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Note

Preparation of lacto-N-neotetraose from human milk by high-performance liquid chromatography

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Human breast milk contains a mixture of various isomeric oligosaccarides that have been isolated by gel filtration and paper chromatography[1]. Paper chromatography, in particular, is time consuming and has limited resolution for the oligoaccharides. The recovery of the oligoaccharides following paper chromatography is also low. High-performance liquid chromatography (HPLC) is an alternative method to paper chromatography, because of its speed of performance and high resolution.

In our studies we found that HPLC can replace paper chromatography in the fractionations of milk oligosaccharides. We report here that lacto-N-neotetraose (LNnT) can readily be separated from lacto-N-tetraose (LNT). The fractionation of other milk oligosaccharides is also described.

EXPERIMENTAL

The oligosaccharides were prepared as described in detail by Kobota[1]. In brief, the lipids were separated from the breast milk by centrifugation at 2°C and the proteins and lactose were removed by precipitation with ethanol. The supernatant, containing the oligosaccharides, was applied to a Bio-Gel P-6 column (130 × 5 cm) and the neutral sugar fraction collected. To remove contaminating sialic acid containing oligosaccharides, the mixture was passed through a mixed bed resin (10 × 1 cm) of Amberlite AG 50 (H^+) and AG 3 (OH^-). The oligosaccharides were then separated by descending chromatography on Whatman 3MM paper into four bands, using ethyl acetate–pyridine–water (12:5:4) as solvent. Each band was subjected to paper chromatography for a second time. The standards for these separations were kindly supplied by Dr. Akira Kobata and Dr. Victor Ginsburg. Band I from the paper chromatography contains LNT and LNnT, band II is LNF-I, band III is LNF-II and LNF-III and band IV is LND-I and LND-II. The structures of the oligosaccharides are shown in Table I. The oligosaccharides of each band were subjected to HPLC.

HPLC analyses were performed using the following Waters Assoc. equipment:

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TABLE I

STRUCTURES OF HUMAN MILK OLIGOSACCHARIDES

Compound	*Abbreviation*	*Structure*
Lacto-N-tetraose	LNT	Gal-β-(1→3)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Lacto-N-neotetraose	LNnT	Gal-β-(1→4)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Lacto-N-fucopentaose I	LNF-I	Fuc-α-(1→2)-Gal-β-(1→3)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Lacto-N-fucopentaose II	LNF-II	Gal-β-(1→3) Fuc-α-(1→4) > GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Lacto-N-fucopentaose III	LNF-III	Gal-β-(1→4) Fuc-α-(1→3) > GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Lacto-N-difucohexaose I	LND-I	Fuc-α-(1→2)-Gal-β-(1→3) Fuc-α-(1→4) > GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Lacto-N-difucohexaose II	LND-II	Gal-β-(1→3) Fuc-α-(1→4) > GlcNAc-β-(1→3)-Gal-β-(1→4) Fuc-α-(1→3) > Glc

M 6000 pump, U6K injector, R401 refractive index detector, radial compression module (RCM 100), Dextropak plastic column (10 × 0.8 cm) which for use was compressed in the RCM 100. Two Dextropak columns were used in series, with water as eluting solvent, unless otherwise stated. Fractions were collected as required, subject to lyophilization and reinjected.

Proton nuclear magnetic resonance (NMR) on LNT and LNnT was carried out a Bruker CXP 300 spectrometer. Samples were repeatedly exchanged with ^2H_2O, followed by lyophilization. Spectra were recorded at 21°C in 0.5 ml of ^2H_2O, and are reported relative to acetone as internal standard ($\delta = 2.216$ ppm below tetramethylsilane).

RESULTS

The retention times of the oligosaccharides are listed in Table II. The HPLC separations of LNT and LNnT are shown in Fig. 1a. Peak 2 contained the earlier and peak 3 the later eluting anomer of LNT. Peak 3 was chromatographed and revealed two larger peaks with an additional peak interposed (Fig. 1b). The ^1H NMR pattern was consistent with LNT as described by Dua and Bush[2] who used "structural reporter" protons in the identification of milk oligosaccharides. There were additional peaks at 5.17 ppm (doublet) and at 4.27 ppm (quadruplet), which could not be assigned to particular proton resonances.

TABLE II

RETENTION TIMES OF MILK OLIGOSACCHARIDES
Two Dextropak columns; in water at 2.0 ml/min.

Compound	*Retention time (min)*
LNnT	7.8, 8.6
LNT	8.4, 10.2
LNF-I	12.3, 16.6
LNF-II LNF III	6.6, 7.2, 7.8
LND-I LND II	6.3, 6.7

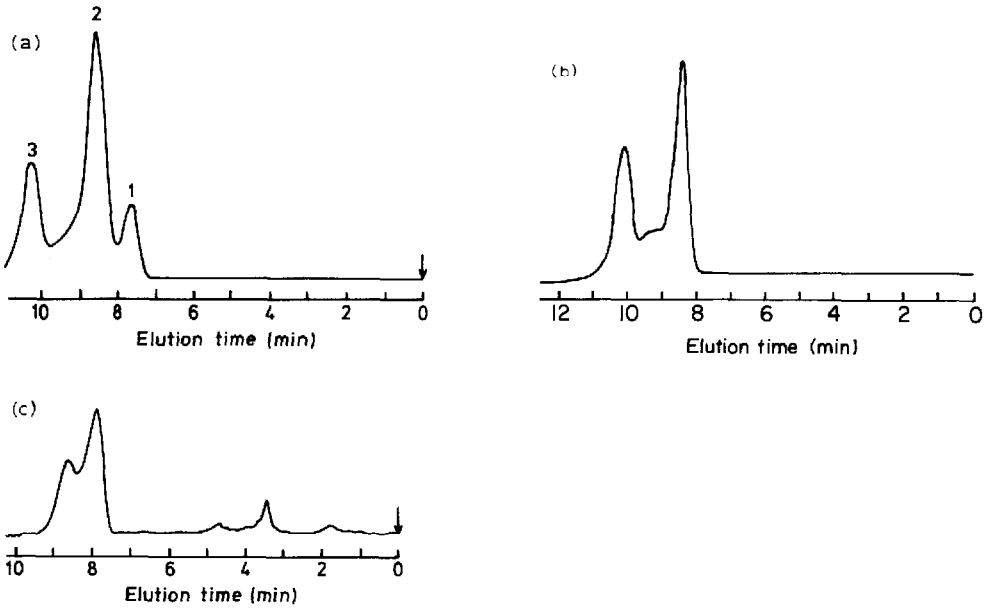

Fig. 1. (a) HPLC profile of LNT and LNnT. Peaks: 1 = LNnT, peak a. 2 = LNT first peak plus LNnT second peak. 3 = LNT second peak. Two Dextropak columns, 2 ml/min, water. (b) Rechromatography of peak 3 from (a). One Dextropak column, 2 ml/min, water. (c) Rechromatography of peak 1 from (a). Two Dextropak columns, 2 ml/min, water.

Peak 1 of Fig. 1a was contributed by the early eluting anomeric LNnT. LNnT could be prepared essentially pure (Fig. 1c) by rechromatography. Its identity and purity was confirmed by comparison with the ^{1}H NMR pattern previously described[2].

The HPLC separations of LNF-I are shown in Fig. 2. This fraction, obtained from band II of the paper chromatography, was pure. Each peak represents one anomer of LNF-I. Each peak was collected and aloowed to re-equilibrate in water to give essentially the same pattern as in Fig. 2.

The separation of LNF-II and LNF-III from the mixture contained in band III of the paper chromatography is shown in Fig. 3. This fraction was contaminated with a small amount of LNF-I (peaks 4 and 5). Because of similar retention times, it was difficult to seperate LNF-II from LNF-III.

The chromatogram of LND-I and LND-II contained in band IV of the paper chromatography is shown in Fig. 4. The separation of these two components was not possible.

DISCUSSION

The Dextropak is essentially a C_{18} reversed-phase column, optimised for separation of oligosaccharides[3]. It may also be used for separation of methyl glycosides[4]. The separation mechanism is unclear, but is certainly structure dependent, as shown by the different retention times of various oligosaccharides of the same molecular weight, but of different linkage type[3]. In general, the higher the molecular weight, the

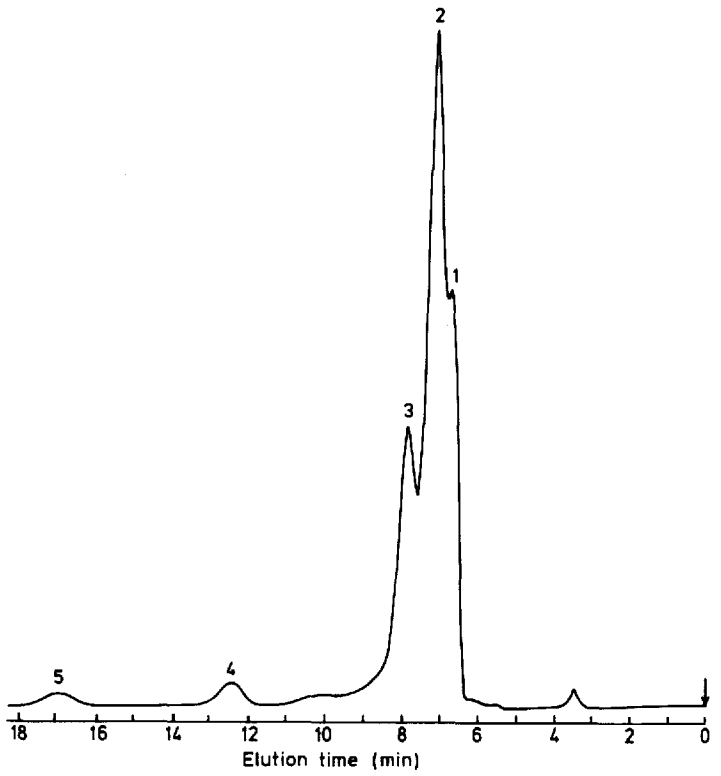

Fig. 2. HPLC profile of pure LNF-I, showing large separation between anomers. Two Dextropak columns, 2 ml/min, water.

Fig. 3. HPLC profile of LNF-II and -III. Peaks: 1, 2, 3 = LNF-II and -III; 4, 5 = LNF-I. Two Dextropak columns, 2 ml/min, water.

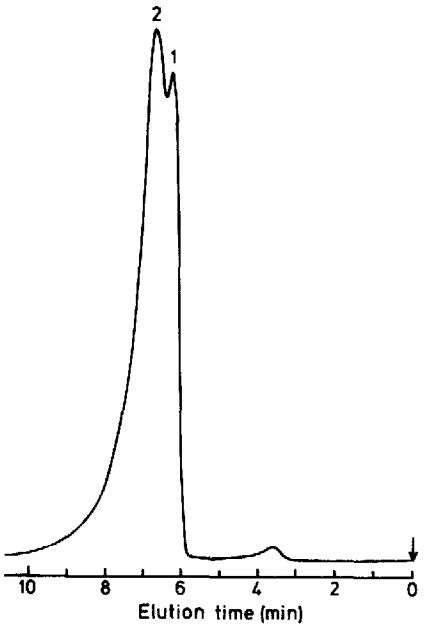

Fig. 4. HPLC profile of LND-I and -II. Two Dextropak columns, 2 ml/min, water.

longer the retention time of structurally similar oligosaccharides. Several exceptions to this generalization occur, *e.g.*, stachyose elutes before raffinose, and $\beta 1 \rightarrow 4$ linked-D-mannose oligomers are not resolved[5]. The stereochemistry at the anomeric linkage is important, *e.g.*, in the separation of LNF-I into two anomers, the separation of anomers of malto oligosaccharides[1], by the resolution of all four methyl glycosides of some monosaccharides[4], and of methyl α- and β-maltosides[6]. Glycosides of the 6-deoxy sugars L-fucose and L-rhamnose elute later than those of the corresponding galactose and mannose[5]. This is consistent with the observation[7] that on reversed-phase columns, highly methylated monosaccharides elute later than those with a lower degree of substitution.

The separation of the differently linked tetrasaccharides LNT and LNnT was not unexpected. LNF-I, a pentasaccharide, elutes later than the tetrasaccharides, as expected, but the separation between anomers is surprisingly large. The early elution of LNF-II and LNF-III was also unexpected, as these pentasaccharides also contain fucose, whose deoxy function would be expected to increase elution times. The most unexpected result was that of LND-I and LND-II, which elute earliest of all, yet are hexasaccharides containing two fucosyl units. Obviously, structure is predominating over molecular weight in the interactions with the C_{18} stationary phase. The most highly branched oligosaccharides elute earliest, leading to the tentative conclusion that "linear" molecules interact most with the C_{18}. This conclusion is supported by observations on the elution times of linear and brached isomaltodextrins, where the branched molecules always elute earlier than linear ones of the same degree of polymerization[8].

Our results indicate that HPLC can replace paper chromatography in the separation of milk oligosaccharides. Because LNT and LNnT had similar retention times as LNF-II and LNF-III. it is advisable to isolate LNT and LNnT by Bio-Gel P-2 filtration from a mixture of milk oligosaccharides prior to HPLC. HPLC will then provide pure LNnT. However, LNT contained an impurity, which may suggest the existence of a yet unknown isometric tetrasaccharide.

LNnT contains the blood group type II precursor chain(Galβ1$\rightarrow$4GlcNAc). The preparation of this component by HPLC will benefit, among many other purposes, the study of acceptor specifications of respective glycosyl transferases. This structure also contains blood group system I activity and will be useful in the determination of antigenic domains of anti-I.

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