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BIOAVAILABILITY, BIODISTRIBUTION AND TOXICITY OF BIOCAL™* A NEW CALCIUM SOURCE. COMPARATIVE STUDIES IN RATS

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ABSTRACT

The purpose of this study is to determine the bioavailability, biodistribution and toxicity of Biocal™, a new calcium source. Biocal™ is a calcium gluconate stabilized with glycine. A comparative study of this compound versus calcium gluconate was performed in Sprague-Dawley rats. Bioavailability studies were carried out by the labeling of both compounds with ^{45}Ca. We administered a dose of 30 mg of Ca per kg of body weight *p.o.* to two groups of 7 male adult rats each. The urine elimination of the ^{45}Ca, expressed as total accumulated percentage of ^{45}Ca activity in urine (Ae^{∞}), between the rats that received Biocal™ (Ae^{∞} = 2.436±1.337 %) and the rats that received calcium gluconate (Ae^{∞} = 1.241±0.473 %) were found to be statistically different ($p<0.05$). Biodistribution studies showed that the calcium from Biocal™ follows the same metabolic pathway as calcium from calcium gluconate. Values of radioactivity concentration of 97.1±1.3% and 98.7±1.6% were found in bone for Biocal™ and calcium gluconate, respectively. Toxicity studies of Biocal™ were carried out with 60 female and 60 male rats. The values of oral LD_{50} for female rats was 13.5 g/kg with a lower limit of 12.8 g/kg and upper limit of 14.3 g/kg. In the case of male rats the LD_{50} was 13.0 g/kg with a lower limit of 12.2 g/kg and upper limit of 13.9 g/kg. These values are higher with regard to the oral LD_{50} for calcium gluconate (10 g/kg). Our results demonstrate that calcium from Biocal™ has a higher bioavailability with the same metabolic behavior than calcium from calcium gluconate. The value of oral LD_{50} shows that the toxicity of Biocal™ is lower than that of the calcium gluconate. Therefore we conclude that Biocal™ has adequate properties to be considered as a promissory calcium compound to be used as dietary supplement or for food fortification.

Key Words: Calcium, Rat, Bioavailability, Metabolism, Toxicity, Solubility.

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INTRODUCTION

Calcium is one of the most important extracelular cations (1), representing between 1.5 and 2 % of the body weight (2). One of the principal functions of this mineral is to support the skeletal integrity. It has other important physiological functions with regard to muscular contractility, nervous excitability and blood clots (3). Two different mechanisms are involved in calcium absorption. Active transport is carried out in the first fraction of duodenum and facilitated diffusion in the rest of the small intestine (4). There are different individual factors that affect its absorption like age, sex, and nutritional status for this element (5).

The calcium intake is critical during the first years of life for the anatomical structure (6). Prospective studies demonstrated that the biggest increase of the bone mass occurs principally during the age of puberty (7). An inadequate calcium intake during the growth period may cause a failure to reach the peak of the bone mass. This situation produces different bone illnesses like osteopenia, osteoporosis, decreased skeletal integrity, increasing the fracture risk in later life (8). Some researches have reported that the increase in calcium intake attenuates the bone mass loss during the age of menopause (9-11). However, calcium plays other important roles with regard to different physiological functions; it has been demonstrated that there is a direct relationship between calcium intake and the regulation of the blood pressure and colon cancer risk (5, 12, 13). Therefore it is important to have an adequate calcium intake during the different ages of life.

The adequate calcium intake was suggested by the National Academy of Science (NAS). This institute recommended between 800 and 1500 mg of calcium per day according to the age and sex of the people (14). The principal calcium intake by western populations comes from foods. Milk is one of the best calcium source because it has about 1200 mg of calcium per liter and the lactose contained in it enhances the calcium absorption in normal subject (3). Other foods like vegetables and cereals have calcium together with other compounds like oxalic acid and phytates, which make insoluble complexes that decreased calcium absorption (15-18). Therefore it is difficult to have an adequate calcium intake only from diet. One way to solve this problem is to increase the calcium intake from other calcium sources like calcium supplements or fortified foods. These calcium sources must have an adequate bioavailability and their metabolic and physiological behavior must be the same as the calcium provided naturally by foods. With this pourpose we studied in this work a new calcium source called Biocal™, that is a calcium gluconate stabilized with glycine.

MATERIALS AND METHODS

Animals: We used 74 male and 60 female Sprague-Dawley rats, with weights ranging from 400 to 450 g for the male and from 300 to 350 g for the female. They were housed in stainless steel cages of 315 mm by 445 mm by 240 mm high with grated floor and a collecting tray of the same material. The animals were maintained with free access to water and nourished with a normalized diet (Nutrimentos™ Diet N° 3). Throughout the experimental period the animals were maintained with cycles of 12 h with light and 12 h in darkness.

Labeling of the compounds: Both compounds were intrinsically labeled with ^{45}Ca (Calcium chloride. Specify Activity 370 GBq/g. Dupont NEZ-013). Calcium gluconate labeled with ^{45}Ca was synthesized by the addition of 117.8 mg of CaO (Anedra # 6481. Argentina) to 748.4 mg of D(+)-Gluconic acid δ-lactone (Fluka # 49120. Switzerland) in presence of 400 μCi of ^{45}Ca in a final volume of 8 mL. Then the mix was heated at 50°C for 30 min. Biocal™ labeled with ^{45}Ca was synthesized in the same way than calcium gluconate with the addition of 315 mg of glycin (Merck # 4201. Germany).

Activity concentration of the compounds: In order to determine the activity concentration of Biocal™ and calcium gluconate solutions, volumes of 0.01, 0.025, 0.05 and 0.1 mL of both compounds, were placed in duplicate in different vials containing 12 mL of scintillation solution (Packard Instrument B.V. Formula 989™). They were measured and the radioactivity as a function of the different solution volumes for each compound was fitted by liner regression analysis. In this way the slope value for each compound represents the activity concentration expressed as μCi/mL (Figure 1).

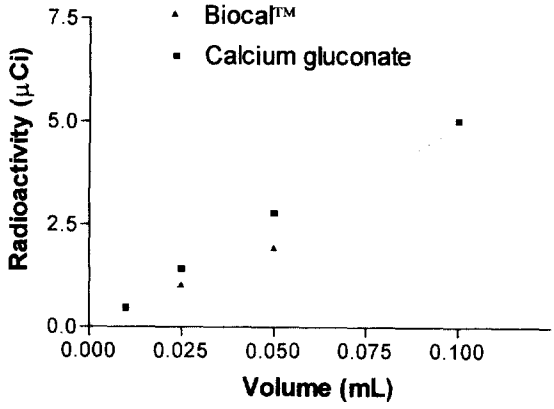

Figure 1. Radioactivity measured as a function of the different solution volumes for Biocal™ and calcium gluconate. In this case the slope value for each curve is the activity concentration, giving a value of 51 μCi/mL and 50 μCi/mL for Biocal™ and calcium gluconate respectively.

Administration of the compounds: Before the administration of the compounds, the animals were deprived of any solid food for 10 h, which was not provided until 1 h after the compounds intake. They were administered by means of a syringe coupled to a gastric catheter, which allowed the intake volume to be standardized to one mL.

Bioavailability studies: They were carried out with two groups of 7 male rats each. One of them received calcium gluconate as reference standard and the other one calcium gluconate stabilized with glycine (Biocal™). In both cases we administered a dose of 30 mg of Ca per kg of body weight. Each rat was placed in stainless steel metabolic cages of 180 mm by 240 mm by 180 mm high (La Tecnica™ model 304), that allow the separation of urine from feces. The urine was collected for a period of 12 days and its volume was daily measured. Samples of 1 mL were taken by triplicate and were kept for subsequent radioactivity determination. The

bioavailability was determined by the measurement of ^{45}Ca urine excretion as a function of time and given as the percentage of the total amount of ^{45}Ca activity in urine (Ae^{∞}) (19).

Biodistribution studies: Biological distribution were carried out 12 days after the administration of the compounds under study, in order to determine the calcium biodistribution. The rats received i.v. 1500 I.U. of heparin per kg of body weight, then they were anesthetized with diethyl ether and finally bled by means of retroorbital sinus puncture, collecting about 15 mL of blood from each rat. Afterward, liver, spleen, bone, the gut with its content, muscle, lungs, heart, brain and kidneys were removed, washed with isotonic saline solution and weighted. The organs were kept for subsequent radioactivity determination. The results were given as percentage of radioactivity concentration, C%. C% = (A/w)%, where A is the radioactivity measurement and w is the weight of each organ; in all the cases the sum of whole organs' activity concentration was considered equal to 100 % for each animal.

Samples processing: One mL of each urine sample was placed in a glass vial and treated with 100 μL of Solvable™ (Packard Instrument B.V.) at room temperature for 24 h. Then they were heated at 50° C for 2 h. Afterward they were decolorized with 200 μL of H_2O_2, 100 vol.; 100 μL of Na_2EDTA 5 %w/v was used as calcium chelate reagent. Then the samples were heated again at 50°C for an hour and finally 12 mL of scintillation solution (Packard Instrument B.V. Formula 989™) were added. For the processing of organ samples, between 100 mg and 200 mg of tissue was treated in the same way as the urine samples, with the exception of bone. This tissue was previously treated with 100 μL of HCl (Merck) at room temperature for 24 h and then at 50° C for 2 h in order to destroy the mineral matrix.

Measurements: Each sample was counted during 5 min. in a liquid scintillation counter (Wallac 1410). Counts were corrected to disintegration per minute (dpm) using an external standard quench program, with a measurement error less than 1%.

Toxicity studies: Toxicity studies of Biocal™ were carried out with six groups of ten female and six groups of ten male rats, which received increasing doses of 10, 11, 12, 13, 14, 15 g of Biocal™ per kg of body weight.

Statistical studies: The data are presented as Mean ± SD. The results were evaluated by a one-way analysis of variance (ANOVA). To test the differences among the means, the Student-Newman-Keuls method was used. Only probability levels <0.05 were considered to be statistically significant (20). The acute oral toxicity results were expressed as LD_{50} with its limits, according to the method proposed by Litchfield and Wilcoxon (21).

RESULTS

Figure 2, shows the curve of the accumulated percentage of ^{45}Ca radioactivity in urine as a function of time. The bioavailability was expressed as total amount of ^{45}Ca excreted in urine (Ae^{∞}), giving a value of 2.436±1.337 % for Biocal™ and 1.241±0.473 % for calcium gluconate. These values are statistically different with $p<0.05$.

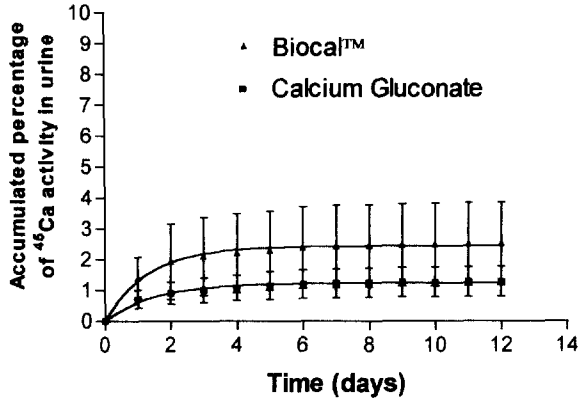

Figure 2. Bioavailability studies of Biocal™ versus calcium gluconate. The value of Ae^{∞} for Biocal™ is statistically higher than the Ae^{∞} for calcium gluconate ($p<0.05$).

Table 1 shows the parameters characterizing bioavailability. The statistical comparison between the pharmacological patterns, demonstrate that Ae^{∞} for Biocal™ is significantly higher than that for calcium gluconate ($p<0.05$), while the others are not significantly different.

(TABLE 1) Statistical comparison of bioavailability parameters for both compounds

PARAMETER	BIOCAL™	CALCIUM GLUCONATE	DIFFERENCE LIMIT $p<0.05$
Ae^{∞} (%)	2.436±1.337	1.241±0.473	Significant
K (day^{-1})	0.749±0.156	0.676±0.121	Not Significant
$T_{1/2}$ (day)	0.956±0.177	1.062±0.243	Not Significant
r^2	0.983±0.008	0.979±0.011	Not Significant

Ae^{∞}: total amount of ^{45}Ca excreted in urine, expressed as percentage of administered dose. K: excretion constant. $T_{1/2}$: half time, time to reach $Ae^{\infty}/2$. r^2: determination coefficient between the experimental data.

Figure 3 shows the results of biodistribution studies for each compound. The highest values of radioactivity concentration was found in bone, giving a value of 97.1±1.3% for Biocal™ and 98.7±1.6% for calcium gluconate. These results agree with the predominant role that this tissue has in calcium metabolism.

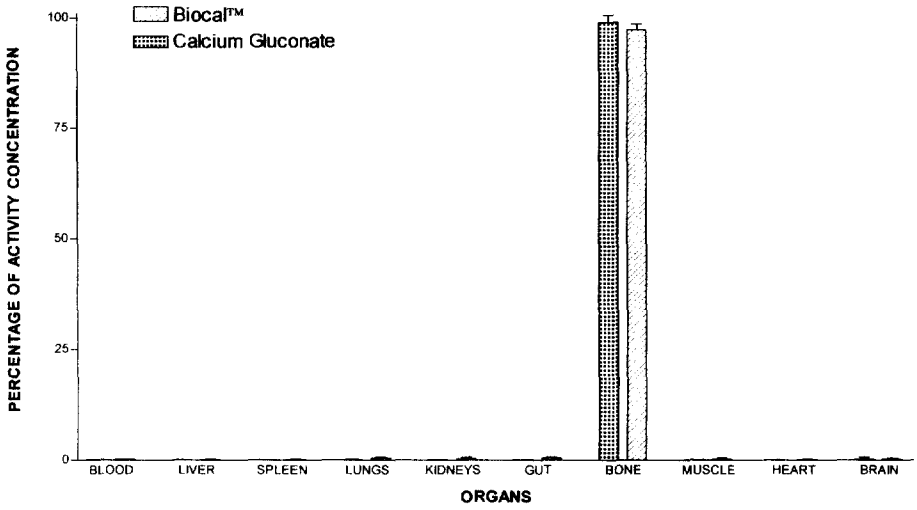

Figure 3. Biological distribution of Biocal™ versus calcium gluconate. In both cases the highest values of radioactivity concentration were found in bone.

Table 2 shows the results found in our studies of the acute oral toxicity for Biocal™. The values of LD_{50} for female rats was 13.5 g/kg with a lower limit of 12.8 g/kg and an upper limit of 14.3 g/kg. In the case of male rats the LD_{50} was 13.0 g/kg with a lower limit of 12.2 g/kg and an upper limit of 13.9 g/kg.

(TABLE 2) Acute oral toxicity of Biocal™. Values of LD_{50} and its confidence limits

LD_{50}*	Lower Limit*	Upper Limit*	Species	N° of animals	Sex
13.5	12.8	14.3	Rat	60	Female
13.0	12.2	13.9	Rat	60	Male

*Expressed as g of Biocal™ per kg of body weight.

DISCUSSION

The multiple biochemical roles that calcium plays in the organism determine its physiological importance (3). Calcium nutritional deficiency affects people that live in developing and developed countries (15). The adequate calcium intake can be covered by an adequate diet, but the calcium contained in the foods is usually not high enough to supply an adequate amount of calcium to cover the daily requirement (22). One way to solve this problem is to increase the calcium intake from other calcium sources. Fortified foods as well as calcium supplements are the most usual ways to solve this deficiency (3). The physicochemical and nutritional properties of the compounds used for this strategy are essential when choosing them (23). Some compounds like calcium carbonate or calcium phosphate are insipid but practically insoluble in water or aqueous environments. Other compounds, like calcium lactate and calcium gluconate are highly soluble in water but the principal disadvantage is their strong taste, that is why people refuse to consume them. Both kinds of compounds are soluble in gastric environment and therefore their bioavailabilities are not so different (23). In the first

case they could be used to fortify solid but not liquid foods because they precipitate. In the last case they could not be used to fortify foods due to their strong taste, and when they are used as calcium supplements, like effervescent tablets, the people that drink them discontinue their consumption for the same reason (24). In order to solve this problem, we studied a new calcium source, called Biocal™ with a high solubility as well as a soft taste. For these reasons this product can be used as calcium supplements as well as a calcium source for food fortification.

The results obtained in the bioavailability studies demonstrate that the value of Ae^{∞} for calcium from Biocal™ is about two folds higher than that of the calcium from calcium gluconate (Figure 2). This result could be explained considering that the solubility of Biocal™ is about ten folds higher than that of calcium gluconate. Other important physiochemical property of Biocal™ is its high solubility in alkaline solutions, that determine its solubility in the intestinal environment and its consequent higher bioavailability. It is interesting to point out that the statistical comparison between the other pharmacological patterns for both compounds did not show any significant difference between them, demonstrating that calcium from both compounds have the same pharmacological behavior (Table 1).

Even though bioavailability is an important parameter for the study of a calcium source, it is also important to determine that this calcium has a metabolic and physiologic behavior similar to that of the calcium naturally provided by foods. With this purpose the biodistribution studies were carried out. In order to solve the influence of the weight of each tissue on the calcium distribution the data are given as activity concentration percentage, C%. Figure 3 shows the C% for each calcium source and tissue. Since only the used calcium source is labeled with ^{45}Ca, the measured radioactivity indicates the metabolic behavior of the calcium provided by this source. The highest percentage is found in the tissue related to calcium metabolism; in bone nearly 98% values were found. These results agree with the predominant role that this tissue has in calcium metabolism. In the other tissues the C% values are negligible for both compounds. Even though in these tissues, their calcium content has important functions since it is included in biomolecules which are metabolically essential for these tissues. In none of the cases we could observe any statistically significant difference in the activity concentration percentage of any organ or tissue with regard to the calcium source.

Taking into account that these kinds of compounds could be massively consumed, their toxicological studies are essential. The LD_{50} for Biocal™ after an oral intake can be observed in Table 2. The obtained result demonstrate that the toxicity of Biocal™ is lower with regard to the value of 10 g/kg assigned to calcium gluconate (25).

Our results demonstrate that calcium from Biocal™ has a higher bioavailability than calcium from calcium gluconate with the same pharmacological behavior. The biodistribution studies show that calcium from both compounds follows the same metabolic pathway, and the value of oral LD_{50} demonstrate that the toxicity of Biocal™ is lower than that of calcium gluconate. Therefore we conclude that Biocal™ has adequate properties to be considered as a promissory calcium compound to be used as dietary supplement or for food fortification.

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