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A multinational study of α-lactalbumin concentrations in human milk

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Abstract

α-Lactalbumin, a 14-kD protein, plays a central biochemical role in the mammary gland as the regulatory subunit of lactose synthase, and also plays a nutritional role for the rapidly growing neonate as the protein in highest concentration in human milk. The current study was undertaken to better characterize α-lactalbumin concentrations in human milk from a variety of countries. Mature human milk (lactation duration ≥1 month) was collected from at least 50 women from nine different countries on five continents. α-Lactalbumin concentration was determined by HPLC. The mean ± SD for 452 samples was 2.44 ± 0.64 g/L. The mean value of the samples from the United States was significantly higher than that from any other country, and the mean in Mexico was significantly lower than that from every country except China and Canada. α-Lactalbumin concentration decreased with increasing duration of lactation and was positively correlated with total nitrogen. On average, α-lactalbumin contributed 16% of the total nitrogen content of human milk and consequently an important part of the amino acid content.

Keywords: Lactalbumin; HPLC; Multi-country variation; Mature human milk

1. Introduction

Concentrations of proteins and the protein profile of human milk change dramatically during early lactation. The concentration of total protein falls continuously during the first month of lactation, with smaller changes occurring in subsequent months [1]. Alterations in protein profiles also occur over the first 2 months of lactation. SIgA levels decrease to one fourth of those in early milk, whereas the concentrations of other proteins decrease less precipitously [2]. The whey-to-casein ratio decreases from as high as 80:20 to approximately 60:40 [3]. Since various proteins have different roles for the mother/infant dyad, this observation is not surprising. For example, immunoglobulins provide protection from infection, whereas lactoferrin may play a role in iron transport or act as a bacteriostatic/bactericidal agent. α-Lactalbumin is important in several ways, including the synthesis of lactose, the driving osmotic force in the movement of water from the mother's circulation to her milk, and a rich source of essential amino acids for the neonate [4].

It is generally agreed that the nutrients provided by breast milk usually meet the requirements of infants with low likelihood of deficiency or excess [5]. The precise protein requirement of breast-fed infants, however, has remained a controversial issue. Although net protein intake of breast-fed infants is relatively high during the first month of life, after that period, it is lower than most recommendations for infants. However, there is no evidence that the protein intake of breast-fed infants is inadequate and it is more likely that the protein requirement has been over-estimated [6,7]. These points are important when considering improvements to the protein components of infant formulas.

The most abundant protein in mature human milk is α-lactalbumin, which constitutes 10–20% of total protein [8]. Published values for the concentration of this protein are highly variable, most likely due to genetic, environmental, and dietary factors. Additional reasons for the variation in concentrations include the fact that the number of samples analyzed in previously published reports have often been limited and various analytical methods have been used, ranging from semi-quantitative methods (e.g., electro-

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phoresis) to more precise immunological methods [9,10]. It is consequently difficult to obtain a "normative" value for α-lactalbumin in mature human milk from the literature that can be used as a target for the manufacture of an improved infant formula [11,12]. We have therefore collected breast milk samples from a large number of subjects in nine different countries from various parts of the world and analyzed α-lactalbumin by state-of-the-art methodology.

2. Methods and materials

2.1. Subjects

The research team leaders at the nine study sites were pediatricians. A staff nurse or dietitian who served as a study coordinator assisted each pediatrician. All mothers in the study had to meet the following inclusion criteria: healthy, well-nourished, 18–40 years of age, delivered a term infant, 1–12 months postpartum, parity less than five, nursing a single healthy infant, no rigorous dietary restrictions, diet consisting of at least three servings daily of fruits and vegetables, and postpregnancy weight no more than 4.5 kg below their prepregnancy weight. All of the women's infants were current patients of the respective clinics and their health status was determined from clinic records. The health status of the mothers was determined by an extensive questionnaire and interviews. Exclusion criteria included use of steroids and alcohol intake greater than one serving per day. The pediatric clinics (Adelaide, South Australia, Australia; Edmonton, Alberta, Canada; Cardiff, Wales, United Kingdom; Santiago, Chile; Tucson, Arizona, USA; Mexico City, Mexico; Chengdu, Sichuan, P.R. China; Manila, Philippines; and Tokyo, Japan) where the mothers were recruited served a middle socioeconomic population except in Manila, Philippines, and Chengdu, China. The clinics at both of these sites served a lower socioeconomic population. Socioeconomic status was determined by financial status as well as living conditions of the subjects. Full expressions of breast milk were collected between 1 PM and 5 PM on the day of sampling. The minimum acceptable milk volume was 50 mL. The milk was collected and stored according to a detailed clinical protocol [13]. Informed consent was obtained from all subjects.

2.2. Samples

All samples were collected in polypropylene bottles at designated locations and stored at −70°C until shipment to the analytical laboratory. Before analysis, the samples were thawed overnight in a refrigerator. The next morning, they were placed in a water bath and brought to 40°C while being stirred to disperse the lipids. A 4-mL aliquot was placed in a 10-mL volumetric flask, diluted to volume with high-performance liquid chromatography (HPLC)–grade water, and mixed thoroughly. The solution was subsequently poured into a 30-mL centrifuge tube and centrifuged for 30 minutes at 5°C and 15,000 × *g*. The lipid layer was removed by siphoning with a pipette, and a portion of the aqueous phase was transferred to an HPLC auto-sampler vial.

2.3. Analysis

HPLC analysis was performed on a Hewlett-Packard 1050 system (Agilent Technologies, Wilmington, DE) equipped with a Phenomenex Jupiter C4 column (Phenomenex, Torrance, CA). The column temperature was 30°C and the mobile phase gradient parameters were 60A/40B to 45A/55B (where A was 99.9% H_2O/0.1%TFA and B was 99.9% CH_3CN/0.1%TFA) in 30 minutes. Detection was monitored using DAD (diode array) at 210 nm and 280 nm. The flow rate was 0.8 mL/min and the injection volume was 20 μL. Samples were not filtered prior to analysis because previous data suggested that α-lactalbumin could adhere to the filter.

Total nitrogen in the samples and reference α-lactalbumin was determined by complete combustion of the sample using the LECO FP-528L (LECO Corporation, St. Joseph, MI). The FP-528L Determinator is a microprocessor based, software-controlled instrument capable of determining nitrogen content in a variety of substances. It follows a three-step process: 1) purging of any atmospheric gases that might have entered the sample during loading; 2) addition of pure oxygen with combustion at 950°C; and 3) passage through scrubbers to remove oxygen, water, and carbon dioxide. A thermoelectric cell measures the remaining combustion product, nitrogen, in a helium carrier. The result represents total nitrogen (protein nitrogen as well as nonprotein nitrogen).

2.4. HPLC method validation

The human α-lactalbumin reference standard was isolated from pooled human milk. The purity was 95.8% and the molecular weight 141,780. Amino acid analysis and nitrogen analysis were used to determine purity. Measurement of the molecular weight of α-lactalbumin as accomplished using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (G2025A MALDI-TOF-MS, Agilent Technologies, Wilmington, DE).

The HPLC method validation included the following: reproducibility of injection (relative standard deviation [SD] 0.27%), reproducibility of analyses (relative 2.2%), analyte recovery at one half and twice the innate concentrations (107% and 102% of theoretical recovery, respectively), and linearity. The relative SD is the percentage of variation between the SD and the mean.

2.5. Data analysis

All chromatographic data were generated from a calibration curve by the HPLC data station. The means of

Table 1
Demographic data for subjects and infants

Country	Mean Age (y)*	Parity 1†	Parity 2†	Parity 3†	Parity 4†	Mean Birth Weight (kg)
Australia	29 ± 4.78	23	14	12	2	3.59 ± 0.51
Canada	32 ± 3.49	10	25	11	3	3.60 ± 0.41
Chile	26 ± 6.62	22	16	6	4	3.30 ± 0.56
China	27 ± 2.82	47	1	0	0	3.32 ± 0.39
Japan	32 ± 2.86	33	12	2	0	3.17 ± 0.33
Mexico	31 ± 4.61	24	16	8	0	3.18 ± 0.34
Philippines	27 ± 5.23	29	21	11	7	3.33 ± 0.50
UK	32 ± 4.56	23	20	6	1	3.38 ± 0.49
USA	31 ± 4.92	26	5	6	1	3.35 ± 1.04
Overall mean	30 ± 5.09	22	14	12	2	3.35 ± 1.04

* Mean ± SD, (N = 444).
† N = 457, (total number of samples analyzed).
‡ N = 453.
Some data points were missing in age and baby weight categories.

α-lactalbumin data by country were analyzed using analysis of covariance, with the period of lactation (lactation days) used as the covariant. Each country's mean was a least-square mean. Analysis of variance was used for the overall mean of the samples because the stages of lactation within each country were different. The amount of nitrogen attributed to α-lactalbumin was calculated from total nitrogen and the percentage of nitrogen in the reference.

3. Results

Table 1 provides information about the mothers who contributed milk to this study, along with information about their infants. Mothers' mean ages varied from 26 years in Chile to 32 years in Canada, Japan, and the UK The mean age of all mothers was 30 years. Most of the infants were first– or second–born children. Of the 48 infants in the China group, only one was not primiparous. Canada was the only country in which there were more second-born children than first. Mean birth weight of all infants was >3.0 kg.

Table 2
Duration of lactation and mean number of lactation days

Country	Range (days)	Duration (days)*
Australia	39–243	120 ± 48.6
Canada	45–277	125 ± 53.0
Chile	30–301	83 ± 74.0
China	30–154	75 ± 35.8
Japan	30–212	99 ± 51.3
Mexico	34–276	125 ± 63.9
Philippines	30–314	90 ± 64.4
UK	33–115	66 ± 18.2
USA	31–330	120 ± 81.6
Overall mean†	30–314	100 ± 61.2

* Values are mean ± SD.
† N = 452.

Table 2 provides information on the duration of lactation. A substantial variation in mean days of lactation was noted with the shortest mean duration being 66 days (UK) and the longest being 125 days (Canada and Mexico). Within each country, a wide variation of lactational days was also apparent, which permitted an assessment of α-lactalbumin variation by duration of lactation.

The mean (±SD) α-lactalbumin concentration for all subjects was 2.44 ± 0.64 g/L. The α-lactalbumin means of the nine countries are illustrated in Fig. 1. The mean for the USA (3.23 ± 1.00 g/L) was significantly greater when compared to all other countries. Mexico had the lowest mean at 2.05 ± 0.51 g/L, which was significantly lower than those in the USA, Australia, Japan, Chile, Philippines, and the UK.

Total nitrogen means ranged from 2.10 g/L in Mexico to 2.79 g/L in Chile (Table 3). There was a positive relationship between total nitrogen and the concentration of α-lactalbumin in all countries, and all were significantly correlated ($P < 0.05$), except for Mexico and Canada, as illustrated in Table 3.

The concentration of α-lactalbumin and duration of lactation were negatively correlated. The extent of the decrease

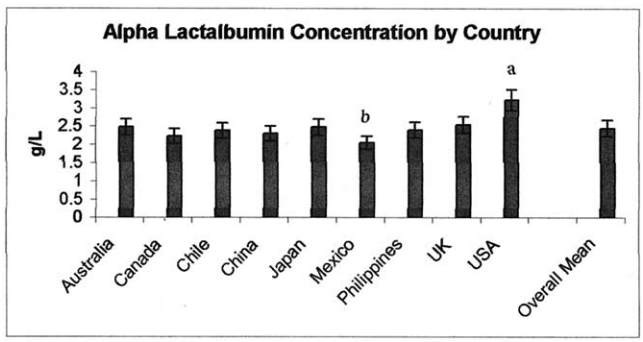

Fig. 1. α-Lactalbumin concentrations by country (mean ± SD). [a]United States was significantly higher than all other countries; [b]Mexico was significantly lower than all countries, except for Canada and China; [a,b]$P < 0.05$.

Table 3
Total nitrogen and human α-lactalbumin

Country	Mean Total, *N* (g/L)	α-Lac, *N* (g/L)	α-Lac N as % of N	Correlation α-Lac to N (*r*)
Australia	2.37	0.38	16.5	0.366*
Canada	2.41	0.34	14.2	0.114
Chile	2.79	0.38	14.0	0.445*
China	2.65	0.38	14.2	0.487*
Japan	2.56	0.39	15.4	0.311*
Mexico	2.10	0.31	16.0	0.172
Philippines	2.31	0.39	16.9	0.488*
UK	2.56	0.41	15.9	0.460*
USA	2.40	0.50	20.8	0.496*
Overall mean	2.46	0.39	16.0	0.315*

Total number of subjects was 452.
* $P < 0.05$.
Lac = lactalbumin; N = nitrogen; r = correlation coefficient.

during the duration of lactation was not the same for all countries. The slope of change for Japan, China, Mexico, and the USA was similar, whereas those for the UK, Philippines, Australia, Chile, and Canada were similar. These relationships are illustrated in Table 4.

4. Discussion

The α-lactalbumin concentrations reported in this work are similar to literature values [8–12]. HPLC has been shown to be a very accurate and reproducible technique for analyzing α-lactalbumin in human milk [14] and is applicable to automation. Since we planned to analyze a large number of samples in a relatively short period of time, HPLC represented the ideal technique. Other techniques such as immunological methods are time consuming and more cumbersome than HPLC.

The protein composition of human milk is different from that of bovine milk [14,15]. One major difference is the level of protein, with mature human milk containing ~9–11 g/L in contrast to bovine milk, which contains ~33 g/L [16–18]. In addition, bovine milk contains some proteins not found in human milk. For example, bovine milk contains β-lactoglobulin and α_{s2}-casein, whereas the human mammary gland does not express these proteins and human milk is devoid of them [19]. Although some of the protein components of human and bovine milk are similar, the concentrations are often different. For example, the concentration of lactoferrin in human milk is 1–2 g/L, whereas that in cow's milk is only ~0.01 g/L [15,16,20]. Since α-lactalbumin is a vital regulatory component of the lactose synthase complex, it will by necessity be found in both human and bovine milk. However, human milk α-lactalbumin is not identical to bovine milk α-lactalbumin, although there is a 72% sequence homology. The complete amino acid sequences for bovine and human α-lactalbumin have been published [21,22].

Table 4
Relationship of data points over duration of lactation

Country	Slope*	Correlation (r)†
Australia	−0.0039	−0.3815
Canada	−0.0017	−0.2155
Chile	−0.0010	−0.2949
China	−0.0052	−0.3595
Japan	−0.0050	−0.4890
Mexico	−0.0040	−0.5267
Philippines	−0.0018	−0.2502
UK	−0.0017	−0.0687
USA	−0.0055	−0.4471

Correlation of β-lactalbumin over duration of lactation.
* Mean slope of actual data points.
† $P < 0.05$ for all countries except UK.

A study from Spain by Sanchez-Pozo et al. [10] demonstrated a lower concentration of α-lactalbumin in subjects with lower socioeconomic status, although Lönnerdal et al. [8] found no difference in milk α-lactalbumin concentration between Ethiopian women of high or low socioeconomic status. Our data did not suggest a correlation between socioeconomic status and α-lactalbumin concentration. Subjects in the current study from the Philippines and China were classified as lower socioeconomic status, whereas women from the other countries were from intermediate socioeconomic status. The mean concentration of α-lactalbumin in milk from the Philippine subjects was 2.40 g/L and in China it was 2.38 g/L, which is very similar to the overall mean of 2.44 g/L for all subjects. A substantial variability was observed within the North American countries. Mexico and Canada had the lowest means, whereas the mean for the USA was significantly greater than for all other countries. In a previous study [8], mature milk from Ethiopian women was found to have an α-lactalbumin concentration of 2.7 g/L, which was similar to that in milk from Swedish women. The reasons for the regional differences in α-lactalbumin concentration are not known.

All milk samples came from mothers who had delivered term infants. The duration of lactation range was 30–330 days. There was a significant interaction effect between stage of lactation and α-lactalbumin concentration. α-Lactalbumin decreased throughout lactation, but the extent of the decrease varied among countries. In a group of eight subjects delivering term infants, Velona et al. reported that α-lactalbumin concentrations decreased from 3.56 g/L at the start of lactation to 2.83 g/L at day 115 [23]. Lönnerdal et al. [8] found an α-lactalbumin concentration of 3.62 g/L in early lactation, and 2.68 g/L at months 3.5–6.5 of lactation. These α-lactalbumin concentrations were somewhat higher than our overall mean, but that could have been related to the population itself or to analytical differences.

α-Lactalbumin and nitrogen both decreased as lactation progressed. There was a significant relationship between total nitrogen and α-lactalbumin concentrations. This correlation was not surprising, as the data showed that 16% of

the total milk nitrogen was contributed by α-lactalbumin. In fact, the proportion of α-lactalbumin to true protein is much higher than this because of the high nonprotein nitrogen found in human milk.

α-Lactalbumin is a rich source of essential amino acids and is therefore of central nutritional importance to the rapidly growing neonate [4]. The high concentration of α-lactalbumin in human milk compared to bovine milk contributes substantially to the relatively high concentrations of amino acids such as tryptophan and cysteine found in human milk [4]. Both of these amino acids play important roles in addition to being required for appropriate protein synthesis. For example, tryptophan is the precursor to the neurotransmitter serotonin, whereas cysteine is a component of glutathione, critical as a component of antioxidant systems [24,25]. Thus, α-lactalbumin plays an important nutritional role for the neonate.

In conclusion, α-lactalbumin concentrations have been determined in >450 samples from nine countries. The mean concentration of samples collected in the USA was significantly higher than all other countries, whereas it was significantly lower in Mexico than in all countries except Canada and China. α-lactalbumin decreased with increasing duration of lactation. This information will be useful when considering future improvements to infant formula.

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