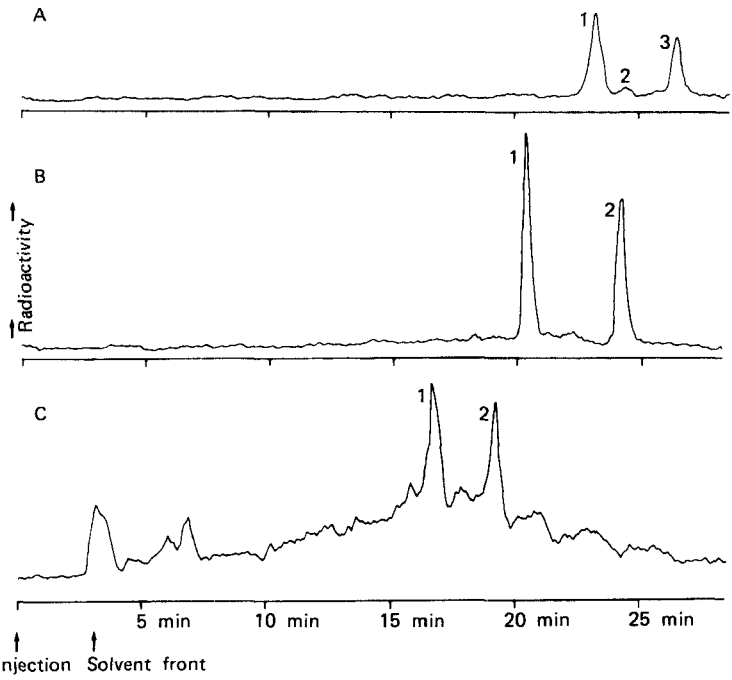


FIGURE 3 HPLC of acetylated tetra- and penta-saccharide alditols. Column, ODS-Hypersil (250x5mm); eluent, 20 min linear gradient elution (30%-60%) acetonitrile in 0.5 M ammonium acetate followed by isocratic elution at 60% acetonitrile for 10 min.
flow rate, 1ml/min; detector, radioactivity monitor.
A1, Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc-ol (LNF II);
A2, Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc-ol (LNF III);
A3, Di-N-acetylated LNF II; B1, Galβ1-3GlcNAcβ1-3Gal β1-4Glc-ol (LNT); B2, Di-N-acetylated LNT; C1, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-ol; C2, presumed di-N-acetylated product of C1.

exploited to purify LNT from LNNT as one of the reducing-end anomers of LNT is separable from its second anomer and the two anomers of LNNT (9,17). With mixtures of unknown isomers a complex chromatographic profile would result and this would be a disadvantage. Secondly, unreduced oligosaccharides form Schiff bases with TEPA and the amino groups bonded to silica, thus decreasing their yields.

In addition, reduction renders oligosaccharides stable to base. This is a high yielding chemical reaction which is often carried out simultaneously with the release of oligosaccharides from glycoproteins.

HPLC of 1-10 mgs of reduced oligosaccharides on the 250x5mm columns used in the present studies resulted in yields of >80%. Recovery of acetylated derivatives was 70-80% and their overall yields were decreased further by the acetylation and de-acetylation reactions. Acetylation at 100°C results in complete O-acetylation, but also yields di-N-acetylated products. Acetylation at ambient temperature is reported not to give rise to di-N-acetylated products (3,8) however this may lead to only partial O-acetylation.

Removal of TEPA and the ions introduced from the de-O-acetylation reaction and by the use of buffers can be achieved in high yield by cation exchange chromatography and solvent evaporation.



## Comparison of Methods of Detection in the HPLC of Oligosaccharides

Oligosaccharides containing N-acetyl, O-acetyl or $CO_2H$ groups can be detected at 195-210 nm UV absorbance when acetonitrile 'S' grade and water are used as the eluents. In analytical studies 1 nmole of oligosaccharides was detected. UV monitoring is convenient although non-oligosaccharide peaks may also be detected.

A tritium group introduced on reduction enables more specific detection of the oligosaccharide alditols to be achieved although background peaks may appear on storing at 4°C or on freezing and thawing the labelled oligosaccharides (Figs. 1,2 and 3C). Routinely $10^5$ cpm/nmole oligosaccharide were detected by a radioactivity monitor. The specific activity could be increased for more sensitive analytical studies or decreased for preparative separations. Monitoring of radioactivity, unlike detection using UV absorbance, enables the use of the column modifiers e.g. TEPA, buffer gradients and organic solvents other than acetonitrile 'S'.

## CONCLUSIONS

HPLC of several oligosaccharides containing acetamido and neutral sugars has shown that more than one column system will usually be required in the purification of multiple oligosaccharide isomers. The different chromatographic properties afforded by hydroxyl, N-acetyl and O-acetyl groups on normal and reverse phase HPLC of native and acetylated derivatives will separate many isomers containing acetamido and neutral sugars. As this separation is never strictly by size an initial gel filtration step is also required. The following scheme is suggested for the preparative separation of oligosaccharide mixtures: (a) Biogel P4 column chromatography (18); (b) chromatography of reduced oligosaccharides on a reverse phase column eluted with buffer/ acetonitrile; (c) acetylation of the separated compounds, extraction into chloroform and removal of salt by washing with water, (d) chromatography of the acetylated derivatives on a reverse phase column using the conditions described in Table 1 followed by removal of ammonium acetate by chloroform/water extraction (e) chromatography of the acetylated derivatives on a silica column eluted with isopropanol/hexane/ dichloromethane; (f) de-O-acetylation followed by chromatography on a silica column eluted with aqueous acetonitrile/TEPA and (g) removal of TEPA and salt by cation exchange chromatography.

The reproducibility of the chromatographic systems described above enables structural information as to size and acetamido/ neutral sugar ratio to be gained from the retention behaviours of the oligosaccharides during purification.



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