LABORATORY METHODS USEFUL IN THE ANALYSIS OF HUMAN BREAST MILK AND MILK POWDER SAMPLES

BOGDAN NEAMȚU[1], OVIDIU TIȚA[2], MIHAI NEAMȚU[3], MIHAELA TIȚA[4], MIRELA HILA[5], IONELA MANIU[6], LUCA LIVIU RUS[7]

[1,2,3,4,6,7]*"Lucian Blaga" University of Sibiu,* [5]*Pediatric Clinical Hospital of Sibiu*

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Abstract: *Human breast milk contains substances with anti-infective roles, immunoglobulins, cells involved in immune responses, prebiotics, and important nutrients for infants. Immunonephelometry, polyacrylamide gel electrophoresis, ultrasound spectroscopy can be used to analyze the composition of human milk. In our research, we have examined the sensitivity of these methods in the quantification of lactose, fat and pH of human breast milk and the sensitivity regarding evaluation of proteins, immunoglobulins (A, G, M) concentrations in both human breast milk (52 samples) and milk powder (48 samples). The results showed the appropriateness of using the ultrasonic spectroscopy only for the analysis of the fat content, pH and lactose. For milk samples immunogram (breast milk and milk powder) immunonephelometry can be considered as a reference method. Polyacrylamide gel electrophoresis remains the method of choice in assessing protein concentration. Breast fed infants have superior immune protection in particular by IgA titers and β-globulins.*

Cuvinte cheie: *metode de laborator, lapte matern, lapte praf, sugari, imunitate*

Rezumat: *Laptele matern conține substanțe cu rol antiinfecțios, imunoglobuline, celule implicate în răspunsuri imune, prebiotice, având totodată și roluri nutritive pentru sugari. Imunonefelometria, electroforeza în gel de poliacril amidă, spectroscopia cu ultrasunete pot fi utilizate și pentru analiza compoziției laptelui uman. În studiul propus am analizat sensibilitatea acestor metode în dozarea lactozei, a grăsimilor și a pH-ului din laptele matern și respectiv sensibilitatea în dozarea proteinelor, a imunoglobulinelor (A, G, M) din laptele matern (52 probe) și din laptele praf (48 probe). Rezultatele au arătat oportunitatea folosirii spectroscopiei cu ultrasunete doar în analiza conținutului în grăsimi, a pH-ului și a lactozei. Pentru imunograma probelor de lapte (matern și lapte praf) imunonefelometria poate fi considerată o metodă de referință, iar pentru evaluarea concentrației în proteine, electroforeza pe gel de poliacrilamidă rămâne metodă de elecție. Sugarii alimentați natural au protecție imună superioară în special prin titrul IgA și β-globuline.*

INTRODUCTION

Breast milk contains substances with antimicrobial and antiviral roles, immunoglobulins, cells involved in immune responses, prebiotics. Breast milk is composed of fats (AG-fatty acids, PUFA polyunsaturated fatty acids-AG), proteins (casein, α-lactalbumin, albumin, β-lactoglobuline, IgA, IgG, lactoferrin, lysozyme), carbohydrates (lactose, oligosaccharides), minerals (calcium, phosphorus, sodium, potassium, chloride), bioactive factors.(1,2,3,4,5)

Although it has a complex composition, human milk can be easily divided by centrifugation into three major parts, namely soluble whey, casein, the mycelia of globules of milk fat (MFGs floating). Breast milk contains different components necessary for newborn for growth and development. Among these components, specific proteins in milk and plasma proteins, such as β-casein, k-casein, α-lactalbumin, serum albumin, lactoferrin, lysozyme, immunoglobulins A, C3, C4 (complement fractions) that have significant nutritional and immunological functions.(1,2,3,5)

Numerous methods have been reported as tests used for the analysis of the composition of human milk: 1.immunonephelometry, 2 polyacrylamide gel electrophoresis, 3.chromatography of the proteins in a liquid medium, 4.ion exchange chromatography, 5. Kjeldahl method. 6. Mojonnier, Gerber, and Babcock methods 7. Ultrasonic Spectroscopy.(6)

Nephelometric and turbidimetric methods of analysis are based on the phenomenon of diffusion or absorption of light by solid particles or colloids by measuring the intensity of scattered light flux by solid particles in a solution. Immunonephelometry is based on conventional nephelometric quantification of the scattered light of antigen-antibody complexes formed during the immunoprecipitation reaction liquid phase, and is usually used for the determination of human serum proteins including IgA, and fractions of the complement C3, C4. This technique allows the measurement of IgA, complement fractions in mature human milk C3, C4 with precision and accuracy.(6) Ultrasonic spectroscopy, a non-destructive analytical technique measures the parameters of low-energy ultrasonic waves propagating through the sample analyzed. It allows probing intermolecular forces in the sample, providing new information on the structure.(7) It is used to analyze the content of milk fats, proteins, lactose, physico-chemical parameters such as density, freezing point, added water, pH, temperature and conductivity in fresh milk (cow, sheep buffalo, goat).(8) An interesting approach with implications concerning the improvement of the milk powder formula composition is to test the reliability and suitability in evaluating the composition of breast milk samples from infants

[1]Corresponding author: Bogdan Neamțu, Str. Lucian Blaga, Nr. 2A, 550169, Sibiu, România, E-mail. bogdanneamtu76@gmail.com
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hospitalized, using predominantly methods of laboratory analysis of human serum (immunonephelometry, polyacrylamide gel electrophoresis) and methods of analysis of milk samples of animal origin (ultrasonic spectroscopy).

METHODS

The proposed study aimed at analyzing the differences in composition between globulin fractions α1, 2, β, γ, immunoglobulins A and G studying samples of milk and milk powder using immunonephelometry and polyacrylamide gel electrophoresis, but also the ultrasonic analyzer performance in the analysis of breast milk samples (used in the food industry for testing milk samples of animal origin). To this end, we have collected biological samples from nursing mothers admitted in Pediatric Clinic of Sibiu in order to study the composition of breast milk and milk powder. We have tried to establish regarding the analysis of samples of human milk and milk powder, the limitations of laboratory methods used in our clinic just for the analysis of serum from blood samples until now.

The samples were collected aseptically and with local antisepsis (areola disinfected, using sterile gloves) following the protocol: 52 control samples (skin disinfected) for verification, 52 breast milk samples and 48 samples of infant formula. We have used sterile gloves and sterile containers for milk. Samples were transported on ice to the laboratory and kept in a refrigerator (-20 ^{0} C) for further analysis.

Biological samples from human milk and milk powder were subjected to centrifugation at 3,000 rotations/ minute for 20 minutes. The supernatant represented by fat was separated and the filtrate was used to determine the Ig G, A, M and to perform electrophoresis of the proteins. Electrophoresis was carried out with the device Genio S, using the cellulose acetate film at a basic pH. The determination of immunoglobulins (IgA, F, G) was carried out on samples of the filtered Hitachi device 912 by immunoturbidimetric method (readings at λ=340 nm). Evaluation of physical and biochemical parameters of human milk was performed using an ultrasonic analyzer. Pearson correlations, statistical tests (Independent Sample Test) were studied in accordance with the objectives of the study.

RESULTS AND DISCUSSIONS

In samples of breast milk protein the content was 3.45 g/dl (mean) with a minimum of 3.24 g/dl and a maximum of 3.58 g/dl after analysis of data provided by ultrasonic Ekomilk Total Analyzer. The same samples were also tested on electrophoresis device Genio S and the values were expressed in g/dl (mean was 1.021 g / dL, with a minimum of 0.268 g / dl and a maximum of 1.48 g/dl). Basically there was a discrepancy between the measured values offered by ultrasound analyzer and the electrophoresis device, regarding the determination of protein. The literature shows that proteic macronutrients of mature breast milk vary between 0.9 and 1.2 g/dl and differs from one mother to another during lactation and nutritional status.(5,6,9) In a recent study, Zachariassen et al (2013).(10) have shown a high degree of variability of protein content in 736 breast milk samples collected from mothers with premature infants. It has been described even a much wider range from 1.06 g / dl to 2.96 g / dl. The data on the concentration of proteins in colostrum showed values of 2% greater than the transitional milk (5%) and mature milk (1%).

The fat content of breast milk samples had an average of 3.85 g / dl with a minimum of 0.79 g/dL and a maximum of 7.64 g/dl. In the specific studies, values of 3.6 g / dl with a minimum of 2.2 g/dl and less than 5 g/dl colostrum are reported.(5,6) Fat content evaluation was performed only on ultrasonic analyzer and it is appropriate. The content of lactose in breast milk samples had an average of 5,021 g/dl with a minimum of 4.76 g/dl and a maximum of 5.25 g/dl. There have been described values 6.6 g / dl in colostrum which increased progressively to values of 6.7 g/dl in human milk even 7.8 g/dl.(9) The values for lactose vary during the lactation although there are several authors who reported the lowest percentage of lactose about 6 g/dl. The differences may arise depending on the method used. The values obtained using ultrasonic analyser are comparable to those in the composition of cow's milk (4.5 g/dl to 5 g/dl). The pH values obtained from samples of breast milk were on average 7.042 with a minimum of 6.8 and a maximum of 7.16. There were reported values for pH of 7.45 for colostrum, 7.01-7.04 for first 3 months postpartum, and then values increased to 7.4 at 10 months.(9) It is noted that low pH with lactose promote intestinal proliferation of lactic acid bacteria level.V IgA values levels of breast milk samples showed positive correlations with β-globulins ($p < 0.05$) and did not show any correlation with lactose ($p = 0.35 > 0.05$). Beta globulins in human serum increase in acute infections, especially protein fractions C3 and C4, so it is interesting a positive correlation of β-globulins IgA in breast milk. In the milk powder formulas, there are statistically significant correlations between the fractions α1, α2, β globulin, and negative correlations between IgA and α2 -globulins.

Figure no. 1. Globulin synopsis breast milk / milk powder

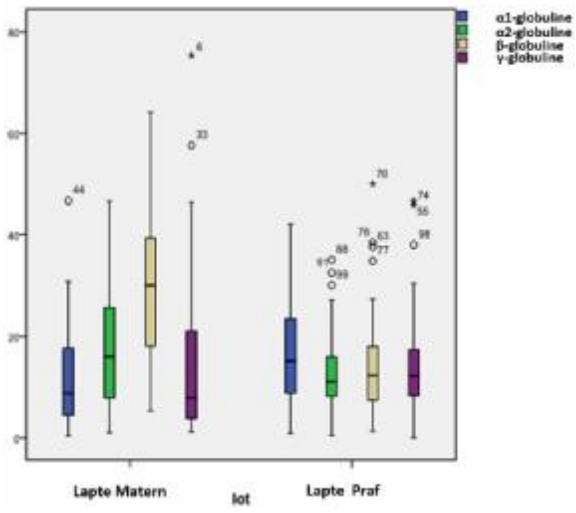

Figure no. 2. IgA levels comparison breast milk / milk powder

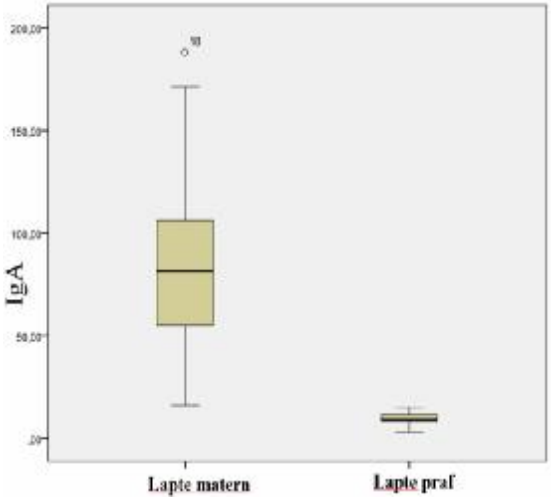

Table no. 1. Breast milk protein levels

Device	Origin of the proteins	No.	Mean g/dl	Standard deviation	Minimum	Maximum	Percentiles		
							25	50 (Median)	75
Electrophoresis device	Total proteins of breast milk	52	1.022	0.2687	0.58	1.48	0.7375	1.075	1.252
Ultrasonic Analyzer	Total protein in breast milk ± 0.2,	52	3.456	660E-02	3.24	3.58	3.4	3.47	3.507

Table no. 2. Breast milk fats

Device	Biomarker type	No.	Mean g/dl	Standard deviation	Minimum	Maximum	Percentiles		
							25	50 (Median)	75
Ultrasonic Analyzer	fats ± 0.1%	52	3.8521	1.8869	79	7.64	2.72	3.45	4.94

Table no. 3. Breast milk lactose and pH levels

Device	Biomarker type	No	Mean g/dl	Standard deviation	Min	Max	Percentiles		
							25	50 (Median)	75
Ultrasonic Analyzer	Lactosis ± 0.2 %	52	5.021	1.219	4.76	5.25	4.95	5.04	5.107
Ultrasonic Analyzer	pH ± 0.02	52	7.042	660E-02	6.8	7.16	7.01	7.06	7.09

Table no. 4. Statistical significant correlations of bioactive factors in milk and milk powder

Breast fed infants		Formula fed infants	
Human milk		Formula	
Positive Pearson correlations	Negative Pearson correlations	Positive Pearson correlations	Negative Pearson correlations
β-globulins with IgA	β-globulins with α1, γ	α1 globulins with α2	α1 globulins with IgA
	γ-globulins with α 2		

Mean concentration of IgA in breast milk samples (83.71 mg / dl, SD = 38.03) was significantly higher ($p < 0.05$) than the average of IgA concentration in milk powder samples (9.45 mg / dl, SD = 2.71). Mean β-globulin for breast milk (M = 29.95%, SD = 15.73) was significantly higher than the mean of β-globulins in milk powder (M = 14.30%, SD = 10.52). No significant differences between groups for γ-globulins ($p = 0.697 > 0.05$) were, meaning that the mean of γ -globulins in breast milk (M = 14.71%, SD = 16.02) was not significantly higher than the mean of γ-globulin for milk powder (M = 13.68%, SD = 9.93). Mean of α1 globulin in breast milk (M = 11.89%, SD = 9.41) was significantly lower than the α1 globulin in milk powder (M = 16.94%, SD = 10.85). Mean of α2 globulin in breast milk (M = 18.41%, SD = 11.87) was significantly higher than the mean for α2 globulin in milk powder (M = 12.81%, SD = 8.18). It can be noticed that there is a superior anti-infective protection of breast fed infants primarily by IgA titer, β-globulin and γ-globulin , α1,2 globulin probably having a secondary role, and in formula fed infants artificial protection is achieved by α1,2 globulin and γ-globulin respectively. Data actually explains why natural diet reduces the risk of respiratory infections in infants. It is known that exclusively breastfed for at least 4 months decreases the risk of upper respiratory infections by 63%; Exclusively breastfed for 6 months reduces the risk of upper respiratory infections by 72%.(11,12,13,14) There was an increase of IgA in breast milk in lactating mothers of infants with very severe infections (sepsis) as follows: colostrum (+/- 183.48 114.91 mg / l - control group; +/- 221.22 115.46 mg / l - study group), mature milk (147.75 +/- 114.6 mg / l - control group; +/- 267.81 77.89 mg / l - study group).(14) Increased concentrations of these biomolecules seems to be protective.

CONCLUSIONS

Analyzing the data presented, the following conclusions are emerging consistent with the objectives of the study on the analysis of samples of breast milk and milk powder:

1. Polyacrylamide gel electrophoresis is suitable for the determination of proteins in breast milk, and analysis devices based on ultrasound spectroscopy used in food technology require specific adaptations.
2. Immunonephelometry can be successfully used to analyze serum samples resulted from the preparation of milk and milk powder respectively.
3. Devices based on ultrasonic spectroscopy used in the food industry can be successfully used in the analysis of fat content, pH and lactose.
4. Breast fed infants with respiratory infections have a superior immune protection to those fed with formula.

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