



Original

Bioavailability of iron measurement in two nutrients multiple solutions by *in vitro* and *in vivo*; a comparative methodology between methods

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Abstract

Objetivos: The bioavailability of dietary iron present in a nutritional formulation may be evaluated by *in vitro* and *in vivo* methods since they provide for a cohesive line study and provided in the literature. The aim of this study was to evaluate the bioavailability of iron targeting a comparative analysis of two nutritional supplement formulations (A and B).

Methods: For this study were using *in vitro* and *in vivo* methods, both described in the literature for availability of iron in an enteral feeding after ingestion supplement nutrition with much nutrients.

Results: The results obtained by *in vitro* simulation of the human gastrointestinal tract were 0.70 ± 0.02 and 0.80 ± 0.01 % iron availability by formulations A and B. *In vivo* studies, as measured by the curves of serum iron in humans after ingestion of formulations allowed the calculation of coefficient of variation $\Delta < 0$, indicating that there was a low absorption of iron. The bioavailability of iron as two multi-nutrients solutions obtained by *in vitro* and *in vivo* showed that there were comparisons of those methodologies used in this study.

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Key words: *Bioavailability. Iron. In vitro. In vivo.*

BIODISPONIBILIDAD DE HIERRO EN DOS SOLUCIONES NUTRITIVAS ESTUDIADAS POR *IN VITRO* Y *IN VIVO*; UNA COMPARACIÓN ENTRE DOS MÉTODOS

Abstract

Objetivos: La biodisponibilidad de hierro presente en una formulación nutricional puede ser evaluada por *in vitro* y *in vivo*, ya que proporcionan para un estudio de línea cohesiva y proporcionado en la literatura. El objetivo de estudio fue evaluar la biodisponibilidad de hierro con *in vitro* y *in vivo*, dirigida a un análisis comparativo de dos formulaciones de suplementos nutricionales (A y B).

Métodos: Fueron utilizados dos métodos descritos en la literatura que para evaluar la biodisponibilidad de hierro. Uno que es la simulación de digestión humana y otro por los niveles de hierro sérico después de la ingestión de la formulación en los seres humanos.

Resultados: Los resultados obtenidos por la simulación *in vitro* de la digestión del tracto gastrointestinal humano fueron $0,70 \pm 0,02$ y $0,80$ dialisibilidad $0,01$ % de hierro, respectivamente, para las formulaciones A y B. Los estudios *in vivo*, según se mide por las curvas de hierro en suero en seres humanos después de la ingestión de las formulaciones mostró coeficiente de variación $\Delta < 0$, lo que indica que había una baja absorción de hierro. La biodisponibilidad de hierro a los dos multi-nutrientes soluciones fueron obtenidos por *in vitro* y *in vivo* mostraron que había una comparación de las metodologías utilizadas en soluciones acuosas de muchos nutrientes.

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Palabras clave: *Biodisponibilidad. Hierro. In vitro. In vivo.*

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Introduction

The bioavailability of iron in many nutrient solutions can be studied by *in vitro* and *in vivo* methods for estimated on iron absorption. *In vitro* methods are relatively simple, rapid and inexpensive and can simulating the digestion gastric and duodenal, followed by dialysis. The proportion of the element diffused through the semi permeable membrane during the process, is the dialysability element after an equilibration period, being used as an estimate of nutrient bioavailability¹⁻³.

Luten et al³. in a collaborative study to compare the methods using *in vitro* and *in vivo* to assess the absorption of nonheme iron, found a statistically significant correlation indicating that the results obtained using the method *in vitro* can be extrapolated to humans. Chipionkar et al⁴. and Narasinga Rao⁵ studying different types of diets and food for the purpose of measuring the iron dialysability components at different concentrations in order to test methods *in vitro* and *in vivo* and have shown a correlation of $r = 0.94$, suggesting the data was reflected nonheme iron absorption in humans.

Conway et al⁶. proposed the serum iron curves obtained after ingestion of food or multiple formulations containing nutrients to be used to assess dietary iron absorption in humans. Conway et al⁶. and Hoppe et al⁷. showed good correlation between the method of the study the area of the curves of iron and serum stable isotopes technique for iron to check on iron absorption at food and it is possible to analyze the absorption and circulation of iron in humans.

In previous studies in our group whose experimental designs were similar to that used in this study showed response of serum iron levels after administration of iron sodium EDTA (NaFeEDTA)⁸ and iron *bis*-glycine chelate⁹, in healthy volunteers. Rosa¹⁰ was observed the iron absorption in obese patients after ingesting 15 mg of elemental iron by ferrous sulfate before and after bariatric surgery.

The objective was to evaluate the bioavailability of iron as two multi-nutrient solutions by *in vitro* simulation of the human gastrointestinal tract and *in vivo* through the response curve of serum iron level by means of a delta (Δ) of variation serum levels of mineral obtained after intake of the formulations A and B in healthy volunteers and obeses, in order to compare the methods *in vitro* and *in vivo* to evaluate the availability of iron absorption in nutritional formulations.

Methodology

Materials and methods

Preparation and Composition of the Nutritional Supplement Formulations

We prepared two multiples nutrient formulation that would reproduce the nutrient composition of products

used for oral supplementation or polymeric enteral diet (table I). All components (protein soy isolate, malt dextrin, canola and corn oils, soy lecithin, partially hydrolyzed guar gum, and a mixture of minerals and vitamins) used to prepare the formulation were purchased. In parallel, we prepared an aqueous ferrous sulphate solution containing 25 mg elemental iron to which the following nutrients were later added: partially hydrolyzed guar gum (25 g); salt mixture (3 g); vitamin mixture (10 g); calcium (800 mg); and vitamin C (135 mg). A total volume of 250 mL and an iron concentration of 25 mg were kept constant regardless of the nutritional composition of the enteral formula or aqueous iron solution.

We prepared two multiples nutrients formulations A e B described by Bueno¹¹ that would reproduce the nutrient composition of products used for oral supplementation or polymeric enteral diet (table II). All components (protein soy isolate, malt dextrin, canola and corn oils, medium-chain-triglycerides (MCTs), soy lecithin, partially hydrolyzed guar gum, and a mixture of minerals and vitamins) used to prepare those formulations were purchased. Regardless of the nutritional composition of the formulations, the iron concentration was maintained constant in all the formulations tested

Table I
Nutritional composition of a multiple nutrient solution A and B

Nutrient Sources and composition	Formulation		Bueno
	A	B	
Protein (g)			
Soy Protein isolate	3.10	3.10	13.34
Carbohydrate (g)			
Malt dextrin	63.10	64.10	59.12
Lipid (g)			
MCT	4.50		
Corn oil		1.00	7.31
Canola oil		3.50	7.74
Soy lecithin	3.00	3.00	1.30
Mineral (g)			
Salt mixture	4.00	3.00	2.15
Fe (mg)	25.00	25.00	2.50
Ca (mg)	1000.00	800.00	80.00
Vitamin (g)			
Vitamin mixture	1.00	1.00	4.30
Fiber (g)			
Partially hydrolyzed guar gum	25.00	25.00	4.30
Total (g)	100.00	100.00	100.00

Salt mixture (4/3g): Mg (15 mg), P (75 mg), K (90 mg), Zn (0.50 mg), I (0.09 mg), Mn (0.11 mg), Cu (0.08 mg), Na (60 mg). Vitamin mixture (1 g): Vitamina A (500 µgRE), Vitamina D (4µg), Vitamina E (8 mg TE), Vitamina K (40 µg), Vitamina B₁ (1 mg), Vitamina B (1 mg), Niacina (10 mg), Ácido Pantotênico (5 mg), Vitamina B₆ (1.5 mg), Ácido Fólico (150 µg), Vitamina B₁₂ (0.5 µg), Biotina (120 µg), Vitamina C (50 mg)

Table II
Different composition by supplement nutrition formulation for this protocol research

Formulations	Partially hydrolyzed guar gum (g)	Salt mixture (g)	Vitamin mixture	Calcium (mg)	Vitamin C (mg)	MCT
Aqueous iron solution	–	–	–	–	–	
Aqueous iron solution + guar goma	25	–	–	–	–	
Solução aquosa de ferro + mistura salina		3				
Aqueous iron solution + salt mixture			1			
Aqueous iron solution + calcium carbonate (A/B)				1000/800		
Aqueous iron solution + vitamin C					135	
Formulation A	25	3	1	1000	50	4,5
Formulation B	25	3	1	800	50	

(25 mg ferrous sulfate). The quantities of nutrients were changed to demarcate the possible effects of calcium, fiber and MCTs on iron availability. A pot of each formulation was selected to be applied *in vitro* in the same manner as formulations A and B were prepared to be given to the study *in vivo*.

Patients

The study was conducted on twenty two volunteers of both genders aged 18 to 50 years and eutrophic (n = 7) and obeses (n = 15) the Center for the Treatment of Bariatric Surgery of the Discipline of Nutrology, University Hospital, Faculty of Medicine of Ribeirão Preto (HCFMRP). The study was approved by the Research Ethics Committee of the Hospital, and the data were collected from November 2007 to December 2008.

Inclusion Criteria: Adult subjects aged 18 to 50 years with no diseases potentially interfering with absorptive capacity and giving informed consent to participate.

Exclusion Criteria: Adult with anemia (hemoglobin level of less than 10.0 mg/dL), with chronic renal insufficiency, alcoholism, intestinal parasitic diseases, diabetes, and chronic diarrhea were excluded from the study.

Experimental design

Those formulations was prepared at the Hospital Pharmacy of HCFMRP and placed in sealed pots containing 100 g powder. For use in the Metabolic Unit of HCFMRP, 100 g powder was diluted in 200 mL fil-

tered water, transferred to a pot with a lid and stored in the refrigerator for 12 hours. The experimental assays involving the research subjects were started in the morning. The formulation of the nutritional supplement provided 25.0 mg elemental iron from heptahydrated ferrous sulfate, and its interaction with 800 mg calcium and 25 g fiber was determined. Those formulations contained 38 additional nutrients for simulation a normal meal in nutrients, whose quality and quantity are listed in table I.

The formulation A and B differ about lipids and A was canola and corn oils, long-chain triglycerides (FNA - MCL) and B medium-chain triglycerides purified (FNB-MCT). Water intake was permitted throughout the experiment. After an overnight fast of 12 hours, a blood sample was collected from each subject. Blood samples were obtained at 0, 1, 2, 3 and 4 hours^{5,12}.

Digestion and dialysability of the samples

The *in vitro* bioavailability of iron in the samples was determined by the method of Miller et al². and modified by Luten et al³. For the simulation of the digestive process, a 250mL sample of the multiple nutrient formulations was homogenized and 6 N HCl was added until a pH value of 2 was reached. Five 20g aliquots were separated and pepsin was added at the proportion of 0.125 g/g protein. The solution was incubated at 37 °C in a water bath with shaking for 2 h. Finally, titration with 0.5 N KOH was performed up to pH 7. A sodium bicarbonate solution was prepared and added to the dialysis tube until pH 5 was reached after 30 min under constant shaking. The pancreatin-bile solution was then prepared at the proportion of 25 mg

pancreatin/g protein in the sample and of 0.4 g pancreatin/2.5 g bile extract. The pancreatin-bile solution (4 mL) was added to 3 beakers containing 20 g of the digest and the mixture was incubated in a water bath with shaking for 2 h.

The process was finalized by removing the dialysis tubes from the solutions and the content of the beakers was transferred to a 25 mL volumetric round-bottom flask and reconstituted to its final volume with deionized water. For the samples of aqueous solutions containing 25.0 mg iron, only pH control was performed by acidification and neutralization with the reagents used in the method, without the addition of digestive enzymes.

For the evaluation of iron dialysability, 20 g of the digest or of the aqueous solutions was placed in a beaker together with the dialysis tube previously hydrated in deionized water for 10 min and filled with 25 mL of NaHCO₃ solution. The flasks were covered and kept in a water bath at 37 °C with shaking for 30 min. Four mL of the bile-pancreatin suspension was added to each flask and incubation was continued for 2 additional hours. At the end of the incubation period the dialyzed content was transferred to volumetric balloons and deionized water was added to complete the volume to 25 mL, followed by storage in a freezer at ~20 °C until the time for reading.

Determination of total and dialyzed iron

For the determination of total iron in the aqueous solutions and in the various formulations tested, 2 g samples were obtained and digested with nitric acid (HNO₃) and hydrogen peroxide (H₂O₂) at a 5:1 proportion at 100 °C in a block digester (Pyrotec®). The material was diluted with deionized water in a 50 mL round-bottom flask. The analyses were performed using a Shimadzu® atomic absorption spectrophotometer model AA 6200 (Shimadzu Corporation, Tokio, Japan) with an air/acetylene oxidant under the following conditions: hollow cathode lamp, 248.3 wavelength for iron and a 0.2 nm slit. The solutions for the standard iron curve were prepared with Tritisol ferric chloride (Merck -9972) at concentrations of 0.5, 2.0, and 4.0 µgFe/mL. All determinations were carried out in triplicate and data are reported as means ± SD.

Iron dialyzability was estimated as the proportion of dialyzed iron in relation to iron concentration at the beginning of the *in vitro* digestion process after a period of equilibrium through the dialysis membrane.

Determination of Serum Iron Levels after the Ingestion of the Nutritional Supplements Formulations

Samples collected at time 0, 1, 2, 3 and 4 hours were spun to separate serum and red blood cells were immediately discarded. Serum samples were placed in dem-

ineralized Eppendorf tubes and stored frozen at -20 °C until the time for analysis. Iron concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS) in the DRC mode according to the method of Palmer et al¹³, with the samples being diluted 1:20 with 0.5 % HNO₃ diluent (v/v) + 0.005 % TRITON X-100 (v/v).

Readings were then obtained with a Perkin Elmer ELAN DRC PLUS instrument equipped with a cyclonic chamber and coupled to a Meinhard nebulizer under conditions of optimization of gas flow of 0.60 mL/min, lens voltage of 6.00 A and radiofrequency power of 1100.00 W.

Determining of delta variations (Δ) to the levels of serum iron obtained in the volunteers

Variations in the time intervals between serum iron levels were made to measure of the iron absorption in volunteers. All intervals of time were found in a number of variations measured 10, called delta (Δ) or coefficient of variation between serum iron and the time that were taken these values. Were made $\sum \Delta_{1-10}$, $X = \sum \Delta_{1-10} / N$ and the classification was $\Delta < 0$ without absorption and $\Delta > 0$ with absorption.

Statistical analysis

Descriptive analysis of experimental data *in vitro* and *in vivo* was made with means and standard deviations. For *in vivo* testing was considered the ratio of the sum of serum levels iron in volunteers. Those spreadsheets were tabulated in EXCEL program.

Results

Studies of these formulations analyzed by *in vitro*, the scope of the methodology is to show the solubility of the chemical binding molecules according to their affinity for electrons, resulting in 0.70 ± 0.02 and 0.80 ± 0.01% of iron dialysability respectively, for the formulations A and B. In an aqueous solution on 25 mg of iron was showed 70 ± 6%. In the same solution in which iron has been added ascorbic acid had increased to 90 ± 3% of iron availability, confirming the positive effect of iron absorption by the method to describe in simulation of the human gut condition. Fibers in an aqueous solution of iron the value of dialysability of iron was showed 1.00 ± 0.01%. This showed that fiber has a binding affinity for hydrogen atoms and due to it is low activation energy and fibers are not capable of forming organ metallic complexes.

Different calcium concentration 800 and 1000 mg/L the low iron dialysability for 0.80 ± 0.01% and 1.30 ± 0.02% showing interaction between calcium and iron in an availability of iron (table III).

Table III
Means and Standard Deviation of percent iron in a pure aqueous solution or in the same solution after addition of individual components and the supplement nutrition formulations A and B

Formulations	Dialysability of iron (%)
Aqueous iron solution	70.00 ± 6.00
Aqueous iron solution + guar goma	1.00 ± 0.01
Aqueous iron solution + salt mixture	2.00 ± 0.06
Aqueous iron solution + vitamin mixture	25.00 ± 0.12
Aqueous iron solution + calcium (A)	0.80 ± 0.02
Aqueous iron solution + calcium (B)	0.70 ± 0.02
Aqueous iron solution + vitamin C	90.00 ± 3.00
Formulation A	0.70 ± 0.02
Formulation B	0.80 ± 0.01
Formulation was described by Bueno ¹¹	7.00 ± 0.40

The results obtained by *in vivo* assays shown by the sum of the variances between the experimental period and level of serum iron measured in the volunteers noted that there was poor absorption of iron by the ingestion of the formulation (table IV). Comparing with the results observed *in vitro* and *in vivo* inhibitory effects of nutrients influencing the bioavailability of iron were potentiated in humans especially because the quantity of fiber and calcium in the formulation.

Discussion

Van Dyck et al¹⁴. studied the influence of the nutritional components of multiple supplement nutrition formulations by iron dialysability and concluded that the fibers are interfering negative because the presence of phytate and calcium in the fibers components. Azevedo¹⁵ showed that the proportion of calcium and iron ranging from 50:1 to 60:1 and the components of the fibers negative strongly influence of iron

dialysability in many formulations of enteral nutrition. The percentages in these formulations of iron dialyzed was 2.34 to 9.67%. These parameters of iron dialysability were classified by low iron availability to < 5%, mean availability 5-8% and good availability > 8%. Bueno¹¹ showed the solution enteral formulation should contain 10 g/ L of fiber, 0 (zero) of MCT and 320 mg / L of calcium and keeping the amount of 10 mg / L iron from ferrous sulfate, to provide a dialysability of 7% iron bioavailability was estimated by mathematical modeling of the ingested amount corresponding to 0.7 mg / L.

Fibers have been added to the formulations of nutritional supplements because of their functional characteristics and benefits for the human organism^{16,17}. On the other hand, studies of the action of fibers on the bioavailability of minerals have demonstrated that these components interfere with the absorption of iron, zinc, copper, calcium and magnesium^{5,17,18}. Gupta et al¹⁹. to assess the bioavailability of calcium and iron in leafy vegetables, by *in vitro* dialysis concluded that the components present in the chemical structure such as food fibers, oxalate, phytic acid and tannins are the primary interfering bioavailability of iron.

Minerals bioavailability was measured by the habitual consumption of foods such as wheat, rice, corn and soy and in a study of the Chinese population showed that the amounts of phytate and fiber in these foods enabled the formation of insoluble compounds that decreased the iron bioavailability²⁰. In cereals, fortified or not, the interaction of iron absorption was reduced in the presence of fibers and other types of foods such as coffee and milk, probability of presence that caffeine and calcium²¹. Kapsokefalou and Miller²² to compare the solubility and dialysability of iron sources (pyrophosphate, 2-glycinate, glutamate, lactate and ferrous sulfate) in samples of milk prior to addition of ascorbic acid they authors can observed that infants products with lower amounts of calcium and total protein in their composition showed an availability of iron

Table IV
Variation of Δ s and somatory by levels serum iron were obtained by *in vitro* and *in vivo* methods in the volunteers after ingestion of A and B supplement nutrition formulations

Variation Δ	n	Sum of Serum Iron Levels ($\mu\text{g/dL}$)	Means of Serum Iron Levels ($\mu\text{g/dL}$)	Standard Deviation ($\mu\text{g/dL}$)
Δ_1 (J-1H)	22	-824.60	-37.50	89.80
Δ_2 (J-2H)	22	-775.10	-35.20	124.90
Δ_3 (J-3H)	22	-831.35	-38.00	122.80
Δ_4 (J-4H)	22	-861.70	-39.20	116.20
Δ_5 (2H-1H)	22	49.50	2.20	63.60
Δ_6 (3H-1H)	22	-11.50	-0.52	58.80
Δ_7 (4H-1H)	22	-37.00	-1.70	66.00
Δ_8 (3H-2H)	22	-61.00	-2.80	60.30
Δ_9 (4H-2H)	22	-86.60	-4.00	77.80
Δ_{10} (4H-3H)	22	-25.50	-1.20	50.00

around 62% higher than the milk suitable for older children.

Velasco-Reynold et al²³. have been showed the mean dialysability of magnesium found in duplicate in hospital meals (daily, lunch, dinner) was 13.2% per meal. The dark green vegetables and vegetables in general are primary sources of bioavailable magnesium in daily diet. The magnesium dialysabilities were significantly influenced only by dialysable calcium, magnesium, zinc, chromium and iron fractions. Consequently, important similarities in the magnesium and calcium in foods and behaviours as well as meals in their absorptive processes exist. The fibre content of duplicate meals did not influence the dialysable calcium fraction and calcium dialysabilities. Dietary fat positively affects perhaps the calcium absorption by the chelating action of fatty acids. Only total magnesium and dialysable magnesium levels and magnesium dialysabilities significantly influenced on dialysable calcium fractions.

The partially hydrolyzed guar gum are important for the production of short chain fatty acids in humans gut and to provide supply energy to the body²⁴ and were selected to be included in the nutritional formulation for maintain their characteristics without altering the viscosity, the solution solubility and can be used in drinks²⁵ and nutritional enteral and supplement nutrition formulations¹¹.

Yoon et al²⁵. discussed the possibility of fiber acting on the human gastrointestinal tract by causing changes in the utilization of nutrients and showed that greater amounts of fiber (> 20 g/day) can affect the bioavailability of minerals. The supplements studied here contained 25 g fiber that may have represented a factor capable of reducing iron absorption.

By studying the interactions of Fe²⁺, Ca²⁺ and Fe³⁺ in the formulation of enteral nutrition by *in vitro* methods in different concentrations of soluble fiber, insoluble fiber and different pHs, simulating physiological different conditions, observed that high amounts of fiber and physical-chemical unsuitable can lead to poor availability of iron²³⁻²⁶. Cook, Dassenko and Whittaker²⁷ assessed the effect of calcium salts commonly used as supplements on iron absorption when administered during the interval between meals and observed that calcium carbonate at the dose of 600 mg did not inhibit the absorption of ferrous sulfate (18 mg), at an iron/calcium proportion of 1:33). When the same assays were repeated using citrate and phosphate salts as a source of calcium at the same concentrations, iron absorption was reduced to 44% and 62%, respectively, showing that the type of salts used can also affect the bioavailability of minerals. Reddy and Cook²⁸ observed that different iron/calcium proportions (above 1:40) and the types of salt sources of the minerals interfere with the bioavailability of iron.

Those MCTs were the nutrient present in the formulation A and absent in the formulation B in agreement with the results showed by Bueno¹¹. They are not stored

in liver and adipose tissue and were used quickly, in conjunction with glucose as energy source for the organism. No need of action with plasma albumin, in cellular metabolism or transport by carnitine when activated in the mitochondria for oxidation²⁹⁻³⁰ and were showed not interference by an iron availability or absorption.

Numerous interactions exist between the different trace elements affecting absorption via the gastrointestinal tract. Factors affecting bioavailability of trace elements include the actual chemical form of the nutrient (eg, organic form of iron is better absorbed than the ionic form), antagonistic ligands (eg, zinc absorption is decreased by phytate and fiber; iron absorption is decreased by fiber), facilitatory ligands (eg, zinc absorption is aided by citric acid or iron absorption is increase by amino acids or fermented products), and competitive interactions (eg, iron depresses the absorption of copper, and zinc; zinc depresses copper absorption and vice versa)³¹.

The bioavailability of iron by *in vitro* and *in vivo* methods by multiple supplement nutrition formulations showed that comparison between these methodologies and by the low iron availability and absorption in humans. Studies aimed at the optimization of iron in nutritional formulations should include *in vitro* methods followed by an assessment of iron absorption *in vivo* in order to better investigate their metabolic behavior.

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