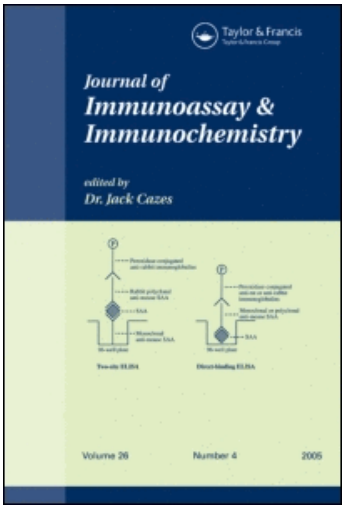

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Microparticle-Enhanced Nephelometric Immunoassay of Alpha-Lactalbumin in Human Milk

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MICROPARTICLE-ENHANCED NEPHELOMETRIC IMMUNOASSAY OF ALPHA-LACTALBUMIN IN HUMAN MILK

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ABSTRACT

A microparticle-enhanced nephelometric immunoassay was developed for alpha-lactalbumin quantitation in human milk. It is based on the nephelometric measurement of the light scattered during the competitive immunoagglutination of a microparticle–alpha-lactalbumin conjugate with an anti-alpha-lactalbumin antiserum. This immunoassay is sensitive (detection limit in reaction mixture, 1.5 µg/L) and could be performed in high dilution of milk, excluding any interference or sample pretreatment. It allowed the quantification of alpha-lactalbumin on a large range of concentrations (0.5-16.9 g/L) with accuracy (linear recovery in dilution-overloading assay) and precision (within- and between-run coefficients of variation from 1 to 7%). Changes in the alpha-lactalbumin concentration of human milk during lactation were determined in 162 samples. The concentration and ratio of alpha-lactalbumin total protein were found to be significantly lower in colostrum (4.9 g/l, 27%) than in transitional milk (5.2 g/L, 40%), then decreased in mature milk (3.4 g/L, 31%).

(KEY WORDS: Alpha-Lactalbumin, Human Milk, Lactation, Immunoassay, Immunonephelometry, Microparticle)

INTRODUCTION

Alpha-lactalbumin (α-LA) is a major protein of human milk. It is a calcium-binding protein (1) of 14 kDa molecular mass, constituted by a single polypeptidic chain of 123 aminoacids, neither glycosylated nor phosphorylated (2). In the mammary gland, α-LA has an activity of lactose synthetase (EC 2.4.1.22), modifying the catalytic site of galactosyltransferase (3). Human α-LA has a high nutritional value with an aminoacid composition adapted to the requirement of newborns (4). In spite of this protein quality and the interest of having a thorough knowledge of α-LA variations in human lactation, very few specific methods of quantitation of α-LA in human milk have been reported (5-8).

Microparticle-enhanced nephelometric immunoassays have been previously described as sensitive and accurate techniques for the determination of various human serum proteins (9). Used for assaying the main components of bovine milk (10-14), in particular α-LA (15), it appeared as an appropriate method for the evaluation of the quality of bovine milk from production up to processing in the cheese industry (16). This immunoassay is based on the nephelometric quantification of the inhibition of microparticle–antigen conjugate immunoagglutination by the antigen to be assayed (17). In the present work, we report the development of a microparticle-enhanced nephelometric immunoassay of α-LA in human milk.

MATERIALS AND METHODS

Reagents

Purified α-LA from human milk (90% in PAGE) was obtained from Sigma Chemical Co. (St Louis, MO, USA, L 7269). Specific (one

precipitation arc with human milk and no reaction against human plasma in immunoelectrophoresis) rabbit anti-α-LA antiserum (anti-α-LA As) was a product of Dakopatts (Glostrup, Denmark, A 579). Polyfunctional hydrophylic microspheres (MS) of 300 ± 12 nm diameter, synthesized as previously reported (18), and the buffer for nephelometry used for all immunonephelometric assays (0.05 M borate buffer, pH 8 containing 0.1 M NaCl, 1.5 mM Na_2-EDTA, 30 mM NaN_3, 2 g/L Triton X-100 and 30 g/L PEG 6000) were supplied by Sanofi-Diagnostics-Pasteur (Marnes, France).

Human milk samples used for precision, recovery and investigation of the changes in milk α-LA concentration during lactation, were collected from 8 voluntary mothers at the maternity hospital of Nancy (France) or at home. These 162 samples were colostrum (15 samples), obtained from 2 to 5 days post partum, transitional milk (80 samples), from 6 to 14 days post partum and mature milk (67 samples), from 15 to 86 days post partum. Milk samples were frozen, immediately after collection, and stored at -20°C until use. They were thawed at 40°C in a water bath and vigorously homogeneized, immediately before their analysis. Total protein in these human milks was determined by Bradford's method (19).

Preparation of Microparticle–Alpha-Lactalbumin Conjugate

Polyfunctional hydrophylic MS were covalently coated with α-LA through the formation of imine bonds between the aldehyde groups on MS and the primary amino groups of α-LA. Human α-LA (8.4 g) and MS (10 g) were mixed in 1 L of 0.05 M borate buffer, pH 8 containing 0.3 M NaCl. After 2 h at room temperature then 18 h at 4°C, 2-aminoethanol (0.12 M in 0.1 M borate buffer pH 8) was added to block unreacted aldehyde groups on MS, and the mixture further incubated for 2 h at room temperature.

MS–α-LA conjugate, unbound α-LA and 2-aminoethanol excess were separated by centrifugation (10000g, 4°C, 1 h) on a discontinuous sucrose gradient (200-800 g/L in 0.05 M borate buffer, pH 8 containing 0.3 M NaCl). MS–α-LA conjugate was recovered at the interface of the sucrose solutions and stored at 4°C, at 0.4 g/L in the binding buffer supplemented by 2 g/L NaN_3.

Assay of Milk Alpha-Lactalbumin

Microparticle-enhanced nephelometric immunoassay of milk α-LA was performed in a one-step reaction : 30 µl of unknown or control milk (15 000-fold diluted) or serial dilutions (from 1/800 to 1/25 600) of the solution of purified human α-LA used as standard (0.9 g/L), MS–α-LA conjugate (30 µl, 0.4 g/L), anti-α-LA As (30 µl, 650-fold diluted) and nephelometry buffer (210 µl) were mixed together in a reaction microcuvette (Nephelia® microcuvette, Sanofi-Diagnostics-Pasteur). All predilutions were performed in the nephelometry buffer, with a Hamilton (Bonaduz, Swizerland) dilutor. The scattered light was measured with the Sanofi-Diagnostics-Pasteur nephelometer Nephelia® N600 (20) after incubation for 1 h at room temperature.

Reproducibility of the calibration curves was estimated by measuring light scattering for each dilution of the α-LA standard in 10 successive assays. The precision of the immunoassay was assessed by measuring α-LA in human milks with low, intermediate and high concentration, 30 times during the same assay (within-run precision) and in 10 successive assays (between-run precision). Analytical recovery was tested in a dilution-overloading experimentation : the dilution assay was performed on 5 serial dilutions (1/50000-1/800000) of a milk sample

containing 3.4 g/L of α-LA ; the overloading assay was performed in 2 milks (3.4 and 3.1 g/L of α-LA), diluted (1/800000) then overloaded with by 8 increasing amounts of purified α-LA (from 1.5 to 13.5 g/L). The slopes calculated by linear regression analysis for the dilution and the overloading assays were compared by using Student's *t* test. For the total recovery, including dilution and overloading assays, the null hypothesis H_0 (intercept = 0 and slope = 1) *v.* the alternative hypothesis H_1 (intercept $\neq$ 0 and slope $\neq$ 1) were tested by *F* (Fisher) and *t* tests respectively.

RESULTS

Microparticle–Alpha-Lactalbumin Conjugate

Preliminary tests, performed with various concentrations of α-LA in the mixture of MS coating, had shown that the most immunoreactive MS–α-LA conjugate was obtained with the conditions given in Materials and Methods (4 10^{-5} moles of α-LA for 10g of MS in 1L of binding mixture). The MS–α-LA conjugate did not autoagglutinate and remained immunoreactive for several months when stored at 4°C. It was agglutinated by serial dilutions (1/100-1/51200) of anti-α-LA As, and the light scattered by conjugate clusters formed during this immunagglutination could be quantified by nephelometry.

Agglutination of MS–α-LA conjugate (40 mg/L) with anti-α-LA As (6500-fold diluted) was progressively inhibited by graded concentrations of free α-LA (1-225 µg/L in the reaction mixture). Inhibition at 50% was observed with 20 µg/L α-LA and a minimal concentration of 1.5 µg/L was detectable in the reaction mixture as giving an intensity of light scattered 3 SD lower than the mean value obtained in the absence of α-LA (0% inhibition).

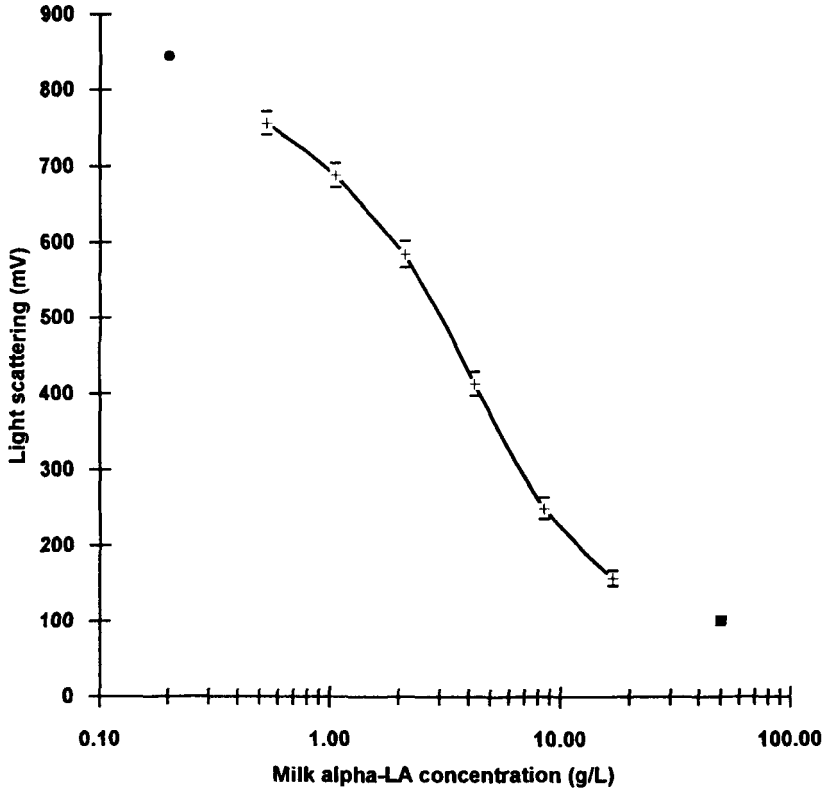

FIGURE 1. Calibration curve of the microparticle-enhanced nephelometric immunoassay of α-LA in human milk. Results are plotted as mean ± SD obtained in ten successive assays; ●, light scattering in the absence of α-LA; ■, light scattering by MS-α-LA conjugate alone. Assay procedure is given in the text.

Assay of Milk Alpha-Lactalbumin

The inhibition of MS–α-LA conjugate immunoagglutination by free α-LA from 3.5 to 112.5 µg/L in reaction mixture was retained for establishing the calibration curve of the milk α-LA assay. Calibration range from 0.5-16.9 g/L of α-LA in whole milk (Figure 1) was thus obtained when assay was performed with milk sample 150 000-fold

TABLE 1

PRECISION of MILK Alpha-LA DETERMINATION

	Within-run precision (n = 30)			Between-run precision (n = 10)		
Min (g/L)	9.27	3.37	1.21	11.59	3.91	1.20
Max (g/L)	10.16	3.53	1.59	12.35	4.28	1.46
Mean (g/L)	9.74	3.45	1.52	12.08	4.09	1.36
SD (g/L)	0.20	0.04	0.07	0.26	0.12	0.09
CV (%)	2.0	1.0	4.6	2.2	2.8	6.7

n, number of determinations; SD, standard deviation; CV, coefficient of variation

diluted in reaction mixture. Reproducibility CVs of this calibration curve ranged from 2.0% for the lowest α-LA concentration to a maximum of 6.3%.

The precision of the immunoassay was assessed by the CVs obtained in within- and between-run studies (from 1.0% to 6.7%) and shown in Table 1. Analytical recovery (Figure 2) was linear for α-LA concentrations in human milk ranging from 0.6 to 14.1 g/L (n = 13, mean percentage of recovery = 98.8%, r = 0.997, $P<0.001$). The slopes of the dilution (0.97) and overloading (0.99) curves were not significantly different ($P>0.05$). The slope (0.99) and the intercept (0.003 g/L) of the total recovery curve, including dilution and overloading assays, were not significantly different ($P>0.05$) from 1 and 0 respectively.

Application of the microparticle-enhanced nephelometric immunoassay of α-LA to 162 human milk samples gave the following global results : minimum = 2.2 g/L, maximum = 8.3 g/L, mean = 4.5 g/L, SD = 1.3 g/L. Changes in α-LA concentration of milk and α-LA ratio in milk total protein were observed according to the stage of lactation (Figure 3).

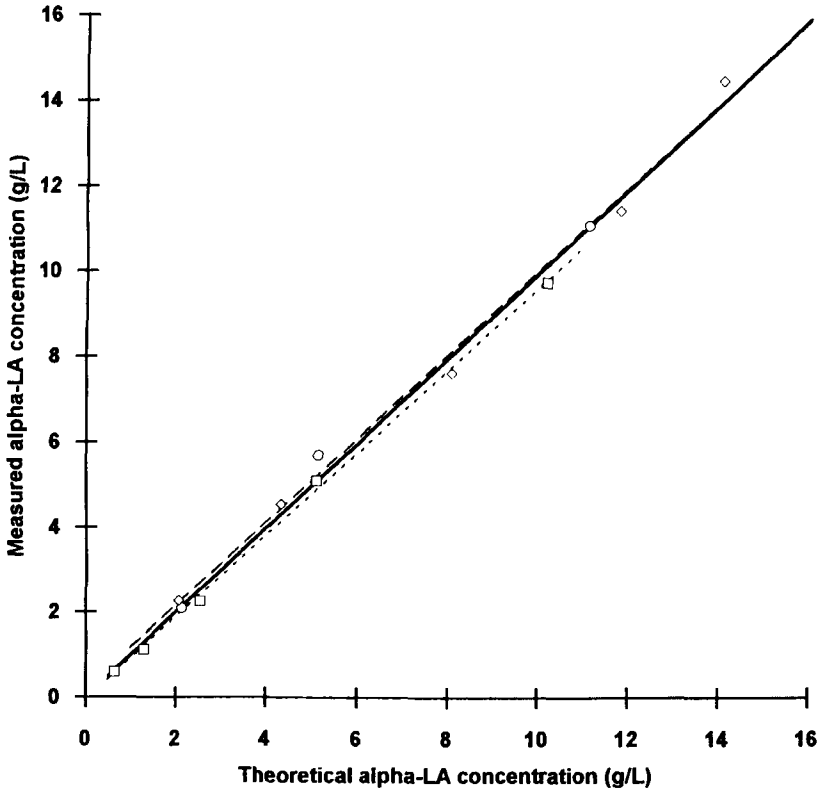

FIGURE 2. Analytical recovery of milk α-LA. □ and ·····, dilution assay; ◇, ○ and – – –, overloading assay; ——, linear regression for total recovery. Experimental details and linear regression parameters are given in the text.

Concentration was significantly higher ($P<0.01$) in transitional milk (n = 80, *minimum* = 3.5 g/L, *maximum* = 8.3 g/L, *mean* = 5.2 g/L, SD = 0.4 g/L) than in colostrum (n = 15, minimum = 4.1 g/L, maximum = 6.8 g/L, mean = 4.9 g/L, SD = 0.2 g/L), then decreased ($P<0.001$) in mature milk (n = 67, minimum = 2.2 g/L, maximum = 6.2 g/L, mean = 3.4 g/L, SD = 0.6 g/L). The percentage of α-LA in total protein varied similarly : it was signicantly

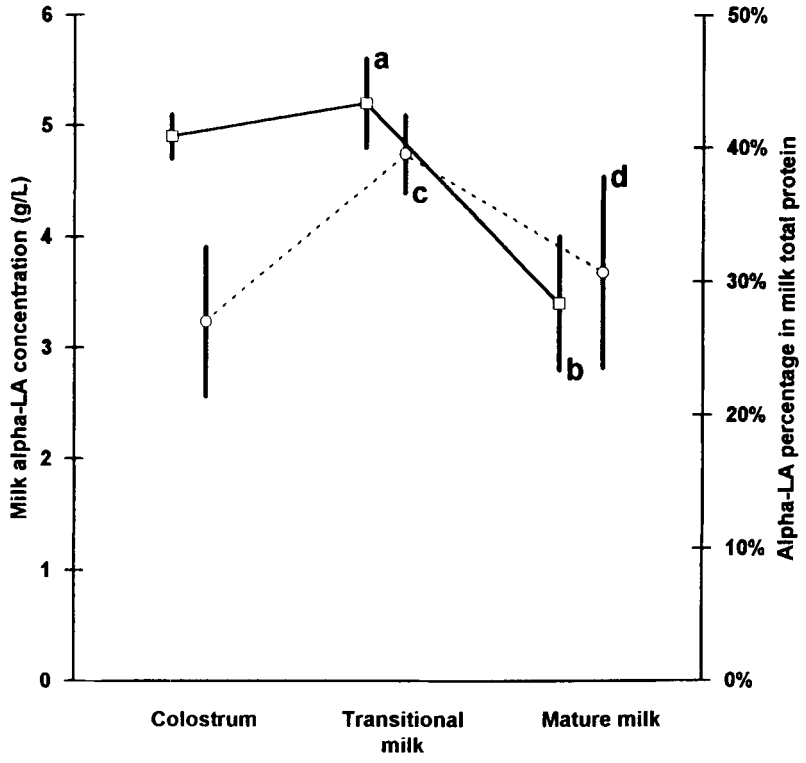

FIGURE 3. Changes in the α-LA concentration of human milk during lactation. Results are plotted as mean ± SD; □ and ——, α-LA concentration; ○ and – – –, α-LA percentage in total protein. Concentration significantly different (P<0.01) compared with colostrum (a) and (P<0.001) compared with colostrum and transitional milk (b); percentage significantly different (P<0.001) compared with colostrum (c) and (P<0.001) compared with transitional milk (d).

higher (P<0.001) in transitional milk (mean = 39.6%, SD = 2.9%) than in colostrum (mean = 27.0%, SD = 5.6%) and signicantly lower (P<0.001) in mature milk (mean = 30.7%, SD = 7.2%) than in transitional milk. This ratio was not significantly different (P>0.05) in colostrum and mature milk.

DISCUSSION

Nephelometric measurement of the inhibition of MS–α-LA conjugate immunoagglutination by free α-LA allowed the development of a microparticle-enhanced nephelometric immunoassay for α-LA in human milk. This immunoassay is easy to perform (one step assay, without washing or phase separation) and rapid (1 h). The concentration of α-LA in milk is measured over a large range (from 0.5 to 17 g/L) with high reproducibility (CVs lower than 7% in within- and between-precision) and accuracy (linear recovery in dilution-overloading assay). The inhibition mode chosen protects against the risk of underestimation by antigen excess, which may be encoutered in immunoassays based on a non-competitive antigen-antibody reaction. The sensitivity (detection limit, 1.5 µg/L) allows to use high dilutions of milk samples, cancelling sample blank measurement and such clarifying pretreatment as skimming or casein precipitation.

The concentrations of α-LA measured by this microparticle-enhanced nephelometric immunoassay in 162 human milk samples, collected from 8 mothers and including colostrum, transitional and mature milks, were distributed over a large range (from 2.2 to 8.3 g/L) and varied depending on the stage of lactation. The mean concentration and percentage among total protein obtained here in mature milk (3.4 g/L, 31%) were close to those previously reported (2.8-4.8 g/L, 28-36%) using other analytical methods such as electroimmunoassay (5), electrophoresis (6, 8) and immunodiffusion (7). Important changes in the absolute and relative α-LA concentration of human milk were observed during lactation. As previously suggested (8), the increase of α-LA concentration observed

from colostrum (4.9 g/L, 27%) to transitional milk (5.2 g/L, 40%) could be related to its lactose synthetase activity and paralleled with the similar variation in lactose milk levels during the days following postpartum (21). Lactose and whey proteins levels then appear negatively correlated in mature milk, when lactation is well established (22).

In addition to the α-LA assay described in this work, the development of microparticle-enhanced nephelometric immunoassays for the other main proteins of human milk could allow the establishement of protein profile of individual milks and permit an exhaustive investigation of the qualitative and quantitative changes in milk proteins during lactation, providing a better knowledge of the lactogenic response.

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