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Rapid Communication

A pilot study on the use of natural calcium isotope ($^{44}Ca/^{40}Ca$) fractionation in urine as a proxy for the human body calcium balance

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ABSTRACT

We explored the possibility of using natural calcium (Ca) isotope variations in the urine ($\delta^{44/40}Ca_{urine}$) as a proxy for the Ca balance in the human body. We chose two test persons extremely different in their health status, gender and age (4-year-old healthy boy and a 60-year-old woman known to suffer from osteoporosis). During a 5 day interval the Ca isotope composition of the individual diet ($\delta^{44/40}Ca_{diet}$) was monitored for both test persons to be in general agreement to the Ca isotope composition of the normal western European diet ($\sim -1.02 \pm 0.1$‰). However, measurements showed that (1) $\delta^{44/40}Ca_{urine}$ of both test persons are ~1.37 and ~2.49‰, respectively, heavier than $\delta^{44/40}Ca_{diet}$ and that (2) the $\delta^{44/40}Ca_{urine\text{-}boy}$ is ~1.1‰ heavier when compared to the value of the woman. The individual offset between diet and test persons is interpreted to reflect individual Ca reabsorption rates in the kidneys being the result of Rayleigh type Ca isotope fractionation related to the partitioning of Ca between the glomerular filtrate and filtered residue. The relative difference between $\delta^{44/40}Ca_{urine\text{-}boy}$ and $\delta^{44/40}Ca_{urine\text{-}woman}$ of ~1.1‰ may reflect individual differences in the balance of bone mineralization and demineralization processes related to age, gender and health status. By arbitrarily defining an equilibrium value for $\Delta^{44/40}Ca_{diet\text{-}urine}$ of −1.93‰ being the arithmetic mean of $\delta^{44/40}Ca_{urine}$ for both test persons the measured $\delta^{44/40}Ca_{urine}$ values may be applied to model the individual bone mineralization and demineralization processes in a qualitative way. Note, second order influences of intestinal Ca absorption during sequestration of Ca between intestine and blood have to be subject of further studies.

Introduction

Calcium (Ca) is composed of six isotopes spanning a mass range from 40 to 48 atomic mass units (u). The natural abundances of the Ca isotopes range from ~97% for ^{40}Ca down to ~0.004% for ^{46}Ca. During the past years an increasing number of studies were published concerning the fractionation of Ca isotopes in geological and biological systems. This is because Ca isotopes may be used to reconstruct sea surface temperatures [1,2] and secular changes of Ca concentration of the oceans [3–5] and to study biomineralization processes (e.g. [6–8]). Besides its importance in the chemical Earth history Ca also plays an important role in the physiology of most organisms. In human physiology Ca is used in biominerals for the formation and maintenance of bones and teeth (e.g. [9]). Furthermore, Ca is a major participant in processes like muscle contraction, signal transmission, cell apoptosis, cell reproduction, and coagulation of blood. In larger concentrations Ca is known to be cell poisoning and, hence, its concentration has to be held constant in very close limits (Ca homeostasis). Mainly, three organs control Ca homeostasis: the gastrointestinal tract where Ca is absorbed from the diet, the skeleton which is the main reversible Ca reservoir and the kidneys controlling the excretion and recycling of calcium from the blood. Disease related organ malfunction, physiological ageing processes and other diseases are known to be associated with disturbances of the calcium homeostasis.

In earlier studies the dynamic of human Ca homeostasis and Ca absorption rates in the gastrointestinal tract has been investigated in great detail in tracer experiments by using stable Ca (e.g. ^{42}Ca and ^{44}Ca) as well as radioactive Ca isotopes (^{41}Ca, ^{45}Ca or ^{47}Ca) (e.g. [10–15]). These kind of studies are carried out by administering tracer enriched Ca orally and intravenously, respectively. In contrast, studies using natural Ca isotope fractionation (e.g. $^{44}Ca/^{40}Ca$, $^{44}Ca/^{42}Ca$) have the advantage that they are noninvasive and less expensive and have no exposure to radioactive Ca isotopes [8,16–18]. However, studies using natural Ca isotope fractionation are rare because the precise measurement of small variations of natural Ca isotope variations is still a challenge, although analytical methods largely improved in recent years as a function of analytical innovation.

Skulan and DePaolo [17] were the first to study stable Ca isotopes in a life science context by presenting a conceptual model describing the fractionation of Ca isotopes between soft and mineralized tissue (bones and shells) in multi-cellular organisms. Their simplified

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physiological box model assumed that (1) Ca isotope fractionation between diet and soft tissue is negligible, (2) no Ca isotope fractionation between soft tissue and excreted Ca, and (3) no Ca isotope fractionation during bone degradation but (4) a Ca isotope fractionation between soft tissue and mineralized tissue occurs. From empirical data Skulan and DePaolo [17] found that there is an isotope difference of −1.3‰ between soft tissue and bone being the bone isotopically lighter (enriched in ^{40}Ca) than the soft tissue (enriched in ^{44}Ca). Hence, the Ca isotopic composition of the excreted Ca in the urine should reflect the ratio between Ca loss and gain in the mineralized tissue. If the degradation of mineralized tissue prevails (Ca loss from the bone) the Ca isotopic composition of soft tissue and of the excreted Ca becomes isotopically lighter. In contrast, if the growth of mineralized tissue prevails (Ca enrichment in the bone) the Ca isotopic composition of soft tissue and thus of the excreted Ca becomes enriched in the heavy Ca isotopes. Therefore, the authors suggested that the isotopic composition of excreted Ca can be used to monitor the calcium balance in vertebrates. Note, that recent progress [18] meanwhile confirmed the earlier assumption (1) of Skulan and DePaolo [17] that Ca isotope fractionation is negligible between diet and soft tissue. Assumption (2) has been tested by Skulan et al. [8] indicating that isotope fraction occurs between soft tissue and excreted Ca probably due to physiological processes in the kidneys. Latter findings of Skulan et al. [8] have to be taken into account in further studies and box modeling.

Based on the model of Skulan and DePaolo [17] the Ca isotope composition of excreted Ca in the urine may be used as a noninvasive tool to detect disturbances of the Ca homeostasis between blood (blood plasma) and mineralized tissues (e.g. bones) in humans. A relative enrichment of the light isotope in the urine would then be indicative for a net Ca loss of the body (degradation of mineralized tissue prevails) as it would be expected for diseases like osteoporosis while a relative enrichment of the heavy isotope in the urine would be indicative for an enrichment of Ca in the skeleton during growth of mineralized tissue. In a recent publication Skulan et al. [8] presented a study on $\delta^{44/40}Ca_{urine}$. They interpreted $\delta^{44/40}Ca_{urine}$ values to reflect changes of the osseous Ca balance. One-week pooled urine samples from a 10-person subset of participants have been measured to study the effectiveness of countermeasures to bone loss in spaceflight. As could be expected from Skulan and DePaolo [17] the results indicate that changes in Ca isotope composition of the soft tissue are associated with changes in bone balance. The Ca isotopic composition of the urine also correlates with bone mineral density and biochemical bone marker data of the study [19]. However, Skulan et al. [8] also stated that processes other than bone formation may fractionate Ca resulting in isotopically different compartments. This statement is supported by the fact that all of the measured $\delta^{44/40}Ca_{urine}$ values are higher than the $\delta^{44/40}Ca_{diet}$ values.

Here we present a simple pilot study on the use of natural Ca isotope fractionation in order to detect Ca isotope fractionation processes on the way of Ca from the diet via the gastrointestinal tract, blood, the bones, the kidneys to the urine. In order to evaluate the dynamic range of Ca isotope fractionation in the humans' physiology we chose to investigate a 4-year-old healthy boy and a 63-year-old woman, known to suffer from osteoporosis. Since the test persons' Ca metabolisms are expected to represent opposite physiological ends their urine Ca isotope composition should differ to a large extend. The daily variations of the test persons dietary Ca were monitored during the sampling period to distinguish physiological induced variations from those induced by the individual diet.

Samples and experimental approach

Following the basic assumption of Skulan and DePaolo [17] and neglecting the influence of any organ the isotopically lightest, in ^{44}Ca depleted Ca can be expected in the urine of those humans showing a net loss of Ca due to mineral dissolution and disintegration of bone mass. In contrast human urine should be isotopically heavier, enriched in ^{44}Ca, for those bodies which tend to accumulate Ca building up bone mass. Following this simplified conceptual approach we may predict that the Ca isotope composition of any person's urine suffering from osteoporosis should isotopically be lighter than the urine of a young child still being in the process of growth and bone mass accumulation. In order to test this simplified assumption we chose a 4-year-old healthy boy (series A) and a 64-year-old woman (series B) known to suffer from osteoporosis. None of the test persons took any drugs, pharmaceuticals, Ca supplements or other not recorded substances during the test period. The urine of both test persons was collected over a period of about 5 days with three samples per day in the sequence morning–noon–evening.

Methods

Experimental setup and performance

Throughout a time period of 170 h (series A) and 109 h (series B) the diet of the two test persons was monitored by taking notes on the type and relative amount of the food. The elderly lady (B) kept note about her daily diet herself whereas the young boy's (A) diet was recorded by his parents. The nutrition was classified into dairy products, vegetables, fat, crop products and meat. We found that there is a general agreement of the test persons' nutrition with the typical western European diet as can be seen from Table 5. Slight differences in the lady's diet and the little boy's diet concerning its composition are related to a somewhat larger contribution of dairy products in the boy's diet, because he was used to drinking about 50 to 100 ml pure milk in the morning. Neither of the test persons is vegetarian nor preferred a specific nutrition due to religious or disease related reasons causing a difference from the typical Western European diet. Also none of the test persons went to a restaurant during the test period nor ate fast food products of which the Ca isotope composition could be obscured. For A the urine was sampled by the parents, whereas person B sampled the urine herself. We requested 3 urine samples per day roughly corresponding to a morning, noon and evening sequence. The samples were collected in pre-cleaned normal laboratory vials.

^{43}Ca/^{48}Ca double spike

During mass spectrometric measurement and chemical purification of a sample Ca isotopes fractionate to a large extent [e.g. [20]]. In order to measure the original Ca isotope composition of a sample we applied the Ca double spike technique. First Russell et al. [21] introduced this technique of Ca isotope measurements using a Ca double spike enriched in ^{42}Ca and ^{48}Ca. In contrast we use a double spike enriched in ^{43}Ca and ^{48}Ca (cf. [22]) being those isotopes with the lowest natural abundance beside ^{46}Ca. A basic request is that the isotope composition of the spike has to be well known and that the spike material is depleted in ^{40}Ca and ^{44}Ca being the isotopes of interest.

The ^{43}Ca/^{48}Ca double spike was added to the sample prior to chemical purification (see Sample preparation). In the mixture (spike and sample) most of the ^{43}Ca and ^{48}Ca comes from the spike solution whereas most of the ^{40}Ca and ^{44}Ca comes from the sample. Hence, any natural variations of the ^{43}Ca/^{48}Ca ratio are negligible allowing quantitatively distinguishing between natural Ca isotope fractionation from fractionation introduced during sample preparation and mass spectrometer measurements.

Sample preparation

In order to achieve optimal double-spiking of the samples prior to chemical purification the Ca concentration of the urine has been

determined by ICP-OES (inductively coupled plasma optical emission spectrometer) prior to the isotope measurements. Then a fraction of urine corresponding to about 3 μg of Ca was taken and diluted with 240 μl of our $^{43}Ca/^{48}Ca$ double spike solution. In addition, 2 ml of ultrapure concentrated HNO_3 and 250 μl of $HClO_4$ (12 mol/l, SEASTAR chemicals) was added to the solution. This solution was put on a heating plate and heated at 150 °C for about 12 h and then evaporated at about 190 °C. Later, about 1 ml of ultrapure HNO_3 was added to the residue and slowly heated up to ~150 °C again and left at this temperature until complete dryness. Finally the residue was dissolved in 0.25 ml of 10 mol/l ultrapure HCl. Used HCl and HNO_3 were reaction grade acids (Merck KGaA, Darmstadt, Germany) cleaned by double sub-boiled distillation.

Following the digestion procedure, a chemical separation of the solutions was performed following the method of Wombacher et al. [23]. For the chemical separation, 1 ml of Biorad AG50W-X8 (200–400 mesh) cation exchange resin was rinsed into ion-exchange columns (cf. [23]). The resin in the columns was cleaned by rinsing with ~30 ml of 4mol/l HCl. The column was then prepared for chemical separation by rinsing with 2 ml of 10 mol/l HCl. The sample solution (0.25 ml of 10 mol/l HCl) was loaded onto the column and washed into the resin by adding 2.5 ml of 10 mol/l HCl onto the column. In a last step the Ca-fraction was separated by loading 10.5 ml of 7 mol/l HBr (suprapure, Merck KGaA, Darmstadt, Germany) onto the column and collecting the eluate. The Ca eluate was dried down on a hot plate and diluted with 2 ml of a 2.25 mol/l HCl solution.

For each measurement about 200 μl of the purified sample solution was evaporated and diluted with 1.5 μl of a 2.25 mol/l HCl solution and then loaded onto a pre-outgassed Rhenium ribbon single filament with a Ta activator and a sandwich loading technique [22]. First, about 0.4 μl of a Ta activator solution was loaded onto the filament and nearly completely dried down at 0.7 A filament current. Then the sample solution containing about 300 ng Ca was put onto the filament and was dried down to near dryness with 0.7 A of filament current. A second layer of Ta activator was loaded onto the filament and dried down completely with 0.7 A. [22,24]. The filament current was slowly increased to 1.5 A and was left at this current for about 20 s. The current was then again slowly increased until a weak red glowing of the filament ribbon was observed followed by an immediate shutdown of the current.

Mass spectrometry

Ca isotope measurements were performed on a TRITON thermal ionization mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) at the mass spectrometer facilities of the "Leibniz-Institut für Meereswissenschaften", IFM-GEOMAR, Kiel (Germany). In the thermal ionization mass spectrometer (TIMS) Ca ions are produced by thermal ionization, accelerated and focused into an ion beam. After passing a magnetic field the Ca ions follow certain trajectories according to their distinct mass to charge ratio. Ca ion intensity is finally measured by multiple Faraday cups coupled to a resistor ($\sim 10^{11}$ Ω). The 9 Faraday cups of the used TIMS are arranged in a way allowing simultaneous measurement of all Ca isotopes in two subsequent steps (Table 1). Latter configuration allows the application of short idle times after each measurement step because each cup is only used once in one of the two steps (Table 1) allowing the cup and resistor to relax during the step where it is not measuring. Filament heating was done using a computer controlled heating routine (Table 2) allowing fully automated measurements. Table 3 shows the used settings for the data acquisition.

Table 1
Used faraday cup configuration for the measurement of Ca isotopes on the TRITON TI.

Line	Faraday cup:	L4	L3	L2	L1	C	H1	H2	H3	H4
#1	Mass:	40				42	43	44		
#2	Mass:			43	44					48

Table 2
Step heating procedure used for Ca isotope measurements.

Line	Estimated start time (min)	Valve open	Function[a]	Value	Slope (mA/min)	Actions[b]
1	0:00	No	FILC	1500 mA	1000	None
2	1:30	No	FILC	2300 mA	500	None
3	3:06	No	FILC	2800 mA	250	None
4	5:06	No	WAIT			None
5	5:36	No	TEMC	1425 °C	125	FF, ZF, F
6	9:00	Yes	TEMC	1475 °C	60	FF, ZF, F, PC
7	13:17	Yes	IONC	7000 mV	40	FF, ZF, F
8	15:51	Yes	IONC	8500 mV	30	FF, ZF, F
9	18:25	Yes	WAIT			None
10	18:55	Yes	IONC	9000 mV	10	FF, ZF, F

[a] FILC = filament current controlled, TEMC = temperature controlled, IONC = intensity controlled.
[b] FF = wheel focus, ZF = Z focus, F = focus, PC = peak center.

For data reduction and spike denormalization we used an iterative algorithm previously described by Compston and Oversby [25] modified for Ca isotope measurements [1,22]. This algorithm calculates the $^{44}Ca/^{40}Ca$ sample ratio from the measured $^{40}Ca/^{48}Ca$, $^{43}Ca/^{48}Ca$ and $^{44}Ca/^{48}Ca$ ratios. All Ca isotope data are reported in the delta notation. The δ value ($\delta^{44/40}Ca$) represents the deviation of the Ca isotopic composition of a sample ($^{44}Ca/^{40}Ca_{sample}$) relative to the Ca isotopic composition of a chosen standard material ($^{44}Ca/^{40}Ca_{standard}$) in parts per thousand or per mill (‰): $\delta^{44/40}Ca$ (‰) = $[((^{44}Ca/^{40}Ca)_{sample} - (^{44}Ca/^{40}Ca)_{standard})/(^{44}Ca/^{40}Ca)_{standard}] * 1000$ following the suggestion of Coplen et al. and Eisenhauer et al. [26,27]. As standard material we used the standard reference material NIST SRM 915a which was measured together with our samples (NIST = National Institute of Standards and Technology; SRM = Standard Reference Material).

Results

The results of the Ca concentration and isotope measurements are presented in Table 4, Figs. 1 and 3. The urine samples were taken in chronological orders in the sequence morning–noon–evening corresponding to roughly a 5-day period. Both $\delta^{44/40}Ca_{urine}$ records show variations in the order of about 1.9‰ (boy, time series A) and 0.4‰ (woman, time series B), respectively, throughout the study period. However, no daily variations or sinusoidal oscillations of our data can be observed, as it might be expected from the sequence of alternating uptake of nutrition related to the morning, noon and evening diet. Rather the $\delta^{44/40}Ca_{urine}$ data are quite uniform without

Table 3
Instrumental setting for Ca isotope measurements.

Accelerating voltage	10 kV
Resistor	10^{11} Ω
Source vacuum	$4–9 \times 10^{-8}$ mbar
Analyzer vacuum	$2–4 \times 10^{-9}$ mbar
Heating	Automatic (see Table 2)
Pilot mass	40
Start intensity	$9–10 \times 10^{-11}$ A (=9–10 V) on pilot mass
Baseline	Defocused, before each block, 63 s
Peak centering	Before acquisition
Interblock actions	None
Data collection	Blocks per run: 6 Scans per block: 26 Integration time: 4 s (both sequences) Idle time: 1 s (both sequences)

Table 4
Results of Ca isotope measurements ($\delta^{44/40}$Ca) and Ca concentrations (mg/l) on urine samples from healthy boy (series A) and the woman suffering from osteoporosis (series B). Note that the Ca isotopic composition is presented in per mill (‰) deviation from a given standard $\delta^{44/40}$Ca (‰) = [(^{44}Ca/^{40}Ca)$_{sample}$ − /(^{44}Ca/^{40}Ca)$_{standard}$/(^{44}Ca/^{40}Ca)$_{standard}$]*1000.

Sample	Time after start of experiment (h)	$\delta^{44/40}$Ca (‰)	±2 SE	n	[Ca] (mg/l)	±1 SD
Boy (series A)						
174-06	0.00	2.87	0.13	7	37.51	0.13
175-06	8.58	1.84	0.09	3	31.62	2.89
176-06	24.25	1.91	0.17	5	61.26	1.68
177-06	37.33	1.73	0.10	7	62.92	0.81
178-06	48.00	1.11	0.11	5	149.80	0.76
179-06	56.50	1.48	0.12	3	51.99	0.42
180-06	61.00	1.80	0.10	3	27.04	1.37
181-06	71.83	1.62	0.12	3	60.28	1.16
182-06	85.25	1.46	0.16	7	82.87	0.89
183-06	96.00	1.45	0.16	5	95.03	0.39
184-06	107.00	1.36	0.10	7	40.25	0.91
185-06	109.00	1.36	0.15	7	89.32	0.93
186-06	120.75	0.96	0.06	3	190.95	0.25
187-06	133.00	1.13	0.15	7	167.23	0.79
188-06	169.25	1.33	0.11	7	112.79	1.35
Mean[a]		1.47	0.29 (1 SD)		87	13
Woman (series B)						
218-06	0.00	0.32	0.07	3	106.81	0.55
219-06	8.75	0.54	0.10	3	151.95	1.19
220-06	14.25	0.22	0.05	3	285.69	0.46
221-06	24.00	0.35	0.08	3	159.39	0.31
222-06	33.25	0.46	0.10	5	75.72	0.92
223-06	37.75	0.42	0.11	5	110.07	1.05
224-06	48.00	0.17	0.09	7	126.93	0.60
225-06	56.75	0.49	0.09	3	130.03	0.56
226-06	62.25	0.36	0.11	5	214.51	0.29
227-06	73.00	0.34	0.12	7	115.74	0.68
228-06	80.75	0.39	0.20	8	118.60	0.66
229-06	85.75	0.31	0.14	7	452.54	0.27
230-06	96.00	0.32	0.16	7	204.99	0.41
231-06	103.25	0.32	0.16	5	128.60	0.25
232-06	108.75	0.25	0.10	5	87.19	0.18
Mean		0.35	0.10 (1 SD)		165	25

[a] Sample 174-06 is not used for the calculations of mean values (see text).

any major excursions except for the first $\delta^{44/40}$Ca$_{urine\text{-}boy}$ value in time series A which is about a factor of two larger then the corresponding average value (sample in brackets). We do not have any explanation

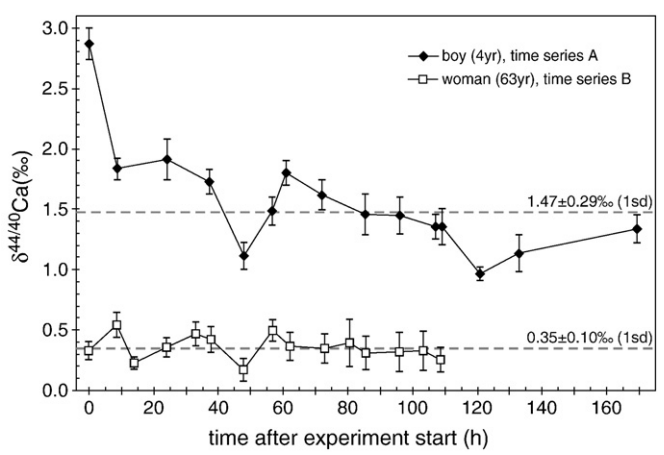

Fig. 1. Time series of Ca isotope measurements of urine from a 4-year-old healthy boy (closed diamonds) and a 63-year-old woman, respectively over a test period of 108 h (woman) and 170 h (boy), respectively. Note that the Ca isotopic composition is presented in per mill (‰) deviation from a given standard ($\delta^{44/40}$Ca (‰) = [((^{44}Ca/^{40}Ca)$_{sample}$ − (^{44}Ca/^{40}Ca)$_{standard}$)/(^{44}Ca/^{40}Ca)$_{standard}$]*1000). The results exhibit a difference of about 1.1‰ between $\delta^{44/40}$Ca$_{urine\text{-}boy}$ and $\delta^{44/40}$Ca$_{urine\text{-}woman}$ most likely reflecting age, gender and individual health status.

for this high value and will therefore neglect it for further discussions. The mean value for $\delta^{44/40}$Ca$_{urine\text{-}boy}$ is 1.47 ± 0.29‰, (1 SD) while the mean value form $\delta^{44/40}$Ca$_{urine\text{-}woman}$ is isotopically lighter averaging around ~0.35 ± 0.10‰ (1 SD). Note, that there is a significant offset of ~1.1 ± 0.3‰ for the mean values of the two data sets.

Discussion

The Ca isotope composition of the diet ($\delta^{44/40}Ca_{diet}$)

As the diet is the only major source of Ca for the human body, variations of the Ca isotope composition of the nutrition influence the urine Ca isotope composition. Our own measurements of the nutrition Ca isotope composition (Table 5) together with previously published Ca isotope ratios of representative food products [16] allow an estimation of the $\delta^{44/40}$Ca-composition of the test person's diet. Calculation (equation 1) of the average $\delta^{44/40}$Ca$_{diet}$ takes the individual isotope compositions of the specific food as well as their relative contribution into account. For the calculation of the average Ca isotope composition of the daily diet we adopted values representing an average value for European diet [28]. The average Ca isotope composition can then be estimated according to Eq. (1):

$$\delta^{44/40}\mathrm{Ca}_{\mathrm{diet}} = \frac{\sum_i \left([\mathrm{Ca}]_i \cdot \delta^{44/40}\mathrm{Ca}_i \cdot x_i\right)}{\sum_i \left([\mathrm{Ca}]_i \cdot x_i\right)} \quad (1)$$

with x_i = relative contribution of the specific food i to the nutrition, $[\mathrm{Ca}]_i$ = Ca concentration of the food i; $\delta^{44/40}\mathrm{Ca}_i$ = Ca isotope composition of food i.

From Eq. (1) we calculated an average Ca isotope value for the average diet consumption of European nutrition to be ~ −1.02 ± 0.1‰. This value is directly pertinent to our test persons because their nutrition is in general accord with this average European diet uptake as outlined in Table 4. However, relative differences occur concerning the amount of dairy products taken up by the two test persons. In contrast to test person B (elderly lady) the nutrition of test person A (boy) was composed of a larger amount of dairy products. In particular, during the test period A was used to drinking about 50 to 100 ml of warm milk in the morning representing a major contribution to his Ca budget. From the data compilation (Table 5) it is obvious that dairy products are most critical for the determination of $\delta^{44/40}$Ca$_{diet}$ because of its relatively high Ca concentrations. Any change of the fraction or the mean isotope composition of the dairy products may change the $\delta^{44/40}$Ca$_{diet}$ values, which is to a smaller degree also true for vegetables. However, a change of the relative contribution of dairy products between 8 and 31% the resulting $\delta^{44/40}$Ca$_{diet}$ will still be within the statistical uncertainties of the Ca isotope measurements and insignificantly deviate from −1.02 ± 0.10‰ (Fig. 2). Consequently, the observed variability in the $\delta^{44/40}$Ca$_{urine}$ records as well as the offset of about ~1.1‰ between the two data sets

Table 5
Compilation of the mean Ca concentration ([Ca]), the fraction (cf. [28]) and the $\delta^{44/40}$Ca values of different food groups of human nutrition.

Type of nutrition	[Ca] (mg/100 g)	European average (%)	$\delta^{44/40}$Ca (‰ SRM 915a)
Dairy products	500	14	−1.20
Vegetables	100	21	−0.68[a]
Fruit	20	14	−0.68[a]
Crop products	35	24	−0.56
Meat	10	6	−0.08[a]
Fats	7	2	−1.00
Water	5	20	+0.68

[a] Recalculated from the $\delta^{44/42}$Ca-based values of Chu et al. [16]. The values were calculated by using the following relationship: $\delta^{44/40}\mathrm{Ca} = \delta^{44/42}\mathrm{Ca} \cdot (\ln(\text{m-}44/\text{m-}40)/\ln(\text{m-}44/\text{m-}42))$; m-44: 43.955 u, m-42: 41.962 u, m-40: 39.962 u.

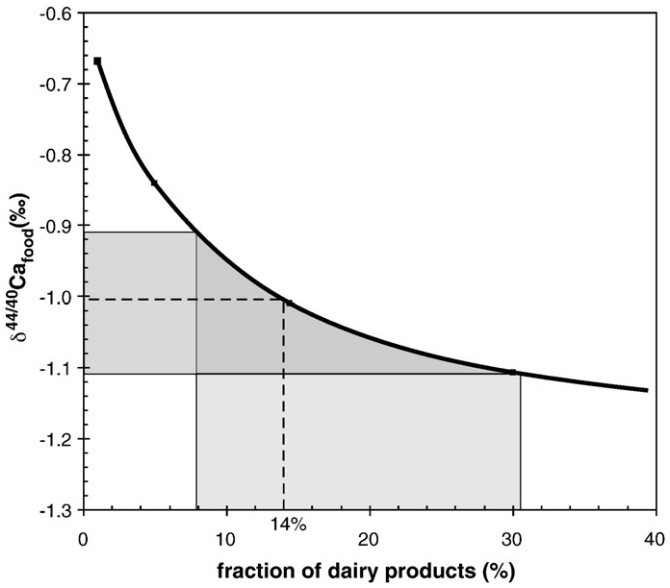

Fig. 2. The $\delta^{44/40}Ca_{diet}$ ratios are presented as a function of the relative contribution of dairy products to the usual European diet. Note that the Ca isotopic composition is presented in per mill (‰) deviation from a given standard $\delta^{44/40}Ca$ (‰) = $[(({}^{44}Ca/{}^{40}Ca)_{sample} - ({}^{44}Ca/{}^{40}Ca)_{standard})/({}^{44}Ca/{}^{40}Ca)_{standard}]*1000$. Any change of the relative contribution of dairy products in the range between 8 and 31% (light gray area) will result in $\delta^{44/40}Ca_{diet}$ variations in the range of $\sim \pm 0.10$‰ around the mean value (dark gray area).

have to be attributed to physiological processes along the Ca pathway from the diet to the urine rather to any change of the nutrition composition.

The Ca isotope composition of the urine ($\delta^{44/40}Ca_{urine}$)

The difference ($\Delta_{diet\text{-}urine}$) between $\delta^{44/40}Ca_{urine}$ and $\delta^{44/40}Ca_{diet}$ is about −2.49‰ for series A (boy) and −1.37‰ for series B (woman), which is in general accord of findings by Skulan et al. [8]. After the incorporation Ca becomes absorbed in the gastrointestinal tract where a certain amount of Ca is partitioned into blood and feces, respectively. Within the blood circulation Ca is redistributed between various organs (e.g. kidneys) and the bones. At the transition from the blood to the bones Ca becomes fractionated where the lighter Ca isotopes are enriched in the bones and the heavier ones are enriched in the blood. The Ca isotope difference between blood and bone ($\Delta_{bone\text{-}blood}$) has been quantified to be about ~−1.3‰ [17]. Depending on health status, age, gender and various other factors Ca stored in the bones may become dissolved again and transferred back from the bones to the blood. However, in contrast to the mineralization the demineralization process is supposed not to cause any fractionation of the Ca isotopes [17]. Eventually, Ca reaches the kidneys where it either become reabsorbed for additional physiological use in the body or excreted via the urine.

It is generally known, that the generation of urine in the kidneys may be described by a two stage process. The primary urine (glomerular filtrate) is extracted from the blood mainly consisting of water, salts and organic compounds, and is then filtered within the kidney to recycle physiologically important salts and organic compounds. The filtered residue (secondary urine) is then collected in the bladder and excreted. Extraction processes across membranes may cause isotope fractionation which can be described by a Rayleigh fractionation process:

$$R = R_0 f^{(\alpha - 1)} \quad (2)$$

where R is the $({}^{44}Ca/{}^{40}Ca)_{urine}$ and R_0 corresponds to $({}^{44}Ca/{}^{40}Ca)_{blood}$, f is the remaining fraction (secondary urine) in the reservoir and α is the isotope fractionation factor. Hoenderop et al. [29] stated that about 98% of the Ca entering the kidneys is recycled and only 2% are directly leaving the body ($f = 0.02$) via the secondary urine. Neglecting any possible fractionation during intestinal absorption of Ca from the diet (cf. [17]) to the kidneys we can calculate the fractionation coefficient α for the fractionation during urine generation. We get $\alpha_{boy} = 0.99936$ (boy, series A) and $\alpha_{woman} = 0.99965$ (woman, series B). As most of the Ca is reabsorbed during urine generation the value of the reabsorbed Ca ($\delta^{44/40}Ca_{readsorbed}$) is nearly undistinguishable from $\delta^{44/40}Ca_{blood}$. In contrast, the relatively small amount of Ca excreted with the secondary urine is heavily enriched in ^{44}Ca showing considerably higher values for $\delta^{44/40}Ca_{urine}$ than for the $\delta^{44/40}Ca_{diet}$.

Effect of renal function

The average Ca concentration of the boy's urine ($[Ca]_{urine\text{-}boy}$, $(87 \pm 51$ mg/l)) is in average a factor of about ~2 lower than for the woman ($[Ca]_{urine\text{-}woman}$, $(165 \pm 97$ mg /l)). In addition there is an inverse correlation of $[Ca]_{urine}$ to $\delta^{44/40}Ca_{urine}$ for both test persons (Fig. 3). However, the gradient of the $\delta^{44/40}Ca_{urine\text{-}boy}$–$[Ca]_{urine\text{-}boy}$ relationship is about one order of magnitude higher and is correlated to a higher degree than the $\delta^{44/40}Ca_{urine\text{-}woman}$–$[Ca]_{urine\text{-}woman}$ relationship.

The $\delta^{44/40}Ca_{urine\text{-}boy}$–$[Ca]_{urine\text{-}boy}$ gradient shows that the Ca isotopic composition of the urine tends to ^{44}Ca enriched values as more light ^{40}Ca is reabsorbed from glomerule filtrate and thus relatively more of ^{44}Ca excreted via urine. This connection between Ca isotopic composition and Ca concentration is not observable for subject B (woman). The observed $\delta^{44/40}Ca_{urine}$–$[Ca]_{urine}$ gradient may also reflect individual differences in age, gender and health status of the test persons.

Based on the knowledge that the Ca homeostasis tends to keep the Ca balance in narrow limits, we may speculate that the observed relationship represents a dependency of the Ca isotope fractionation on the amount of available Ca in the blood ($\delta^{44/40}Ca_{urine} \sim 1/[Ca]_{blood}$). The more Ca is available in the soft tissue the less will be recycled back from the primary urine to the blood and the isotopically lighter will be the secondary urine.

5.4. The influence of bone mineralization on $\delta^{44/40}Ca_{urine}$

The amount of Ca stored in the bones ($[Ca]_{bones}$) is not constant; bone decomposition and formation occur during the lifetime as a

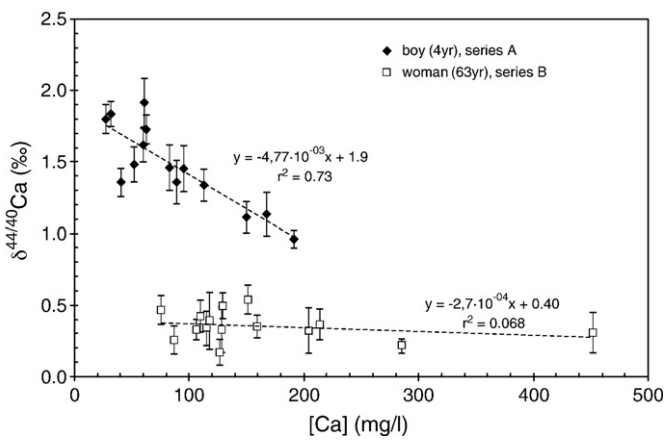

Fig. 3. The $\delta^{44/40}Ca_{urine}$ values are plotted as a function of their corresponding Ca concentration ($[Ca]_{urine}$). Note that the Ca isotopic composition is presented in per mill (‰) deviation from a given standard $\delta^{44/40}Ca$ (‰) = $[(({}^{44}Ca/{}^{40}Ca)_{sample} - ({}^{44}Ca/{}^{40}Ca)_{standard})/({}^{44}Ca/{}^{40}Ca)_{standard}]*1000$. The $\delta^{44/40}Ca_{urine\text{-}boy}$–$[Ca]_{urine\text{-}boy}$ gradient is one order of magnitude larger than for the $\delta^{44/40}Ca_{urine\text{-}woman}$–$[Ca]_{urine\text{-}woman}$ gradient.

dynamic process [30]. During childhood and adolescence bone formation exceeds bone decomposition. With increasing age this ratio is shifted toward bone decomposition (e.g. [31]). In an attempt to quantify the effect of age, gender and health status on $\delta^{44/40}Ca_{urine}$ we follow a simple box model presented earlier by Skulan and DePaolo [17] (Fig. 4). The subscripts denote the Ca isotopic composition of the various Ca fluxes (V) in and out and between the different Ca bearing boxes like diet, blood, bones and the kidneys. The δ values represent the isotopic characteristics of the Ca reservoirs and the Δ values the isotopic difference or offset between the boxes due to possible fractionation processes during the transition from one box to the other. In particular, Skulan and DePaolo [17]) showed earlier that Ca isotopes become fractionated during bone mineralization having a $\Delta_{bone\text{-}softtissue}$ value of ~ −1.3‰. As blood plasma is the central Ca compartment from where Ca is exchanged with soft tissue we split the original soft tissue box into blood and soft tissue. Soft tissue is comparatively small and thus negligible for our model. We, therefore, used a $\Delta_{bone\text{-}blood}$ value of ~ −1.3‰ which corresponds to $\Delta_{bone\text{-}softtissue}$ value of [17]. For our rough calculations the Ca isotope composition of the bones ($\delta^{44/40}Ca_{bone}$) can be estimated ($\delta^{44/40}Ca_{bone} = \delta^{44/40}Ca_{diet} + \Delta_{bone} = -1.02‰ + (-1.3‰)$) to be ~ −2.3‰.

In order to account for the Ca extraction processes in the kidneys we extended the earlier model of Skulan and DePaolo [17] with an additional box representing the kidneys in order to account for their large effect on the Ca isotope fractionation which we found during this pilot study. Although we cannot yet quantify $\Delta_{blood\text{-}urine}$ we may assume that $\delta^{44/40}Ca_{blood}$ is close to $\delta^{44/40}Ca_{diet}$ in accordance with earlier statements [17,18]. Hence, $\Delta_{diet\text{-}urine}$ is about equal to $\Delta_{blood\text{-}urine}$. Following this approach the $\Delta_{blood\text{-}urine}$ for the woman and the boy is approximated to be −1.37‰ and −2.49‰, respectively. Hence, the Ca isotope balance for the kidneys (Fig. 4) can be expressed as:

$$0 = \left(V_{ex} - V_{kidney}\right) \cdot \delta^{44/40}Ca_{blood} - V_{urine} \cdot \left(\delta^{44/40}Ca_{urine} + \Delta_{blood-urine}\right)$$
$$\text{with } \left(V_{ex} - V_{kidney}\right) \approx V_{urine} \text{ follows :}$$
$$\delta^{44/40}Ca_{urine} = \delta^{44/40}Ca_{blood} - \Delta_{blood-urine} \quad (3)$$

where subscripts denote the compartment, V are Ca fluxes in and out of a compartment, δ values represent the Ca isotopic composition of the compartment and Δ values denote the isotopic difference or offset between them. Together with the equation for $\delta^{44/40}Ca_{blood}$ presented by Skulan and DePaolo [17] Eq. (3) can be rewritten to Eq. (4)

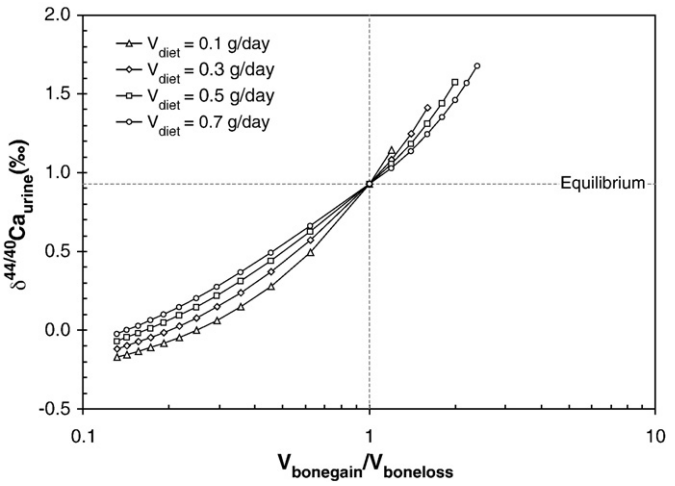

Fig. 5. The $\delta^{44/40}Ca_{urine}$ model values are presented as a function of the ratio of $V_{bonegain}$ and $V_{boneloss}$. Note that the Ca isotopic composition is presented in per mill (‰) deviation from a given standard $\delta^{44/40}Ca$ (‰) = [(($^{44}Ca/^{40}Ca)_{sample}$ − ($^{44}Ca/^{40}Ca)_{standard}$)/($^{44}Ca/^{40}Ca)_{standard}$]*1000. The values are calculated for different V_{diet} values representing Ca absorption from 10 to 70%. The mean value for $\delta^{44/40}Ca_{urine\text{-}boy}$ and $\delta^{44/40}Ca_{urine\text{-}woman}$ to be −0.93‰ is arbitrarily defined to reflect equilibrium where $V_{bonegain}$ equals $V_{boneloss}$.

allowing a quantitative investigation of the influence of bone gain to bone loss ratios on the $\delta^{44/40}Ca_{urine}$:

$$\delta^{44/40}Ca_{urine} = \delta^{44/40}Ca_{diet} + \frac{V_{boneloss} \cdot \left(\delta^{44/40}Ca_{bone} - \delta^{44/40}Ca_{diet}\right) - V_{bonegain} \cdot \Delta_{bone}}{V_{diet} + V_{boneloss}} - \Delta_{blood-urine} \quad (4)$$

Our test persons show considerable differences in their $\delta^{44/40}Ca_{urine}$ values which reflect our selection of individuals being extremely different in their gender, age and health status. The young boy is supposed to be in a healthy status gaining Ca whereas the elderly woman is known to suffer from osteoporosis having a net loss on Ca. Hence, both persons and their individual $\delta^{44/40}Ca_{urine}$ ratios represent extreme values being off from equilibrium where $V_{bonegain}$ equals $V_{boneloss}$. However, we may define equilibrium by arbitrarily assuming that the mean value of $\delta^{44/40}Ca_{urine\text{-}boy}$ and $\delta^{44/40}Ca_{urine\text{-}woman}$ constrains an equilibrium value

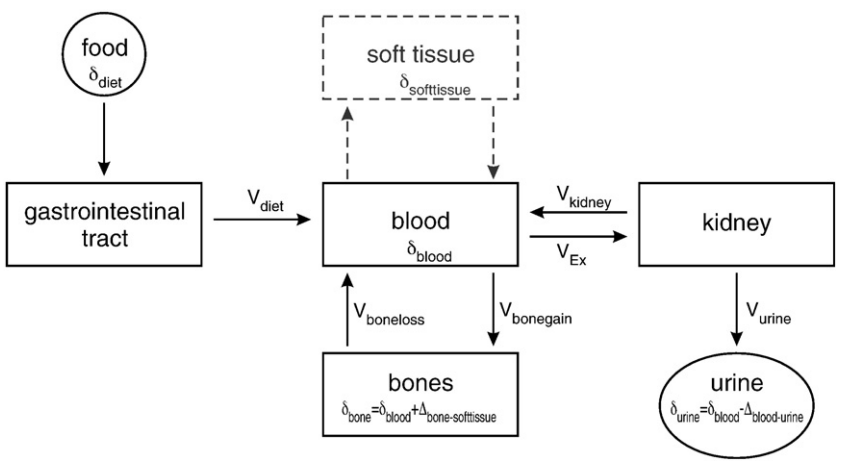

Fig. 4. Simplified box model representing the transport of Ca along its pathway from the diet via the blood to the soft tissue, the bones and the kidneys. This model is based on a model presented earlier by Skulan and DePaolo [17] but extended by additional boxes for the gastrointestinal tract and the kidneys. The original soft tissue box of Skulan and DePaolo [17] was split into a blood and a soft tissue box to better match human physiology. Please note that the central calcium compartment is blood and therefore the soft tissue compartment can be neglected. V values label the Ca fluxes between compartments, the δ values correspond to the specific Ca isotopic composition of a certain box and the Δ values correspond to the isotope difference between compartments due to fractionation processes.

($\delta^{44/40}Ca_{urine\text{-}equilibrium} \sim -0.91‰$) where $V_{bonegain}$ equals $V_{boneloss}$. Latter equilibrium value corresponds to a $\Delta_{blood\text{-}urine}$ of $\sim -1.93‰$.

In this regard, it is interesting to note that the $\Delta_{blood\text{-}urine}$ of $\sim -1.93‰$ is very close to the $\Delta_{blood\text{-}urine}$ value measured at so called "Göttingen" mini-pigs [32] where the Ca isotopic composition of blood and urine from primiparous mini-pigs was determined. Mini-pigs serve as a model organism for the human physiology and are used in medical research for the study of Ca related diseases like osteoporosis. Ca isotope studies on these mini-pigs, which will be presented elsewhere, showed that the $\Delta_{blood\text{-}urine}$ value of mini-pigs representing a healthy control group shows $\Delta_{blood\text{-}urine}$ values of $\sim -2‰$ being close to the value arbitrarily defined in this study.

In Fig. 5 we plotted model values for $\delta^{44/40}Ca_{urine}$ for different values of V_{diet}, $V_{bonegain}$ and $V_{boneloss}$ ratios as a function of the arbitrarily defined equilibrium value. The average daily V_{diet} flux for Ca corresponds to a value of 0.3 g Ca/day, which is ~30% of the normal daily Ca uptake of 1 g Ca/day [33], and $V_{bonegain} = V_{boneloss} = 0.5$ g Ca/day ($V_{bonegain}/V_{boneloss} = 1$). However, in order to study the influence of varying V_{diet} value we also modeled $\delta^{44/40}Ca_{urine}$ for V_{diet} values of 0.1, 0.5 and 0.7 g Ca/day representing absorption rates of 10, 50 and 70%, respectively. In order to account for $V_{bonegain}/V_{boneloss} > 1$, $V_{bonegain}$ was changed but $V_{boneloss}$ was kept constant at 0.5 g Ca/day. However, for $V_{bonegain}/V_{boneloss} < 1$ $V_{boneloss}$ was varied but $V_{bonegain}$ kept constant at 0.5 g Ca/day. Please note that maximum $V_{bonegain}$ cannot exceed $V_{diet} + V_{boneloss}$ as diet is the only source of Ca for the body in addition to Ca lost from the bones.

From our model calculations it can be seen that the differences of calculated $\delta^{44/40}Ca_{urine}$ for the same gain/loss ratio at different Ca fluxes from the diet are small compared to the changes of $\delta^{44/40}Ca_{urine}$ with varying gain/loss ratios. According to the definition of an equilibrium value $\delta^{44/40}Ca_{urine\text{-}boy}$ is associated with a $V_{bonegain}/V_{boneloss}$ ratio being larger than 1 whereas $\delta^{44/40}Ca_{urine\text{-}woman}$ is associated with a $V_{bonegain}/V_{boneloss}$ ratio being lower than 1. Qualitatively, the two values reflect net loss and net gain of Ca from the bones corresponding to the gender, age and health status of the two test persons.

Beside the qualitative information relative to the arbitrarily defined equilibrium value there is yet no quantitative information in this model without further detailed knowledge of other Ca isotope fractionation processes of the human physiology and the calibration of the Ca fluxes to absolute values. In particular, the better empirical knowledge of a $\delta^{44/40}Ca_{urine\text{-}equilibrium}$ value might be important if one would like to use natural Ca isotope fractionation as a diagnostic tool to test the human Ca balance.

Summary of the results

Our Ca isotope data show that the Ca isotope composition of human urine ($\delta^{44/40}Ca_{urine}$) is higher than the Ca isotope composition of the diet ($\delta^{44/40}Ca_{diet}$) which may be attributed to Ca isotope fractionation during the sequestration of Ca between secondary and primary urine in the kidneys.

The mean $[Ca]_{urine}$ value is twice as large for the woman than for the boy. In addition there is an inverse correlation between $\delta^{44/40}Ca_{urine}$ and $[Ca]_{urine}$. This may probably reflect the dependency of the amount of available Ca and the Ca sequestration between primary and secondary urine.

Probably, as a consequence of Rayleigh type isotope fractionation processes in the kidneys related to Ca sequestration between primary and secondary urine the light Ca becomes reabsorbed into the blood whereas the excreted urine becomes enriched in the heavy Ca isotopes.

Applying a box model the isotope differences in the two time series may be modeled to reflect differences in the Ca balance of bone mineralization and demineralization. By arbitrarily defining an equilibrium value the measured $\delta^{44/40}Ca_{urine}$ values can be attributed to a certain $V_{bonegain}/V_{boneloss}$ ratio in a qualitative way.

Conclusions

This study shows that the Ca isotopes of urine sensitively reflect human Ca balance. The advantages of the applied methods are: that it is non-invasive because only urine samples are needed and the use of large amounts of enriched and expensive tracers or even radioactive Ca is not necessary. These advantages over traditional methods have to be evaluated further in clinical studies. Although the precise measurement of Ca isotopes is still challenging this study may help to generate further developments in mass spectrometer techniques to enhance accuracy, precision and sample throughput for clinical application.

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