Variations in Urine Calcium Isotope Composition Reflect Changes in Bone Mineral Balance in Humans

By

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Summary

Changes in bone mineral balance cause rapid and systematic changes in the calcium isotope composition of human urine.

Abstract

Urine from subjects in a 17 week bed rest study was analyzed for calcium isotopic composition. Comparison of isotopic data with measurements of bone mineral density and metabolic markers of bone metabolism indicates the calcium isotope composition of urine reflects changes in bone mineral balance. Urine calcium isotope composition probably is affected by both bone metabolism and renal processes. Calcium isotope analysis of urine and other tissues may provide information on bone mineral balance that is in important respects better than that available from other techniques, and illustrates the usefulness of applying geochemical techniques to biomedical problems.

The movement of calcium between organisms and their environments, and between different compartments within an organism, is strictly regulated and essential to life. In humans, acute disruptions of calcium metabolism can lead to serious health problems such as neurologic and muscle disorders. Persistent disruptions can be manifested in skeletal disorders such as osteomalacia and osteoporosis. Vertebrate calcium intake and excretion are controlled largely by the linked processes of intestinal calcium absorption and urinary and fecal calcium excretion. The skeleton, being the largest reservoir for calcium in the body, is responsible for the short term control of blood levels of this element. Thus, the development of techniques for quantifying bone resorption, bone formation and skeletal mass play a key role in our search for understanding calcium movements in the bone compartment of humans. Because measures of bone resorption and formation are relative estimates of processes that are not quantitatively comparable, it remains very difficult to determine changes of mineral balance in the bone. However, in this report we show that *natural* variations in calcium isotope composition can be detected in human urine, and present evidence that these variations indicate changes, including rapid changes, in bone mineral balance.

Natural calcium is a mixture of six isotopes (1). It is known that many processes, including biological processes, can "fractionate", or alter the relative abundances of calcium isotopes. Calcium isotopic compositions are expressed as $\delta^{44}Ca$, which can be defined as the difference, in parts per thousand (‰), between the $^{44}Ca/^{40}Ca$ (2) of a sample and a laboratory reference material (here, dissolved calcium in seawater). There appears to be no isotope fractionation during intestinal absorption and secretion of

calcium (3), or during bone resorption (4, 5). However, there is a approximately -1.3‰ $\delta^{44}Ca$ fractionation factor associated with bone formation (6), resulting in the $\delta^{44}Ca$ of skeletal calcium being about 1.3 ‰ lower (i.e., isotopically lighter) than the diet and soft tissue average (4, Figure 1).

A simple theoretical model relates soft tissue $\delta^{44}Ca$ to bone mineral balance (4). The basis of this model is that calcium isotope fractionation during bone formation preferentially moves isotopically light calcium into the skeleton, thus enriching soft tissue in heavy calcium. Conversely, bone resorption returns isotopically light skeletal calcium to soft tissues. Thus, positive bone mineral balance (where the rate of bone formation exceeds the rate of bone resorption) causes positive excursions in soft tissue $\delta^{44}Ca$, and negative bone mineral balance causes negative excursions in soft tissue $\delta^{44}Ca$. If bone formation is the only process in the body that fractionates calcium isotopes, at "steady state" (when the rate of bone formation and bone resorption are equal), $\delta^{44}Ca$ of soft tissue will be the same as $\delta^{44}Ca$ of diet. Given the short residence time of calcium in soft tissue (i.e., days) and the fact that soft tissue calcium concentrations are strictly regulated, soft tissue $\delta^{44}Ca$ should respond quickly to changes in bone mineral balance. These changes should be reflected in both blood and urine calcium isotope composition.

A correlation between soft tissue $\delta^{44}Ca$ and bone mineral balance has been inferred in non-human vertebrates (4). To determine whether the same correlation is detectable in humans, $\delta^{44}Ca$ of two food samples and 49 urine samples from 10 subjects in a 17- week bed rest study (7, 8, Table 1) were measured (9). This original study was designed to determine the effectiveness of two countermeasures-- resistive exercise and the anti-

resorptive bisphosphonate drug alendronate—against (simulated) weightlessness-induced bone loss. Subjects were assigned to one of three groups: a group treated with alendronate, a group subjected to a regimen of resistive exercise, and a control (untreated) group. Effectiveness of treatments was evaluated by comparing measurements of bone mineral density (BMD), net calcium balance, and biochemical markers of bone resorption (n-telopeptide, NTX(10)) and bone formation (bone-specific alkaline phosphatase, BSAP (11)) in the alendronate and exercise subjects with those of the control subjects. These data have been published and interpreted elsewhere (7, 8).

Relative to initial values measured for each subject, $\delta^{44}Ca$ values were generally negative for the control group, positive for the alendronate group and unchanged for the exercise group (Figure 2). These data indicate that control subjects lost bone during bed rest while alendronate subjects gained bone and exercise subjects maintained bone mass balance. These results confirm the prediction of bone loss in the control group, and generally are consistent with published interpretations of the BMD and biochemical marker data (7, 8) Table 2). The results are also consistent with theoretical predictions. Given reasonable estimates of intestinal calcium absorption and bone turnover rate in control subjects (12), the predicted change in urine $\delta^{44}Ca$ of control subjects during bed rest is -0.20‰ to –0.48‰. The observed change was –0.58‰. Considering quantitative uncertainties in the data, and uncertainties about key aspects of calcium isotopic behavior in humans, such as the calcium isotope fractionation factor during bone mineralization, the agreement between predicted and observed change in $\delta^{44}Ca$ is good.

Measurements of metabolically induced variations in calcium isotope composition may provide better data on short-term changes in bone mineral balance than other current techniques. Changes in BMD over the course of the experiment were small, varied considerably between different parts of the skeleton, and are difficult to interpret in terms of changes in total skeletal mass. Net calcium balance calculations are notoriously inaccurate, because they ignore dermal calcium loss (13, 14), and because net calcium balance is typically small compared to calcium intake and excretion measurements from which it is calculated, so that relatively small errors in measuring intake or excretion can translate into large errors in net calcium balance (15, 16). NTX and BSAP reflect bone resorption and bone formation, respectively, but there is no reliable way to combine these measurements into a quantitative measure of bone mineral balance. This difficulty is compounded by the fact that changes in bone turnover rate can affect rates of bone formation and resorption in similar ways. Thus NTX and BSAP are often positively correlated, as is the case with the alendronate and exercise subjects in this study (Table 2). In contrast $\delta^{44}Ca$ is directly related to bone mineral balance. Unlike BMD, large changes in $\delta^{44}Ca$ can occur quickly and do not require large changes in bone mineral mass. Unlike NTX and BSAP, $\delta^{44}Ca$ responds to the relative rates of bone formation and resorption, not by the absolute rate of bone turnover. Moreover, methods utilizing a measurement of $\delta^{44}Ca$ have the potential to discriminate between low bone density due to loss of skeletal mineral (i.e. osteoporosis) versus low bone density caused by a decrease in the mineralization of existing osteoid (i.e. osteomalacia and rickets). For these reasons $\delta^{44}Ca$ measurements may be broadly useful in the study of bone mineral metabolism.

If bone formation were the only process that fractionated calcium isotopes in humans, urine $\delta^{44}Ca$ of soft tissues should be lower than dietary $\delta^{44}Ca$ when bone loss exceeds bone formation. However, $\delta^{44}Ca$ is uniformly higher than dietary $\delta^{44}Ca$, even in control subjects who experienced net bone loss. Urine $\delta^{44}Ca$ also is strongly and inversely correlated with the rate of urinary calcium excretion (Figure 3). These observations indicate that calcium isotopes are fractionated during urine formation. Fractionation during renal reabsorption of calcium would almost certainly deplete urine of isotopically light calcium (17), and reasonably could account for both the correlation between urinary $\delta^{44}Ca$ and calcium excretion rate, and the fact that urine calcium is isotopically heavier than dietary calcium. Alternatively, or in addition, high $\delta^{44}Ca$ of urine calcium may reflect isotope fractionation between the ultra-filtratable and the protein-bound fractions of calcium in blood. Only the ultra-filtratable fraction is a source for calcium in urine, and if calcium in this fraction is isotopically heavier than the protein-bound fraction (e.g., due to differences in Ca-bond strengths), this difference would be reflected in urine. It appears that measurements of $\delta^{44}Ca$ may also be useful in studying aspects of calcium metabolism other than bone mineral balance, such as renal calcium processing.

There is great potential for fruitful and truly interdisciplinary research into biologically induced variations in the isotopes of calcium and other elements. By using knowledge of the isotopic behaviors of elements such as carbon and oxygen, the natural isotope chemistry of these elements is exploited in geology and other sciences (18). However, such tools are rarely applied to medically important intra-organismal processes. The ability of calcium isotopes to track biologically and medically significant changes in the human body demonstrates that isotope analysis in general is a promising area for

biomedical research. The intra-organismal behaviors of the isotopes of elements such as lead, mercury, selenium (19), iron (20), copper (21, 22), zinc (21, 22) chromium (23) and molybdenum (24), await investigation.

NOTES AND CITATIONS

1. ^{40}Ca (96.94%), ^{42}Ca (0.65%), ^{43}Ca(0.15%), ^{44}Ca (2.09%), ^{46}Ca (>0.01%), and ^{48}Ca (0.19%).

2. The isotope fractionation discussed in this paper is mass-dependent, such that the magnitude of fractionation is directly proportional to the mass differences between isotopes. In this study, the $^{44}Ca/^{40}Ca$ ratio was used for analytical reasons.

3. J. L. Skulan, D. J DePaolo, T. L Owens, *Geochim. Cosmochim. Acta* **61**, 2505 (1997).

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5. Mass-dependent isotope fractionation is possible during bone formation because Ca^{++} ions can freely move and compete for sites in growing crystals. Less massive ions move more quickly than heavier ions and are more likely to be incorporated into the crystals. Once in bone Ca atoms are locked in a crystal lattice and thus whether or not they dissolve depends on their position, not their mass.

6. A fractionation factor is the instantaneous isotopic difference between the reactant and the product (in this case Ca^{++} and Ca in bone mineral, respectively) in a reaction.

7. A. D. LeBlanc *et al., Musculoskel. Neuron. Interact.*, **4**, 335 (2002).

8. A. D. Leblanc et al., *J. Appl. Phys.*, in revision.

9. Urine samples were diluted in 2% Teflon-distilled HNO3, while food samples were first totally dissolved in concentrated Teflon-distilled HNO3 on a warm plate (30°C) and then diluted with distilled water to 2% HNO3 for analysis of Ca concentration by inductively coupled plasma mass spectrometry. Calcium was purified from the diluted samples using cation-exchange chromatography and analyzed for isotopic composition using thermal ionization mass spectrometry. Prior to sample purification, an aliquot of each sample sufficient to provide 750ng of Ca was mixed with a set amount of a calibrated ^{42}Ca-^{48}Ca double spike solution. Use of the double spike allows correction for both instrumental mass discrimination and possible isotope fractionation associated with non-quantitative recovery during column chemistry. The double spike procedure allows $^{44}Ca/^{40}Ca$ to be determined with an internal precision of ± 0.15‰ or better at the 2-sigma level. Further details of column chemistry, mass spectrographic techniques and data reduction procedures are available elsewhere (3,14).

10. Enzyme-linked immunosorbent assay, Ostex Int., Seattle WA

11. Enzyme-linked immunosorbent assay, Metra Biosystems, Palo Alto, CA

12. The theoretical relationship between soft tissue $\delta^{44}Ca$ and bone mineral balance is given by:

$$\delta_s = \delta_d + (V_l(\delta_b - \delta_d) - V_b\Delta_b)/V_d + V_l$$

Where δ_s, δ_d and δ_b are $\delta^{44}Ca$ of soft tissue, dietary Ca and bone, respectively, V_l, V_b and V_d are the fluxes of Ca resorbed from bone, incorporated into bone and absorbed from diet, respectively, and Δ_b is the Ca isotope fractionation factor during bone formation.

Average dietary Ca for all subjects was about 1000 mg/day. Assuming 20% absorption of dietary Ca, a skeletal Ca mass of 1200g, and a 1.25% loss of bone mass over 17 weeks, the flux of Ca into the body from diet was 200mg/day, and the net loss of calcium from bone was 131mg/day. Given a fractionation factor of –1.3‰ $\delta^{44}Ca$ during bone formation, if the rate of Ca incorporation into new bone was 500mg/day and the rate of bone loss was 630mg.day, the predicted change in $\delta^{44}Ca$ is –0.20‰. Doubling the rate of bone loss and using a fractionation factor of –1.8‰ $\delta^{44}Ca$ yields a predicted change in $\delta^{44}Ca$ of –0.48‰.

13. J. T. Irving, *Calcium & Phosphorus Metabolism.* (Academic Press, New York, 1973)

14. B. Isaksson, B. Sjogren, *Proc. Nutr. Soc.* **26**, 106 (1967)

15. R. P. Heaney, *J. Nutr.*, **131**, 1344S (2001).

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17. Isotope fractionations associated with unidirectional processes such as filtration and irreversible chemical reactions almost always select against heavy isotopes. If calcium

was fractionated during renal excretion, the expected result would be isotopically light urine (lower $\delta^{44}Ca$).

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Table 1. Isotope, NTX, and BSAP data from urine samples. Week numbers refer to the number of weeks of bed rest. Week 0 values are those at the start of bed rest. Before bed rest started, subjects were acclimated to a uniform diet for 3 weeks. For subject 2, these weeks are designated with negative numbers. Bed rest ended at week 17, after which subjects were allowed to return to normal activity but were monitored for an additional 2 weeks. Week 19 data are from the end of this ambulatory period.

Sample no.	Subject	Group	Weeks of bedrest	$^{44}Ca/^{40}Ca$	$\delta^{44}Ca$ (‰)	NTX (nmol/day)	BSAP (U/L)
28	Subject 1	A	0	0.021683	-1.38	621	15.7
44	Subject 1	A	4	0.021692	-0.97	455	15.8
48	Subject 1	A	10	0.0216959	-0.79	659	15.3
12	Subject 1	A	17	0.0216984	-0.67	594	14.3
47	Subject 1	A	19	0.021678	-1.61	579	12.5
41	Subject 9	A	0	0.0216757	-1.72	630	22.8
5	Subject 9	A	4	0.021709	-0.18	555	21.7
50	Subject 9	A	10	0.0216891	-1.10	580	19.1
33	Subject 9	A	17	0.0216782	-1.60	591	17.0
23	Subject 9	A	19	0.0216868	-1.21	586	18.9
3	Subject 10	A	0	0.0217161	0.14	680	24.5
40	Subject 10	A	4	0.0217177	0.22	612	20.1
31	Subject 10	A	17	0.0217207	0.35	497	15.3
10	Subject 10	A	19	0.0217195	0.30	431	16.9
35	Subject 2	C	-2	0.0217048	-0.38	462	nm
38	Subject 2	C	-1	0.0217075	-0.25	379	nm
8	Subject 2	C	0	0.0217138	0.04	456	14.3
19	Subject 2	C	4	0.0216947	-0.84	472	nm
4	Subject 2	C	6	0.021686	-1.24	730	16.0
45	Subject 2	C	10	0.0216773	-1.64	608	nm
30	Subject 2	C	12	0.0216781	-1.61	537	nm
42	Subject 2	C	15	0.0216896	-1.08	611	nm
16	Subject 2	C	17	0.0216873	-1.18	612	14.4
24	Subject 2	C	18	0.0216925	-0.94	623	nm
20	Subject 2	C	19	0.0217064	-0.30	535	14.7

29	Subject 5	C	0	0.0216794	-1.55	909	20.3
27	Subject 5	C	4	0.0216795	-1.54	1239	17.7
49	Subject 5	C	10	0.0216884	-1.13	1127	19.5
7	Subject 5	C	16	0.0217	-0.60	1088	18.2
25	Subject 5	C	19	0.021687	-1.20	785	20.6
18	Subject 7	C	0	0.0217035	-0.44	418	15.9
26	Subject 7	C	4	0.0216857	-1.26	662	15.5
54	Subject 7	C	10	0.0216866	-1.22	681	17.0
36	Subject 7	C	17	0.0216783	-1.60	765	16.3
21	Subject 7	C	19	0.0216921	-0.96	615	19.4
15	Subject 8	C	0	0.0217032	-0.45	434	17.1
9	Subject 8	C	4	0.021685	-1.29	764	nm
53	Subject 8	C	10	0.0216899	-1.06	852	15.8
46	Subject 8	C	17	0.021693	-0.92	850	16.8
43	Subject 8	C	19	0.0216981	-0.69	586	17.7
17	Subject 3	E	0	0.021694	-0.88	710	21.4
11	Subject 3	E	4	0.0216947	-0.84	851	32.0
51	Subject 3	E	10	0.0216972	-0.73	977	30.1
2	Subject 3	E	17	0.0216898	-1.07	1075	27.3
37	Subject 3	E	19	0.0216792	-1.56	1051	28.0
22	Subject 4	E	0	0.0216965	-0.76	697	15.2
6	Subject 4	E	4	0.0216888	-1.11	511	16.6
52	Subject 4	E	10	0.0217014	-0.53	740	20.1
34	Subject 4	E	17	0.0216972	-0.73	620	22.7
14	Subject 4	E	19	0.0216903	-1.05	806	21.5
32	Subject 6	E	0	0.0216765	-1.68	614	25.0
13	Subject 6	E	4	0.021686	-1.24	698	51.7
1	Subject 6	E	17	0.0216902	-1.05	949	37.8
39	Subject 6	E	19	0.0216915	-0.99	819	30.8

Table 2. Mean data for all subjects at all times for each group. 1 sigma errors are shown for $\delta^{44}Ca$ data. BMD data from the axial skeleton were used because this is the part of the skeleton most likely to show mineral loss during inactivity, and should most clearly reflect experimentally induced changes in bone mineral balance. NTX and BSAP are metabolic markers of bone resorption and formation, respectively. Note that in the exercise and alendronate groups these markers co-varied, reflecting the fact that bone formation and resorption are metabolically coupled. This coupling makes it difficult to explain changes in overall bone mineral balance in terms of changes in NTX and BSAP. Net calcium balance are published values for whole experimental groups (6, 7), not just the subsets of these groups selected for urine calcium isotope analyses.

Group	Mean change in BMD from starting value (%)	Mean change in urinary $\delta^{44}Ca$ from starting value (‰)	Mean change in NTX (nmol/day)	Mean change in BSAP (U/L)	Net Ca balance (mg/day)
Control	-1.25	-0.580 (+/-.35)	210.66	0.02	-199 ± 33
Exercise	0.06	0.072 (+/-.13)	153.06	9.36	22 ± 54
Alendronate	1.61	0.483 (+/- 0.18)	-89.40	-3.97	-62 ± 38

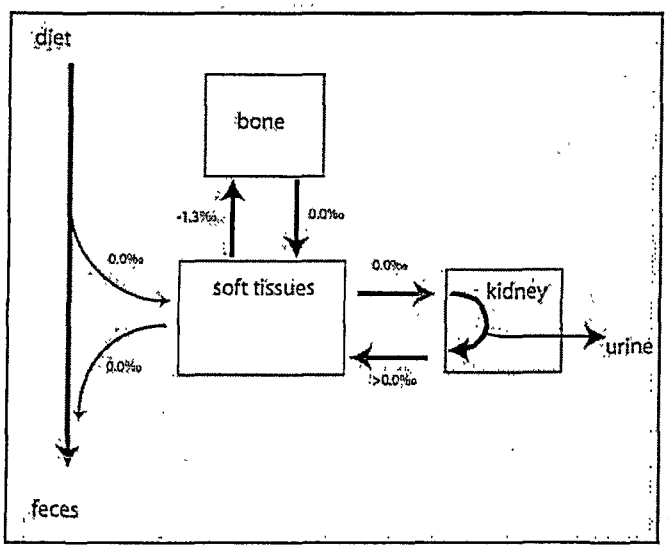
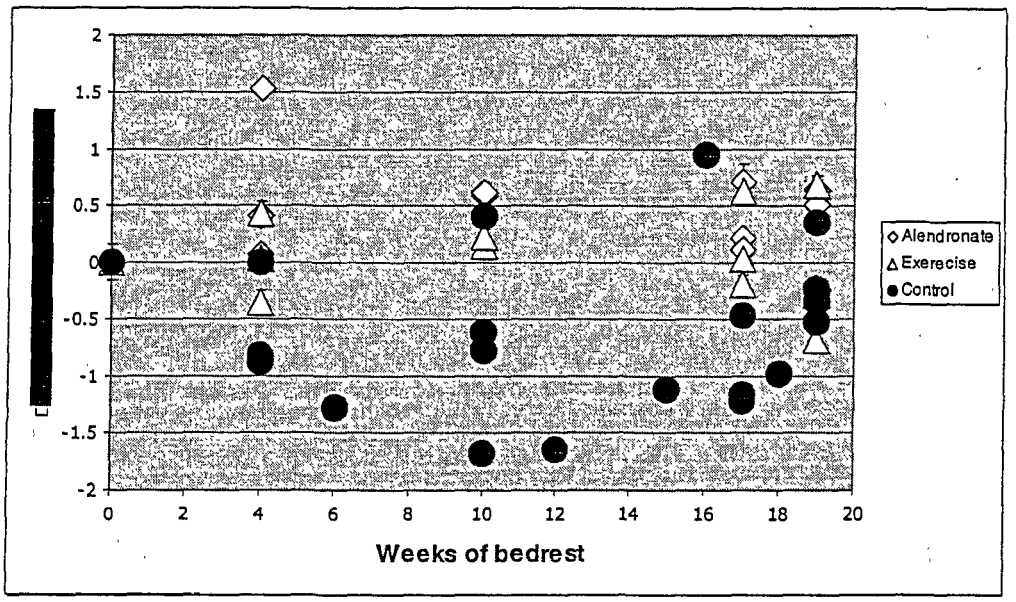


Figure 1. Simplified schematic representation of vertebrate calcium metabolism. Arrows indicate routes of calcium movement between compartments, and numbers with arrows show calcium isotope fractionation factors. The calcium isotope difference between soft tissue and bone, and the ability of soft tissue and urinary $\delta^{44}Ca$ to track changes in bone mineral balance, is the result of there being a 1.3‰ fractionation during bone formation, but no fractionation during bone resorption. This model assumes that renal fractionation occurs during calcium re-uptake.


2
1.5
1
0.5
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-1
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16
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Weeks of bedrest
Alendronate
Exerecise
Control

Figure 2a

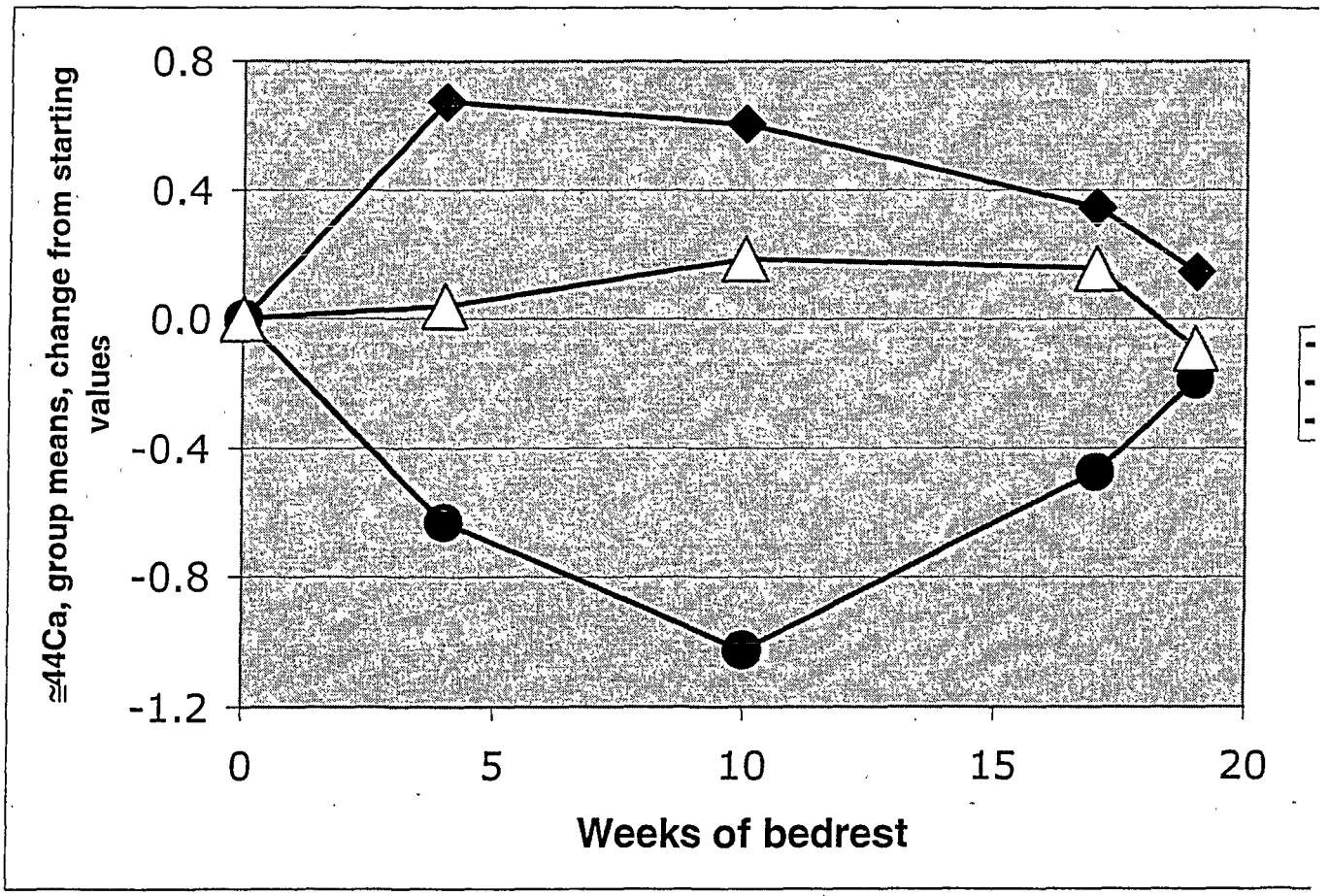
0.8
0.4
0.0
-0.4
-0.8
-1.2
0
5
10
15
20
Weeks of bedrest
≅44Ca, group means, change from starting values

Figure 2b

Figure 2. Change in urine d44Ca during and after bedrest, relative to week zero values, by subject. Because starting $\delta^{44}Ca$ of subjects varied widely, $\delta^{44}Ca$ is referenced to the starting value of each patient. During the study, patients were fed identical diets. Two samples of homogenized diet yielded the same $\delta^{44}Ca$ (-1.88‰ and –1.90‰), indicating that dietary $\delta^{44}Ca$ was approximately constant during the study. Variations in dietary $\delta^{44}Ca$ would have affected all subjects, and could not explain the isotopic differences between subjects that are of interest here. Figure4 2a shows data for all patients with 1 sigma errors. Figure 2b shows group means. If changes in $\delta^{44}Ca$ are positively correlated with bone mineral balance, these data indicate that during bed rest, on average, alendronate subjects maintained positive bone mineral balance, exercise subjects neutral bone mineral balance, and control subjects negative bone mineral balance. Note that because the true bone mineral status of any individual subject is unknown. Because there is no expectation that the bone mineral status of all subjects in a particular group at a particular time would be the same, errors are not shown for the group means in Figure 2b.

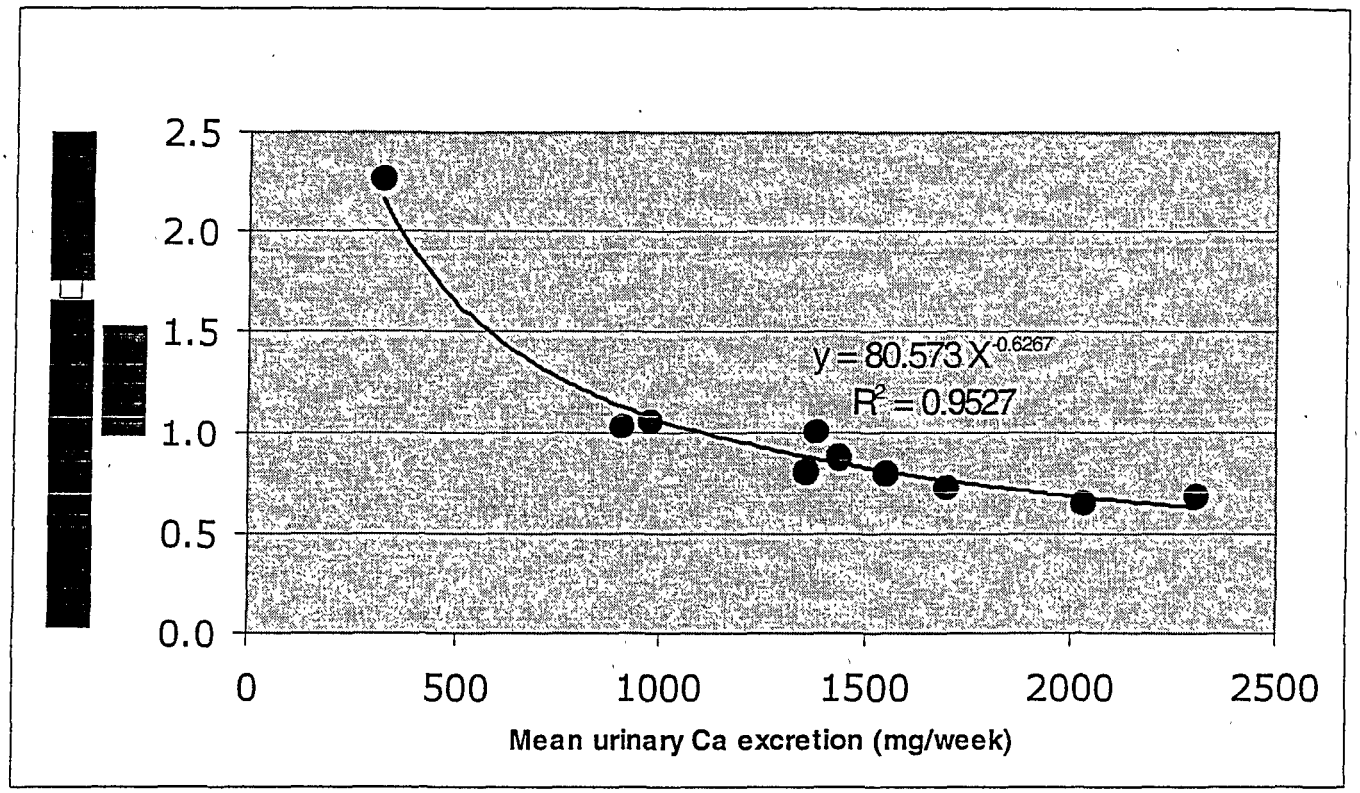

Figure 3. Ca excretion rate versus the mean isotopic offset between urine and diet ($\delta^{44}Ca_{urine}$ minus $\delta^{44}Ca_{diet}$). Each point represents the mean values for one subject through the period of bed rest. Urinary $\delta^{44}Ca$ of all subjects was higher than dietary $\delta^{44}Ca$ regardless of bone mineral balance. Isotopic offset was also inversely correlated with urinary calcium excretion rate. This correlation would be most easily explained if renal reuptake depletes urine of isotopically light calcium; the higher the fraction of isotopically light calcium that is absorbed, the more istopically heavy the calcium remaining in urine would become. Even small fractionation factors can produce large isotope effects when they operate continuously on a diminishing and closed pool of

calcium. As about 98% of calcium originally filtered from blood is reabsorbed, fractionation during reuptake probably is quite small.