Solubility and Bioavailability of Stabilized Amorphous Calcium Carbonate

Oren E Meiron,[1,5] Elad Bar-David,[1,5] Eliahu D Aflalo,[1,3] Assaf Shechter,[5] David Stepensky,[4] Amir Berman,[2,3] and Amir Sagi[1,3]

[1]Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[2]Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[3]National Institute for Biotechnology in the Negev (NIBN), Ben-Gurion University of the Negev, Beer-Sheva, Israel
[4]Department of Pharmacology, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[5]Amorphical, Ltd., Beer Sheva, Israel

ABSTRACT

Since its role in the prevention of osteoporosis in humans was proven some 30 years ago, calcium bioavailability has been the subject of numerous scientific studies. Recent technology allowing the production of a stable amorphous calcium carbonate (ACC) now enables a bioavailability analysis of this unique form of calcium. This study thus compares the solubility and fractional absorption of ACC, ACC with chitosan (ACC-C), and crystalline calcium carbonate (CCC). Solubility was evaluated by dissolving these preparations in dilute phosphoric acid. The results demonstrated that both ACC and ACC-C are more soluble than CCC. Fractional absorption was evaluated by intrinsically labeling calcium carbonate preparations with ^{45}Ca, orally administrated to rats using gelatin capsules. Fractional absorption was determined by evaluating the percentage of the administrated radioactive dose per milliliter that was measured in the serum, calcium absorption in the femur, and whole-body retention over a 34-hour period. Calcium serum analysis revealed that calcium absorption from ACC and ACC-C preparations was up to 40% higher than from CCC, whereas retention of ACC and ACC-C was up to 26.5% higher than CCC. Absorbed calcium in the femurs of ACC-administrated rats was 30% higher than in CCC-treated animals, whereas 15% more calcium was absorbed following ACC-C treatment than following CCC treatment. This study demonstrates the enhanced solubility and bioavailability of ACC over CCC. The use of stable ACC as a highly bioavailable dietary source for calcium is proposed based on the findings of this study. © 2011 American Society for Bone and Mineral Research.

KEY WORDS: ABSORPTION; AMORPHOUS CALCIUM CARBONATE; BIOAVAILABILITY; RATS; SOLUBILITY

Introduction

Calcium is considered to be one of the most important minerals in the human body. It is required for maintaining bone mass, is essential for exocytosis of neurotransmitters, takes part in the contraction of muscle cells, replaces sodium as the depolarizing mineral in the heart, and participates in many other physiologic functions.[1] Studies have shown that a lack of adequate dietary calcium can induce many bone-related diseases such as osteoporosis, especially in postmenopausal woman, as well as other calcium-related deficiencies, such as hypocalcaemia and poor blood clotting.[1,2] The U.S Institute of Medicine recommends a daily dietary intake of 1000 to 1300 mg of calcium (depending on age and gender) as the adequate amount needed to prevent calcium deficiency–related diseases.[3] Despite these recommendations, most adults do not achieve these levels and must enrich their diet by calcium supplementation.[1]

Calcium supplements generally are divided into two groups according to the nature of their chelating counterion. Organic calcium supplements include negatively charged organic molecules, such as malate, citrate, fumarate, and gluconate, whereas inorganic calcium supplements rely on inorganic chelating molecules, such as carbonates, phosphates, and chlorides.

Calcium carbonate has six known polymorphs, three of which are anhydrous crystalline (ie, calcite, aragonite, and vaterite), two of which are hydrated (ie, crystalline monohydrocalcite and ikaite), and one of which is hydrated amorphous, namely,

amorphous calcium carbonate (ACC).[4] The most thermodynamically stable of these forms is calcite, whereas the least stable is ACC. ACC is a transient polymorph that precipitates out of a supersaturated solution following Ostwald's step rule; if not stabilized by any element, ACC will crystallize rapidly and completely into one of the five more stable polymorphs within seconds.[5] The amorphous polymorph is characterized by distinctive 40 to 120 nm spherules, in contrast to the 1 to 10 μm crystals typical of the other polymorphs.[4] Solubility studies suggest dramatic differences between the calcium carbonate polymorphs. While crystalline phases are considered poorly soluble, the amorphous polymorph is approximately 120 times more soluble than calcite.[6] Several techniques have been reported for the synthesis and stabilization of ACC,[4,7] but all known methods use either toxic materials or various organic polymers to stabilize ACC for more than 3 days.[7–11] To avoid use of such compounds, a novel method for synthetic production of stabilized ACC using phosphoaminoacids was developed recently[12] and was shown to stabilize ACC for more than 4 months under ambient conditions.

Macromolecules that inhibit crystallization of calcium carbonate and which stabilize the amorphous phase have been found in several organisms.[10,13,14] In most cases, these organisms use calcium in the amorphous phase as a precursor for one of the crystalline phases.[15] In other instances, organisms construct specialized transient mineral storage sites composed of stabilized amorphous calcium carbonate embedded on an organic matrix comprising dense chitin fibers and proteins. The role of the chitin and the proteins in ACC stabilization, and possibly also in calcium absorption, has been investigated only recently.[16,17] When needed, the thermodynamic instability of ACC can be exploited to dissolve calcium and allow for fast transport of this ion across the intestinal epithelium and into the bloodstream.[18,19]

The bioavailability of calcium in both humans and rats has been suggested to be governed by factors such as dose quantity, the nature of the chelating molecule, solubility of the salt, and the hormonal state of the receiving subject.[1] Furthermore, calcium in humans is absorbed by at least two and possibly three different pathways along the gastrointestinal tract. The first route corresponds to active transport across the intestinal epithelium, mainly through the duodenum and jejunum, a pathway that is vitamin D–dependent.[20,21] The second route involves passive paracellular transport of free calcium ions, which depends on the free calcium concentration in the small intestine.[1] A possible third pathway involves passive absorption of intact calcium-based complexes such as calcium oxalate without dissociation.[22]

To date, reports on calcium bioavailability in both animals and humans have been inconclusive largely because small differences in experiment parameters can lead to significantly different results.[1,23,24] Taking this into account, evaluation of calcium bioavailability in the rat model is commonly performed using one of the following four assays: (1) analyzing secreted calcium in feces and urine (balance studies) following Ca^{2+} administration,[25,26] (2) using double-isotope labeling techniques, such as used commonly with humans, despite suffering from technical difficulties and inaccurate results when per-

formed in rats,[27,28] (3) multiple administrations of ^{45}Ca-labeled test meals throughout the duration of the experiment,[29] and (4) single administration of a ^{45}Ca-labeled preparation directly into the rat stomach by gavage. This last method was introduced in 1961[30] and was found to be the most accurate and cost-effective of the four.[27] After careful review of these methods, we decided to use a single administration of ^{45}Ca-labeled material in this study, aimed at assessing Ca^{2+} bioavailability when presented as the amorphous polymorph.

To the best of our knowledge, the bioavailability of stable ACC has never been evaluated, nor has it been compared with that of other calcium salts or polymorphs. This study thus compares the solubility and bioavailability of two ACC-based products versus crystalline calcium carbonate (CCC).

Materials and Methods

Synthesis of amorphous and crystalline CCC for analytical analysis

Stable ACC and stable amorphous calcium carbonate embedded in a chitosan matrix (ACC-C) were synthesized based on proprietary technology developed by Amorphical, Ltd., and Ben-Gurion University.[12] Crystalline calcium carbonate (CCC) was synthesized by mixing 0.1 M $CaCl_2$ (Acros, supplied by Holland Moran Ltd., Yahud, Israel) with 0.1 M Na_2CO_3 (Acros) in 0.3 M Tris-HCl (Sigma, St Louis, MO, USA), pH 9. The suspension was stirred at 40°C for 2 hours. The CCC slurry was filtered using a Buchner funnel and washed twice with absolute ethanol (BioLab, Jerusalem, Israel).

Analytical analysis

The calcium carbonate preparations were characterized and analyzed by high-resolution scanning electron microscopy (HR-SEM; JEOL JSM-7400F, Tokyo, Japan), a polarized optical microscopy (Axiovert 135 Zeiss, Oberkochen, Germany), Raman spectroscopy (Jobin-Yvon LabRam HR 800 Micro-Raman, Edison, NJ, USA), and Fourier-transformed infrared spectroscopy (FTIR; Thermo Scientific Nicolet 6700, Waltham, MA, USA) performed on KBr pellets. The percentage of elemental calcium in the calcium carbonate preparations was evaluated by atomic absorption (Varian AA240, Palo Alto, CA, USA).

Dissolution assay

Dissolution of calcium carbonate was performed in 0.01 M phosphoric acid ($n = 5$; BioLab) according to a previously reported assay.[31] The clustered ACC and ACC-C preparations were mortared to powder prior to dissolution analysis, whereas both CCC and commercial calcite were obtained as fine powders. Dissolution was achieved by rapidly adding 40 mg of elemental calcium as CCC (100 ± 1 mg, $n = 5$), commercial calcite (100 ± 1 mg, $n = 5$; Sigma), ACC (121 ± 1 mg, $n = 5$), or ACC-C (148 ± 1 mg, $n = 5$) to 50 ± 1 mL of stirred 0.01 M phosphoric acid solution. The pH was monitored using a gel pH electrode (Thermo) connected to a controller (Eutech, Nijkerk, The Netherlands). The controller was directed by MatLab (MathWorks, Boston, MA, USA) programmed to record one measurement per second for 240 seconds. Dissolution rates were

evaluated according to the time needed for each preparation to reach 50% of its final pH. Final pH was determined at the end of the assay when the pH change was less than 0.05% during a 10-second period. One-way ANOVA was performed to evaluate the significance of the results.

Intrinsic radioactive labeling of calcium carbonate

Three different radiolabeled preparations of calcium carbonate were synthesized using the intrinsic labeling method employing 1 mCi/mL of radioactive $^{45}CaCl_2$ stock solution (PerkinElmer, supplied by Eisenberg Bros. Ltd., Airport City, Israel). Radiolabeled $^{45}CaCl_2$ from the stock solution was diluted with $CaCl_2$ solution prepared for the calcium carbonate preparations to yield radioactivity of approximately 1 ± 0.3 μCi/5 mg of elemental calcium in each one of the preparations. Syntheses of all three preparations from this point on were identical to the nonradioactive syntheses described under "Materials and Methods" for the synthesis of amorphous and crystalline calcium carbonate for analytical analysis. Qualitative polymorph assurance was performed on each radiolabeled preparation using a polarized optical microscope.

Capsule preparation

The radiolabeled calcium carbonate preparations (12.5 ± 0.05 mg of CCC, 15.15 ± 0.04 mg of ACC, and 18.5 ± 0.03 mg of ACC-C), all containing 5 mg of elemental calcium with approximately 1 μCi as dry powder, were inserted into mini-gelatin capsules (Harvard Apparatus, Holliston, MA, USA)

Experimental design

Animals and acclimation

All animals were treated according to the Israel Animal Welfare Act under the supervision of the Ben Gurion University Animal Care and Use Program. Fifty-one 2-month-old male Wistar rats (Harlan-Teklad, Jerusalem, Israel) weighing 240 ± 15 g were randomly housed in 12 stainless steel cages in an environmentally controlled room (23°C, 12:12-hour light/dark cycle). The rats were fed laboratory rat chow pellets adequate in nutrients *ad libitum* (Koffolk, Petah-Tikva, Israel) and had free access to water for 48 hours. Four days before the beginning of the experiment, the regular diet was replaced with a low-calcium diet containing $0.24\% \pm 0.05\%$ calcium ($0.675\% \pm 0.05\%$ phosphate), specially prepared by mixing two food types containing, respectively, 0.01% calcium (0.3% phosphate; Harlan-Teklad) and 1% calcium (0.8% phosphate; Koffolk). The two food types were separately ground in a mill to yield two powders that were dry mixed in a ratio of 4:1, respectively, until fine homogenization. The homogenized powder was extruded to form new food pellets.

Seventeen hours prior to capsule administration, the rats were weighed, and blood samples were taken (baseline). The rats then were placed into individual metabolic cages and deprived of food and water until 3 hours after capsule administration. Then 3 and 24 hours after dosing, approximately 10 g of low-calcium food pellets (0.01% calcium; Harlan-Teklad) were given to each

rat. Distilled water was allowed *ad libitum* starting 3 hours after capsule administration.

Gelatin capsule administration

Each rat was lightly sedated for 30 seconds with isoflurane)-Minrad, Buffalo/Niagara, NY, USA) diluted 1:4 (v/v) with propylene glycol (BioLab). A single capsule containing a specific calcium carbonate preparation (ie, CCC, ACC, or ACC-C; $n = 17$ for each group) was administered intragastrically to each of the experimental rats using a stainless steel rat administration syringe (Harvard Apparatus).

Blood sampling and chemical analysis

Blood samples of 120 to 150 μL were taken from each rat's tail vein 17 hours prior to capsule administration (time 0) and at 2, 3, 6, 10, 24, and 34 hours after administration. The blood samples were immediately centrifuged for 10 minutes at 3000*g* using a tabletop centrifuge (Hettich Zentrifugen, Bach, Switzerland). Duplicate samples (30 μL) of the supernatant serum were transferred into plastic vials containing scintillation liquid (Zinsser Analytic, Berkshire, UK), and radioactivity was measured using a liquid scintillation counter (Tri-Carb 2100TR, PerkinElmer, Boston, MA, USA). Plasma radioactivity from the given dose was normalized according to the measured radioactivity and specific radioactive dose that each rat received [(serum cpm $\times$ 100)/(total cpm $\times$ sample volume)].

Pharmacokinetic calculations

Noncompartmental analysis of an individual rats' calcium concentration versus time data was performed using WinNonLin 5.2 Software (Pharsight, Mountain View, CA, USA).

Feces and urine sampling and analysis

Feces and urine were collected during the 17-hour starvation phase during the acclimation period (baseline) and during the entire 34 hours of the experiment to evaluate calcium. Samples of urine (500 μL) were transferred into plastic vials filled with scintillation liquid. Feces were dried overnight at 70°C in an oven. The samples were ground in a mortar until finely homogenized. Feces samples (200 mg) were placed into 5 mL of 1 N NaOH solution (Gadot, Netanya, Israel), incubated for 3 hours at 80°C, and centrifuged for 10 minutes at 3600*g*. Duplicate samples of the supernatant (30 μL) were transferred into plastic vials containing scintillation liquid. Radioactivity of the urine and feces samples was measured using a liquid scintillation counter. Retention values were calculated by subtracting the radioactivity measured in the feces and urine from the given dose [(intake – feces and urine excretion)/intake $\times$100%].

Femur sampling and analysis

Thirty-four hours after dosing, the rats were euthanized by exposure to CO_2. The right and left femurs were removed and cleaned of soft tissue. The femurs were oven dried at 70°C overnight. Each femur was weighed and separately decalcified at room temperature for 24 hours in 4 mL ofl decalcifying solution (Rapid Calci-Clear, National Diagnostic, Atlanta, GA,

USA). Duplicate samples of the resulting solution (50 µL) were transferred into plastic vials containing scintillation liquid. The calcium content was normalized according to the specific radioactivity and calcium dose of each preparations and then calculated per 100 mg of dry bone content [(total femur cpm × total calcium dose)/(total cpm × femur weight) × 100%].

Statistical analysis

One-way analysis of variance (ANOVA) was performed on retention values, calcium concentrations in the femur, pharmacokinetic results, and solubility results using Statistica 6.1 software (StaSoft, Tulsa, OK, USA). A p value of less than .05 was deemed significant.

Results

Following stabilization of ACC,[12] we produced nonradioactive ACC and CCC and started to verify the composition of the different preparations using the following methods: Differences in the morphology of the three calcium carbonate preparations considered here, as revealed by HR-SEM, are presented in Fig. 1. CCC consists of a mixture of two crystalline forms (Fig. 1a), namely, calcite (rhomboids) and vaterite (spherules) particles, both ranging in size from 1 to 10 µm. The observed spherules contain smaller nanosized crystallites, a formation that, together with its 1 to 10 µm size, is typical of vaterite. The ACC particles (Fig. 1b) are 1 to 2 orders of magnitude smaller than the calcite and vaterite crystals (Fig. 1a), thus increasing the effective surface area of the material by up to 1×10^6. The amorphous nature of ACC is depicted by its 40- to 100-nm particles presenting varying morphologies. ACC embedded in a chitosan matrix (ACC-C; Fig. 1c) also present 40 to 100 nm particles with varying morphologies. ACC and ACC-C cannot be differentiated using HR-SEM because it appears that the chitosan matrix of ACC-C is covered by ACC particles and therefore is not visible in the SEM images.

After obtaining the HR-SEM results that showed that the CCC preparation consists of two crystalline phases, we used several spectroscopic methods to determine the polymorph composition of the different preparations. The first method used was Fourier transform-infrared (FTIR) spectroscopy (Fig. 2), where the carbonate vibrational shifts from one polymorph to another allow for affirmation of the polymorphic nature of the compound based on the wave number of the vibrations. Specifically, the CCC preparation spectrum reveals the presence of calcite, as reflected in a sharp peak at approximately $870 \, cm^{-1}$ and a peak at approximately $712 \, cm^{-1}$. The presence of vaterite was detected by a vibration at approximately $745 \, cm^{-1}$ (Fig. 2a). The presence of both calcite and vaterite in the CCC preparation is also supported by the HR-SEM image shown in Fig. 1. The spectrum of the ACC preparation is characterized by a typical broad peak at approximately $865 \, cm^{-1}$ (Fig. 2b). The spectrum of the ACC-C preparation, with a broad peak at approximately $864 \, cm^{-1}$, also suggests the presence of ACC (Fig. 2c). The presence of chitosan in the ACC-C preparation was verified by peaks at 1028, 1150, and $2877 \, cm^{-1}$, previously identified in the FTIR spectra of chitosan alone (indicated by arrows). No distinct $CaCl_2$ or $NaCO_3$ peaks were
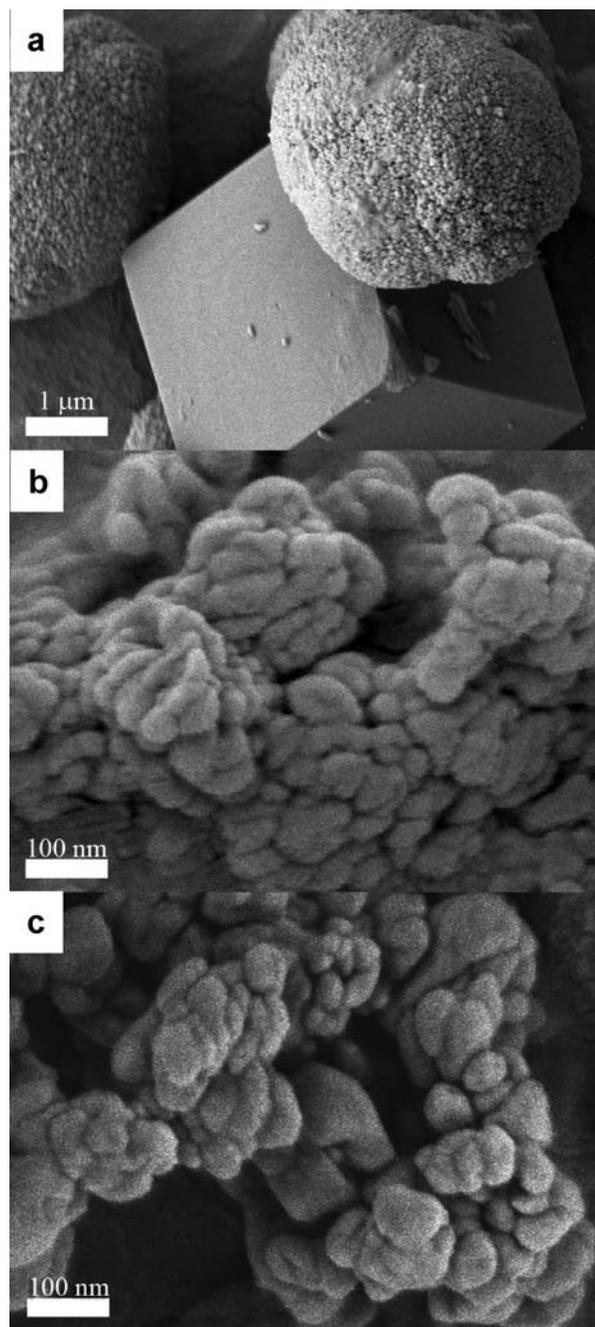

Fig. 1. Scanning electron microscope (SEM) images of three calcium carbonate preparations: (a) Crystalline calcium carbonate (CCC; magnification ×15,000); (b) amorphous calcium carbonate (ACC; magnification ×100,000); (c) amorphous calcium carbonate with chitosan (ACC-C; magnification ×100,000). Bars represent scale.

detected, suggesting that there is no residual $CaCl_2$ or $NaCO_3$ in the different calcium carbonate preparations.

The second spectroscopy approach employed to verify the composition of the different preparations was Raman spectroscopy. Raman analysis of the CCC preparation (Fig. 3a) yielded a sharp peak at $1085.6 \, cm^{-1}$ and a small peak at $713.3 \, cm^{-1}$, representing the presence of calcite. Vaterite is not visible in this spectrum because of the high intensity of the major calcite peak, which masks the vaterite peak that typically appears as a doublet
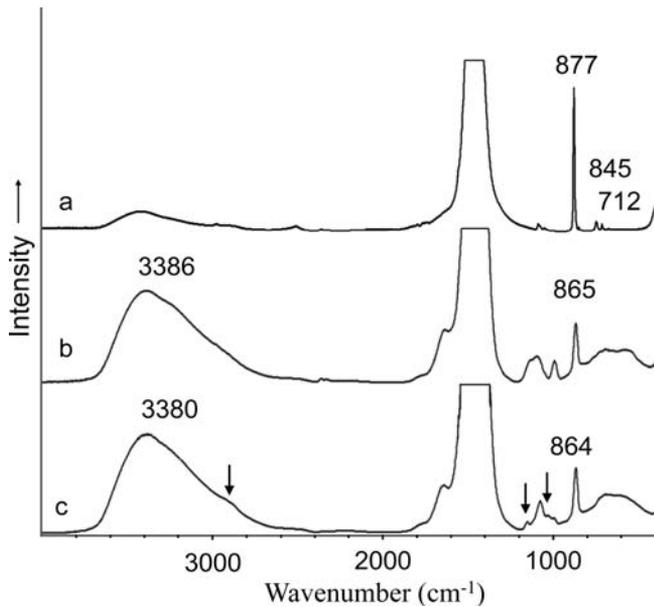
Fig. 2. Representative FTIR spectra of the three calcium carbonate preparations: (a) Crystalline calcium carbonate (CCC); (b) amorphous calcium carbonate (ACC); (c) amorphous calcium carbonate with chitosan (ACC-C). Note that the intensity is scaled differently for each spectrum; that is, the relative height of each spectrum corresponds to the strongest peak. Arrows point to specific chitosan peaks.

at 1087 cm^{-1}. The ACC preparation is characterized by a broad Raman peak at 1079.8 cm^{-1}, revealing the presence of ACC (Fig. 3b). Similarly, the ACC-C preparation presented a broad peak at 1080.4 cm^{-1} (Fig. 3c). Chitosan, present in the ACC-C preparation, did not produce a known peak in Raman spectro-
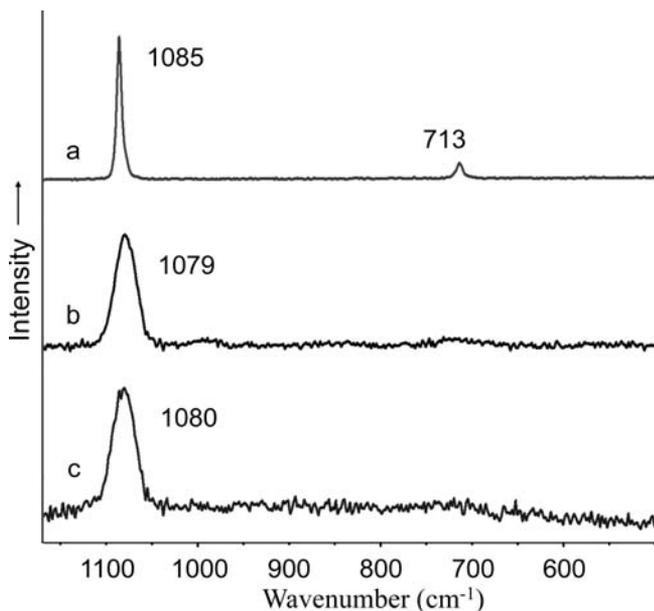
scopy, although a distinctive background reading was obtained during measurement of ACC-C, possibly caused by the presence of chitosan in the sample (data not shown).

After verifying the polymorph composition of the calcium carbonate preparations, we studied the dissolution rate and solubility of the synthetic ACC prepared for this assay compared with the crystalline forms of calcium carbonate. pH measurements represent the number of unbound H$^+$ ions, which directly correlates with the number of dissolved CO$_3^{2-}$ ions. The more CaCO$_3$ dissolves to ionic Ca^{2+} and CO$_3^{2-}$, the higher pH values are obtained. When dissolving the calcium carbonate preparations in phosphoric acid (Fig. 4), both the final pH values of the ACC and ACC-C solutions were higher than the final pH observed for the calcite solution by more than 1 pH unit, corresponding to a 20% increase ($p < .05$). The final pH values of both the ACC and ACC-C solutions were higher than the final pH value of the CCC preparation by more than 0.5 pH unit, reflecting a 9% increase. Furthermore (Table 1), it took commercial calcite 49 seconds to reach 50% maximal pH, whereas it took 20, 21, and 22 seconds for CCC, ACC-C, and ACC to reach 50% maximal pH, respectively. In all tested samples, a remnant precipitate still was present at the end of the analysis.

After verifying the different preparation properties, we preformed a bioavailability experiment to test the relative absorption of the previously mentioned preparations according to the single-isotope, single-administration method.[30] It is important to note that despite our best efforts to ensure minimal stress to the tested rats, we still could not prevent a certain degree of mortality during the experiment. During the course of the bioavailability experiment, 4 rats died in each group (25%); representative postmortem examinations performed on the rats
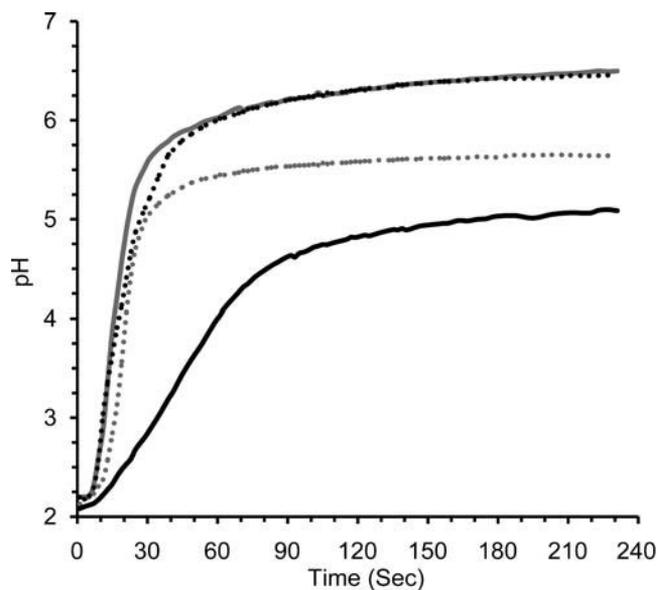

Fig. 3. Representative Raman spectroscopy spectra of the three calcium carbonate preparations: (a) Crystalline calcium carbonate (CCC); (b) amorphous calcium carbonate (ACC); (c) amorphous calcium carbonate with chitosan (ACC-C). Note that the intensity is scaled differently for each spectrum; that is, the relative height of each spectrum corresponds to the strongest peak.

Fig. 4. Changes in pH of a 0.01 M phosphoric acid solution following dissolution of calcium carbonate preparations: ▬▬▬ represents amorphous calcium carbonate with chitosan (ACC-C); •••••• represents amorphous calcium carbonate (ACC); •••••• represents crystalline calcium carbonate (CCC); and ▬▬▬ represents commercial calcite. Each line represents the average of five repeats.

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Table 1. Dissolution Parameters

Calcium carbonate preparation	pH_{MAX}	$T_{50\%}$ (s)
Calcite	5.11 ± 0.02^a	49^a
CCC	5.64 ± 0.01^b	20^b
ACC	6.46 ± 0.14^c	22^b
ACC-C	6.51 ± 0.21^c	21^b

Note: pH_{MAX} represents the final pH that was reached after 240 seconds (pH_{MAX} results are presented as means $\pm$ SE). $T_{50\%}$ represents the time it took the pH to reach 50% of its maximum value. Different superscript letters represent statistical significances, as determined by ANOVA.

Table 2. Pharmacokinetic Parameters of Calcium in the Serum Following Oral Administration of Radioactive Calcium Carbonate Preparations

Compound	C_{max} (µg/mL)	T_{max} (h)	AUC (µg × h/mL)
CCC	5.8 ± 0.7^a	3.0 ± 0.3^a	109.7 ± 6.4^a
ACC	8.1 ± 0.8^b	2.8 ± 0.2^a	134.5 ± 7.0^b
ACC-C	$6.5 \pm 0.6^{a,b}$	3.4 ± 0.2^a	131.5 ± 9.7^b

Different superscript letters represent statistical significance ($p < .05$), as determined by ANOVA.

that died revealed damage to the esophagus or bladders on the stomach wall presumably caused by the metal syringe used in the administration procedure. Figures 5 and 6 present results from bioavailability experiments of rats that survived through the entire experiment, testing the fractional absorption of the preceding preparations intrinsically labeled with ^{45}Ca following a single oral administration.

Figure 5 presents the changes in serum Ca concentration, as calculated by the radioactive readings normalized to the administered dose. The C_{max} values in rats that received ACC were significantly higher (up to 40%) than those in the CCC group (Fig. 5 and Table 2). The C_{max} values in rats that received ACC-C also were higher (up to 12%) than in rats that received CCC, although this difference was not statistically significant (Table 2). Pharmacokinetic analysis indicates that the bioavailability of the ACC and ACC-C is significantly higher than that of CCC (AUC values are higher by 22.5% and 20%, respectively, $p < .05$), whereas the time required to reach the maximal concentration (T_{max}) did not differ between groups (Table 2).
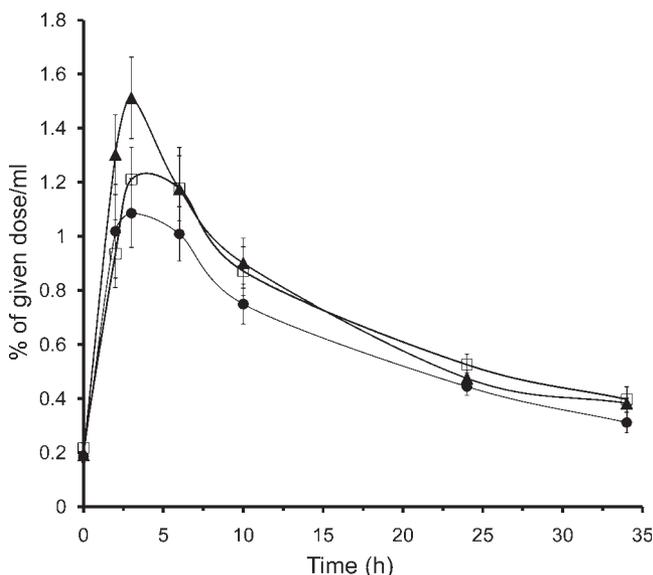

Fig. 5. Serum radioactivity following oral administration of calcium carbonate preparations. Measured radioactivity concentration was divided by the total given radioactivity to calculate the radioactivity percentage of the given dose in the serum. ● = crystalline calcium carbonate (CCC, $n = 13$); ▲ = amorphous calcium carbonate (ACC, $n = 13$); □ = amorphous calcium carbonate with chitosan (ACC-C, $n = 12$). Bars indicate SEM in each case. Each line represents the average of n repeats. Pharmacokinetic values of the graph are presented in Table 2.

The results of calcium content assessment in femurs presented in Fig. 6A are normalized to 100 mg of dry bone weight. Calcium content in the femurs of rats that received ACC was 30% higher ($p < .05$) than that in rats that received CCC. The calcium content in the femurs of rats that received ACC-C was 15% higher than that in rats that received CCC, although these changes were not statistically significant.

Finally, the retention values presented in Fig. 6B suggest that rats that received CCC-containing capsules retained 48.5% $\pm$ 1.3% of the received dose. On the other hand, rats that received ACC- and ACC-C-containing capsules retained 61.4% $\pm$ 2.0% and 60.6% $\pm$ 2.1% of the received dose, respectively. This corresponds to a significant increase in retention of 26.6% and 25%, respectively, compared with retention by the CCC-treated group ($p < .05$).

Discussion

In recent years, our research group has been studying a unique biomineralization process of calcium deposition and mobilization in the freshwater crayfish, *Cherax quadricarinatus*.[18] Based on our findings, phosphoproteins in this remarkable organism were found to be capable of stabilizing one of the least stable salts in nature, ACC.[17] The unique physicochemical properties of ACC enable the crayfish to store large quantities of calcium in transient storage sites and, when needed, to rapidly and efficiently reabsorb calcium through the intestinal epithelia. These observations led us to the hypothesis that ACC also may be absorbed effectively in higher organisms. In order to test this hypothesis and the bioavailability of ACC in such models, a novel technology inspired by the same biologic principals used by the crayfish was developed to produce stable ACC synthetically using selected phosphoaminoacids.[12] Within the scope of this study, we set to investigate the solubility and bioavailability of these synthetic ACC products and compare them with the commonly available CCC.

Analytical assessments using FTIR, Raman spectroscopy, and HR-SEM of our preparations confirmed the amorphous nature and the nanometric structure of the synthetic ACC. Solubility was evaluated according to Economou's procedure from 1996.[31] Since the pH values are correlated with the amount of dissolved CO_3^{2-} ion, the higher the pH, the more soluble is the calcium preparation and the more calcium carbonate was dissolved. Our findings suggest that the solubility of ACC is at least over 10
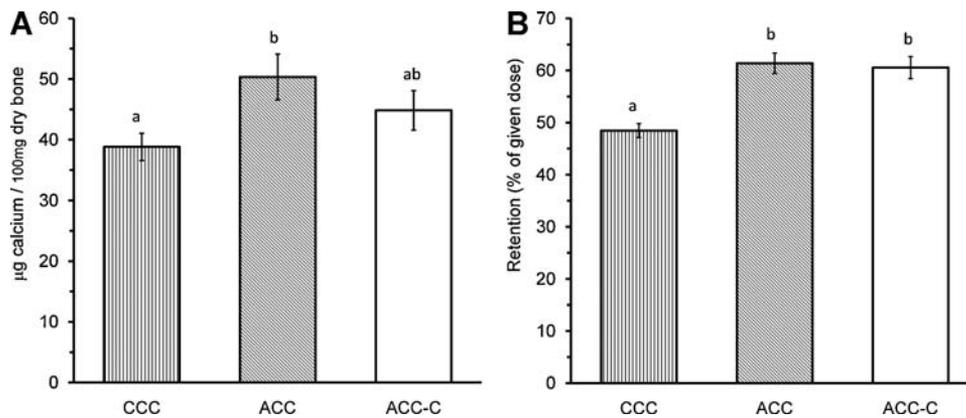
Fig. 6. Calcium content in the femur and whole-body retention following oral administration of radioactive calcium carbonate preparations. (*A*) Calcium content in the femur. Calcium content was normalized to 100 mg of dry bone weight. *From left to right:* Crystalline calcium carbonate (CCC, $n = 13$), amorphous calcium carbonate (ACC, $n = 13$), and amorphous calcium carbonate with chitosan (ACC-C, $n = 12$). (*B*) Total-body retention of calcium. *From left to right:* Crystalline calcium carbonate (CCC, $n = 13$), amorphous calcium carbonate (ACC, $n = 13$), and amorphous calcium carbonate with chitosan (ACC-C, $n = 12$). Bars indicate SEM. Different superscript letters represent statistical significance as determined by ANOVA.

times higher than that of CCC. Interestingly, CCC synthetically produced in our lab, comprising of a mixture of calcite and vaterite, yielded faster dissolution rates and demonstrated higher solubility than did commercial calcite probably owing to the different polymorph composition of the former. The precise role of solubility in fractional absorption of calcium remains under debate, with several publications suggesting that solubility has little or no correlation with bioavailability.[24,32] Others, however, suggest that solubility plays a crucial role in intestinal absorption.[33–38] Our data support the latter viewpoint, where solubility and fractional absorption are closely linked, although this observation was not proven.

Following a thorough examination of available assays, the fractional absorption of ACC was evaluated using a slightly modified protocol of the single-isotope technique.[30] To prevent immediate crystallization of ACC during predissolution in the suspension gavage, we adopted a technique that uses mini-gelatin capsules,[39,40] which allow administrating the preparations directly into the rat's stomach. Acclimation of the rats prior to the administration was performed using a 0.2% calcium diet instead of 1% calcium, which, according to several reports, is suggested to be the optimal diet for calcium bioavailability assays.[41,42] The analysis of calcium content in the feces and femurs was performed using a decalcification solution instead of ashing the radioactive substances in a muffle furnace, as reported previously,[33,42,43] owing to environmental restrictions. This method proved to be highly accurate (~95%) when compared with the ashing technique (data not shown).

The differences in bioavailability between the six polymorphs of calcium carbonate have never been addressed. As such, this study is the first to compare bioavailability of different calcium carbonate polymorphs. Our results suggest that polymorphism of calcium carbonate, clearly demonstrated in the case of ACC, can affect bioavailability of calcium in rats. It is plausible that the differences in particle size, surface area, and solubility[4] affect calcium ion absorption by the intestine, a situation that would enhance passive diffusion of calcium, as described previously by Weaver and colleagues.[1] Another possible explanation for the

higher calcium absorption from ACC is the nanometric nature of ACC particles; these might be small enough to pass the intestinal epithelia as intact calcium carbonate complexes. This hypothesis was suggested previously by the observation of rats' ability to absorb small, intact complexes such as calcium oxalate.[44] Although not proven, absorption of intact calcium carbonate complexes could explain in part the absorption of calcium in humans afflicted with achlorhydria (a state where the production of gastric acid in the stomach is absent or low).[2] The elevated levels of calcium detected in the femurs of ACC-administrated rats also may be linked to the high levels of calcium detected in the serum.

In an attempt to further increase the similarity of synthetically produced ACC to the ACC produced by the crayfish, embedded in a chitin matrix and serving as our biologic inspiration,[17,18] chitosan, the commonly used deacetylated derivative of chitin, was added during the synthesis of ACC-C. Chitosan is also suggested to increase intestinal absorption of macromolecules such as octreotide, insulin, and low-molecular-weight heparin (LMWH).[45–47] It is proposed that polycations such as chitosan increase molecule absorption by enhancing permeability through tight junctions in the intestine.[48,49] However, our findings show that the presence of chitosan in ACC-C did not enhance calcium bioavailability.

Comparison of the results obtained in this study with previous reports highlights a few significant differences. In contrast to previous studies, where T_{max} values were close to 1 hour from administration,[33,50] values obtained in this study were closer to 3 hours. We suggest that our T_{max} values are affected by use of the gelatin capsules, which prolonged dissolution of the samples, thus shifting T_{max}. To the best of our knowledge, there is only a single report of a similar study where T_{max} values were close to 3 hours,[34] although these differences were not addressed.

While the differences between the amorphous and crystalline preparations were clearly demonstrated in all the parameters tested in rats, namely, in the serum, femur, and in terms of retention values, the retention results in all tested preparations

Table 3. Elemental Calcium Weight Percentage in Common Calcium Compound Formulations

Chelating molecule	Elemental calcium
Carbonate	40%
Amorphous calcium carbonate[a]	33%
Citrate	21%
Citrate-malate	15% to 23%[b]
Fumarate	19%
Gluconate	9.3%
Lactate	18%
Lactate gluconate	12%

Note: Calcium weight percentage was calculated by dividing the calcium molecular weight by the total molecular weight of the salt.

[a]Amorphous calcium carbonate elemental calcium was evaluated by atomic absorption.

[b]Exact percentage depends on the complex used.

obtained here were notably higher than most previous reports of similar calcium carbonate single-dose experiments.[51–53] The enhanced retention results might be attributed to the low calcium acclimation diet used and the use of direct administration via gelatin capsules.[54] The effect of the different polymorph compositions also may be attributed to high retention, especially in the CCC preparation, comprising a mixture of vaterite and calcite. The polymorph composition of calcium carbonate tested in previous reports was never addressed, thus limiting comparability to our results.

While difficult to compare between different calcium formulations with or without a reference salt, the retention values obtained in this study are as high as those observed for calcium ascorbate[50] and higher than those reported for other calcium formulations, such as calcium lactate, fumarate, citrate, citrate-malate, carbonate, and TBE.[34,51,52] Moreover, when considering the effectiveness of dietary calcium sources, one also must take into account the relatively low elemental calcium content of these formulations. In this respect, ACC contains a relatively high elemental calcium content of 33%, as shown in Table 3.

Bioavailability assessments depend strongly on age, gender, and metabolic state of the rats, and small changes in the experimental model can significantly influence calcium bioavailability. Further investigation into amorphous calcium carbonate bioavailability and its incorporation in the bone in other models including osteoporotic models should be addressed in future studies.

In conclusion, this study revealed the higher solubility and bioavailability of amorphous calcium carbonate versus commonly used crystalline calcium carbonate. The significant improvements in all tested parameters support the use of ACC as a highly available dietary source of calcium.

Disclosures

Acknowledgments

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