ELSEVIER

Food Chemistry 65 (1999) 353–357

Food Chemistry

Calcium bioavailability in human milk, cow milk and infant formulas—comparison between dialysis and solubility methods

M.J. Roig*, A. Alegría, R. Barberá, R. Farré, M.J. Lagarda

Nutrition and Food Chemistry, Faculty of Pharmacy, University of Valencia, Avda. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain

Received 7 July 1998; accepted 30 September 1998

Abstract

The percentages of total, soluble and dialysable calcium of human milk, cow milk and milk and soy based infant formulas were determined in order to detect possible differences in the calcium bioavailability of the samples. For this purpose an in vitro method was applied to these four calcium sources. The ranking of the analysed samples in terms of calcium bioavailability depends on the criteria applied. Calcium ranked dialysis percentage was: cow milk > human milk > soy based formula > milk based formulas. Calcium ranked solubility percentage was: human milk > cow milk > soy-based formula > milk-based formulas. Comparison of the results of the in vitro assay with the information available on in vivo calcium absorption showed that the total soluble calcium contents agree with the in vivo absorption values better than with calcium dialysis percentages.

1. Introduction

The in vitro estimation of the bioavailability of minerals and trace elements involves simulating gastrointestinal digestion of the food with pepsin–HCl and pancreatin–biliary salts and then measuring the fraction of the soluble element (Narasinga Rao & Prabhavathi, 1978; Schwartz, Belko, & Wein, 1982; Crews, Burrel, & McWeeny, 1983; Kim & Zemel, 1986; Nadeau & Clydesdale, 1991) or the fraction of element that dialyses through a membrane of a certain pore size. In the dialysis procedures dialysability can be measured in equilibrium (Miller, Schricker, Rasmussen, & Van Campen, 1981; Keane, Potter, & Sherbon, 1988) or continuously (Minihane, Fox, & Fairweather-Tait, 1993; Wolters et al., 1993; Shen, Luten, Robberecht, & Deelstra, 1994). In fact these methods estimate only the fraction of the element available for absorption and therefore only the first step of the in vivo process of absorption is taken into account.

To be absorbed, the elements need to be in a soluble form or at least bound to a compound that can be absorbed and can then release the element (Miller et al., 1981; Keane et al., 1988; Schnepf & Madrick, 1991). The fact that, in some elements, as for instance iron, there is a good correlation between the soluble fraction and in vivo absorption supports the use of the solubility value as an estimate of bioavailability (Narasinga Rao & Prabhavathi, 1978).

The in vitro methods, based on the dialysability of the element differ from those based on solubility measurements, in that the in vitro dialysability techniques incorporate a passive diffusion step that allows a differentiation between the soluble compounds of high and low molecular weight (Hazell & Johnson, 1987).

In the case of calcium there is no agreement among the authors with respect to the relationship between the in vitro solubility and in vivo bioavailability of the element. Some authors (Pantako & Amiot, 1994) indicate that there is a relationship, whereas the contrary is reported by other authors (Schwartz et al., 1982; Wien & Schwartz, 1983; Zemel, 1984).

The aim of this study is to estimate the percentages of total soluble and dialysable calcium in human milk, cow milk and milk- and soy-based infant formulas in order to detect possible differences in the calcium bioavailability of the samples studied, and also to compare the results of the in vitro assays with the information available on in vivo calcium absorption.

* Corresponding author.

0308-8146/99/$—see front matter
PII: S0308-8146(98)00232-5

2. Material and methods

2.1. Apparatus

- Perkin–Elmer Model 2380 atomic absorption spectrophotometer fitted with a calcium hollow cathode lamp.
- Heraeus M1100/3 muffle furnace fitted with a Eurotherm 821 temperature programmer.

2.2. Material and reagents

The digestive enzymes and biliary salts were provided by Sigma Chemical Co. (St. Louis MO, USA). The working solutions of these enzymes were prepared immediately before use. The pepsin solution was obtained by dissolving 1.6 g of pepsin (P-7000, from porcine stomach) in 10 ml of HCl (0.1 M). The solution of pancreatin and biliary salts was prepared by dissolving 0.2g of pancreatin (P-1750, from porcine pancreas) and 1.25 g of salt biliary extract (B-8631 porcine) in 50ml of 0.1 M $NaHCO_3$.

The dialysis membranes, with a pore size (MMCO) of 10 000–12 000 Da (Visking 3—20/32″, 15.9 Medicell, London, UK), were boiled for 10 min in a solution of 0.01 M EDTA—Na_2, 2% $HNaCO_3$ and 0.1% sodium dodecylsulphate to remove trace element impurities. Then the membranes were rinsed five times with deionized water and boiled for 5–10 min with deionized water. The ready-to-use membranes were kept in a 20% ethanol solution in the refrigerator (4°C). They were rinsed several times with deionized water before use.

Calcium standard solutions were prepared immediately before use by dilution with deionized water of a standard solution of 1000 mg/litre (Titrisol, Merck). The lanthanum solution (5 g/100 ml) was prepared with La_2O_3 (Merck).

All reagents used were of reagent grade, and Millipore–MilliQ distilled deionized water was used throughout.

Glass and polyethylene material was soaked in HNO_3 (sp. gr. 1.40) for 10 min and then rinsed three times with deionized water.

2.3. Samples

A pool of human milk (frozen immediately after sampling) provided by lactating women of the area, whole cow milk and milk- and soy-based infant formulas, that had not been supplemented with minerals and vitamins, were provided by a manufacturer of infant formulas.

2.4. In vitro digestion

The Miller et al. (1981) in vitro method with slight modifications (Luten et al., 1996) was applied to human milk (95 g), cow milk (95 g) and non-supplemented milk and soy-based infant formulas (10 g).

2.5. Calcium determination

Calcium was measured by flame atomic absorption spectroscopy (FAAS). Instrumental conditions: $\lambda = 422.8$ nm, slit = 0.7 nm, acetylene flow = 1.75 litre/min, air flow= 14 litre/min, nebulizer = spoiler. Determinations were carried out directly in the dialysate obtained by applying the in vitro method, and also in human and cow milks and non-supplemented infant formulas, after mineralization of the organic matter. In all cases lanthanum was added to the solutions to obtain a content of 0.1% (w/v).

2.6. Dry ashing

Two grammes of human milk, 10 g of cow milk and 2 g of non-supplemented infant formulas were ashed in a muffle furnace at 450°C for 48 h (temperature was slowly increased at a rate of 50°C/h). After cooling, the residue was dissolved with 0.4 ml of HCl (sp. gr. 1.19) and water to 10 ml.

2.7. Estimation of dialysis and solubility percentages

The dialysis percentage is calculated as follows: $\%\text{dialysis} = 100 \times D/C$, where: D = calcium dialysate (mg Ca /100 ml sample), and C is the total calcium content of the sample (mg Ca /100 ml sample).

Estimating the percentage of soluble calcium implies splitting the in vitro gastrointestinal digestion products (non dialysate fraction) by centrifugation at 3000 rpm at 4°C for 1 h. The sediment is rejected and the calcium content of the supernatant is measured (*Snd*). The soluble calcium percentage is calculated as follows: $\%S = [D + Snd/C] \times 100$, where D is the content of dialysate calcium (mg Ca/ 100 ml sample), *Snd* (mg Ca/100 ml sample) the calcium content of the supernatant obtained by centrifugation of the gastrointestinal digestion products, and C is the total Ca content of the sample (mg Ca /100 ml sample).

3. Results and discussion

The calcium dialysability values (D) obtained by applying the above-mentioned in vitro method and the soluble non-dialysate calcium contents (*Snd*) of the supernatant obtained by centrifugation of the intestinal digestion products are reported in Table 1. The sum of $D + Snd$ corresponds to the total soluble calcium. All values are also related to the total calcium content of the samples, and expressed as percentages.

Table 1
Calcium: total, dialysate, soluble non dialysate and total soluble contents[b]

Samples	Total content (mg/100 ml)[a]	Dialysate		Soluble non dialysate		Soluble total	
		(mg/100 ml)	(%)	(mg/100 ml)	(%)	(mg/100 ml)	(%)
Human milk	29.2 ± 1.4	3.98 ± 0.24	13.6 ± 0.8	16.3 ± 0.72	55.8 ± 2.5	20.3 ± 0.62	69.4 ± 2.0
Cow milk	98.0 ± 4.2	19.80 ± 1.34	20.2 ± 1.4	24.7 ± 0.93	25.1 ± 1.0	44.5 ± 1.86	45.3 ± 1.9
Milk based formula	42.3 ± 4.6	3.43 ± 0.36	8.1 ± 0.9	4.82 ± 0.43	11.4 ± 1.0	8.25 ± 0.53	19.5 ± 1.3
Soy based formula	4.1 ± 0.2	0.54 ± 0.05	13.0 ± 1.3	1.04 ± 0.06	25.2 ± 1.4	1.58 ± 0.09	38.2 ± 2.4

[a] Results expressed as mg calcium /100 ml are obtained considering that the product is reconstituted at 14%.
[b] Mean values ± standard deviation.

The results obtained show that the ranking of the analysed samples in terms of calcium bioavailability depends on the criterion used. When the dialysis percentage is used, the ranking is cow milk > human milk > soy-based formula > milk based formula, whereas if the solubility percentage is used, it is human milk > cow milk > soy-based formula > milk-based formula.

In all the samples the total soluble calcium contents are higher than the values corresponding to the dialysability (calcium dialysate). This means that some of the soluble calcium does not dialyse in the in vitro assay conditions applied.

The values of the dialysis percentages obtained in this study are similar to the ones reported for the same kinds of samples (human milk, milk- and soy-based infant formulas)—that is, 19.6 ± 2.1%, 6.7 ± 0.2% and 11.4± 1.0%, respectively,—by Shen et al. (1994) and Shen, Robberechet, Van Dael, and Deelstra (1995) when they applied the same in vitro method.

The soluble calcium content of cow milk estimated in this study is 45.3 ± 1.9%, which differs from 60.8 ± 9.2%, and of 23.5 ± 1.3%, reported by Kim and Zemel (1986) and Reykdal and Lee (1991), respectively. In both these studies the same method was applied but with slight modifications.

Keane et al. (1988) reported a higher calcium dialysis percentage in an isolate of soy proteins (66.8%) than in non fat dry milk (53.1%). The same is observed in our study: the calcium dialysis percentage of the soy based formula (13.0%) is higher than the one corresponding to the milk based formulas (8.1%). These differences are probably due to the different calcium contents of the two types of formulas, higher in milk-based formulas than in soy-based ones.

Reykdal and Lee (1991) measured the soluble and dialysable calcium of whole and skimmed cow milk, and of spinach. The dialysable (8.21 ± 0.17%) and soluble calcium percentages (23.5 ± 1.3%) of cow milk were lower than the values obtained in our study (see Table 1). However, the conditions of the assay are not the same. Reykdal and Lee adjusted the pH value to 6.5 before the intestinal digestion whereas, in our assay, the pH changed gradually during the intestinal digestion. There are also differences in the pore size of the dialysis membranes; they use 6000–8000 Da membranes and we use 10 000–12 000Da membranes. Reykdal and Lee concluded, from their study, that the dialysis method is more adequate than the solubility one to estimate the calcium bioavailability of non-skimmed milk products.

Sarria, Vaquero, and Navarro (1997) measured the total soluble and dialysate calcium of cow milk and of an infant formula by applying an in vitro method of bioavailability estimation, and obtained higher values in cow milk than in formulas. Their results agree with ours.

Solubility may be only one of several factors useful in predicting potential bioavailability (Brennan, Duncan, Wartofsky, Butler, & Wray, 1991). The soluble elements found in the supernatant after applying an in vitro method based on solubility are potentially available for absorption, but bioavailability depends, not only on physiological factors, but also on the element and the type of food (Crews et al., 1983). Therefore, although nearly all the calcium of human milk, cow milk and milk-based formulas is potentially available for absorption in the gastrointestinal tract, the amount of calcium actually absorbed is determined by physiological factors (Flynn, 1992).

In the case of calcium there is no agreement on the usefulness of the in vitro methods based on solubility for determining bioavailability. Some authors (Zemel, 1984; Schwartz & Nevins, 1989) have reported that the correlation between the in vitro solubility of calcium and the in vivo bioavailability of the element is not good. Zemel (1984) attributed the discrepancies between the two types of procedures to the formation of very stable soluble calcium complexes, which are not absorbed. However, later studies (Pantako & Amiot, 1994) reported a good correlation between the percentages of soluble calcium in diets with milk or soy proteins and the calcium absorption percentages in rats, estimated by means of porto-aortic differences ($r = 0.829 - 0.999$). They therefore concluded that solubility is the main indicator of potential calcium absorption in vivo.

It has been suggested that the ionic soluble calcium of gastrointestinal digestion could be a better indicator of

calcium bioavailability than total soluble calcium (Zemel, 1984). Kim and Zemel (1986) measured total soluble and ionic calcium in cow milk and spinach, and indicated that both values make it possible to establish qualitative differences in the bioavailability of calcium coming from different foods. Soluble ionic calcium seems to be the best indicator of the calcium bioavailability of foods having high contents of soluble chelating agents like EDTA and polyphosphates. Given that parallel in vivo assays were not carried out, conclusions as to which parameter best reflects the in vivo bioavailability of calcium cannot be drawn.

A review of the in vivo calcium absorption percentages reported by different authors show that the values for human milk in infants are 65% (Shaw, 1976), $67.2 \pm 3.6\%$ (Bronner et al., 1992) 76% (Hillman, Johnson, Lee, Vieira, & Yergey, 1993) and $61.3 \pm 22.7\%$ (Abrams, Wen, & Stuff, 1996) higher than those reported for cow milk in healthy women ($46.3 \pm 9.5\%$, Heaney, Weaver, Hinders, Martin, & Packard, 1993; $45.1 \pm 8.8\%$, Weaver, Heaney, Proulx, Hinders, & Packard, 1993). The ranking according to the value of calcium absorption is the same as the one obtained with calcium solubility percentages.

On the other hand, when calcium absorptions from human milk and infant formulas are compared, significantly lower values are reported for the latter (Bronner, Salle, Putet, Rigo, & Senterre, 1992; De Vizia, Fomon, Nelson, Edwards, & Ziegler, 1985; Hillman et al., 1993; Rudloff & Lönnerdal, 1990). In this case, the percentages of both, total soluble and dialysed calcium are lower in infant formulas than in human milk. It must be pointed out that, as reported by De Vizia et al. (1985) in a balance study in infants fed formulas differing only in their calcium content, the percentage of calcium absorption decreases when the intake of calcium increases.

Therefore, the difference in the calcium absorption percentages between human milk and infant formulas mentioned by different authors can be partially due to the differences in the calcium contents, because human milk has a lower calcium content than infant formulas.

In conclusion, both the calcium solubility and calcium dialysis percentages obtained by applying the in vitro digestion method make it possible to establish trends in the bioavailability of calcium from cow milk, human milk and infant formulas but, when it comes to ranking the products, there is better agreement between the in vivo percentages of calcium absorption and in vitro soluble calcium percentages than between the former and the dialysed calcium percentages.

Acknowledgements

This study is part of a project ALI 94-0224 financially supported by the CICYT (Spain).

References

Abrams, S. A., Wen, J., & Stuff, J. E. (1996). Absorption of calcium, zinc, and iron from breast milk by five- to seven-month-old infants. *Pediatr. Res., 39* (3), 384–390.

Brennan, M. J., Duncan, W. E., Wartofsky, L., Butler, V. M., & Wray, H. L. (1991). In vitro dissolution of calcium carbonate preparations. *Calcif. Tissue Int., 49*, 308–312.

Bronner, F., Salle, B. L., Putet, G., Rigo, J., & Senterre, J. (1992). Net calcium absorption in premature infants: results of 103 metabolic balance studies. *American Journal of Clinical Nutrition, 56* (6), 1037–1044.

Crews, H. M., Burrell, J. A., & McWeeny, D. J. (1983). Preliminary enzymolysis studies on trace element extractability from food. *Journal of Science of Food and Agriculture, 34*, 997–1004.

De Vizia, B., Fomon, S. J., Nelson, S. E., Edwards, B. E., & Ziegler, E. E. (1985). Effect of dietary calcium on metabolic balance of normal infants. *Pediatr. Res., 19*, 800–806.

Flynn, A. (1992). Minerals and trace elements in milk. *Adv. Food Nutr. Res., 36*, 209–252.

Hazell, T., & Johnson, I. T. (1987). In vitro estimation of iron availability from a range of plant foods: influence of phytate, ascorbate and citrate. *The British Journal of Nutrition, 57*, 223–233.

Heaney, R. P., Weaver, C. M., Hinders, S. M., Martin, B., & Packard, P. T. (1993). Absorbability of calcium from brassica vegetables: broccoli, bok choy, kale. *Journal of Food Science, 58* (6), 1378–1380.

Hillman, L. S., Johnson, L. S., Lee, D. Z., Vieira, N. E., & Yergey, A. L. (1993). Measurement of true absorption, endogenous fecal excretion, urinary excretion, and retention of calcium in term infants by using a dual-tracer, stable-isotope method. *J. Pediatr., 123*, 444–456.

Keane, L. A., Potter, N. N., & Sherbon, J. W. (1988). Estimation of calcium status in selected food systems. *Journal of Food Science, 53*, 1111–1112.

Kim, H., & Zemel, M. B. (1986). In vitro estimation of the potential bioavailability of calcium from sea mustard (*Undaria pinnatifida*), milk, and spinach under simulated normal and reduced gastric acid conditions. *Journal of Food Science, 51* (4), 957–959.

Luten, G., Crews, H., Flynn, A., Van Dael, P., Kastenmayer, P., Hurrell, R., Deelstra, H., Shen, L., Fairweather-tait, S., Hickson, K., Farre, R., Schlemmer, U., & Frohlich, W. (1996). Interlaboratory trial on the determination of the in vitro iron dialysability from food. *Journal of the Science of Food and Agriculture, 72*, 415–424.

Miller, D. D., Schricker, B. R., Rasmussen, R. R., & Van campen, D. (1981). An in vitro method for estimation of iron availability from meals. *American Journal of Clinical Nutrition, 34*, 2248–2256.

Minihane, A. M., Fox, T. E., & Fairweather-Tait, S. J. (1993). A continuous flow in vitro method to predict bioavailability of Fe from foods. In U. Schlemmer (Ed.), *Bioavailability '93. Nutritional, chemical and food processing implications of nutrient availability*, (pp. 175–179). Proceedings, part 2: Ettlingen. Karlsruhe: Bundesfoschunganstalt für Ernährung.

Nadeau, D. B., & Clydesdale, F. M. (1991). Effect of Zn concentration, Ca source, percent milkfat and homogenization on Ca, Fe and Zn solubility in wheat cereal systems under simulated gastrointestinal pH. *Journal of Food Science, 56* (1), 146–150.

Narasinga Rao, B. S., & Prabhavathi, T. (1978). An in vitro method for predicting the bioavailability of iron from foods. *American Journal of Clinical Nutrition, 31*, 169–175.

Pantako, T. O., & Amiot, J. (1994). Correlation between in vitro solubility and absorption in rat of calcium, magnesium and phosphorus from milk protein diets. *Sciences des Aliments, 14* (2), 139–158.

Reykdal, O., & Lee, K. (1991). Soluble, dialyzable and ionic calcium in raw and processed skim milk, whole milk and spinach. *Journal of Food Science, 56* (3), 864–866.

Rudloff, S., & Lönnerdal, B. (1990). Calcium retention from milk-based infant formulas, whey-hydrolysate formula, and human milk in weanling Rhesus monkeys. *Am. J. Dis. Child., 144*, 360–363.

Sarria, B., Vaquero, M. P., & Navarro, M. P. (1997). In vitro and in vivo studies on calcium bioavailability from milk and infant formula subjected to domestic microwave heating and conventional heating. In *Bioavailability '97*. Wageningen, The Netherlands: Book of abstracts.

Schnepf, M., & Madrick, T. (1991). The solubility of calcium from antiacid tablets, calcium supplements and fortified food products. *Nutr. Res., 11*, 961–970.

Schwartz, R., Belko, A. Z., & Wien, E. M. (1982). An in vitro system for measuring intrinsic dietary mineral exchangeability: Alternative to intrinsic isotopic labeling. *The Journal of Nutrition, 112*, 497–504.

Schwartz, R., & Nevins, P. (1989). Effects of phytate reduction, fat extraction, and level of calcium on calcium and zinc bioavailability. Compared in vitro and in vivo. *Biol. Trace Elem. Res., 19* (1–2), 93–106.

Shaw, J. C. L. (1976). Evidence for defective skeletal mineralization in low-birth weight infants: the absorption of calcium and fat. *Pediatrics, 57*, 16–25.

Shen, L., Luten, J., Robberecht, H., Bindels, J., & Deelstra, H. (1994). Modification of an in vitro method for estimating the bioavailability of zinc and calcium from foods. *Zeitschrift für Lebensmitteluntersuchung und Forschung, 199*, 442–445.

Shen, L., Robberecht, H., Van dael, P., & Deelstra, H. (1995). Estimation of the bioavailability of zinc and calcium from human, cow's, goat, and sheep milk by an in vitro method. *Biol. Trace Elem. Res., 49*, 107–118.

Weaver, C. M., Heaney, R. P., Proulx, W. R., Hinders, S. M., & Packard, P. T. (1993). Absorbability of calcium from common beans. *Journal of Food Science, 58* (6), 1401–1404.

Wien, E. M., & Schwartz, R. (1983). Comparison of in vitro and in vivo measurements of dietary Ca exchangeability and bioavailability. *The Journal of Nutrition, 113*, 388–393.

Wolters, M. G., Schreuder, H. A., Van den Heuvel, G., Van Lonkhuijsen, H. J., Hermus, R. J., & Voragen, A. G. (1993). A continuous in vitro method for estimation of the bioavailability of minerals and trace elements in foods: application to breads varying in phytic acid content. *Br. J. Nutr., 69* (3), 849–861.

Zemel, M. B. (1984). In vitro evaluation of the effects of ortho-, tripoly- and hexametaphosphate on zinc, iron and calcium bioavailability. *Journal of Food Science, 49*, 1562–1565.