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CHARACTERIZATION OF LACTO-N-HEXAOSE AND TWO FUCOSYLATED DERIVATIVES FROM HUMAN MILK BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PROTON NMR SPECTROSCOPY

VIRENDRA K. DUA
Department of Chemistry, Illinois Institute of Technology, Chicago, IL 60616 (U.S.A.)
KAZUE GOSO and VOLKER E. DUBE
Department of Pathology, Evanston Hospital, Evanston, IL 60201 (U.S.A.)
and
C. ALLEN BUSH*
Department of Chemistry, Illinois Institute of Technology, Chicago, IL 60616 (U.S.A.)
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SUMMARY

The branched hexasaccharide whose trivial name is lacto-N-hexaose (LNH) as well as a mono- and a difuco-derivative were isolated from human breast milk of a Lewis a positive donor by high-performance liquid chromatography (HPLC). Reversed-phase HPLC on C_{18} columns which have been used previously in our laboratory for successful separations of the smaller milk oligosaccharides were not effective in this system. However, normal-phase HPLC on an amino-bonded silica column (Varian AX-5) gave both analytical and preparative separations. The identity of the LNH core structure was verified both by HPLC and by NMR spectroscopy of the fucosidase digestion products of the fucosylated oligosaccharides. LNH was identified by NMR spectroscopy of the reduced and acetylated derivative whose high field spectrum has been previously reported. The positions of fucosylation of β-GlcNAc residues were determined by the characteristic chemical shifts of the resonances assigned to α-fucose H1 and H5. The assignment of many of the resonances to sugar ring protons was possible using difference decoupling methods. The observation of nuclear Overhauser effects between the anomeric protons and the aglycone protons of adjacent residues confirmed the assignment of the glycosidic linkages.

INTRODUCTION

Human milk contains numerous complex oligosaccharides containing N-acetyl amino sugars which are known as "bifidus factors" as a result of their influence on the intestinal flora of breast-fed infants[1]. Although no other biological function has been ascribed to these oligosaccharides, whose structures are similar to those of naturally occurring glycolipids and glycoproteins, they are an important source of substrates for studies of the acceptor specificities of glycosyl transferases, the specificities

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of glycosidases and as antigenic determinants both for the production and characterization of polyclonal and monoclonal antibodies[2–4].

The free oligosaccharides have been isolated from human milk by gel filtration and preparative paper chromatography[5,6]. Recent advances in high-performance liquid chromatography (HPLC) have provided the tools for expedient and more refined separation of complex oligosaccharides such as those from human milk. Using reversed-phase HPLC, we have fractionated the small neutral milk oligosaccharides and have provided reference ^1H and natural abundance ^{13}C NMR spectra for these compounds[7–9]. Egge and co-workers[10–12] have isolated some milk oligosaccharides as the reduced and per-O-acetylated derivatives using HPLC and have identified the derivatives by mass spectrometry and high field proton NMR spectroscopy.

In this communication, we report the isolation of the branched hexasaccharide lacto-N-hexaose (LNH)*, and of a mono- and a difucosylated derivate in high purity from breast milk of a Lewis a positive donor by normal-phase HPLC (See Table I for oligosaccharide structures). In addition to the chromatographic properties of the native underivatized oligosaccharides on normal- and reversed-phase HPLC, we report high field proton NMR spectra which will serve as reference spectra for future characterization of similar oligosaccharides from various biological sources.

TABLE I

STRUCTURES OF THE MILK OLIGOSACCHARIDES

Designation	*Compound*	*Structures*
M1	Lacto-N-hexaose (LNH)	Gal(β-1,4)GlcNAc (β-1,6) ↘ Gal(β-1,4)Glc Gal(β-1,3)GlcNAc (β-1,3) ↗
M2	Monofuco lacto-N-hexaose	Fuc(α-1,3) ↓ Gal(β-1,4)GlcNAc(β-1,6) ↘ Gal(β-1,4)Glc Gal(β-1,3)GlcNAc(β-1,3) ↗
M3	Difuco lacto-N-hexaose	Fuc(α-1,3) ↓ Gal(β-1,4)GlcNAc(β-1,6) ↘ Gal(β-1,4)Glc Gal(β-1,3)GlcNAc(β-1,3) ↗ ↑ Fuc(α-1,4)

* Abbreviations used: LNH, lacto-N-hexaose (see Table I for structure); LNT, lacto-N-tetraose [gal(β-1-3)glcNAc(β-1-3)gal(β-1-4)glc]; LNnT, lacto-N-neotetraose [gal(β-1-4)glcNAc(β-1-3)gal(β-1-4)glc]; LNF-II, lacto-N-fucopentaose II {gal(β-1-3)[fuc(α-1-4)]glcNAc(β-1-3)-gal(β-1-4)glc}; LNF-III, lacto-N-fucopentaose III {gal(β-1-4)[fuc(α-1-3)]glcNAc(β-1-3)-gal(β-1-4)glc}.

MATERIALS AND METHODS

Human breast milk from a Lewis a positive donor was processed as described by Kobata and Ginsburg[2,3]. The milk was centrifuged at 4°C and the lipid layer removed by filtration through glass wool. Lactose and proteins were precipitated by the addition of ethanol and the supernatant was lyophilized. The lyophilized material was fractionated on a Bio-Gel P-6 column (140 × 4 cm) using water as eluent and the fractions were monitored for neutral sugar by the phenol–sulfuric acid test[13].

The HPLC apparatus consisted of an LDC Constametric III pump, a Rheodyne Model 7125 injector and a Kratos SF 770 variable-wavelength ultraviolet detector. Normal-phase HPLC was accomplished using a Varian Assoc. MicoPak AX-5 column (300 × 4.0 mm) eluted with acetonitrile–1.0 m*M* phosphate buffer (pH 5.4). An octadecyl silica column (Alltech Associates 605 RP 5) with dimensions 250 × 4.6 mm was eluted with water for reversed-phase chromatography[7,14,15]. The column effluent was monitored by ultraviolet absorbance at 202 nm. Preparative HPLC was carried out on the same columns by collecting the fractions manually at the outlet of the detector.

For ^{1}H NMR spectroscopic analysis, samples (approx. 5 μmoles) were dissolved in ^2H_2O and then repeatedly exchanged with ^2H_2O at room temperature and lyophilized. The samples were dissolved in 0.3 ml high purity ^2H_2O (Merck, Sharp and Dohme) in a 5-mm NMR tube and studied at 300 MHz in a Nicolet spectrometer at IIT and at 470 MHz at the Purdue Biological Magnetic Resonance Center. The observed chemical shifts are reported relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) using acetone as an internal standard (δ = 2.225 ppm downfield from DSS). The spin difference decoupling experiments (SDDS)[16] were carried out at 24°C while the nuclear Overhauser enhancement (n.O.e.) experiments were done a 5°C.

LNH was reduced with borohydride and per-O-acetylated as described by Dabrowski *et al.*[11]. The spectrum recorded in C^2HCl_3 was compared with the data reported in ref. 12 to verify the identity of the hexasaccharide.

Fucosidase digestion was used to assist in the oligosaccharide structure identification. An amount of 5 mg of a mixture of the mono- and difuco-derivatives of LNH were dissolved in 1 ml of 1.0 m*M* sodium acetate buffer (pH 5.0) and 200 μl (one unit) of bovine kidney α-fucosidase (Sigma) were added. The reaction mixture was incubated at 37°C for 16 h and one additional unit of α-fucosidase was added followed by an additional 16 h incubation at 37°C. The hydrolysis products were monitored by normal-phase HPLC of aliquots drawn directly from the incubation mixture. The reaction products of the fucosidase digestion were isolated by preparative HPLC and identified by ^{1}H NMR spectroscopy.

RESULTS

In the Bio-Gel P-6 fractionation of milk oligosaccharides from the Lewis a positive donor (Fig. 1), fraction A contains mostly lactose while fraction B contains a mixture of oligosaccharides ranging up to pentasaccharides which have been analyzed previously by HPLC[7,8]. The constituents of fraction C in the Bio-Gel chromatogram ranging from hexa- to octasaccharides were freeze dried and studied by

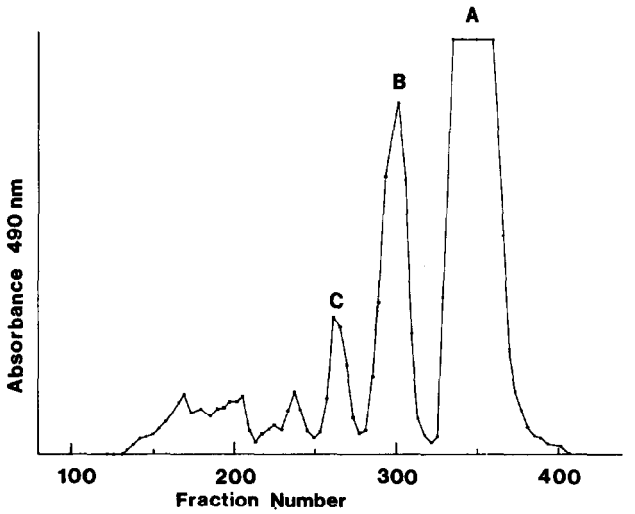

Fig. 1. Bio-Gel P-6 chromatogram of human milk oligosaccharides from the Le^a donor detected by the phenol–sulfuric acid method. Elution was with water; 3.0-ml fractions were collected.

HPLC. Because of short retention times, this mixture could not be fractionated in reversed-phase HPLC as will be explained below. However, Fig. 2 shows that three peaks are clearly resolved in normal-phase HPLC using acetonitrile–phosphate buffer (50:50, v/v) as eluent. Preparative HPLC of the three peaks labeled M1, M2 and M3 was carried out and re-chromatography showed little detectable cross contamination.

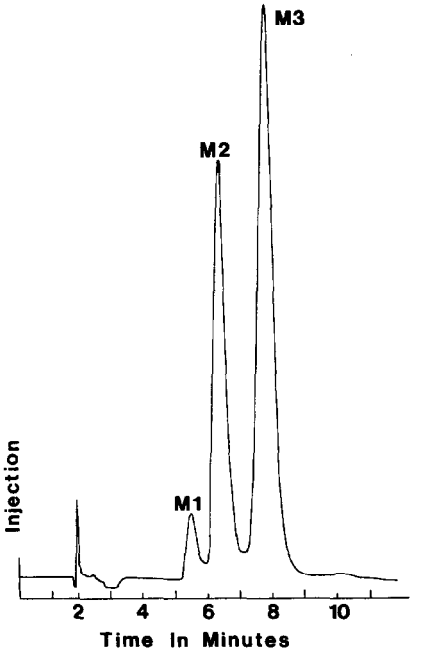

Fig. 2. Normal-phase HPLC of Bio-Gel P-6 fraction C eluted with acetonitrile–1 m*M* KH_2PO_4 (50:50) at a flow-rate of 1 ml/min.

Furthermore, the peaks run true in re-chromatography showing that this normal-phase system does not separate the anomeric forms of the reducing terminal residues as does reversed-phase HPLC of the milk oligosaccharides reported previously[7,8].

The data for system C in Table II, which gives the reversed-phase HPLC results for M1, M2 and M3 as isolated by normal-phase HPLC, illustrates clearly the reason for our failure to fractionate the mixture by reversed-phase chromatography. The short retention times coupled with broadening of the two reducing terminal anomeric peaks for each oligosaccharide result in substantial overlapping of the components.

The peaks M1, M2 and M3 as isolated by normal-phase HPLC (Table II and Fig. 2) were shown to contain pure oligosaccharides and their structures were determined by ^{1}H NMR spectroscopy in conjunction with enzyme digestion. The NMR spectrum (Fig. 3), which will be discussed in detail below, shows M1 to be a hexasaccharide and suggests the LNH structure shown in Table I, while the spectrum of M2 shows it to be a heptasaccharide containing a single α-fucose residue in (1 → 3)

TABLE II

RELATIVE RETENTION TIMES OF MILK OLIGOSACCHARIDES

Chromatographic conditions: system A: MicroPax AX-5 eluted with acetonitrile–1 m*M* phosphate at pH 5.4 (50:50, v/v), 1.3 ml/min; system B: MicroPax AX-5 eluted with acetonitrile–1 m*M* phosphate at pH 5.4 (55:45, v/v), 1.0 ml/min; system C: Alltech C-18 605 RP-5 eluted with water at 1.0 ml/min.

*Oligosaccharide**	*System A*	*System B*	*System C*
M1 Lacto-N-hexaose	2.8**	4.3	2.5, 2.9***
M2 (fuco LNH)	3.1	5.2	2.0, 2.4
M3 (difuco LNH)	3.7	6.7	1.8, 2.0

* See Table I for oligosaccharide structures.

** Retention time relative to that of the elution volume of the column.

*** In reversed-phase (system C) each reducing oligosaccharide gives two peaks corresponding to the anomers[7,14].

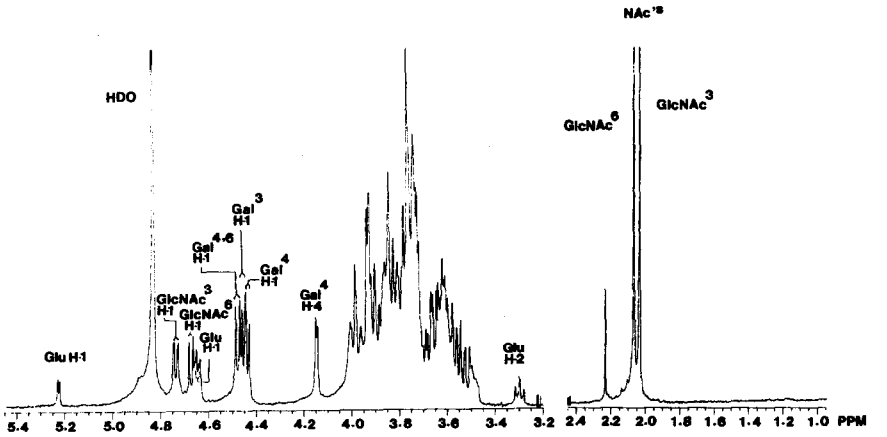

Fig. 3. 470-MHz ^{1}H NMR spectrum of lacto-N-hexaose in ^2H_2O at 24°C.

linkage to glcNAc (Fig. 4). The spectrum of M3 (Fig. 5) shows it to be an octasaccharide containing two α-fucose residues one in (1 → 3) and the other in (1 → 4) linkage to glcNAc. Exhaustive fucosidase digestion of M2 and M3 gave a single oligosaccharide product which was characterized both by HPLC retention time and by its ^{1}N NMR spectrum as M1, showing that M2 and M3 are fucosylated derivatives of M1. The identity of M1 as LNH was established by comparison of the NMR spectrum of the reduced and per-O-acetylated M1 with that reported for LNH in ref. 12. These data firmly establish the structures of all three oligosaccharides since the only possible positions of fucosylation of glcNAc in the LNH core are those which are shown in Table I.

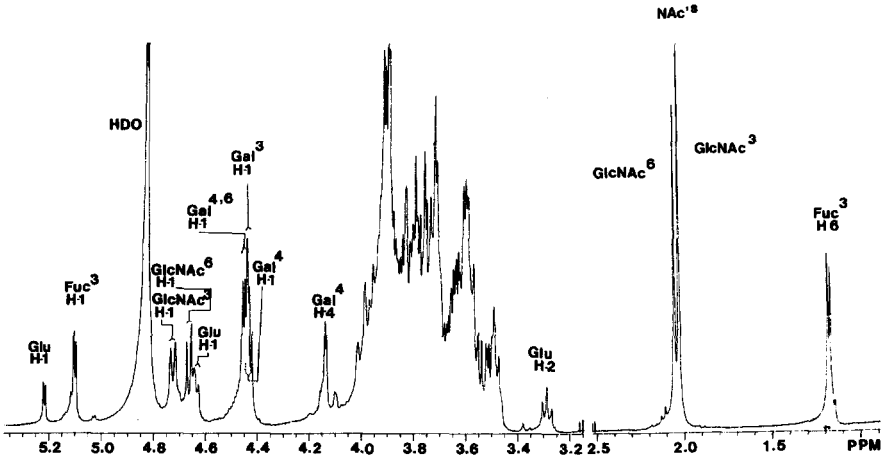

Fig. 4. 470-MHz ^{1}H NMR spectrum of monofuco lacto-N-hexaose in ^2H_2O at 24°C.

A more detailed analysis of the NMR spectra, including decoupling and n.O.e. data, confirms the oligosaccharide structures of Table I and also allows assignment of many of the resonances to individual protons in the structures. Integration of the anomeric region of the ^{1}H NMR spectrum of M3 (Fig. 5) shows eight anomeric protons expected for an octasaccharide. The reducing terminal glucose residue is indicated by its characteristic resonances at 5.214 (H1 α), 4.693 (H1 β) and 3.287 ppm (H2 β)[7]. Integration of the amide methyl region indicates six protons corresponding to two acetamido sugars (glcNAc) while integration of the fucose H6 region shows two fucose residues. Comparison of Fig. 5 with the spectra of LNF-II and LNF-III[7] allows assignment of the resonances at 5.098 (H1), 4.873 (H5) and 1.170 (H6) to the α-fucose 3 linked to glcNAc and assignment of the resonances at 5.025 (H1), 4.915 (H5) and 1.178 (H6) to the α-fucose 4 linked to glcNAc.

Assignments of the anomeric and some of the other proton resonances in Fig. 5 may be made with data from decoupling and n.O.e. experiments. Selective decoupling irradiation at each of the three doublets assigned to the β-galactose H1 at 4.424, 4.453 and 4.504 ppm identified the resonances of the corresponding H2 at 3.546, 3.489 and 3.496 ppm, respectively. Selective decoupling irradiation of the narrow doublet at 4.145 ppm, which is assigned to H4 of a β-galactose which is glycosylated

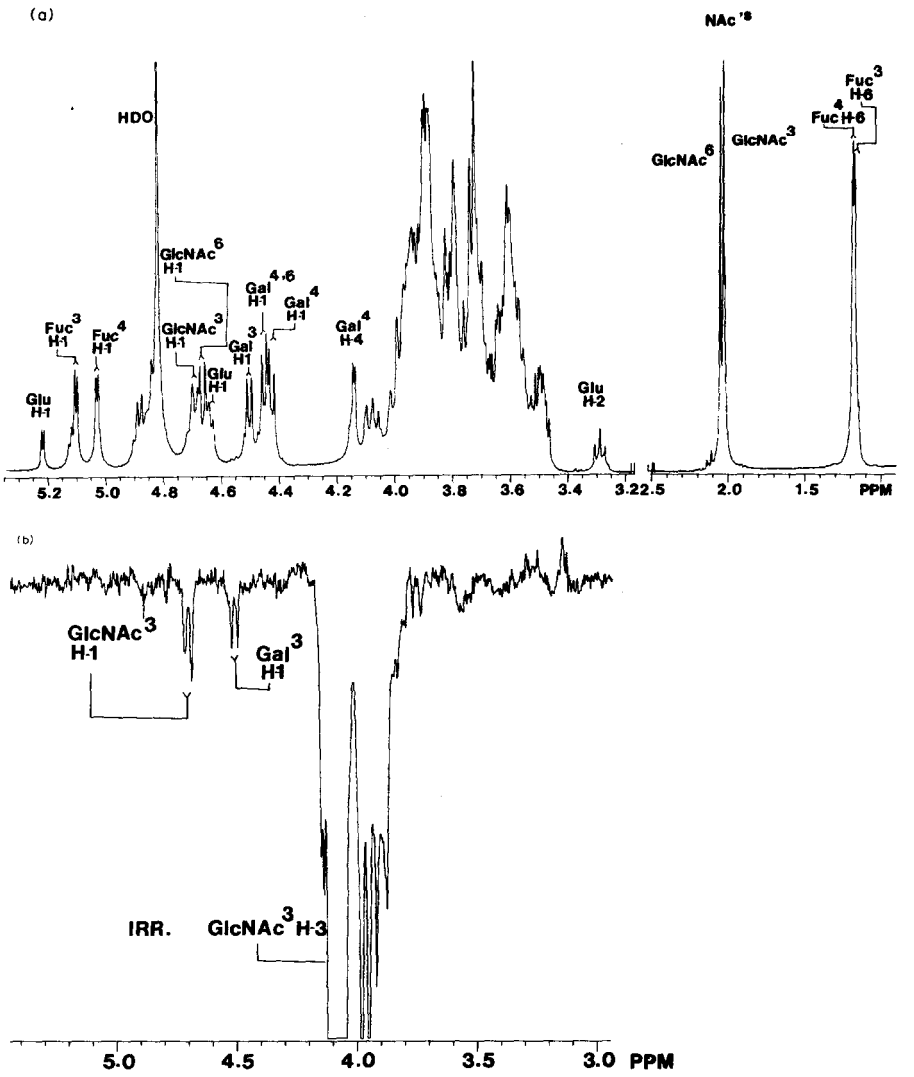

Fig. 5. (a) 470-MHz ^{1}H NMR spectrum of difuco lacto-N-hexaose in ^2H_2O at 24°C. (b) Difference n.O.e. spectrum (300 MHz) of difuco lacto-N-hexaose at 5°C. Irradiation at β-GlcNAc^3 H3 (4.077 ppm).

at C-3[7], identifies the H3 resonance (3.695 ppm) of β-galactose which is C-3 substituted (the branching galactose). Decoupling at the gal H2 resonance at 3.546 ppm identifies this same gal H3 resonance thus completing the assignment of H1, H2, H3 and H4 of the branching galactosyl residue indicated in Table III.

Of the two β-anomeric resonances assignable to the glcNAc residues, that at 4.692 ppm as well as the amide methyl resonance at 2.030 ppm may be assigned to β-glcNAc^3 by analogy to the assignments in LNT[7]. Confirmation of this assignment

TABLE III

^{1}H NMR CHEMICAL SHIFTS OF SOME MILK OLIGOSACCHARIDES

Chemical shifts are given relative to internal (DSS) using acetone as the internal standard (δ = 2.225 ppm downfield from DSS).

Residue		*LNH*	*Monofuco LNH*	*Difuco LNH*
α-Glc	H1	5.221	5.218	5.213
	H2	3.589	3.585	3.585
β-Glc	H1	4.637	4.639	4.629
	H2	3.290	3.287	3.284
	H3	3.635	3.629	3.632
β-Gal^4	H1	4.431	4.424	4.424
	H2	3.573	3.583	3.546
	H3	3.732	3.705	3.695
	H4	4.143	4.147	4.145
β-Gal^{4,6}	H1	4.472	4.450	4.453
	H2	3.510	3.527	3.489
	H3	3.673	–	3.620
β-Gal^3	H1	4.446	4.440	4.504
	H2	3.512	3.512	3.496
	H3	–	–	3.627
β-GlcNAc^3	H1	4.780	4.712	4.692
	H2	3.880	3.881	3.937
	H3	–	–	4.077
	H4	–	–	3.749
	N-Acetyl	2.026	2.024	2.030
β-GlcNAc^6	H1	4.666	4.670	4.663
	H2	3.741	3.901	3.913
	H3	–	–	3.706
	N-Acetyl	2.057	2.050	2.050
α-Fuc^3	H1	–	5.105	5.098
	H2	–	3.680	3.683
	H4	–	3.900	3.910
	H5	–	4.866	4.873
	H6	–	1.170	1.170
α-Fuc^4	H1	–	–	5.025
	H2	–	–	3.785
	H4	–	–	3.880
	H5	–	–	4.915
	H6	–	–	1.178

of β-glcNAc^3 H1 is found in n.O.e. observed at that doublet on irradiation of β-glcNAc^3 H3 at 4.077 ppm (Fig. 5b). The appearance of β-glcNAc^3 H3 as a structural reporter group results from fucosylation at C-4 as was observed in the case of LNF-II[7]. The data of Fig. 5b show that irradiation at 4.077 ppm also gives n.O.e. at the doublet at 4.504 ppm allowing its assignment to β-gal^3 H1. Completion of the assignment of the remaining β-gal^3 signals is done by decoupling (Table III). Irradiation at α-fuc^4 H1 gives n.O.e. at β-glcNAc^3 H4 (3.749 ppm) due to inter-ring

n.O.e. and at α-fuc^4 H2 (3.683 ppm) due to intra-ring n.O.e. (data not shown). Selective decoupling at β-glcNAc^3 H4 confirms the assignment of β-glcNAc^3 H3 and decoupling at H1 identifies H2 completing the assignment of the resonances of β-glcNAc^3 as summarized in Table III. By elimination the remaining glcNAc anomeric proton resonance is assigned to β-glcNAc^6 and H2 and H3 of that residue are identified by decoupling. Likewise, the remaining β-gal H1 resonance at 4.453 ppm is assigned to β-gal^{4,6} on the 6 arm of the LNH core. These assignments of the galactose anomeric proton resonances of the type I and type II arms of M3 are consistent with the assignments of LNF-II and LNF-III[7].

The 470-MHz ^{1}H NMR spectrum of oligosaccharide M2 is given in Fig. 4 and the assignments are summarized in Table III. Integration of the anomeric region shows seven anomeric protons with resonances characteristic of glucose at the reducing terminal[7]. Integration of the amide methyl region of the spectrum indicates two acetamido sugars while the integration of the fucose H6 region shows a single residue. The signals at 5.105(H1), 4.866(H5) and 1.170(H6) are characteristic of α-fucose linked to the C-3 position of β-glcNAc by analogy to LNF-III[7]. Of the three anomeric doublets assignable to β-galactose at 4.424, 4.440 and 4.450, the most upfield is assigned to the branching galactose residue by the same decoupling relations to β-gal^4 H4 (4.147 ppm) which were used for this assignment in oligosaccharide M3. Assignment of the remaining two β-galactose anomeric doublets of M2 is suggested by analogy with the fucosylated type II arm galactose anomeric resonances of M3 and of LNF-III[7] for which the β-gal H1 are at 4.453 and 4.464 ppm. Assignment of the resonance at 4.450 to β-gal^{4,6} H1 of M2 leaves the doublet at 4.440 to be assigned to β-gal^3 H1. This tentative assignment is consistent with the assignments of galactose anomeric protons of the non-fucosylated type I and type II arms of LNH, LNT and LNnT as will be discussed below. Chemical shift analogies may be used to assign the β-glcNAc^3 anomeric (4.712 ppm) and amide methyl (2.024 ppm) resonances as well as the β-glcNAc^6 resonances at 4.670 and 2.050 ppm (Table III).

The 470 MHz spectrum of M1 (LNH) is given in Fig. 3 and the assignments are summarized in Table III. Integration of the anomeric region implies a hexasaccharide with glucose at the reducing terminal and the amide methyl region shows two acetamido sugars. Assignment of the anomeric proton of the branching galactose can be done by sequential decoupling to establish the relationship with β-gal^4 H4 at 4.143 ppm. The β-galactose anomeric resonance at 4.446 ppm is assigned to β-gal^3 by analogy with M2 and with LNT[7] leaving the resonance at 4.472 ppm to be assigned to β-gal^{4,6} H1 which is consistent with the spectrum of LNnT[7]. Since the effect of fucosylation on the β-glcNAc anomeric resonances is small, their assignments are readily made by analogy to M2 and M3. The influence of fucosylation is more apparent in the chemical shifts of glcNAc H2 to which the anomeric signals can be related by decoupling (Table III).

DISCUSSION

Although the three milk oligosaccharides which we have studied in this work have been previously isolated by preparative paper chromatography and characterized by chemical degradation[17] the preparation by HPLC is considerably faster and the assay of identity and purity by HPLC and NMR spectroscopy is more precise.

The proton assignments in the NMR spectrum will be useful in the characterization of related structures isolated from glycolipids or glycoproteins as well as in studies of the conformation of the oligosaccharides.

Although reversed-phase HPLC has been found to be useful in separating the small milk oligosaccharides[7,8], the short retention times of these three oligosaccharides (M1,M2,M3) prevented preparative separation. Oligosaccharides containing α-fuc(1 → 2)gal linkages generally show long retention times and excellent separations in reversed-phase HPLC[7,15] while oligosaccharides containing the α-fucosyl 3 and 4 substitutents elute rapidly with poor separation. Normal-phase chromatography on AX-5 columns, which was first reported by Mellis and Baenziger[18] for oligosaccharide alditols gave a single chromatographic peak for each reducing oligosaccharide. The order of elution in normal-phase HPLC follows molecular size as has been observed in other oligosaccharide systems[15,18].

From 1 l of Lewis negative human milk, Kobata and Ginsburg[17] isolated, in addition to 15 mg LNH, 5 mg of an isomeric hexasaccharide, lacto-N-neohexaose which has type II linkages [gal(β-1-4)glcNAc] on both the 3 and 6 arm. Neither is normal-phase HPLC of the type we have used in this study expected to fractionate such linkage isomers nor does the chromatogram of Fig. 2 indicate any partial resolution of closely related structures. Therefore the oligosaccharides isolated from our Lewis a positive sample could be contaminated by structures whose core is the lacto-N-neohexaose isomer. If the difucosylated derivative of the isomeric LNneoH core were present and co-chromatographs with M3, spectral lines due to the contaminant would appear in the NMR spectrum of M3 in Fig. 5. If the chemical shifts of the protons of the isomeric octasaccharide differ from those of the major constituent, extra lines should appear. If the chemical shifts of the protons of both isomers are identical, the fuc^3 H1 peak at 5.098 ppm would be larger than the fuc^4 H1 peak at 5.025 ppm. Fig. 5 shows no detectable contamination by isomeric LNnH core at the level of 10%. No similar argument can be made for M2 which could in principle, be a complex mixture of isomeric heptasaccharides, all of which have identical 470-MHz NMR spectra.

If the LNH sample were contaminated by LNnH, there should be a minor peak in the NMR spectrum of Fig. 3 for the $gal^{1,3}$ H1 in the region of the $gal^{4,6}$ H1 peak at 4.472 ppm if the chemical shifts of the two gal^4 H1 differ. No spectrum of LNnH from which to estimate the difference in chemical shifts of $gal^{4,3}$ and $gal^{4,6}$ H1 has been reported but Dr. D. Baker of Chem-Biomed (Edmonton, Canada) has kindly provided us with a 400-MHz proton NMR spectrum of the synthetic pentasaccharide, gal(β-1-4)glcNAc(β-1-6)[gal(β-1-4)glcNAc(β-1-3)]gal-β-O-$(CH_2)_8$-CO_2CH_3. This compound shows two distinct resonances assigned to gal^4 H1 whose chemical shifts differ by 0.007 ppm. Therefore we would expect contamination of our LNH sample by LNnH to result in additional distinct spectral lines in the 4.472 ppm region. Since Fig. 3 shows no such lines, the contamination cannot be more than 10%.

Although it might appear that the proton assignments indicated in Table III, which were made with the assistance of extensive spin decoupling and n.O.e. difference spectroscopy, might have been made more easily with 2-dimensional methods such as 2-d spin correlated spectroscopy and 2-d nuclear Overhauser spectroscopy (NOESY), several considerastions dictated our use of the traditional 1-d difference

method. Since only a few μmoles of the samples studied here were available, rather long accumulations were needed for good signal-to-noise ratio, a measure which is impractical in 2-d spectroscopy where many (*e.g.* 512) individual spectra must be recorded. This problem is further exacerbated by the apodization methods used in 2-d spectroscopy which degrade the signal-to-noise ratio. Moreover, the limited digital resolution of 2-d methods generally obscures multiplet structure which can be detected in 1-d difference spectroscopy and is often useful in carbohydrate resonance assignments for which the coupling constants of axial and equatorial protons can be quite characteristic.

Thirdly, since 2-d spectra are usually displayed in magnitude mode, small peaks are attenuated causing some cross peaks to go undetected. This problem is especially severe for NOESY spectroscopy of oligosaccharides of modest size for which all n.O.e. are small due to the unfavorable rotational correlation time. Although NOESY may be effective for small proteins which show many n.O.e. of −30% or more, the oligosaccharides in this study had negative n.O.e. of a lesser magnitude.

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