

In Vitro Availability of Calcium, Iron, and Zinc from First-Age Infant Formulae and Human Milk

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ABSTRACT

Background: Variation in the bioavailability of calcium (Ca), iron (Fe), and zinc (Zn) occurs because of interactions of food components in the gastrointestinal microenvironment. Bioavailability is preferably determined by *in vivo* tests, but these are expensive, labor-intensive, time consuming, and often unethical. As an alternative, *in vitro* methods can be used to predict bioavailability of nutrients from foodstuffs.

Methods: A continuous-flow dialysis model with preliminary intraluminal digestive phase, adapted to the gastrointestinal conditions of infants younger than 6 months, was used. Human milk was the reference standard. Ca, Fe, and Zn content of samples and dialysates after digestion were analyzed by atomic absorption spectrometry.

Results: Ca availability is similar in human milk ($13.1\% \pm 0.8\%$), whey ($13.3\% \pm 1.2\%$), and soy-based formulae ($13.0\% \pm 1.2\%$; $P > 0.05$), and higher in casein-predominant formula

($21.2\% \pm 0.6\%$; $P < 0.05$). Availability of Fe is highest in human milk ($8.12\% \pm 0.27\%$; $P < 0.05$). Fe availability in whey ($1.28\% \pm 0.28\%$) and soy formulae ($1.48\% \pm 0.28\%$) is similar ($P > 0.05$), but availability is lower in casein-predominant formula ($0.48\% \pm 0.22\%$; $P < 0.05$). Zn availability is also highest in human milk ($13.1\% \pm 0.7\%$; $P < 0.05$). However, Zn availability is similar in whey ($6.7\% \pm 0.6\%$) and casein formulae ($8.5\% \pm 1.6\%$; $P > 0.05$), but lower in soy formula ($2.3\% \pm 0.4\%$; $P < 0.05$).

Conclusions: Our observations are in agreement with previous data from *in vivo* studies in term infants. This *in vitro* procedure is an inexpensive, simple, rapid, and reliable method that predicts the bioavailability of Ca, Fe, and Zn in foods. *JPGN* 32:54–58, 2001. **Key Words:** Bioavailability—Calcium, iron, and zinc—Human milk—Infant formulae. © 2001 Lippincott Williams & Wilkins, Inc.

Although human milk is usually recommended as the reference standard, more than 70% of the infants in Western Europe are formula fed from 12 weeks on (1). These adapted first-age formulae approximate the composition of human milk as much as possible, but they contain ligands that may inhibit mineral and trace element absorption. Therefore, the element concentration of formulae has been increased in comparison with human milk. This can cause precipitation of unabsorbable complexes and great variation in the bioavailability of essential elements, such as calcium (Ca), iron (Fe), and zinc (Zn).

Bioavailability is preferably determined by *in vivo* techniques, but these experiments are often unethical, rather complicated to perform, and yield variable results. *In vitro* methods are simple and rapid, and minor varia-

tions in food composition can be evaluated promptly and thoroughly. Therefore, these methods are useful to predict the bioavailability of nutrients (2–6). Considering, however, the limitations of these methods, we propose to narrow the term to “availability.”

The purpose of this study was to adapt the procedure to the gastrointestinal microenvironment of the young infant. By measuring *in vitro* availability of Ca, Fe, and Zn from infant milks and correlating the results with previous data from *in vivo* studies on term infants, reliability of the *in vitro* procedure can be determined. Human milk was used as reference.

MATERIALS AND METHODS

Materials and Reagents

Pooled mature human milk was studied. The infant formulae were whey, casein, and soy protein-predominant milks used during the first months of infancy. The whey and soy-based first-age formulae are commercially available, whereas the ca-

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sein-based first-age formula was prepared by the manufacturer especially for this study. The nutrient composition of the samples is given in Table 1. The protein content of the infant formulae and mature human milk were taken from, respectively, producer's information and Macy and Kelly (7).

All chemicals (Merck, Darmstadt, Germany) were of analytical grade, and MilliQ water (Millipore, Bedford, MA, U.S.A.) was used throughout the study. Pepsin (P-7000, from porcine stomach mucosa) and bile salts (B-8631, porcine) were purchased from Sigma (St Louis, MO, U.S.A.), and pancreatin (107133 0500, porcine) from Merck. A pepsin solution was prepared by dissolving 10 g pepsin in 100 mL of 0.1 mol/L HCl. The pancreatin-bile mixture contained 3 g pancreatin and 7 g bile in 1 L of 0.1 mol/L NaHCO₃.

In Vitro Dialysis Procedure with a Preliminary Intraluminal Digestive Phase

The entire procedure was undertaken four times for each infant milk tested. The method used is a modification of the continuous-flow dialysis in vitro model (4,5) with a preliminary intraluminal digestive phase adapted to the upper gastrointestinal tract of infants less than 6 months of age (8). The method consists of two phases: a gastric and an intestinal stage. Figure 1 illustrates the different steps of the in vitro procedure.

Intraluminal Digestive Phase

Before the gastric stage, the food sample is brought to pH 4.0 with hydrochloric acid (6 mol/L). Pepsin is added (3 mL), and the food sample is placed in a shaking water bath for 2 hours at 37°C. Titratable acidity is the number of equivalents of NaOH required to titrate the amount of gastric digest to 7.5 after addition of the pancreatin-bile mixture and is measured in an aliquot of the gastric digest.

Continuous-Flow Dialysis Method

The intestinal stage is performed in a stirred cell (Amicon, Beverly, MA, U.S.A.), with a dialysis membrane of 1000 molecular weight cut-off (MWCO), and takes 2.5 hours. During the first 30 minutes a gradual pH adjustment from acid to neutral occur. (5). After 30 minutes, a pancreatin-bile mixture (15 mL) is added to the neutralized food sample and dialysis is continued for another 2 hours.

Analytical Methods

Ca, Fe, and Zn content of the reagents, samples, dialysate, and retentate fractions was determined in triplicate by flame atomic absorption spectrometry (Perkin Elmer, AAnalyst 300, Norwalk, CT, U.S.A.), or electrothermal atomic absorption spectrometry (model 4100 ZL; Perkin Elmer) for the dialysate and retentate fractions of Fe. Blanks for Ca and Zn were determined in pepsin and pancreatin-bile extract and subtracted from the results. Before analysis, four portions of approximately 0.4 g of the samples were subjected to a destruction process as described by Hendrix et al. (9)

Calculation of Availability

The availability of the element is calculated from the amount of element that passed the dialysis membrane related to the total elemental content of the original food sample:

$$\text{Availability (\%)} = \frac{D}{W \times A} \times 100$$

where D is the total content of element in the dialysate after an intraluminal digestive phase (in micrograms), W is the weight of food sample used for the intestinal stage (in grams), and A is the concentration of element in the food sample (in micrograms per gram).

Assessment of the Analytical Performance of the In Vitro Procedure

Initial standardization was achieved by preparing two test samples with a low and a high mineral content (e.g., human milk and infant formula) at concentrations of, respectively 0.27, mg/g and 4.10 mg/g for Ca, 0.3 µg/g and 41.4 µg/g for Fe, and 1.0 µg/g and 42.3 µg/g for Zn. These mixtures were used to determine the recovery of Ca, Fe, and Zn in the test system after simulated digestion. Recovery was calculated by the amount of element found in the dialysate and retentate fraction. Repeatability of the procedure was calculated from the availability of Ca, Fe, and Zn from human milk and infant formula on four occasions during 1 day (inbatch precision). The reproducibility was obtained from 16 digestions of human milk and infant formula during a 4-day period (interbatch precision).

TABLE 1. Nutrient composition of food samples

	Content per 100 ml*				
	Protein		Calcium (mg)	Iron (mg)	Zinc (mg)
	Total (g)	Casein/Whey ratio			
Human milk	1.1	40/60	27.0 ± 0.6	0.03 ± 0.00	0.10 ± 0.01
Adapted formulae					
Whey based	1.4	40/60	54.2 ± 1.6	0.55 ± 0.02	0.56 ± 0.01
Casein based	1.7	80/30	70.7 ± 2.1	0.59 ± 0.02	0.58 ± 0.01
Soy based	1.8	—	66.0 ± 1.6	1.30 ± 0.02	0.80 ± 0.03

* 100 ml infant formulae: calculations based on normal reconstitution of infant formula powder with water (3 spoons/90 ml water = 100 ml).

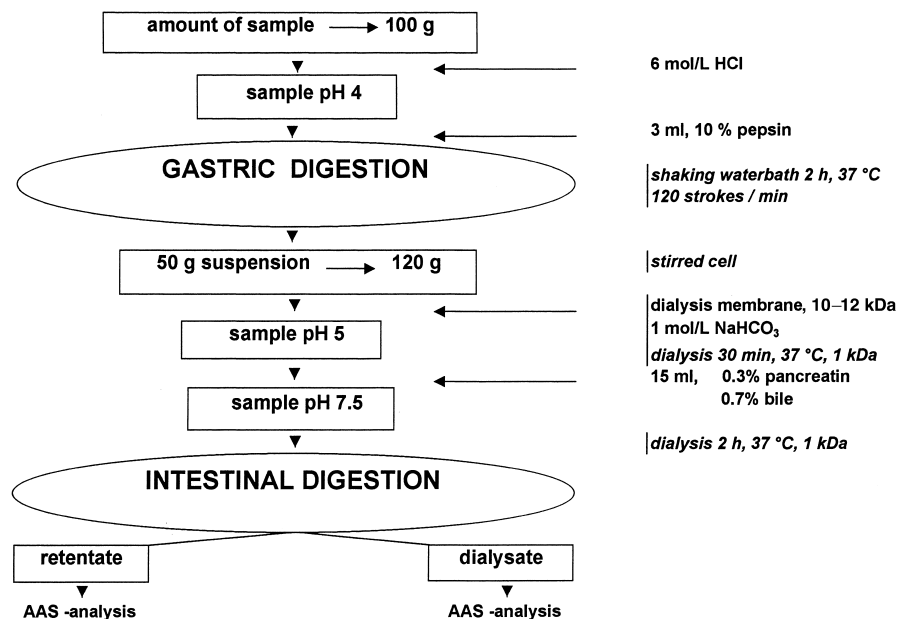


FIG. 1. Different steps of the gastrointestinal digestion protocol.

Statistical Analyses

The availability data for Ca, Fe, and Zn are expressed as mean values \pm SD (Table 2). Statistical evaluation of the data was performed on computer (SigmaStat, ver. 2.0 for Windows; Jandel Scientific, San Rafael, CA, U.S.A.). After testing for normality and equal variances, the mean availability from the food samples was compared by one-way analysis of variance, with a Tukey posttest for multiple comparisons. The difference was considered significant at $P < 0.05$.

RESULTS

Assessment of the Analytical Performance of the In Vitro Procedure

Recovery of the dialysis procedure with preliminary digestion was $90\% \pm 4\%$ for Ca, $90\% \pm 24\%$ for Fe, and $86\% \pm 5\%$ for Zn in human milk and $86\% \pm 2\%$ for Ca, $105\% \pm 5\%$ for Fe and $90\% \pm 4\%$ for Zn in formula. Four replicate determinations of the same food sample during

a single day show a variation coefficient (mean value of both test samples) of 4.1% for Ca, 14.5% for Fe, and 4.0% for Zn. When four times four replicates of a food sample were processed independently and measured in one assay, the mean coefficient of variation was 6.6% for Ca, 19.2% for Fe, and 13.6% for Zn.

Availability of Calcium, Iron, and Zinc from Infant Formulae

Values for Ca, Fe, and Zn content of the food samples are given in Table 1. The availability data for Ca, Fe, and Zn from the food samples are given in Table 2. The total availability per 100 mL formula was calculated by multiplication of the mean elemental content of the sample with the availability (Table 2).

Ca availability was similar in human milk ($13.1\% \pm 0.8\%$), whey ($13.3\% \pm 1.2\%$), and soy-based infant formulae ($13.0\% \pm 1.2\%$; $P > 0.05$) and was significantly

TABLE 2. Results of availability study for calcium, iron, and zinc

	Availability					
	Calcium		Iron		Zinc	
	%	mg/100 ml*	%	μ g/100 ml*	%	μ g/100 ml*
Human milk	13.1 ± 0.8	3.5 ± 0.2	8.12 ± 0.27	2.2 ± 0.1	13.1 ± 0.7	13.4 ± 0.7
Adapted formulae						
Whey based	13.3 ± 1.2	7.2 ± 0.6	$1.28 \pm 0.28^\dagger$	$7.0 \pm 1.5^\dagger$	6.7 ± 0.6	37.4 ± 3.4
Casein based	21.2 ± 0.6	15.0 ± 0.4	0.48 ± 0.22	2.8 ± 1.3	8.5 ± 1.6	49.0 ± 9.4
Soy based	$13.0 \pm 1.2^\dagger$	$8.6 \pm 0.8^\dagger$	$1.48 \pm 0.28^\dagger$	$19.3 \pm 3.6^\dagger$	$2.3 \pm 0.4^\dagger$	$18.1 \pm 2.8^\dagger$

* 100 ml infant formulae: calculations based on normal reconstitution of infant formula powder with water (3 spoons/90 ml water = 100 ml). Values are given as mean \pm SD (n = 4).

† n = 3.

higher in casein-predominant formula ($21.2\% \pm 0.6\%$; $P < 0.05$). The availability of Fe was highest in human milk ($8.12\% \pm 0.27\%$) when compared with all the infant formulae tested ($P < 0.05$). Fe availability in whey ($1.28\% \pm 0.28\%$) and soy protein-predominant formulae ($1.48\% \pm 0.28\%$) was similar ($P > 0.05$) and was significantly lower in casein-predominant formula ($0.48\% \pm 0.22\%$; $P < 0.05$). Zn availability was also highest from human milk ($13.1\% \pm 0.7\%$) when compared with the infant formulae tested ($P < 0.05$). However, Zn availability was similar in whey ($6.7\% \pm 0.6\%$) and casein-based infant formulae ($8.5\% \pm 1.6\%$; $P > 0.05$) but was significantly lower in the formula made of soy proteins ($2.3\% \pm 0.4\%$; $P < 0.05$).

DISCUSSION

The dialysis procedure, with preliminary digestive phase, indicates good recovery (approximately 86–105%) for Ca, Fe, and Zn over the range of concentrations tested. The in vitro procedure also fulfills the requirement of adequate repeatability and reproducibility for the availability data of Ca, Fe, and Zn.

Bioavailability can be described as that portion of a nutrient that can be used (10). Consequentially, the term bioavailability can be used in a large concept including digestion, absorption, and incorporation into metabolic processes. It can also be used in a narrow sense, meaning that any potentially available part of a nutrient after gastrointestinal digestion should be attributed to its bioavailability (10,11). To avoid confusion we propose to use the term availability in the restricted sense of the word.

Our results (Table 1) show values for Ca, Fe, and Zn concentration in mature human milk similar to those found in the study performed by Fransson et Lönnnerdal (12). The elemental concentrations in the infant formulae are high compared with those in human milk (e.g., Fe content). The mineral and trace element composition of infant formulae is often subjected to alterations, including increasing elemental content, to obtain higher uptake levels. This in turn may affect the bioavailability of other nutrients, and research in this area is necessary. Therefore, in vitro methods could be very useful in predicting bioavailability of essential elements from foodstuffs (2,4,5,13–15). Our technique consists of an intraluminal digestion phase followed by continuous-flow dialysis (Fig. 1). It is suspected that the dialysate is completely absorbed in the upper gastrointestinal tract. The gradual pH change from acid to neutral and the continuous removal of dialyzed components mimics the passage of chyme through the gut and the one-way lumen to the mucosal cell pathway for absorption of nutrients in the upper gastrointestinal tract (4,5). A very close simulation of the intraluminal digestive conditions of the infant gut was obtained by changing stomach pH, pepsin output, pancreatin output, and bile acid concentration in the in

vitro assay, because maturation of the gastrointestinal tract in earliest life stage is not yet complete (8).

In the present study (Table 2) Ca availability is highest from cow's milk casein-based formula, compared with the other investigated milk sources. These findings are supported by Grur et al. (16) who reported higher bone mineral content (BMC) in infants fed cow's milk formulae compared with infants fed soy formulae or human milk, suggesting that BMC in cow's milk-fed children is hypermineralized (17).

According to this in vitro assay, Fe availability is highest from human milk and lowest from high casein infant formula (Table 2). Our data therefore suggest that a high casein-to-whey ratio could negatively affect Fe availability in vitro. Hurrell et al. (18) demonstrated that both bovine casein and whey proteins are responsible at least in part for the poor bioavailability of Fe from infant formulae. Casein is the most inhibitory protein: it forms hard curds in the stomach of the young human infant and may pass through the gastrointestinal tract in an indigestible solid form, trapping cations (19). In addition cow's milk casein may bind Fe by the presence of phosphoserine groups on the casein molecule (20–22). In agreement with our findings, Hertrampf et al. (23) also reported optimal Fe status in term healthy infants fed human milk compared with infants receiving cow's milk-based or soy-based formulae.

The data on the availability of Zn (Table 2) are in agreement with the results obtained by Sandström et al. (24) and Hambidge et al. (25), who found highest absorption of Zn from human milk and lowest from soy-based formulae compared with cow's milk-based formulae.

Our availability results obtained with this in vitro assay are in good agreement with previous data from term healthy infants. Therefore, we can conclude that this in vitro method is inexpensive, simple, rapid, and reliable and can be used as an index to predict the bioavailability of essential elements from different foods.

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