Isotopic tracer techniques for assessing calcium absorption in rats

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The femur uptake and whole body retention methods (two isotopic tracer techniques for assessing calcium absorption) were compared using $CaCl_2$ as the salt. The two methods gave identical values for calcium absorption. Tricalcium phosphate, doubly labeled with ^{45}Ca and ^{47}Ca, was used to evaluate extrinsic labeling techniques for determination of calcium bioavailability. The extrinsic ^{47}Ca to intrinsic ^{45}Ca absorption ratio from a test meal ranged from 1.139 ± 0.097 to 1.183 ± 0.087 (mean $\pm$ SD), depending on the procedure for extrinsic labeling. For the femur uptake method, intraperitoneal and intravenous injections of ^{45}Ca were accumulated identically by femurs. We conclude that calcium absorption can be conveniently measured by either whole body retention or femur uptake methods, and that comparisons of oral uptake can be made to either intraperitoneally or intraveneously administered calcium tracers. However, care should be taken in using the extrinsic labeling approach, which tends to overestimate calcium absorption.

Keywords: Isotopic tracers; calcium absorption; rats

Introduction

Isotopic tracer methodologies have been used for measuring calcium bioavailability in animals [1–7] and in humans.[8–12] These tracer techniques are being used to overcome the disadvantages of the traditional balance technique because the fate of the label from specific foods can be determined. These isotropic tracer procedures for measuring calcium absorption from foods are based on the assumption that the tracer is absorbed with the same efficiency as the endogenous food calcium.

Food calcium exchangeability can be determined by comparing the absorption rate of an extrinsic tracer added to a food with that of a second isotope incorporated endogenously into a test food. Extrinsic tracers have been shown to incompletely exchange with intrinsic food calcium in some situations, including low gastric acid conditions[1] and high oxalate foods.[6] On the other hand, extrinsic tracers were absorbed similarly to an intrinsic tracer for milk and its products[4] and kale.[6] In many studies,[5,8–10] the extrinsic labeling approach was used for assessing calcium bioavailability without attempting to determine whether the tracer had exchanged completely with the food calcium.

Further testing of extrinsic labeling techniques is necessary to determine when it is a valid approach for determining calcium bioavailability. A variety of methodologies have been employed to assess calcium bioavailability without direct comparison of the methods. The femur uptake method compares femur accumulation of an oral dose of an isotope tracer with an intraperitoneally injected dose. This method assumes that the intraperitoneal injection behaves as an oral dose with 100% absorption. By contrast, absorption of calcium from an oral dose of an isotopic tracer, as assessed by whole body counting techniques, extrapolates the linear portion of a retention curve to time zero.

The objectives of the research reported here were to compare the utilization by rats of intrinsic and extrinsic calcium tracers of calcium salts, to compare different methods for administering extrinsic isotopes, to compare whole body and femur radioassay procedures for assessing calcium bioavailability, and to compare femur accumulation of intraperitoneally and intravenously injected calcium tracers.

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Materials and methods

Preparation of labeled calcium sources

Tricalcium phosphate (TCP) was prepared from calcium nitrate. Calcium nitrate was prepared by neutralizing Na_2CO_3 with an equimolar quantity of $CaCl_2$ and then adding concentrated nitric acid. TCP was prepared by simultaneously reacting equivalent molar quantities of the prepared $Ca(NO)_3$, KH_2PO_4, and NaOH in a large volume of hot water at pH 9. The precipitate was washed in a Buchner funnel and dried.

Intrinsically labeled TCP was prepared using 0.5 or 5 μCi ^{45}Ca/15 mg calcium added to the initial $CaCl_2$ solution. Extrinsically labeled TCP was prepared by adding $CaCl_2$ (5 μCi ^{45}Ca/15 mg Ca) to TCP by thorough grinding in a mortar and pestle prior to preparation of the test meal (mix method) or by dropping 5 μCi $^{45}CaCl_2$ directly on the test meals containing 15 mg calcium (drop method) (experiment 3). Extrinsically labeled TCP (experiments 2 and 3) and $CaCl_2$ test meals (experiment 2) were prepared by dropping 0.127 μCi $^{47}CaCl_2$ (Amersham, Arlington, Heights, IL USA) directly on the test meals.

Rat feeding studies

Male Sprague Dawley rats (100–125 g) (Harlan Industries, Indianapolis, IN USA) were on reverse day-night cycles for 12 hours each and were fed the American Institute of Nutrition semipurified diet[13] for 8 days before administration of test meals or solutions containing radiotracers. Rats were meal trained for 3 days before receiving the test meal or gavage solution by fasting for 12 hours each day. The experimental protocol for experiments 1 and 2 is given in *Figure 1*. The protocol for experiments 3 and 4 was the same as *Figure 1* except animals were sacrificed at 2 days post dosing.

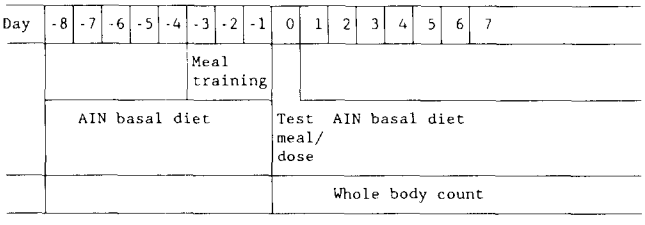

Figure 1 Experimental protocol for experiments 1 and 2.

Whole body counting of ^{47}Ca

Analysis of whole body retention and absorption of ^{47}Ca was determined using a photopeak of 1297 Kev on the whole body counter described previously.[14] Whole body activity at 3-hr post dosing was used as the value for ^{47}Ca activity in each dose. Absorption of the ^{47}Ca tracer was calculated from the whole body retention data by extrapolating the linear terminal portion of the curve to time zero. Absorption of ^{47}Ca and ^{45}Ca from the test meals or solution using the femur uptake method compared accumulation of the radiotracer in the left femur with that accumulated by rats receiving an intraperitoneal injection following the procedure of Wien and Schwartz.[3] All counting results were corrected for decay and contamination of ^{47}Ca and ^{45}Ca. Counting data were statistically analyzed using one-way analysis of variance. Differences between means were compared by least squares differences method.

Experiment 1

The purpose of this experiment was to compare whole body and femur radioassay procedures. Whole body counters require use of a gamma emitter (^{47}Ca), whereas the femur uptake method usually employs a beta emmitter (^{45}Ca) due to its more convenient half life and commercial availability. Therefore, both isotopes were given by two routes to allow for comparison of the methods. One group (n = 10) of rats was injected intraperitoneally with 5 μCi $^{45}CaCl_2$ and 0.172 μCi $^{47}CaCl_2$ in 0.5 mL isotonic saline 2 hours after gavaging 1 mL $CaCl_2$ solution containing 15 mg Ca as $CaCl_2$. The other group was gavaged with 1 mL solution containing 15 mg Ca, 5 μCi^{45}Ca, and 0.172 μCi $^{47}CaCl_2$.

All rats were analyzed individually for the initial ^{47}Ca dose in a whole body counter. The rats were then placed on their original basal diet for 7 more days and monitored for whole body ^{47}Ca activity. All rats were killed with CO_2 after 7 days whole body counting. Upon removal of femurs, ^{47}Ca activity of the femurs was assayed in a whole body counter.

Femur and test meals were ashed in a muffle furnace at 600° C and dissolved in 1 mL 3N HCl and assayed for ^{45}Ca activity after 60 days to allow ^{47}Ca to decay.

Experiment 2

The purpose of this experiment was to compare the utilization by rats of intrinsic and extrinsic calcium tracers of a calcium salt using whole body and femur radioassay procedures for assessing calcium bioavailability. Three-gram test meals *(Table 1)* were fed to 10 rats per group at 9:00. One

Table 1 Composition of test meals

	Ca Salt	Amount %	Alphacel* %	Casein %	Corn oil %	Sucrose %	^{45}Ca μCi	^{47}Ca μCi
Exp. 1	IL TCP	1.25	5	20	10	63.75	5	0.127
	$CaCl_2$	1.39	5	20	10	63.61	5	0.127
Exp. 3	IL TCP	1.25	5	20	10	63.75	5	
	EL TCP (mix)	1.25	5	20	10	63.75	5	
	EL TCP (drop)	1.25	5	20	10	63.75	5	

IL, intrinsically labeled.
EL, extrinsically labeled.
*ICN Nutritional Biochemicals, Cleveland, OH USA.

group of rats was injected intraperitoneally with 0.5 μCi $^{45}CaCl_2$ in 0.5 mL 0.9% saline 2 hours after receiving an extrinsically ^{47}Ca-labeled 3-g test meal of $CaCl_2$. The other group received intrinsically (^{45}Ca) and extrinsically (^{47}Ca) labeled test meals containing TCP.

Experiment 3

The purpose of this experiment was to compare different methods for labeling test meals with isotopic tracers. Test meals *(Table 1)* were fed to 10 rats per group. One group of rats was injected intraperitoneally with 0.5 mL 0.9% saline containing 5 μCi $^{45}CaCl_2$. Another group received a 3-g meal containing intrinsically ^{45}Ca labeled-TCP. Two groups received TCP test meals that were extrinsically labeled with $^{45}CaCl_2$ by thorough grinding or TCP test meals extrinsically labeled by dropping on a $^{45}CaCl_2$ solution. The rats were sacrificed 48 hours after dosing. Femurs and test meals were dried and dissolved in 2 mL concentrated HNO_3 and aliquots were diluted with 15 mL PCS™ liquid scintillation cocktail for determination of ^{45}Ca activity with a Beckman LS 1800 scintillation counter (Fullerton, CA USA)

Experiment 4

The purpose of this experiment was to assure that ^{45}Ca administered as a salt by intraperitoneal injection was accumulated by femurs identically to ^{45}Ca administered intravenously as the salt or as prelabeled rat serum. One group of rats (n = 8) was given 12.7 μCi ^{45}Ca as $CaCl_2$ in 0.5 mL isotonic saline by intraperitoneal injection. A second group of rats (n = 8) was given 7.5 μCi ^{45}Ca as $CaCl_2$ in 0.3 mL isotonic saline by intravenous tail injection. The third group of rats (n = 8) was given 0.24 μCi ^{45}Ca in 0.3 mL prelabeled rat serum by intravenous tail injection. Prelabeled rat serum was obtained by gavaging a rat with $^{45}CaCl_2$, collecting blood, and freezing serum until needed. Femurs were dissolved in 2 mL concentrated HNO_3, diluted to 25 mL, and aliquots counted in a liquid scintillation counter.

Results

Experiment 1

The comparison of whole body and femur radioassay procedures for $CaCl_2$ is given in *Table 2*. The absorption of ^{47}Ca and ^{45}Ca, determined by the femur uptake method, and absorption of ^{47}Ca, determined by whole body counting techniques, were almost identical. The ratio of intrinsic ^{47}Ca determined by whole body counting to intrinsic ^{45}Ca absorption determined by femur uptake by gavage was 1.026 ± 0.077.

Experiment 2

The extrinsic ^{47}Ca and intrinsic ^{45}Ca retention and absorption from TCP as determined by whole body counting and femur uptake methods are given in *Table 3*. The ratio of extrinsic ^{47}Ca absorption, determined by whole body counting, to intrinsic ^{45}Ca absorption, determined by femur uptake, was 1.183 ± 0.087 for TCP; the difference in mean absorption between the intrinsic and extrinsic absorption means was statistically significant. The intrinsic ^{47}Ca absorption (68%) of $CaCl_2$ *(Table 2)* in experiment 1 was significantly lower than absorption of $CaCl_2$ using an extrinsic label of ^{47}Ca (83%) determined by whole body counting *(Table 3)*.

Experiment 3

The effect of tracer labeling techniques on ^{45}Ca retention and absorption is shown in *Table 4*. There was a significant difference ($p < 0.01$) in ^{45}Ca retention and absorption among the labeling techniques.

The extrinsic labeling method in which the label was thoroughly mixed with the salt and radiotracer to prepare the test meal resulted in significantly higher absorption of ^{45}Ca than for the intrinsic labeled salt ($p < 0.01$). The ratio of Ca absorption of the extrinsic label by the mix method to absorption of the intrinsic label was 1.139 ± 0.097. When the extrinsic label was dropped onto a prepared test meal (drop method), calcium absorption was slightly lower than when the extrinsic label was first mixed with the salt and higher than for the intrinsically labeled salt, but not significantly different from either procedure.

Experiment 4

There was no difference in femur accumulation of ^{45}Ca among groups of rats dosed intraperitoneally with $^{45}CaCl_2$ (2.73 ± 0.17), or intravenously with $^{45}CaCl_2$ (2.66 ± 0.16) or with prelabeled rat serum (2.67 ± 0.33).

Table 2 Comparison of whole body and femur radioassay procedures of $CaCl_2$ for assessing calcium bioavailability (experiment 1)*

	Whole body assay		Femur assay			
	IL ^{47}Ca		IL ^{47}Ca		IL ^{45}Ca	
Treatment	retention	absorption	retention	absorption	retention	absorption
	% dose		% dose	% of intraperitoneal injection	% dose	% of intraperitoneal injection
$CaCl_2$†	66.4 ± 7.6	68.5 ± 9.1	2.5 ± 0.4	68.0 ± 0.2	2.3 ± 0.3	66.8 ± 8.0
$CaCl_2$‡	89.3 ± 2.1		3.6 ± 0.1		3.4 ± 0.1	

IL, intrinsically labeled.
*Mean ± SD. No statistical differences among means were observed.
†Radiotracers were administered by gavage.
‡Radiotracers were administered by intraperitoneal injection.

Table 3 Comparison of the utilization of intrinsic ^{45}Ca and extrinsic ^{47}Ca tracers of TCP and $CaCl_2$ by rats (experiment 2)*†

Treatment	Whole body assay		Femur assay		
	EL ^{47}Ca		EL ^{47}Ca	IL ^{45}Ca	
	retention	absorption	femur uptake	femur uptake	absorption
	% dose	%	% dose	% dose	% of IP‡
TCP	80.8 ± 5.2	84.4 ± 4.8[a]	2.9 ± 0.2[A]	4.0 ± 0.4[B]	71.6 ± 7.7[b]
$CaCl_2$	80.5 ± 3.6	83.5 ± 4.6[a]	3.0 ± 0.2[A]		

IL, intrinsically labeled.
EL, extrinsically labeled.
*Mean ± SD.
†Different superscripts indicate significant differences among means ($P < 0.01$). Upper and lower case letters denote comparisons analyzed statistically.
‡The percent of the intraperitoneally injected dose that reached the femur was 5.6 ± 0.3.

Table 4 The effect of tracer labeling techniques on femur ^{45}Ca retention and absorption (experiment 3)*†

Treatment	^{45}Ca retention in femur	^{45}Ca absorption
	% dose	% of intraperitoneal injection
IL TCP	2.5 ± 0.2[a]	70.4 ± 6.0[a]
EL TCP (mix method)	2.9 ± 0.2[b]	80.3 ± 5.7[b]
EL TCP (drop method)	2.7 ± 0.3[a,b]	74.7 ± 7.3[a,b]

IL, intrinsically labeled.
EL, extrinsically labeled.
*Means ± SD, $n = 10$.
†Different superscripts within columns indicated significant differences among means ($P < 0.01$).

Discussion

Whole body counting and femur uptake techniques gave identical results for calcium absorption from the intrinsically doubly labeled $CaCl_2$ gavage solution *(Table 2)*. That these two methods would give similar results was expected from reports in the literature that have partially compared these methods previously. Marsh et al.[5] observed that radiotracer calcium uptake by incisor teeth and excretion data, which reflected whole body counting activity, were almost identical in 5-month-old rats fed control or phosphate and vitamin D-deficient diets. Buchowski et al.[4] also measured Ca tracer activities in whole body and femur and teeth. Both femur and teeth reflected similar activities on a per gram basis. Miller et al.[15] tested calcium fractional absorption from gavage doses containing different lactose levels by measuring calcium in femurs and teeth. They found similar absorption of calcium at each level of lactose as measured by teeth or femur analysis. Kochanoski and Jacobs[7] observed a significant correlation between whole body Ca and tibia Ca by day 7 post dosing over a wide range of calcium doses. Luhrsen et al.[2] observed that the newly absorbed calcium was rapidly distributed to the skeletal system by autoradiographic analysis of the femurs in rats.

Extrinsic labeling of TCP-containing test meals by dropping a $^{47}CaCl_2$ solution on the prepared meal gave significantly different results from an intrinsically labeled test meal (experiment 2). Therefore, experiment 3 was conducted to determine if the technique used for extrinsic labeling affected the results. Extrinsic labeling accomplished by dropping the tracer solution on the test meal appeared to give a closer approximation to endogenous calcium absorption than when the tracer was mixed with the salt prior to preparation of the test meal. However, in experiment 2 when the extrinsic label was dropped on the test meal, the value of calcium absorption was similar to that of the well-mixed method used in experiment 3. In experiment 2, whole-body activity at 3 hr post dosing was used as the value for ^{47}Ca activity in each test dose. In experiment 3, the actual dose could not be determined; it was assumed that the rats ate the whole test meal, and calculation of tracer calcium absorption was based on that meal's ^{45}Ca activity. The standard deviation in the group of rats fed the test meal extrinsically labeled by the drop method was larger than for the other groups. Therefore, we speculate that some of the solution dropped onto the test meal in experiment 3 may have adhered to the glass test meal cup, which would have resulted in a lower estimate of calcium absorption than for experiment 2. If our hypothesis is correct, extrinsic labeling by either the mixing or dropping technique for assessing calcium bioavailability could overestimate calcium absorption from a test meal.

Results from extrinsic labeling techniques have been reported to overestimate results from intrinsic labeling techniques in several situations. Smith et al.[1] observed that extrinsically labeled calcium carbonate released more Ca than intrinsically labeled calcium carbonate under low acid conditions. Their in vitro and in vivo

results suggest that calcium carbonate surface concentrations of extrinsically labeled calcium were disproportionately high. Luhrsen et al.[2] also found in the rat that the extrinsically labeled calcium from milk was better absorbed than the intrinsic calcium, but not for goat milk or calcium carbonate. Chromatographic analysis of labeled milk indicated a difference in the distribution of the extrinsic tracer. Another study[16] using $CaCl_2$, yogurt, milk, and cheese in rats confirmed that an extrinsic calcium tracer was absorbed more efficiently than an intrinsic calcium tracer. Wein and Schwartz[3] reported that for spinach and soy beans, extrinsic Ca tracers were absorbed more efficiently than intrinsic tracers. Our previous study[6] also found extrinsic labeling overestimated calcium bioavailability from spinach, but not for kale.

In contrast to these reports, other investigators have found that extrinsic labeling techniques adequately reflect endogenous calcium absorption. Buchowski et al.[4] using $CaCl_2$, yogurt, milk, and cheese curd prepared from caprin milk found that the extrinsic ^{47}Ca to intrinsic ^{45}Ca ratio in bone and teeth averaged about 1.00 with either milk, yogurt, or $CaCl_2$, but somewhat higher with cheese curd (1.04).

The results of experiment 4 demonstrate that absorption of ^{45}Ca calculated by femur uptake of an oral dose relative to an injected dose is valid for either intraperitoneal or intravenous administration of the injected dose. Furthermore, femur accumulation of the radiotracer was identical when intravenously injected as a salt or as its natural chemical speciation in the blood.

Use of tracers to evaluate absorption may not demonstrate what is occurring in a chronic condition. However, we conclude that the measurement of radiotracer activities of calcium in femur or whole body are equally accurate in comparing bioavailability of calcium among sources and are all convenient ways to evaluate calcium absorption without the collection of excreta or multiple blood samples. Femurs are probably preferable to teeth because calcium tracer retention in teeth was about one-twelth that of femurs in one study[4] and because of the experimental difficulty in removing a consistent proportion of a tooth. Intraperitoneal injection of the radiotracer for the femur uptake method is more convenient and equally valid as intravenous administration. The choice of using the femur uptake versus whole body counting approaches depends on the instrumentation available, the choice of isotopes, and time constraints. The beta emitter, ^{45}Ca, which can only be used in the femur uptake method, is less expensive and has a longer half life than ^{47}Ca. Use of extrinsic labeling techniques requires testing of complete label exchange prior to adoption of a protocol and for different sources of calcium.

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