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Determination of neutral oligosaccharide fractions from human milk by gel permeation chromatography

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ABSTRACT

A gel permeation chromatographic method for quantifying neutral oligosaccharide fractions from human milk has been developed. Oligosaccharides from monofucosyllactoses to trifucosyllacto-N-hexaoses were separated according to size on a Fractogel TSK HW 40 (S) column. Refractive index detection of monofucosyllactoses to difucosyllacto-N-tetraoses yielded a constant mass response factor of *ca.* 1 relative to glucose. After the addition of glucose as an internal standard, oligosaccharides were isolated from human milk by ethanol precipitation or two ultrafiltration procedures. The oligosaccharide concentrations found by the ultrafiltration procedures were significantly lower (significance level 0.05) than those determined by the ethanol precipitation procedure.

INTRODUCTION

Studies of the analysis of oligosaccharides from human milk resulted in the isolation and identification of about a hundred different oligosaccharides [1–3]. The biological effects of these oligosaccharides remain to be elucidated. Several *in vitro* studies produced some evidence that oligosaccharides and glycoproteins from human milk may play a protective role during viral and bacterial infections of the respiratory, gastrointestinal or urogenital tract [4–6]. Because they are structural analogues of receptor glycan chains, oligosaccharides and glycans from human milk are supposed to prevent viruses and bacteria from sticking to epithelial cells. Moreover, oligosaccharides from human milk have been reported to promote growth of *Bifidobacterium bifidum* [7]. A so-called bifidus-dominated gut flora is assumed to be beneficial for a healthy development of human infants [8].

Quantitative data for oligosaccharides in human milk are rare. Mostly, research groups investigating the structures of new oligosaccharides determined the yield of the isolated substances, but did not quantify the individual oligosaccharides in the milk [9]. However, Montreuil and Mullet [10] and Viverge and coworkers [11,12] have analysed systematically the concentration of oligosaccharides from human milk in the course of lactation, as well as diurnal variations of oligosaccharides [13]. They either used colorimetric methods to quantify the

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monosaccharide residues of the oligosaccharides or determined gravimetrically the amounts of isolated oligosaccharide fractions.

This paper describes a method for the quantitative determination of neutral oligosaccharide fractions from human milk by means of gel permeation chromatography and refractive index detection. This method is more convenient than the methods used before [10–12], and yields more detailed information.

EXPERIMENTAL

Isolation and characterization of gel chromatographic fractions

Oligosaccharides were isolated as described in detail by Kobata [9], with some modifications. Human milk was heated for 30 min at 70°C to inactivate possible contamination with hepatitis B and human immunodeficiency viruses [14]. After removal of lipids by centrifugation, proteins were precipitated with cold ethanol [9]. Neutral oligosaccharides were separated from acidic sialic acid containing oligosaccharides by ion-exchange chromatography on Dowex 1-X4 (200–400 mesh) in acetate form. Neutral oligosaccharides were eluted with distilled water, and acidic oligosaccharides with 200 m*M* ammonium acetate. Neutral oligosaccharides were further separated by gel permeation chromatography [2] on a 190 cm × 4.4 cm I.D. Fractogel TSK HW 40 (S) column (Merck, Darmstadt, Germany) at a water flow-rate of 1.25 ml/min. After rechromatography on the same column, the oligosaccharide fractions were evaporated, lyophilized and dried over P_2O_5 in a vacuum desiccator.

Oligosaccharide fractions were analysed by gas chromatography (GC) after methanolysis and trifluoroacetylation [15], on a Chrompack CP 9000 gas chromatograph equipped with a flame ionization detector. The fused-silica capillary column DB210 (30 m × 0.25 mm I.D., 0.25 μm film thickness, J. & W., Carlo Erba, Nordheim, Germany) was operated in split mode with a carrier gas (helium) flow-rate of 1 ml/min. Quantitative GC analyses of oligosaccharide fractions were performed with mannitol as internal standard.

Isomeric oligosaccharides from fractions IV–VIII were separated by high-performance liquid chromatography (HPLC) [16,17] using an LKB 2249 gradient pump and an LKB 2141 variable-wavelength UV–VIS detector (monitoring wavelength 195 nm). Two Nucleosil 100 RP-18 columns (250 mm × 8 mm I.D., 5 μm particle size, Knauer, Berlin, Germany) and a corresponding pre-column (30 mm × 8 mm I.D.) were used in series, with deionized water as eluting solvent at a flow-rate of 2 ml/min. Oligosaccharide standards from human milk were kindly supplied by Dr. G. Strecker (University of Lille, Villeneuve, d'Ascq, France) or purchased from Biocarb (Lund, Sweden).

Fast atom bombardment mass spectrometric (FAB-MS) measurements of reduced and peracetylated oligosaccharides were kindly performed by Professor H. Egge (University of Bonn, Bonn, Germany) [18].

Amino acid analyses were performed on a Biotronic LC 5001 amino acid

analyser using a BTC 2710 ion-exchange column and post-column ninhydrin detection.

The sodium, potassium and calcium contents of the gel permeation chromatography fractions were measured with a Perkin Elmer 1100 atomic absorption spectrophotometer, operated in the flame mode at 589.1 nm (sodium), 766.5 nm (potassium) and 422.7 nm (calcium). La_2O_3, HCl and CsCl were added to standard and sample solutions in order to suppress ionic interferences.

Determination of neutral oligosaccharide fractions

Determination of the milk oligosaccharides was performed as follows. As an internal standard 10 mg of anhydrous glucose (dried over P_2O_5) were dissolved in 2 ml of human milk, and 1 ml of this solution was delipidated and deproteinized by centrifugation at 1500 *g* and 20°C for 1.5 h, with the Amicon MPS-I ultrafiltration system (W. R. Grace, Amicon Division, Witten, Germany) using YM10 or XM50 membranes. Alternatively, lipids from a mixture of 4 ml of human milk and 20 mg of anhydrous glucose were removed by centrifugation at 3000 *g* and 4°C for 10 min. Proteins were precipitated overnight at 4°C by adding 4.25 ml of cold ethanol to 2 ml of skimmed milk. After centrifugation at 3000 *g* and 20°C for 5 min, the clear supernatant was decanted, evaporated to dryness and resuspended in 2 ml of deionized water. A 200-μl aliquot of milk sera, produced either by ultrafiltration or by ethanol precipitation, was injected with an LKB V-7 injection valve into a Fractogel TSK HW 40 (S) column (190 cm × 1.6 cm I.D.), driven by an LKB P1 peristaltic pump at a flow-rate of 0.25 ml/min with deionized water, containing 0.02% NaN_3, as eluent. Substances were detected by an LKB 2142 refractive index detector; peak areas were calculated with a Shimadzu C-R5A integrator. When the resolution became insufficient, the colum was regenerated by rinsing with 0.5 *M* NaOH, deionized water, 0.5 *M* HCl and deionized water at a flow-rate of 0.25 ml/min.

Enzymic determination of glucose was performed by using a test combination of lactose–glucose (Boehringer Mannheim, Mannheim, Germany). Glucose was phosphorylated and reduced to gluconate-6-phosphate by means of hexokinase and glucose-6-phosphate dehydrogenase. The resulting reduced nicotinamide-adenine dinucleotide phosphate was determined photometrically at 340 nm.

RESULTS AND DISCUSSION

Isolation and characterization of gel permeation chromatography fractions

A human milk fraction containing large amounts of carbohydrates was obtained after removal of lipids and proteins by centrifugation and ethanol precipitation [9]. Oligosaccharides and other hydrophilic compounds were fractionated by gel permeation chromatography on a Fractogel TSK HW 40 (S) column. Fig. 1 shows the gel permeation chromatography profile of a human milk sample from a secretor Lewis (a– b+) individual. The elution profiles from fractions III–X on

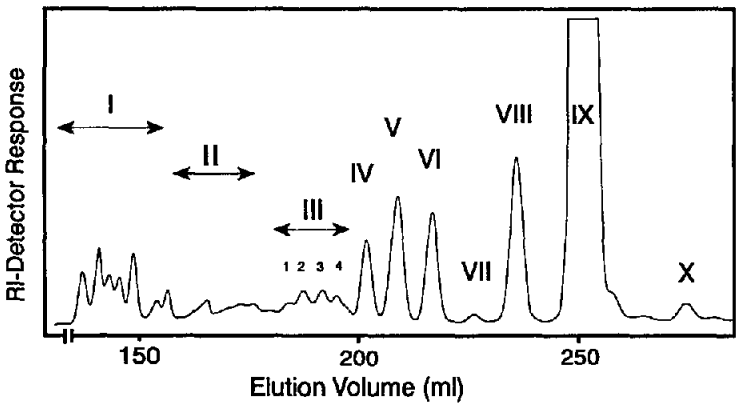

Fig. 1. Gel permeation chromatography profile of human milk oligosaccharides from a secretor Lewis (a − b+) individual. The milk sample was ultrafiltered through an Amicon XM50 membrane and eluted with distilled water on a 190 cm × 1.6 cm I.D. Fractogel TSK HW 40 (S) column at a flow-rate of 0.25 ml/min. The pattern was obtained by monitoring with a refractive index detector.

the small 190 cm × 1.6 cm I.D. Fractogel TSK HW 40 (S) column and on the preparative 190 cm × 4.4 cm I.D. column were identical.

The heterogeneous fraction I near the void volume mainly represents sialylated oligosaccharides, as well as peptides, amino acids and mineral salts. The negatively charged sialylated oligosaccharides elute near the void volume, probably because of repulsive forces directed against the gel matrix. Fraction II mainly contains fucosylated lacto-N-octaoses. GC analysis of the monosaccharide residues yielded a 2:4:1:3 molar ratio of fucose, galactose, glucose and N-acetylglucosamine. The subgroups from fraction III could be isolated by recycling chromatography on Fractogel TSK HW 40 (S). According to the molar monosaccharide ratios, found by GC analyses, fractions III/1 to III/4 are tri-, di-, mono- and non-fucosylated lacto-N-hexaoses.

Fractions IV–VIII, which are the quantitatively predominant oligosaccharides in human milk, were studied in more detail; the corresponding structures are shown in Table I. The molar ratio of fucose, galactose, glucose and N-acetylglucosamine being 2:2:1:1, fraction IV was shown to consist of difucosyllacto-N-tetraoses. HPLC and FAB-MS analyses revealed that fraction IV from milk of a secretor Lewis (a − b+) donor predominantly consists of lacto-N-difucohexaose I; lacto-N-difucohexaose II was detected in trace amounts. Fraction V, a mixture of monofucosylated lacto-N-tetraoses, consists of major amounts of lacto-N-fucopentaose I and medium amounts of lacto-N-fucopentaoses II and III, according to GC, HPLC and FAB-MS analyses. Trace amounts of lacto-N-fucopentaose V were also detected. Lacto-N-tetraose is the main compound of fraction VI; lacto-N-neotetraose was present in trace amounts. Difucosyllactose was detected as the major substance in fraction VII; there were also some unidentified peaks in the HPLC profile of this fraction. Fraction VIII, representing mono-

TABLE I

STRUCTURES OF HUMAN MILK OLIGOSACCHARIDES

Compound	Structure
Fraction VIII: monofucosyllactoses	
3-Fucosyllactose	Gal-β-(1→4) Fuc-α-(1→3) > Glc
2'-Fucosyllactose	Fuc-α-(1→2)-Gal-β-(1→4)-Glc
Fraction VII: difucosyllactose	
Difucosyllactose	Fuc-α-(1→2)-Gal-β-(1→4) Fuc-α-(1→3) > Glc
Fraction VI: lacto-N-tetraoses	
Lacto-N-tetraose	Gal-β-(1→3)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Lacto-N-neotetraose	Gal-β-(1→4)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Fraction V: monofucosyllacto-N-tetraoses	
Lacto-N-fucopentaose I	Fuc-α-(1→2)-Gal-β-(1→3)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Lacto-N-fucopentaose II	Gal-β-(1→3) Fuc-α-(1→4) > GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc
Lacto-N-fucopentaose III	Gal-β-(1→4) Fuc-α-(1→3) > GlcNAc-β-(1→3)Gal-β-(1→4)-Glc
Lacto-N-fucopentaose V	Gal-β-(1→3)-GlcNAc-β-(1→3)-Gal-β-(1→4) Fuc-α-(1→3) > Glc
Fraction IV: difucosyllacto-N-tetraoses	
Lacto-N-difucohexaose 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	Gal-β-(1→3) Fuc-α-(1→4) > GlcNAc-β-(1→3)-Gal-β-(1→4) Fuc-α-(1→3) > Glc

fucosyllactoses, mainly consists of 2'-fucosyllactose, minor amounts of 3-fucosyllactose and traces of an unknown substance. The main fraction IX of the gel permeation chromatographic profile is lactose. No carbohydrates could be detected in fraction X, coeluting with monosaccharides.

The elution pattern on Fractogel TSK HW 40 (S) demonstrates a reasonable group separation of oligosaccharides according to size from trisaccharides (monofucosyllactoses) to nonasaccharides (trifucosyllacto-N-hexaoses). The oligosaccharide structures assigned to the peaks from the gel permeation chromatography profile were in accordance with the assignments made by Kobata *et al.* [1] and Bruntz *et al.* [2].

The purity of fractions III–VIII to be quantified was examined. Besides lactose and oligosaccharides, the following water-soluble substances occur in significant amounts in deproteinized skimmed milk: peptides, amino acids, mineral salts, citric acid, inositol and urea [19,20]. Citric acid elutes before the neutral oligosaccharides, and urea and inositol elute after lactose, so do not interfere with

TABLE II

RELATIVE MASS RESPONSE FACTORS OF HUMAN MILK OLIGOSACCHARIDE FRACTIONS

Data are based on five measurements with independently weighed oligosaccharide fractions and glucose.

Fraction	Mean response[a]	Confidence interval[b]
Monofucosyllactoses	0.97	0.95–0.99
Lacto-N-tetraoses	1.03	0.96–1.10
Monofucosyllacto-N-tetraoses	1.00	0.98–1.02
Difucosyllacto-N-tetraoses	0.97	0.94–0.99

[a] Relative to glucose.

[b] Estimation of the confidence interval calculated on a significance level of $p = 0.05$, based on the assumption that data are normally distributed.

oligosaccharide analysis. The sodium, potassium, calcium and amino acid contents of fractions III–VIII were less than 3%. The GC method for analysis of the monosaccharide residues was used to determine the oligosaccharides contents of fractions III–VIII. The oligosaccharide contents of these fractions were found to be between 88 and 100%.

Mass response factors

A prerequisite for quantifying milk oligosaccharide fractions by gel permeation chromatography with refractometric detection is the knowledge of their relative mass response factors. Exactly weighed amounts of fractions III–VIII and the standard glucose, dried over P_2O_5, were eluted on a 190 cm × 1.6 cm I.D. Fractogel TSK HW 40 (S) column. Relative mass response factors (Table II) were determined by a comparison of the peak areas from the oligosaccharides fractions with the peak area of glucose. The confidence intervals of the response factors of the lacto-N-tetraoses and the monofucosyllacto-N-tetraoses, calculated at a significance level of 0.05 are around factor 1; the mass response factors of the monofucosyllactoses and the difucosyllacto-N-tetraoses are close to 1. Therefore, the masses of these oligosaccharide fractions in human milk should be calculated with factor 1. Exact response factors of difucosyllactose are missing, because only minor amounts of this substance could be isolated. The various fucosylated and non-fucosylated lacto-N-hexaoses were integrated as a whole because of the relatively poor resolution. Reliable mass response factors for these substances were difficult to obtain because integration of the broad base of the peak cluster led to widely scattered peak areas.

The composition of the oligosaccharide fractions probably varies between different samples and is clearly different in milk samples from donors with different blood groups. As a consequence, the corresponding mass response factors may also vary. Monofucosyllacto-N-tetraoses and monofucosyllactoses from milk of

a non-secretor Lewis (a+ b−) individual were analysed in order to test this hypothesis. The monofucosyllacto-N-tetraose fraction from non-secretor Lewis (a+ b−) individuals lacks lacto-N-fucopentaose I, whereas the monofucosyllactose fraction lacks 2′-fucosyllactose. Again, the response factors were close to 1 (data not shown).

The results mentioned above demonstrate that response factors of oligosaccharides fractions from human milk vary around the factor of glucose, independent of their composition. It can be predicted that the response factors of difucosyllactose and of fucosylated and non-fucosylated lacto-N-hexaoses, which could not be determined experimentally, are probably also close to 1, because these structures are built up from the same monosaccharide residues as the oligosaccharides analysed.

In order to investigate the relationship between the concentration of the oligosaccharide fractions and the response of the refractive index detector, various amounts of monofucosyllactoses, lacto-N-tetraoses, mono- and difucosyllacto-N-tetraoses and glucose were eluted on a 190 cm × 1.6 cm I.D. TSK HW 40 (S) column and detected. For all these fractions and for glucose, the refractometric response was linear from 0.1 mg per 200-μl injection volume (50 mg per 100 ml) to 1 mg per 200-μl injection volume (500 mg per 100 ml). This concentration range corresponds to the usual concentrations of these oligosaccharide fractions in human milk.

Comparison of ethanol precipitation and ultrafiltration procedures for isolation of oligosaccharides

The gel permeation chromatographic method was applied to the analysis of oligosaccharides in human milk from a secretor Lewis (a− b+) individual. Since the unknown substance X coeluted with glucose, a relatively large amount of glucose (10 mg) was added to 2 ml of human milk (500 mg glucose per 100 ml milk). By this procedure the analytical error due to substance X could be reduced below 5%.

Three procedures for isolating oligosaccharides from human milk were investigated. Lipids and proteins were removed from human milk by centrifugation followed by ethanol precipitation (method EtOH), by centrifugation using an XM50 ultrafiltration membrane (method XM50) or by centrifugation using a YM10 ultrafiltration membrane (method YM10). The isolated oligosaccharides were analysed by gel permeation chromatography. Table III shows the concentrations of the main oligosaccharide fractions from human milk: calculations are based on the internal standard glucose. The various procedures yielded reproducible results; the relative standard deviations were less than 5%. Using method EtOH, *ca.* 5–15% higher values for oligosaccharide concentrations were found than by using methods XM50 or YM10. These differences are significant on a 0.05 significance level, except for the monofucosyllactoses when comparing method EtOH and method YM10. The higher oligosaccharide concentrations found

TABLE III

QUANTITATIVE ANALYSIS OF OLIGOSACCHARIDE FRACTIONS: COMPARISON OF ETHANOL PRECIPITATION AND ULTRAFILTRATION METHODS

	Monofucosyl-lactoses	Lacto-N-tetraoses	Monofucosyl-lacto-N-tetraoses	Difucosyl-lacto-N-tetraoses
Ethanol precipitation (EtOH)				
Mean[a] (mg per 100 ml)	257.6 (100%)	152.6 (100%)	190.4 (100%)	109.2 (100%)
R.S.D. (%)	2.4	1.8	2.2	3.4
Ultrafiltration XM 50				
Mean[a] (mg per 100 ml)	245.8 (95.4%)	140.6 (92.1%)	175.6 (92.2%)	98.6 (90.3%)
R.S.D. (%)	2.8	3.3	1.7	1.3
Ultrafiltration YM 10				
Mean[a] (mg per 100 ml)	243.2 (94.4%)	139.4 (91.4%)	171.0 (89.8%)	94.0 (86.0%)
R.S.D. (%)	4.6	3.5	3.6	3.5
Mann Whitney test significance level				
EtOH/XM 50	0.037	0.012	0.012	0.012
EtOH/YM 10	0.075	0.012	0.012	0.012
XM 50/YM 10	0.676	0.674	0.249	0.043

[a] The mean values, based on five independent analyses, are calculated on the internal standard glucose.

by method EtOH could be due to either losses of oligosaccharides when methods XM50 and YM10 are used, or precipitation of glucose when method EtOH is used. In order to examine whether glucose is partially precipitated when method EtOH is used, the concentration of the internal standard glucose was determined enzymically in the supernatant after ethanol precipitation. Since no glucose was lost during the precipitation procedure, the lower oligosaccharide concentrations found by methods XM50 and YM10 can be explained only by losses of oligosaccharides, especially the higher ones, during ultrafiltration. The concentrations of the monofucosyllactoses and lacto-N-tetraoses determined using methods XM50 and YM10 are rather similar (Table III). However, there is a tendency towards lower values of the larger mono- and difucosyllacto-N-tetraoses, when method YM10 is used. The difference is significant on a 0.05 significance level for the difucosyllacto-N-tetraoses.

The concentrations of difucosyllactose and of fucosylated and non-fucosylated lacto-N-hexaoses are not shown in Table III because difucosyllactose occurs in small amounts and does not yield reproducible results. The concentrations of the lacto-N-hexaoses also vary widely, because of the integration problems mentioned above. Nevertheless, this gel permeation chromatographic method also allows a rough estimation of the concentration of these two oligosaccharide fractions.

In summary, concentrations of oligosaccharide fractions determined by the

ultrafiltration methods are significantly lower (0.05 significance level) than the concentrations found by method EtOH. These differences can be explained only by losses of oligosaccharides, especially the larger ones, during ultrafiltration. As a consequence, method EtOH is recommended for the quantification of oligosaccharide fractions in milk. For analyses requiring a lower degree of precision, the faster and more convenient method XM50 could be an alternative. Since ultrafiltration membranes with a nominal exclusion limit of less than 10 000 daltons (method YM10) partically cut off the larger oligosaccharides, they are not recommended for the quantification of oligosaccharides.

The gel permeation chromatographic method described here allows the quantification of neutral oligosaccharide fractions from human milk according to size, and can be applied to the study of oligosaccharides in the course of lactation. Since the response factors of the oligosaccharides tested are rather similar, this method could probably be expanded to the analysis of similar oligosaccharides in other matrices, such as other mammalian milks or urine.

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