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Breast Milk Iron Concentrations may be Lower than Previously Reported: Implications for Exclusively Breastfed Infants

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Abstract

Objective: Atomic Absorption Spectrophotometry (AAS) is the most common technique for detecting iron in human milk. Quantifying the iron in human milk is essential for determining the amount of dietary iron available to the exclusively breastfed infant. To determine whether more sensitive procedures for iron analyses would yield different values than those obtained using the AAS, flame atomic absorption, we investigated and compared three different analytical techniques using AAS.

Methods: The present experiment was conducted as part of a larger study on the iron supplementation of 77 exclusively breast-fed infants. Breastmilk samples were collected from 10 mothers of full-term infants at one and 3½ months of age. Each sample was analyzed with a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer by the following analytical methods: flame atomic absorption spectrophotometry (FAAS), direct sample analysis with graphite furnace atomic absorption spectrophotometry (GFAAS) and the method-of-standard-additions with GFAAS.

Results: While all values were within reported ranges, there was significant inter-method variability. The decrease in iron concentration between 1-month and 3.5-month milk samples found in the present study is in agreement with a previous report. GFAAS methods produced consistently lower values than did FAAS: 1 month: direct GFAAS, 0.50 (0.33-0.86) μg/ml; method-of-addition GFAAS, 0.45 (0.31-0.66) μg/ml; FAAS 0.76 (0.25-1.60 μg/ml), mean (range): 3.5-month: direct GFAAS, 0.29 (0.13-0.46 μg/ml; method-of-addition GFAAS, 0.29 (0.11-0.44 μg/ml); FAAS method 0.78 (0.19-1.64) μg/ml. There was no difference between the two GFAAS methods at either time point.

Conclusion: The findings obtained from this study suggest that much of the variability seen in the reported values of human milk iron concentrations could be due to the use of different analytical procedures. GFAAS results are consistently lower than those determined by FAAS suggesting that the exclusively breastfed infant consumes less iron than previously thought.

Keywords: Iron; Breast milk; Accuracy, Full-term; Flame atomic absorption spectrophotometry; Graphite furnace atomic absorption spectrophotometry; Infant

Introduction

Iron is a vital nutrient which is critical for the mental and physical development of infants [1]. For exclusively breastfed infants, breastmilk is their sole source of iron. The concentration of iron in human breast milk has been shown to be relatively constant among different races and across a variety of socio-economic backgrounds [2]. Iron concentration in human milk does not vary greatly with respect to maternal dietary iron intake nor maternal iron status [3,4]. The absolute concentration of iron in breast milk will vary through the course of lactation and has been reported by a number of researchers to be anywhere between 5-16 μM (0.27- 0.90 mg/L), with the iron concentration of colostrum being higher than mature milk [4-8]. The bioavailability of iron from human milk has been demonstrated to be in the area of 20% and may have been overestimated in previous reports [9,10]. The challenge for the exclusively breastfed infant is adequately maintaining proper iron nutrition through the first year of life.

There are currently no worldwide statistics for the prevalence of ID and IDA in infants before 12 months. The World Health Organization (WHO) estimates that 47% of preschool children (aged 0 to 5 years) suffer from IDA and it can be assumed an even higher number suffer from ID without anaemia, which goes undiagnosed [11]. In Canada, 10-15% of exclusively breast fed infants have been reported to have IDA by 6 months of age [12,13]. Fomon [14] estimates to prevent the depletion of iron stores at birth; the infant must absorb 0.55-0.75 mg Fe/day from dietary sources. This amount of iron will allow the infant to meet its developmental needs in the first year of life. This estimate is based on the allocation of iron at birth (birth weight of 2.5-3.5 kg) and the desired allocation for a 12-month old infant weighing 10.0-10.5 kg. While this is not a challenge for infants consuming iron-fortified formulas which contain 12-14 mg Fe/L, the exclusively breastfed infant cannot achieve this goal consuming breast milk alone [15]. However, some researchers and health authorities assume that stores of iron and the iron content of human milk based on currently reported values are adequate to meet iron needs until 6 months of age [16-18].

The purpose of current study was to analyze human milk iron concentrations by three separate analytical procedures using atomic absorption spectrophotometry (AAS) to determine if more sensitive procedures (GFAAS) for iron analyses would yield lower values than those obtained using the most common AAS flame procedure.

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Methods

Subject selection

The present experiment was conducted as part of a larger study on the iron supplementation of exclusively breast-fed infants [19]. Breast milk was obtained from 10 mothers randomly selected from 77 mothers. Women were asked to provide between 10-15 ml of breast milk at 1 and 3.5-month of age at a healthy baby clinic. All procedures were approved by the Research Ethics Board of Memorial University of Newfoundland.

Breast milk collection and storage

To reduce cross contamination each mother expressed milk manually into 10 ml acid-washed polypropylene capped test tubes. The test tubes were transported on ice to the laboratory where they were aliquoted to 1.5 ml acid washed Eppendorf tubes and stored at -20°C until analyzed. The same samples were analyzed by three different procedures, as outlined below.

Diluted direct graphite furnace atomic absorption spectrophotometry

The direct analysis was carried out with a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer (Norwalk, Connecticut, USA) with a Perkin-Elmer Graphite Furnace (GFAAS) and Perkin-Elmer HGA-300 Programmer according to Liang and associates with modifications for breast milk [20]. Frozen samples were placed in a 37°C water bath and allowed to come to temperature. The samples were then taken from the water bath, vortexed and diluted 20X using 1% HNO_3 to eliminate matrix effects and obtain an iron concentration that fell within the range of prepared standards used (10-60 ng/ml). Ten micro litres of sample were then injected into the GFAAS in triplicate and was preceded and followed by blanks of 1% HNO_3. The certified reference material, Analytical Quality Control Services (*AQCS) A-11 trace elements in milk powder* [International Atomic Energy Agency (IAEA), Austria], was used and treated in the same fashion as the samples to ensure the precision of the procedure. Results for the reference material were within 15% of the certified iron concentration (0.40 +/− 0.03 µg Fe/ml; 0.47 µg Fe/ml expected). Instrumental programs and experimental parameters are described in Table 1.

Method of addition graphite furnace atomic absorption spectrophotometry

Frozen samples were placed in a 37°C water bath and allowed to reach temperature. Fifty micro litres of breast milk were aliquoted into each of three acid washed Eppendorf tubes and diluted 20X with 1% HNO_3, 10 ng Fe/ml in 1% HNO_3 and 20 ng Fe/ml in 1% HNO_3. The samples were then injected in triplicate and absorbance obtained. Blanks and certified controls were injected after running 5-8 samples. Certified material, *AQCS A-11 trace elements in milk powder* (IAEA, Austria), was used and treated in the same fashion as the samples to ensure the precision of the procedure and were within 15% of the accepted iron concentration (0.40 +/− 0.03 µg Fe/ml; 0.47 µg Fe/ml expected). Results were analyzed graphically for the concentrations of iron in the diluted samples and then corrected by multiplying by the dilution factor.

Parameter	Procedure		
	Direct GFAAS	Standard Additions GFAAS	Flame AAS
Wavelength (nm)	248.3	248.3	248.3
Slit-width (nm)	0.2	0.2	0.2
Lamp current (mA)	30	30	30
Gas	Nitrogen	Nitrogen	Air/Acetylene
Flow rate (ml/min)	50	50	Not applicable
Injection volume (µL)	10	10	1-2 ml
Furnace type	Standard	Standard	Not applicable
Program - (Temp/Ramp time/Hold Time) (°C/s/s)			
Drying	100/5/30 120/5/25	100/5/30 120/5/25	Not applicable
Ashing	900/20/40	900/20/40	Not applicable
Atomization	2500/1/8	2500/1/8	Not applicable

Table 1: Atomic absorption spectrophotometer experimental parameters.

Flame atomic absorption spectrophotometry

The Perkin-Elmer flame atomic absorption spectrophotometer (FAAS) was optimized and set to measure the concentration of iron using wavelength λ = 248.3 nm and internally set references. The spectrophotometer was calibrated to calculate the concentration based on the mean concentration of a triplicate set of samples. The samples were prepared for the flame analysis in a 5-day digestion at 70°C in concentrated ultra pure nitric acid using our standard procedure [21]. Briefly, 500 micro litres of human breast milk was placed in a Teflon tube with 1000 µl of ultra pure HNO_3. The samples were placed on a hotplate and digested for 5 days at 70°C. When the digestion was complete, heating the Teflon tubes to 150°C rapidly evaporated off the acid. One thousand microliters of ultra pure H_2O were added to wash the samples. The samples were heated until they were dry. The dried samples were brought up to a final volume of 1000 µl with 0.2 N ultra pure HNO_3 [21]. The samples were aspirated into the FAAS and concentrations were calculated. Certified material, *AQCS A-11 trace elements in milk powder* (IAEA, Austria), was used and treated in the same fashion as the samples to ensure the precision of the procedure and were within 6% of the accepted iron concentration (0.44 +/− 0.17 µg Fe/ml; 0.47 µg Fe/ml expected).

Statistical analysis

A comparison of the 3 procedures at two different collection periods and over time was performed using analysis of variance with Bonferroni pair wise post hoc test to isolate differences. Paired student t-tests were used to compare within technique differences at different sample times. The level of significance was set at $p<0.05$. The analysis was performed using SPSS version 11 statistical computer software (SPSS, Chicago, IL).

Results and Discussion

A difference was found to exist in results between direct GFAAS and FAAS at 1-month (direct GFAAS, 0.50 µg/ml; FAAS: 0.76 µg/ml, $p<0.05$) while both GFAAS methods differed from the FAAS at 3.5-months (direct GFAAS, 0.29 µg/ml; method-of-addition GFAAS, 0.29 µg/ml; FAAS method 0.78 µg/ml) (Figure 1). FAAS yielded a consistently higher concentration of iron in both milk samples at 1-month and 3.5-month (Figure 1). There was no difference between the two GFAAS methods at either time point. Table 2 illustrates each individual mothers breast milk iron concentration over time by each of the three methods investigated. Differences between means are outlined both in Figure 1 and Table 2.

The concentrations of many human breast milk constituents do not vary greatly between populations of mothers throughout the world [22]. However, the published values for iron vary by as much as 66% which may be due to the different analytical procedures used for trace

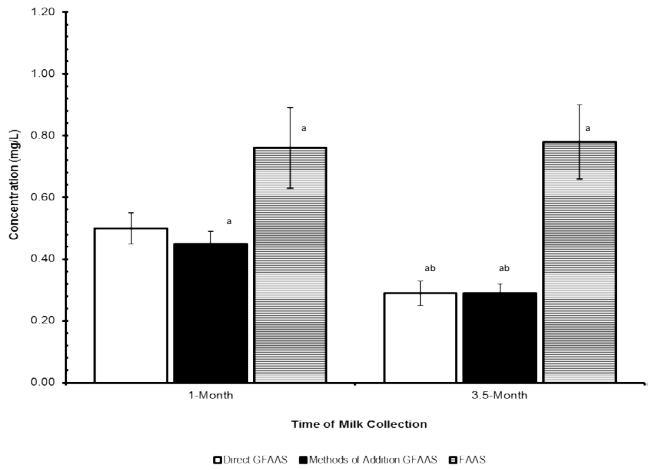

Figure 1: Iron concentration of human breast milk measured at 1 and 3.5 months by direct GFAAS, methods of addition GFAAS and FAAS (N=10).

Concentration is expressed as µg/ml of human breast milk. Mean +/− SEM (error bars). a=Difference between method at same time; p<0.05. b=Difference over time within same method; p<0.05.

Mother	1-Month Milk Sample (mg/L)[1]			3.5-Month Milk Sample (mg/L)		
	Direct	S. Addition	Flame	Direct	S. Addition	Flame
1	0.34	0.31	1.06	0.29	0.34	0.67
2	0.51	0.39	0.28	0.27	0.28	0.76
3	0.46	0.42	0.80	0.46	0.44	0.83
4	0.65	0.66	0.63	0.21	0.27	0.19
5	0.51	0.54	0.86	0.18	0.26	0.69
6	0.33	0.31	0.54	0.18	0.19	0.60
7	0.48	0.40	0.94	0.34	0.27	0.68
8	0.50	0.53	1.60	0.13	0.11	0.80
9	0.86	0.54	0.66	0.42	0.38	0.97
10	0.39	0.37	0.25	0.38	0.41	1.64
Mean	0.50^{b}	0.45^{ab}	0.76^{a}	0.29^{ab}	0.29^{ab}	0.78^{a}
SEM	0.05	0.04	0.13	0.04	0.03	0.12
Mean+/-SEM	0.57+/-0.05			0.46+/-0.06		

[1]Differences between method within same sample time (a; p<0.05) and between same methods at different sample times (b; p<0.05).

Table 2: Comparison of individual breast milk iron concentrations by method.

mineral determination. Trugo and associates reported mature human breast milk contained 0.90 µg Fe/ml in Brazilian women while a value of 0.30 µg Fe/ml has been reported in lactating women in both Finland and the United States [22,23]. Fransson and associates demonstrated that geographic region and socio-economic status has little effect on the concentration of iron in human milk, comparing Swedish women to privileged and underprivileged Ethiopian women [2]. If the higher reported values of breast milk iron concentrations are accepted as representative of all human milk fed infants, then the breastfed infant population should be receiving adequate dietary iron and ID and IDA should be rare among this population. By contrast, if the lower values were considered representative then the breastfed infant population would not be able to achieve the optimal dietary intake of iron and would be expected to have increased the risk of ID and IDA. Because of the wide range of reported values and the potential consequences for the breastfed infant we decided to compare different analytical procedures of AAS for determining iron concentrations in biological samples. Atomic absorption spectrophotometry is the most widely used tool to determine iron content in milk samples [24]. To our knowledge, there has been no study to date, which examined differences in human milk iron concentrations as a function of analytical procedure within the same milk samples.

The decrease in iron concentration between 1-month and 3.5-month milk samples found in the present study is in agreement with change reported by Siimes et al. [25] and those summarized by Casey et al. [22]. Our findings indicated an iron concentration ranging from 0.31 to 0.86 mg/L in 1-month milk samples, and iron concentrations ranging from 0.13 to 0.46 mg/L in mature milk at 3.5-months using the GFAAS methods. However, the FAAS method produced a consistently higher concentration of iron in milk samples ranging from 0.25 to 1.60 mg/L and 0.19 to 1.64 mg/L at 1 and 3.5 months, respectively.

The discrepancy between the results obtained by GFAAS and those by the FASS method illustrate potential problems with the reporting of breast milk mineral concentrations. The variety of ashing techniques, dilution protocols and analytical methods used by different researchers suggest a possible reason for the wide range of results for iron content of human milk seen in the literature. For example, Arnaud and associates [7,26] produced two different ranges for early breast milk iron simply by changing the analytical procedure used. The FAAS method used in our experiments, and used by other researchers for mineral determinations in milk products, yielded consistently higher values for breast milk iron.

However, it should be noted that the values obtained in our study from each of the techniques, while differing between themselves, are within the reported ranges of breast milk iron concentrations [2,22,25].

We think that the exclusively breastfed infant is at risk of poor iron nutrition within the first year of life. This population should be closely monitored using conventional clinical indicators of iron status to ensure proper intervention is provided where needed to prevent ID. It should be noted that the iron concentration of breast milk is lower than the requirements of most exclusively breastfed infants [14]. Indeed, there are researchers who are already recommending infants be supplemented with iron and several other vitamins and minerals [27,28]. The negative effects associated with ID in infancy have been well documented and the cognitive and psychomotor developmental setbacks may last well into childhood [29,30]. Indeed, the American Academy of Pediatrics recommends exclusively breastfed infants receive from irons at 4 month of age [31]. These outcomes are preventable when ID and IDA are prevented rather than treated after the fact. Therefore, there may be a need to re-evaluate the breastfed infant iron requirements during the first few months of life.

The findings obtained from this study suggest that much of the variability seen in the reported values of human milk iron concentrations could be due to the use of different analytical procedures. Reported iron concentration in human milk should be qualified by the analytical procedure and equipment used to determine these concentrations.

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