The Mechanisms for Regulating Absorption of Fe Bis-Glycine Chelate and Fe-Ascorbate in Caco-2 Cells Are Similar[1]

Dora Inés Mazariegos, Fernando Pizarro, Manuel Olivares, Marco T. Nuñez* and Miguel Arredondo[2]

*Micronutrients Laboratory, Institute of Nutrition and Food Technology (INTA) and *Millenium Institute for Advanced Studies in Cell Biology and Biotechnology and Department of Biology, Faculty of Sciences, University of Chile, Santiago, Chile*

ABSTRACT Inorganic iron (Fe) absorption from the diet is controlled mainly in the intestinal tract where apical Fe uptake is inversely related to the Fe content in the enterocyte. Iron bis-glycine chelate is an iron compound that may be absorbed by a mechanism different from the regulated nonheme Fe pathway. Because Fe bis-glycine chelate is used increasingly as an Fe fortificant in foods, the critical question is whether this compound is a safe Fe supplement. We compared apical Fe uptake and transepithelial transport offered either as ^{59}Fe bis-glycine chelate or a ^{59}Fe-ascorbate (Fe-AA) complex in Caco-2 cells, as a model of human intestinal epithelia, grown in different Fe concentrations in the media (0.5, 5 and 20 μmol/L Fe). Apical Fe uptake from ^{59}Fe-AA and ^{59}Fe bis-glycine chelate did not differ nor did transepithelial transport rates. The rate of ^{59}Fe uptake decreased with increasing intracellular Fe concentration (*P* < 0.001), an indication of a common absorption regulatory mechanism. We also evaluated the effect of an excess of Fe (100 μmol/L) provided as Fe bis-glycine chelate or Fe-AA on the incorporation of 1 μmol/L ^{55}Fe-AA into Fe-replete Caco-2 cells. The inhibition of Fe bis-glycine chelate on the absorption of the extrinsic tag of ^{55}Fe-AA (87.5%) did not differ from that of Fe added as Fe-AA (86.8%). These results suggest that Fe derived from Fe bis-glycine chelate and Fe-AA have similar regulatory absorption mechanisms. J. Nutr. 134: 395–398, 2004.

KEY WORDS: • Fe absorption • bioavailability • Fe bis-glycine chelate • Caco-2 cells

Iron (Fe) deficiency is often associated with poor Fe bioavailability in foods, predominantly cereals, with high fiber and phytate content, which are inhibitors of Fe absorption (1,2). Fe fortification is a strategy employed in countries in which Fe deficiency anemia is still highly prevalent (3). Iron compounds used in food fortification should be readily available for absorption but they also should not induce organoleptic changes in the food vehicle chosen. Compared with ferrous sulfate, Fe offered as Fe bis-glycine chelate is better absorbed from maize-based Venezuelan breakfasts, even in the presence of polyphenols from coffee and tea (4) and from a whole maize–based porridge (5). However, Fe absorption from Fe bis-glycine chelate did not differ from that from ferrous sulfate when these compounds were added to a vegetable infant weaning food (6). The absorption of Fe bis-glycine chelate was the same as ferrous ascorbate (Fe-AA),[3] when given alone with water (7). Fe bis-glycine chelate appears to be at least as well absorbed as Fe-AA and it has certain advantages in stability and organoleptic properties.

Inorganic Fe is absorbed in the intestine by a process that includes solubilization in the acid milieu of the stomach, entrance to a common nonheme Fe pool, reduction of Fe^{3+} to Fe^{2+} and transport of Fe^{2+} into the absorptive duodenal cells. Fe absorption is a process regulated by the levels of intracellular Fe (8,9), through the transcriptional and translational regulation of the apical Fe divalent metal transporter 1 (DMT1) (10–12). Phytates and polyphenols decrease Fe bis-glycine chelate absorption (6). This observation supports the hypothesis of Fe bis-glycine chelate entering at least partially into the nonheme Fe pool, where it is subject to the formation of irreversible ligands with phytate, thus reducing its bioavailability. Another indirect observation supporting this hypothesis is the fact that Fe bis-glycine chelate absorption is regulated by Fe stores (13,14). Unregulated absorption of Fe bis-glycine chelate could cause Fe overload. Therefore, we investigated whether the Fe bis-glycine chelate has the same regulated pathway of absorption as Fe-AA in Caco-2 cell cultures.

MATERIALS AND METHODS

Cells. The Caco-2 cell line (American Type Collection, HTB-37, Rockville, MD) provides a model with which to study intestinal Fe absorption, metabolism and regulation (15) and has been used extensively as an in vitro model to estimate Fe bioavailability (16–19). Caco-2 cells were cultured in DME Medium (Gibco BRL, Carlsbad, CA), 10% fetal bovine serum (FBS), 10 kU/L penicillin/streptomycin and 25 mg/L fungizone. Cells were seeded in 25-cm^2 flasks and incubated at 37°C, 5% CO_2. After 1 wk, the cells were trypsinized, reseeded at a density of 1×10^5 cells/flask and cultured to reach 80% confluence. FBS, MOPS buffer pH 7.4 (mmol/L: 50 MOPS, 94 NaCl, 7.4 KCl, 0.74 $MgCl_2$, 1.5 $CaCl_2$ and 5 glucose) and PBS were filtered through Chelex-100 (Bio-Rad Laboratories, Hercules, CA) to minimize contamination from Fe (8). Fe concentration was measured in all solutions by atomic absorption spectrometry (Perkin Elmer, SIMAA 6100, Shelton, CT).

Intrinsic labeling of iron compounds. The day before the uptake experiment, the radioactive Fe compounds were prepared from $^{59}FeCl_3$/HCl or $^{55}FeCl_3$/HCl 0.5 mol/L (NEN, Life Science Products,

[1] Funded in part by grant 1981217 from Fondo Nacional de Ciencia y Tecnología, Chile.

[2] To whom correspondence should be addressed. E-mail: marredon@inta.cl.

[3] Abbreviations used: AA, ascorbate/ascorbic acid; DMT1, divalent metal transporter 1; FBS, fetal bovine serum; NTA, nitrilotriacetate.

Boston, MA). Fe concentration was estimated from the specific activity supplied by the producer and analyzed in 100 μmol/L Fe solutions. Unlabeled and radioactive stock of $FeCl_3$ solutions were combined in a Fe to ascorbic acid (AA) molar ratio of 1:2. ^{59}Fe bis-glycine chelate (100 μmol Fe/L; 3.7 kBq) and ^{55}Fe bis-glycine chelate (100 μmol Fe/L; 3.7 kBq) were prepared according to Olivares et al. (7). ^{59}Fe bis-glycine or ^{55}Fe bis-glycine chelate intrinsically labeled were diluted to 100 μmol/L Fe with 0.5 mol/L HCl, and then to 1 μmol/L with MOPS buffer.

Equilibrium Fe loading of Caco-2 cells. Caco-2 cells (1×10^5) from the cultures prepared as above were incubated for 7 d in Iscove's medium, 10% low-Fe FBS, supplemented with different Fe concentrations (0.5, 5 or 20 μmol/L ^{55}Fe-nitrilotriacetate (NTA) in a 1:2.2 Fe:NTA molar ratio or ^{55}Fe bis-glycine chelate). Equilibrium Fe loading was performed according to Arredondo et al. (8). Inserts were used when they attained stable resistance values between 240 and 280 $\Omega \cdot cm^2$. Inserts with $<240\ \Omega \cdot cm^2$ were discarded.

Uptake and transepithelial transports studies in Caco-2 cells. The day of the experiment, Caco-2 cells cultured in inserts as above, were incubated with 700 μL of MOPS and 50 μmol/L desferrioxamine on the basolateral side. Fe uptake was started by the addition of 200 μL of ^{59}Fe-AA or 1 μmol/L ^{59}Fe bis-glycine chelate in MOPS buffer in the apical side with incubation for 5, 15, 30 and 60 min at 37°C. The uptake was stopped by washing the inserts three times with ice-cold 1 mmol/L EDTA in PBS. Cell-associated radioactivity (apical ^{59}Fe uptake and intracellular ^{55}Fe content), apical and basolateral media and apical transport solutions were measured in a liquid scintillation dual channel counter (Beckman LS 5000 TD. Beckman Instruments, Fullerton, CA). Kinetic data were expressed as nmol Fe/(mg protein · h).

Competition studies of Fe bis-glycine chelate and Fe-ascorbate in Caco-2 cells. Caco-2 cells were seeded onto 24-well tissue culture plates (Costar, MA), as above for 10–12 d. Each well was washed with warmed MOPS buffer. Fe-AA (1 μmol/L), 100 μmol/L Fe-AA or 100 μmol/L Fe bis-glycine chelate uptake was started by the addition of 1 mL of transport solution to each well with incubation at 37°C. Uptake was stopped as above, and a cellular extract was prepared with lysis buffer (mmol/L: 10 Hepes, pH 7.5,3 $MgCl_3$, 40 KCl, 1 phenylmethylsulfonyl fluoride; 1 dithiothreitol, and 10 mg/L leupeptin, 0.5 mg/L aprotinin, 0.7 mg/L pepstatin A, 5% glycerol, 0.5% Triton X-100). Protein determination (20) and cell associated radioactivity were measured in the cellular extract.

Statistical analysis. Values in the text, table and figures are given as the mean ± SEM of three independent measures. Statistical analysis included two-way ANOVA for repeated measures (main effects tested were Fe concentrations and type of Fe compounds) and Sheffé's post-hoc test when the F-test was significant (Student's t test and Pearson correlations). Differences with $P < 0.05$ were considered significant. Statistical analysis was performed by the program SAS (SAS Online Doc 8.0, SAS Institute, Cary, NC).

RESULTS

Intracellular Fe content. Caco-2 cells grown in increasing concentrations of ^{55}Fe bis-glycine chelate or Fe-AA acquired more Fe when the Fe in culture media increased from 0.5 to 20 μmol/L. ($P < 0.001$; **Table 1**). The relationship between Fe in culture media and intracellular ^{55}Fe was linear ($r = 0.998$; $P < 0.01$), showing that incorporation of Fe into cells continues if available Fe is offered in the apical side. Iron uptake was significantly affected by the interaction between iron concentration and the type of ferrous compound ($P < 0.004$) but not by the type of ferrous compound per se.

Apical iron uptake by Caco-2 cells. In a comparison of the uptakes at different extracellular Fe concentrations, the apical uptake from Fe:AA and Fe bis-glycine chelate did not differ (**Fig. 1**). In both cases, uptake decreased as the intracellular Fe increased ($P < 0.05$). In both, there was an inverse relationship between Fe uptake and intracellular Fe ($r = -0.89$, $P < 0.03$).

TABLE 1

Intracellular ^{55}Fe content in Caco-2 cells cultured for 2 wk with different types and concentrations of radioactive Fe

Fe in the culture	Fe-ascorbate	Fe bis-glycine chelate
μmol/L	*nmol Fe/mg protein*	
0.5	0.051 ± 0.002[a]	0.049 ± 0.001[a]
5.0	0.516 ± 0.042[b]	0.577 ± 0.055[b]
20.0	2.771 ± 0.170[c]	2.568 ± 0.150[c]

[1] Values are means ± SEM, $n = 3$. Two-way ANOVA: iron concentration ($P < 0.001$), type of ferrous compound (NS), and interaction ($P < 0.004$). Means without a common letter differ, $P < 0.05$ (Sheffé post-hoc test).

Transepithelial iron transport by Caco-2 cells. Transepithelial transport rates were ~20% of the apical transport rates, consistent with the intracellular handling of Fe in these cells (**Fig. 2**). Transepithelial transport rates were faster in media with low intracellular Fe concentration and slower in media with high intracellular Fe concentration. At each media iron concentration, transepithelial transport rates did not differ between the two Fe sources. There was a negative correlation between intracellular Fe and transepithelial rates ($r = -0.85$; $P < 0.03$), for both Fe bis-glycine chelate and Fe-AA.

Competition of Fe bis-glycine chelate and Fe-AA for the absorption of ^{55}Fe-ascorbate. Unlabeled Fe, added as Fe bis-glycine chelate or Fe-AA, competed with the labeled pool for incorporation into the cells, as shown by a lower uptake of ^{55}Fe, at each time point measured (**Fig. 3**). When 1 μmol/L ^{55}Fe-AA was offered to the cell, the uptake rate [92.5 ± 2.5 nmol Fe/(mg protein · h)] was greater than the rates of 10.6 ± 1.1 and 13.4 ± 1.5 in the presence of Fe-AA and Fe bis-glycine chelate, respectively ($P < 0.01$). The inhibition of unlabeled Fe bis-glycine chelate on the absorption of the extrinsic tag of ^{55}Fe-AA (87.5%) did not differ from that of Fe added as Fe-AA (86.8%). The curves of Fe uptake for Fe bis-glycine chelate and Fe-AA were not different (Fig. 3).

DISCUSSION

This work was designed to compare Fe bis-glycine chelate and Fe:AA intestinal absorption using Caco-2 cells grown in bicameral inserts. This model allows us to eliminate the effect of some variables such as day-to-day and interindividual variations in gastric and intestinal processes, as well the effects related to interactions with food chelators. The apical Fe uptake from Fe bis-glycine chelate was similar to that of Fe-AA, when Caco-2 cells with similar intracellular Fe content were compared. The similarity of uptake rates suggests a common pathway of incorporation into the cell. One simple hypothesis would be that the Fe in the Fe-glycine complex is incorporated into the absorbable Fe iron pool and transported by DMT1. This seems a likely pathway because Fe is derived from Fe-AA chelate in a similar way.

Iron bis-glycine is well absorbed, as demonstrated by several absorption studies in humans. The bioavailability data give us an estimation of the total amount of Fe absorbed through the enterocyte and incorporated into RBC (4,21). However, absorption studies cannot give a mechanistic explanation of the process of absorption. Our hypothesis to explain the high bioavailability of the compound was that it represents a source of inorganic Fe, and as such, its absorption is regulated by

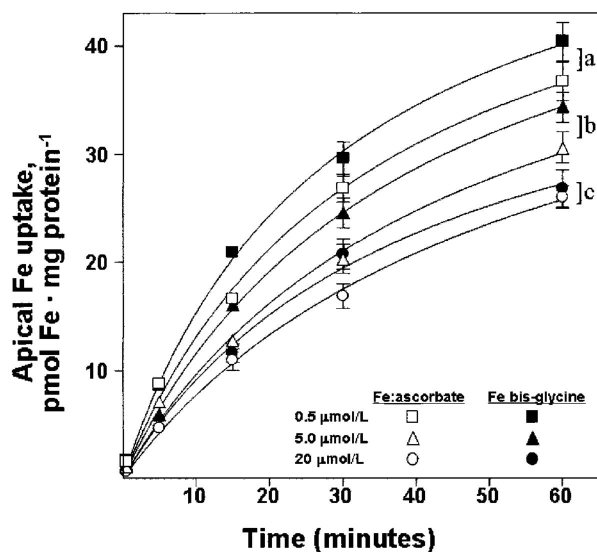

FIGURE 1 Apical ^{59}Fe uptake from Fe:AA and Fe bis-glycine chelate in Caco-2 cells grown in 0.5, 5 and 20 μmol/L Fe. Values are means ± SEM, $n = 3$. Means without a common letter differ, $P < 0.05$).

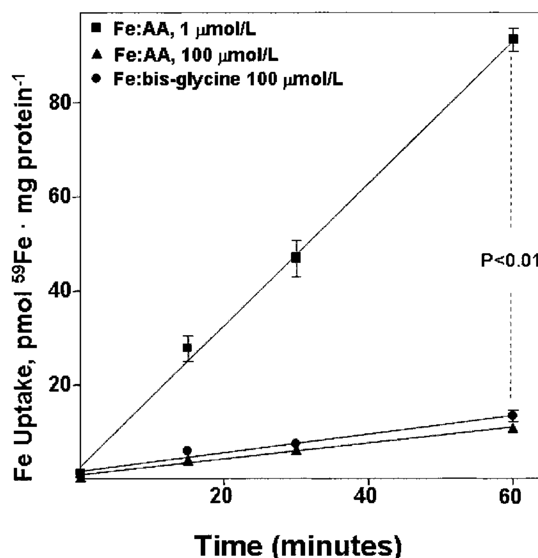

FIGURE 3 Competition of Fe bis-glycine chelate and Fe-AA for the absorption of 1 μmol/L ^{55}Fe-AA in Caco-2 cells. Values are mean ± SEM, $n = 3$.

corporal stores. This regulation was demonstrated in humans using a Fe bis-glycine chelate (7). We showed previously (22) that Fe bis-glycine competed for the absorption of ferrous sulfate. Human volunteers were given a labeled dose with 0.5 mg of nonheme Fe, and the absorption of this dose was measured in the same subjects with increasing amounts of unlabeled Fe given as Fe:AA or Fe bis-glycine chelate. The inhibition curves were similar, showing competition from both the nonheme Fe and from Fe bis-glycine chelate. Using this approach, we could not determine whether the competition between Fe bis-glycine chelate and nonheme Fe arose from a full exchange in the intestinal Fe pool or if some Fe escaped from the chelates as a result of hydrolysis.

We first demonstrated that the dose-dependent competition shown in human studies (22) was also observed in the cell model. A baseline level was set by exposing Caco-2 cells grown in normal culture medium to 1 μmol/L Fe-AA labeled with ^{55}Fe. Unlabeled Fe, added as Fe-AA, entered the common nonheme Fe pool and competed for the absorption of the labeled Fe. Fe bis-glycine chelate produced the same inhibiting effect. Thus, Fe bis-glycine chelate, presented intact to the brush border of the enterocyte, was as good a donor of nonheme Fe as Fe-AA. This suggests that Fe bis-glycine chelate also enters the common nonheme Fe pool, and as such, is taken by the cell through the same transporter. We do not yet understand where the Fe^{2+} breaks from the chelate. However, if the chelate behave similarly to nonheme Fe at the brush border, previous hydrolysis during digestion would not affect the degree of inhibition, as was previously described for the absorption in humans (22).

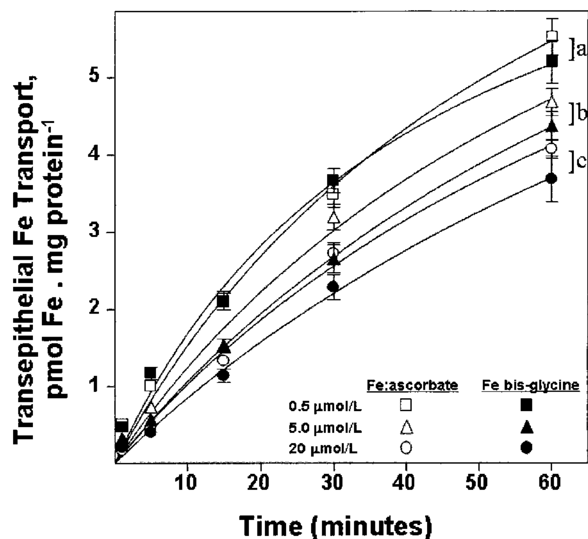

FIGURE 2 ^{59}Fe transepithelial transport from Fe:AA and Fe bis-glycine chelate and in Caco-2 cells grown in 0.5, 5 and 20 μmol/L Fe. Values are means ± SEM, $n = 3$. Means without a common letter differ, $P < 0.05$).

It is also worthwhile to note that in Fe-replete Caco-2 cells, more Fe was taken up by the cells as more Fe was offered in the apical medium. These cells retain a high capacity for Fe uptake; this points to an important role for them in the transepithelial transport because the regulatory mechanism that prevents Fe overload should include the inhibition of this pathway to lose excess Fe during the epithelial desquamation process. In this experiment, cells were exposed to a constant and high amount of Fe in the apical medium; thus, it is not possible to extrapolate to an in vivo situation. In that case, Fe absorption would clearly depend on other factors such as gastric transit time, solubility of the form of Fe and the presence of inhibitors.

We did not find differences between Fe:AA and Fe bis-glycine chelate uptake and transepithelial transport rates at various intracellular Fe concentrations. As we expected, apical uptake and transepithelial transport rates were higher in Fe-deficient cells and lower in Fe-sufficient and Fe-replete cells.

Better absorption of Fe bis-glycine chelate in diets with a high content of inhibitors can be explained by a protective effect of the glycine moiety, both chelating and protecting Fe from inhibitors of the diet, but keeping it soluble and readily available (7) for incorporation into the cell. Once liberated in the gut, despite different sources, the iron utilizes common pathways for absorption.

In summary, we presented strong evidence indicating that Fe bis-glycine chelate is absorbed as nonheme Fe. Fe bis-glycine chelate competes for the absorption of nonheme Fe from Fe-AA, and its uptake and transepithelial transport follow the same pattern as Fe-AA.

LITERATURE CITED

1. Hallberg, L., Rossander, L. & Skanberg, A. (1987) Phytates and the inhibitory effect of bran on iron absorption in man. Am. J. Clin. Nutr. 45: 988–996.
2. Reddy, M. B., Hurrell, R. F., Juillerat, M. & Cook, J. D. (1996) The influence of different protein sources on phytate inhibition of nonheme-iron absorption in humans. Am. J. Clin. Nutr. 63: 203–207.
3. Hurrell, R. (1997) Preventing iron deficiency through iron fortification. Nutr. Rev. 55: 210–222.
4. Layrisse, M., García-Casal, M. N., Solano, L., Barón, M., Arguello, F., Llovera, D., Ramírez, J., Leets, I. & Tropper, E. (2000) Iron bioavailability in humans from breakfasts enriched with iron bis-glycine chelate, phytates and polyphenols. J. Nutr. 130: 2195–2199.
5. Bovell-Benjamin, A., Viteri, F. & Allen, L. (2000) Iron absorption from ferrous bis-glycine and ferric trisglycine in whole maize is regulated by iron status. Am. J. Clin. Nutr. 71: 1563–1569.
6. Fox, T., Eagles, J. & Fairweather-Tait, S. (1998) Bioavailability of iron glycine as a fortificant in infant foods. Am. J. Clin. Nutr. 67: 664–668.
7. Olivares, M., Pizarro, F., Pineda, O., Name, J., Hertrampf, E. & Walter, T. (1997) Milk inhibits and ascorbic acid favours ferrous bis-glycine bioavailability in humans. J. Nutr. 127: 1407–1411.
8. Arredondo, M., Orellana, A., Gárate, M. & Núñez, M. T. (1997) Intracellular iron regulates iron absorption and IRP activity in intestinal epithelial (Caco-2) cells. Am. J. Physiol. 273: G275–G280.
9. Gárate, M. & Núñez, M. T. (2000) Overexpression of the ferritin iron-responsive element decreases the labile iron pool and abolishes the regulation of iron absorption by intestinal epithelial (Caco-2) cells. J. Biol. Chem. 275: 1651–1655.
10. Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Golan, J. L. & Hediger, M. A. (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature (Lond.) 388: 482–488.
11. Fleming, M. D., Trenor, C. C., Su, M. A., Foernzler, D., Beier, D. R., Dietrich, W. F. & Andrews, N. (1997) Microcytic anemia mice have a mutation in Nramp2, a candidate iron transporter gene. Nat. Genet. 16: 383–386.
12. Hubert, N. & Hentze, M. (2002) Previously uncharacterized isoforms of divalent metal transporter (DMT)-1: implications for regulation and cellular function. Proc. Natl. Acad. Sci. U.S.A. 99: 12345–12350.
13. Ashmead, H. (1999) Bioavailability of iron glycine [Letter]. Am. J. Clin. Nutr. 69: 737–738.
14. Fox, T. (1999) Reply to H. D. Ashmead (Bioavailability of iron glycine) [Letter]. Am. J. Clin. Nutr. 69: 738.
15. Alvarez-Hernández, X., Nichols, G. & Glass, J. (1991) Caco-2 cell line: a system for studying intestinal iron transport across epithelial cell monolayers. Biochim. Biophys. Acta 110: 205–208.
16. Glahn, R., Wien, E., Van Campen, D. & Miller, D. (1996) Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: use of a novel in vitro method for rapid estimation of iron availability. J. Nutr. 126: 332–339.
17. Glahn, R., Lai, C., Hsu, J., Thompson, J., Guo, M. & Van Campen, D. (1998) Decreased citrate improves iron availability from infant formula: application of an in vitro digestion/Caco-2 cell culture model. J. Nutr. 128: 257–264.
18. Tapia, V., Arredondo, M. & Nuñez, M. T. (1996) Regulation of Iron absorption by cultured intestinal epithelia (Caco-2) cell monolayer with varied Fe status. Am. J. Physiol. 271: G443–G447.
19. Flanagan, P. (1990) Mechanisms and regulation of intestinal uptake and transfer of iron. Acta Pediatr. Scan. Suppl. 361: 21–30.
20. Lowry, O., Rosebrough, N. & Farr, A. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
21. Garcia-Casal, M. & Layresse, M. (2001) The effect of change in pH on the solubility of iron bis-glycinate chelate and other iron compounds. Arch. Latinoam. Nutr. 51: 35–36.
22. Pizarro, F, Olivares, M, Hertrampf, E, Mazariegos, D. I., Arredondo, M., Letelier, A. & Gidi, V. (2002) Iron bis-glycine chelate competes for the nonheme-iron absorption pathway. Am. J. Clin. Nutr. 76: 577–581.