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Variations of neutral oligosaccharides and lactose in human milk during the feeding

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Variation von neutralen Oligosacchariden und von Lactose in Humanmilch während des Stillvorganges

Summary: There exist only few data concerning the variation of oligosaccharides in human milk. In this study the variations of neutral oligosaccharides and of lactose in human milk during the feeding were determined from five women at day 8 and at day 57 post partum. The milk of the investigated feedings was divided in four parts of equal volumes during sampling; the concentrations of neutral oligosaccharide fractions were determined by gel permeation chromatography on Fractogel TSK HW 40 (S) columns. No significant differences in the concentrations of the neutral oligosaccharide groups monofucosyllactoses, difucosyllactose, lacto-N-tetraoses, monofucosyllacto-N-tetraoses and difucosyllacto-N-tetraoses and of lactose were found in the four milk parts. The results of this study favor the use of so-called mid-feed samples, a simple and convenient sampling method for analytical studies of human milk. Mid-feed samples are representative of the whole feeding as concerned for neutral oligosaccharides.

Zusammenfassung: Über die Variation von Oligosacchariden in Humanmilch liegen nur wenig Daten vor. In dieser Studie werden die Änderungen von neutralen Oligosacchariden und von Lactose während des Stillvorgangs bei 5 Frauen am Tag 8 und am Tag 57 nach der Geburt bestimmt. Die Milchproben der untersuchten Stillvorgänge wurden zu jeweils 4 Teilen gleichen Volumens gesammelt. Die Konzentration der neutralen Oligosaccharide wurde durch Gelchromatographie mit Fractogel TSK HW 40 (S) Säulen bestimmt. In den jeweils 4 Milchteilen der Stillvorgänge waren bei den Konzentrationen der neutralen Oligosaccharidgruppen Monofucosyllactosen, Difucosyllactose, Lacto-N-tetraosen, Monofucosyllacto-N-tetraosen und Difucosyllacto-N-tetraosen und bei Lactose keine signifikanten Unterschiede festzustellen. Die Ergebnisse dieser Studie stützen die sogenannte „mid-feed" Sammeltechnik als eine einfache und praktische Probennahmemethode für analytische Milchstudien. Die „mid-feed" Proben sind bezüglich der Oligosaccharide repräsentativ für die gesamte Milch eines Stillvorganges.

Key words: Human milk – oligosaccharides – feeding – gel permeation chromatography – mid-feed sample

Schlüsselwörter: Humanmilch – Oligosaccharide – Stillvorgang – Gelpermeationschromatographie – „mid-feed" Proben

Introduction

Investigations concerning oligosaccharides in human milk resulted in the isolation and identification of about 100 different oligosaccharides (9, 4, 12). In respect to the large variety of oligosaccharides, human milk seems to be unique among all mammalian spe-

cies. The biological functions of the oligosaccharides remain to be elucidated. Antiviral and antibacterial effects (1, 2, 6) of these compounds are discussed as well as bifidogenic properties. In this context bifidogenic properties are the potential to promote growth of *Bifidobacterium bifidum* (3).

Quantitative data for oligosaccharide concentrations in human milk are rare. Until now, only Montreuil and Mullet (10) and Viverge et al. (14, 16) systematically studied the concentration of oligosaccharides in dependence on the lactation period, especially. The variations of oligosaccharides during the feeding have solely been investigated by Viverge et al. (15). Knowledge about variations of milk constituents during the feeding is not only of biochemical interest, but can also determine the choice of appropriate sampling techniques for further studies, e.g., lactational investigations. To obtain representative samples for milk studies, several authors recommend to collect the whole milk of all feedings from both breasts within a 24-h period (8). Though the scientific value of this technique is undoubted, it has disadvantages. When collecting the whole milk of one study day the infants cannot get their own mother's milk as usual. Breast-feeding is disturbed on the study day and even on the days thereafter.

A study aiming at investigating the variations of the concentrations of neutral oligosaccharides and lactose in human milk during the feeding is described. Gel permeation chromatography was used in this study for determination of various oligosaccharide fractions as it provides sufficient information about the individual carbohydrate fractions (13).

Materials and methods

Collection of samples

All mothers participating in our study were living in the region of Dresden, FRG. They were between 20 and 35 years old and gave birth to healthy infants who were exclusively breast-fed. Variations of oligosaccharides within the feeding for five women were analyzed twice: 8 days after birth as well as 57 days after birth. All the milk of the feeding in the morning was pumped off using an electric breast-pump (NUK BP 1000). During sampling the milk was divided into four parts of equal volume. From each of these four parts a 10 ml aliquot was immediately frozen and stored at –20 °C until analysis. The infants were fed with the remaining milk.

Analytical methods

The concentration of lactose was determined enzymatically with a commercial lactose/galactose test kit (Boehringer Mannheim, FRG). This assay is based on the enzymatic cleavage of lactose into glucose and galactose by β-galactosidase and the subsequent oxidation of galactose by β-galactose dehydrogenase. The formation of NADH (dihydronicotinamide adenine dinucleotide) is determined photometrically at 340 mm.

Determination of neutral oligosaccharides was performed as published (13) and is briefly described. Human milk samples were heated in screw-capped tubes for 30 min at 70 °C to inactivate possible contamination with hepatitis B and human immunodeficiency viruses (7). One ml of human milk and 100 µl of the internal standard (5 % (w/v) glucose solution) were mixed. The lipid and protein content of the mixture were reduced by centrifugation at 1500 g and 20 °C for 1.5 h, (Centrifree ultrafiltration system, W.R. Grace, Amicon Division, Witten, FRG) using 30000 YMT membranes. Subsequently,

200 µl of milk serum were injected into a Fractogel TSK HW 40 (S) column (190 × 1.6 cm I.D.), driven by a LKB pump P500 at a constant flow-rate of 0.3 ml/min with deionized water as eluent. Substances were detected by an LKB 2142 refractive index detector. The concentrations of the individual oligosaccharide fractions were calculated based on the amount of glucose as the internal standard. The relative mass response factors of the oligosaccharides and of glucose have been shown to be identical (13).

Ethical consideration

The study protocol has been approved by the Ethical Committee of the Carl Gustav Carus Medical Academy in Dresden, FRG. The study was performed according to the regulations of the Declaration of Helsinki (5).

Results

The quantitatively dominating neutral oligosaccharide fractions were separated and a typical chromatogram is shown in Fig. 1. The following neutral oligosaccharide fractions were quantified in this study: monofucosyllactoses (fraction VIII), difucosyllactose (fraction VII), lacto-N-tetraoses (fraction VI), monofucosyllacto-N-tetraoses (fraction V) and difucosyllacto-N-tetraoses (fraction IV). The concentration of oligosaccharide fraction III, comprising fucosylated and non-fucosylated lacto-N-hexaoses, could not be determined with the same accuracy, since the individual peaks were not sufficiently resolved. Due to the high concentrations of lactose in all samples, as shown in Fig. 1, lactose concentrations were determined by an enzymatic method instead of using

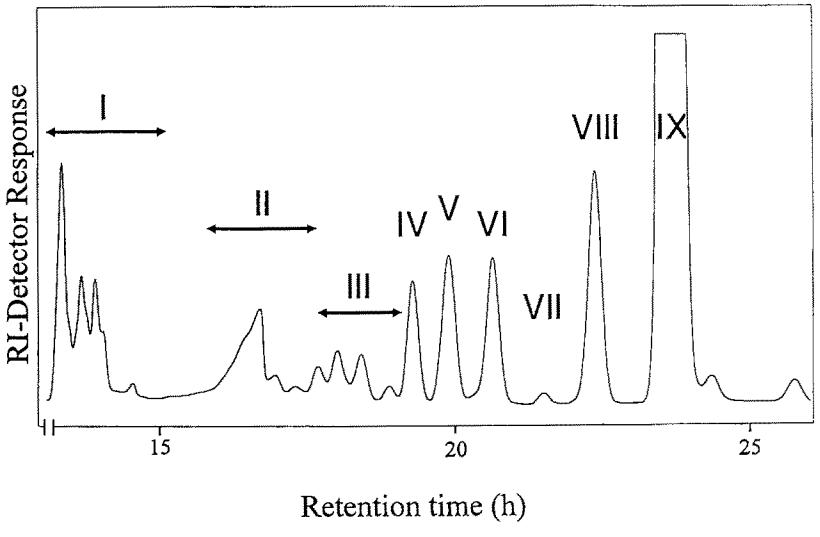

Fig. 1. Gel permeation chromatography profile of human milk oligosaccharides from a secretor Lewis (a- b+) individual. The milk sample was ultrafiltered through an Amicon 30000 YMT membrane and eluted on a 190 cm x 1.6 cm Fractogel TSK HW 40 (S) column at a flow-rate of 0.3 ml min^{-1}.
Fraction XI: lactose; fraction VIII: monofucosyllactoses; fraction VII: difucosyllactose; fraction VI: lacto-N-tetraoses; fraction V: monofucosyllacto-N-tetraoses; fraction IV: difucosyllacto-N-tetraoses; fraction III: fucosylated and non-fucosylated lacto-N-hexaoses; fraction II: higher oligosaccharides; fraction I: acidic oligosaccharides, peptides, amino acids, mineral salts.

Tab. 1. Structures of the major neutral oligosaccharides from human milk

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

The numbers of the carbohydrate fractions correspond to the numbers of the fractions from gel permeation chromatography (Fig. 1).

the chromatographic method. The application of high performance liquid chromatography, gas liquid chromatography, and mass spectroscopy led to the determination of the various isomeric oligosaccharides building up the major fractions of this gel permeation chromatography method as previously published (13). Table 1 shows the result of these investigations adapted to this study. In most cases the quantified oligosaccharide fractions of the gel permeation chromatography method consist of several isomeric carbohydrates.

Analysis of the oligosaccharide pattern from milk samples of the five women revealed that four women could be assigned to blood type secretor Lewis (a– b+). Milk samples from these individuals contain all oligosaccharide structures shown in Table 1. One individual obviously expressed blood type non-secretor Lewis (a+ b–). In this case, peak VII, corresponding to difucosyllactose was absent, whereas all other fractions occurred. Milk samples from non-secretor Lewis (a+ b–) individuals lack oligosaccharide struc-

Tab. 2. Mean values and ranges of the concentrations of neutral oligosaccharides and of lactose from the four milk sample parts at day 8

	Milk sample part			
	1.	2.	3.	4.
Monofucosyllactoses (VIII)	**347** (257-530)	**360** (265-549)	**336** (229-472)	**329** (218-428)
Difucosyllactose (VII)	**40** (9-71)	**42** (7-77)	**37** (13-60)	**30** (18-43)
Lacto-N-tetraoses (VI)	**118** (77-171)	**119** (79-165)	**118** (76-165)	**115** (73-161)
Monofucosyllacto-N-tetraoses (V)	**215** (184-260)	**224** (187-271)	**211** (168-259)	**211** (164-257)
Difucosyllacto-N-tetraoses (IV)	**115** (39-207)	**123** (38-224)	**113** (46-216)	**104** (64-174)
Lactose	**5986** (5620-6300)	**6022** (5530-6440)	**6122** (5850-6360)	**5166** (4100-6250)

All values correspond to mg/100 ml. Number of samples is 5, except for difucosyllactose that only occurs in the four secretor Lewis (a- b+) individuals. Values in brackets indicate the ranges of the carbohydrate concentrations.

tures with fucose α (1–2) linkages. As a consequence among the gel permeation fractions listed in Table 1 only fraction VII is missing in this case.

The concentrations of the oligosaccharide fractions monofucosyllactoses, difucosyllactose, lacto-N-tetraoses, monofucosyllacto-N-tetraoses, difucosyllacto-N-tetraoses and of lactose were determined separately for the four milk parts. In Tables 2 and 3 the mean values and the ranges of all concentrations of the oligosaccharide fractions at study days 8 and 57 are shown. The ranges of the oligosaccharide concentrations are rather wide and reflect the interindividual variations between milk samples of different women.

The concentrations of the oligosaccharide fractions corresponding to the four different milk parts were compared statistically by a non-parametric test procedure described by Wilcoxon and Wilcox for multiple comparisons of linked data sets (17). In all of these tests no significant differences of the concentrations in neutral oligosaccharides and of lactose in the four milk sample parts were found at day 8 and at day 57. This means that the null hypothesis "the oligosaccharide concentrations in the four parts are not different" has to be maintained.

Discussion

Although no significant differences in the concentrations of neutral oligosaccharides and of lactose during feeding could be detected, there existed a tendency towards higher values in the second parts and towards lower values in the fourth parts of the milk sam-

Tab. 3. Mean values and ranges of the concentrations of neutral oligosaccharides and of lactose from the four milk sample parts at day 57

	Milk sample part			
	1.	2.	3.	4.
Monofucosyllactoses (VIII)	**314** (275-376)	**303** (268-365)	**301** (281-341)	**308** (273-361)
Difucosyllactose (VII)	**28** (15-66)	**24** (15-45)	**25** (12-51)	**25** (12-46)
Lacto-N-tetraoses (VI)	**81** (39-131)	**72** (31-116)	**76** (32-120)	**785** (33-117)
Monofucosyllacto-N-tetraoses (V)	**150** (102-230)	**144** (100-229)	**141** (98-213)	**149** (96-231)
Difucosyllacto-N-tetraoses (IV)	**85** (41-155)	**85** (45-154)	**84** (40-153)	**83** (42-140)
Lactose	**6636** (6240-7510)	**6524** (5780-7240)	**6708** (6330-7140)	**6836** (6340-7210)

All values correspond to mg/100 ml. Number of samples is five, except for difucosyllactose that only occurs in the four secretor Lewis (a- b+) individuals. Values in brackets indicate the ranges of the carbohydrate concentrations.

ples at day 8 (Table 2). At day 57 the concentrations in the first part of the milk samples tended to higher values (Table 3).

Since our data did not exhibit significant variations of the oligosaccharide concentrations during the feeding, it should not matter whether one collects a small aliquot during the feeding or a sample out of the whole feeding. However, taking into account the tendency towards higher oligosaccharide concentration at the beginning and lower concentrations at the end of the feeding, we recommend to use the so-called mid-feed sampling procedure for analytical studies on oligosaccharides (11). Concerning neutral oligosaccharides mid-feed samples are representative of the whole feeding samples.

In Table 4 the concentrations of oligosaccharides are shown at study day 8 after transforming the concentrations into relative data. The arithmetic mean values of the second and the third milk sample parts of all women were chosen as reference values, arbitrarily set to a value of 100, in order to mimic a mid- feed sample. The ranges of the relative oligosaccharide concentrations are clearly smaller than the corresponding ranges of the absolute concentrations (Table 2). The mean values of the relative concentrations of the oligosaccharides of all milk parts are close to the value 100 which corresponds to a mid-feed sample. This demonstrates that a sample taken in the middle of the feeding is representative of the whole feeding. Besides, this technique is convenient for the mothers and their infants.

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Tab. 4. Mean values and ranges of the concentrations of neutral oligosaccharides and of lactose from the four milk sample parts at day 8 after transforming all analytical data into relative data

	Milk sample part			
	1.	2.	3.	4.
Monofucosyllactoses (VIII)	**99** (93 - 104)	**103** (96 - 108)	**97** (92 - 104)	**95** (83 - 127)
Difucosyllactose (VII)	**100** (90 - 108)	**98** (70 - 113)	**102** (87 - 130)	**146** (47 - 430)
Lacto-N-tetraoses (VI)	**99** (94 - 104)	**101** (99 - 103)	**99** (97 - 101)	**97** (91 - 104)
Monofucosyllacto-N-tetraoses (V)	**99** (95 - 104)	**103** (96 - 109)	**97** (91 - 104)	**98** (87 - 120)
Difucosyllacto-N-tetraoses (IV)	**99** (93 - 105)	**103** (90 - 111)	**97** (89 - 110)	**101** (72 - 176)
Lactose	**99** (93 - 103)	**99** (95 - 103)	**101** (97 - 105)	**85** (66 - 107)

Reference values are the mean values of the carbohydrate concentrations of milk sample parts 2 and 3 from every women. Values in brackets indicate the ranges of the relative carbohydrate concentrations.

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