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RAPID COMMUNICATIONS

INTRINSIC LABELING OF BOVINE MILK WITH ENRICHED STABLE ISOTOPES OF ZINC[1]

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Abstract. Bovine milk was labeled intrinsically with enriched stable isotopic zinc for human bioavailability studies. Intrajugular administration of zinc isotopes temporarily increased the plasma zinc concentration of Ayrshire cows by as much as 76%, but milk zinc concentration and the distribution of zinc between casein and whey did not change appreciably. Milk zinc isotopic enrichment reached 105 and 613 atom % excess for ^{67}Zn and ^{70}Zn, respectively within 4-12 hr of zinc administration and decreased gradually over several days. This degree of isotopic enrichment is sufficient for testing bioavailability to infants of intrinsic zinc from milk-based formulas.

Introduction. Zinc absorption by human beings from foods has been measured by using the method of fecal monitoring, after feeding formulas or meals to which enriched stable isotopic zinc had been added (1). The assumption is made that the extrinsic zinc label exchanges with the zinc intrinsic to the meal before absorption of all zinc from one common pool. An initial exploration of the validity of the stable isotope, extrinsic-tag approach for measurement of bioavailability of dietary zinc in human adults fed intrinsically labeled chicken meat has been reported (2). Validation of this method is needed because of reports that zinc bioavailability to infants is affected adversely by some dietary substances, including components of cows' milk. Accordingly, the feasibility of intrinsically labeling bovine milk with the stable isotopes ^{67}Zn and ^{70}Zn for use in human feeding experiments was investigated.

Materials and Methods. Three Ayrshire cows between 496 and 504 kg body weight were fed a ration that conformed to National Research Council guidelines for lactating dairy cattle (3). A trace-mineralized salt that contained 0.35% Zn was included in the ration at 0.5% of dry matter. A silastic catheter (0.040" i.d., 0.085" o.d., Dow Corning Corp., Midland, MI) was installed into the right external jugular vein of each cow. The catheter was used for periodic withdrawal of blood into heparinized polyethylene syringes and for administration of enriched stable isotopic zinc. The catheter was flushed before and after each use with heparinized saline.

Enriched stable isotopic zinc (93.11 atom % ^{67}Zn or 88.61 atom % ^{70}Zn as oxide, Oak Ridge National Laboratory, Oak Ridge, TN) was converted to the

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chloride by addition of several drops of 12 M HCl followed by heating to dryness. Addition of deionized water (25 ml), 1.00 mmole of trisodium citrate, and 1.25 to 1.75 mmole of sodium bicarbonate brought pH to 7.36. In this way, the zinc was soluble at physiologic pH, and the solution was isosmotic to blood plasma. The first cow received ^{67}Zn (0.40 mmole Zn) through its catheter in a single dose within 10 sec at time zero. The second cow received ^{70}Zn (0.25 mmole Zn) in three equal doses at time zero, 4.02 hr, and 8.02 hr. The third cow received ^{70}Zn (0.40 mmole Zn) in one dose at time zero.

Blood (50 ml each time) was drawn, and the cows were milked at intervals shown in the Results. During most milkings, oxytocin (1.5 ml at 20 USP units/ml, Anpro Pharmaceuticals, Inc., Arcadia, CA) was administered intravenously to stimulate emptying of the udder. The bucket milker and the cream separator were constructed of stainless steel. Milk and cream were separated immediately, and the skim milk was frozen and lyophilized. Portions of dry milk were reconstituted in deionized water, and the whey and casein were separated by using ultracentrifugation (134,000*g* for 60 min at 4°C), for subsequent measurements of zinc distribution. Heparinized blood plasma was separated from cellular elements by centrifugation (1000*g* for 30 min), small portions were saved for measurement of plasma zinc, and the remainder of each sample was frozen and lyophilized. Lyophilized milk and blood plasma were prepared for isotope ratio measurements and were analyzed by using inductively coupled plasma/mass spectrometry (ICP/MS). Measurements on samples from the first two cows were obtained by using a prototypic instrument constructed in the Ames Laboratory (4); the remainder were performed by using a commercial ICP/MS instrument (ELAN Model 250 with 1986 ion optics upgrade, Sciex Ltd., Thornhill, Ontario, Canada)(5). Ratios for samples have been corrected for mass discrimination by comparison with standards run on the same day that contained zinc at natural abundance. Zinc concentrations were determined by using flame atomic absorption spectrophotometry. Student's t test for unpaired data was used for statistical comparisons of means, and significance was accepted if $P<0.05$.

Results. Ratios of plasma ^{67}Zn referenced to ^{68}Zn, and plasma zinc concentrations vs. time are shown in Table I for the cow given enriched ^{67}Zn in one dose at time zero. Plasma zinc was 73% greater at 30 min than the normal predose value of 1.27 μmole/dl and declined to near normal values by 8 hr. The decrease in plasma $^{67}Zn/^{68}Zn$ from 30 min to 8 hr was parallel to the decline in plasma zinc concentration.

TABLE I

Effect of intravenous ^{67}Zn on zinc isotope ratios of blood plasma[a]

Time (hr)	Plasma Zinc (μmole/dl)	Plasma Ratio $^{67}Zn/^{68}Zn$ (mean)[b]	(%RSD)[c]
0	1.27	0.221	2.1
0.5	2.20	1.358	1.0
1.0	2.02	0.932	1.5
2.0	1.84	0.658	0.9
4.0	1.68	0.488	2.1
8.0	1.53	0.462	1.4

[a]400 μmole zinc given at time zero.
[b]Means of 40-50 measurements (4).
[c]%RSD = 100(standard deviation/mean).

The $^{67}Zn/^{68}Zn$ ratios of the skim milk samples from the cow that was given ^{67}Zn are shown in Table II. The 4- and 8-hr milkings were the most enriched, with $^{67}Zn/^{68}Zn$ of 2.05 times the predose ratio. The appearance of ^{67}Zn in milk gradually declined during the subsequent 35 hr. The mean %RSD of $^{67}Zn/^{68}Zn$ for these milk samples was 1.7%. The zinc content (mean±SD) of extracts of the

TABLE II

Effect of intravenous ^{67}Zn on zinc isotope ratios of dried skim milk[a]

Time (hr)	Milk Zinc Content (nmole/g)	Milk Ratio $^{67}Zn/^{68}Zn$ (mean)	(%RSD)
0	606	0.221	2.0
4	652	0.453	1.7
8	676	0.453	1.8
12	610	0.404	1.7
16	639	0.375	1.3
20	569	0.370	1.0
24	544	0.350	2.4
31	620	0.309	2.2
43	609	0.289	1.4

[a]Milkings were oxytocin-stimulated except at 31 and 43 hr. Other footnotes same as Table I.

labeled milk samples, per gram of solids (615±43 nmole/g) was similar to the value (606 nmole/g) for the extract of the predose milk sample. Both these values are similar to the reference value (mean±SE) for zinc content (624±16 nmole/g) of nonfat milk solids (6).

Plasma zinc concentrations and $^{70}Zn/^{67}Zn$ vs. time are shown in Table III for the cow given three doses of enriched ^{70}Zn at zero, 4.02, and 8.02 hr.

TABLE III

Effect of multiple doses of ^{70}Zn on zinc isotope ratios of blood plasma[a]

Time (hr)	Plasma Zinc (μmole/dl)	Plasma Ratio $^{70}Zn/^{67}Zn$ (mean)	(%RSD)
0	1.39	0.1509	0.2
0.25	1.74	2.37	1.5
0.5	1.70	1.55	0.7
1.0	1.70	0.988	0.4
2.0	1.59	0.704	0.8
4.0	1.59	0.505	0.7
5.0	1.82	1.33	1.3
6.0	1.77	0.943	0.9
8.0	1.67	0.745	0.7
9.0	1.97	1.81	2.9
10.0	1.85	1.31	2.2
12.0	1.77	1.021	1.2
24.0	1.62	0.497	1.0

[a]83 μmoles zinc given at zero, 4.02, and 8.02 hr. Other footnotes same as Table I.

The three ^{70}Zn doses resulted in successive increases in plasma zinc concentration and isotope ratios, followed by gradual decreases. The maximal plasma Zn, at 9 hr (1.97 μmole/dl), was 42% above the predose value (1.39 μmole/dl). The plasma Zn concentration and the ratio had not returned to normal at 24 hr.

The $^{70}Zn/^{67}Zn$ ratios of the skim milk samples from the cow given three doses of ^{70}Zn are shown in Table IV. The ratios of the 12- and 23-hr samples (0.612±0.009, 0.605±0.004) were approximately four times the predose ratio. The milk ratios declined gradually; significant enrichment was observed even after 68 hr. The mean %RSD of these $^{70}Zn/^{67}Zn$ determinations was 0.8%. Zinc content of the dry skim milk was significantly greater at 6 hr than before zinc administration and continued to increase. Zinc in the pellet (casein fraction), expressed as a percentage of the sum of pellet and supernatant (whey) zinc, was significantly higher in the

TABLE IV

Effect of multiple doses of ^{70}Zn on zinc in lyophilized skim milk[a]

Time (hr)	Milk Zinc[b] Content (nmole/g)	Milk Ratio[c] $^{70}Zn/^{67}Zn$ (mean)	(%RSD)	Pellet[d] Zinc (%)
0	465± 6[e]	0.151	0.4	90±2[e]
6	581±11[f]	0.368	1.1	96±1[f]
12	561± 3[f]	0.612	1.6	96±1[f]
23	664±40[fgh]	0.605	0.6	96±1[f]
32	650± 7[g]	0.507	0.9	89±1[e]
44	684± 6[g]	0.398	0.4	87±2[e]
68	742±11[h]	0.285	0.7	85±1[e]

[a]See Table III. Zero-hr, 6-hr, and 12-hr milkings were oxytocin-stimulated.
[b]Data are means±SD; duplicate samples.
[c]Means of 40-50 measurements (4).
[d]Data are means±SD; triplicate samples.
[efgh]Means with different superscripts differ significantly ($p<0.05$).

6-, 12-, and 23-hr milkings than at other times. Recovery of zinc in pellet and whey (mean±SD) was 100.3±5.4% of the zinc in the reconstituted milk samples before ultracentrifugation.

Plasma zinc concentrations and ratios of $^{70}Zn/^{68}Zn$ are shown in Table V for the cow given one dose of ^{70}Zn. Plasma zinc concentration was 76% greater at 15 min than the normal predose value and decreased to near normal by 24 hr.

TABLE V

Effect of intravenous ^{70}Zn on zinc isotope ratios of blood plasma[a]

Time (hr)	Plasma Zinc (μmole/dl)	Plasma Ratio[b] $^{70}Zn/^{68}Zn$ (mean)	(%RSD)
0	1.41	0.0335	1.5
0.25	2.48	1.38	1.3
0.5	2.26	0.850	0.4
1.0	2.13	0.555	0.7
2.0	2.00	0.384	0.7
4.0	1.89	0.288	0.7
8.0	1.91	0.209	0.9
12.0	1.98	0.174	1.2
24.0	1.60	0.1239	0.6
72.0	1.25	0.0703	1.4

[a]400 μmole total zinc given at time zero.
[b]Means of 10-13 measurements (5).

The $^{70}Zn/^{68}Zn$ ratios of the skim milk samples from the cow given one dose of ^{70}Zn are shown in Table VI. The 12-hr milking was enriched to 613 atom % excess of ^{70}Zn. Useful (>200%) enrichment

was evident even at the 48-hr milking. The mean %RSD of the $^{70}Zn/^{68}Zn$ measurements was 0.8%. Zinc content of the dried skim milk was significantly less at zero time than afterwards. The values for milk zinc after zinc administration did not differ significantly and are similar to the reference value (624±16 nmole/g) for zinc content of nonfat milk solids. The percentage of zinc in the pellet was significantly less in the zero-, 24-, and 84-hr milks than in the 12-hr milk. The values for pellet zinc at other times were not significantly different from either extreme. Recovery of zinc in the pellet and supernatant fluid (mean±SD) was 99.3±8.0% of the zinc in reconstituted milk samples before ultracentrifugation.

TABLE VI

Effect of intravenous ^{70}Zn on zinc isotope ratios of dried skim milk[a]

Time (hr)	Milk Zinc[b] Content (nmole/g)	Milk Ratio[c] $^{70}Zn/^{68}Zn$ (mean)	(%RSD)	Pellet Zinc (%)
0	526±30[d]	0.0334	1.1	95±1[d]
12	620±21[e]	0.2381	0.8	93±1[e]
24	604±10[e]	0.1678	0.6	95±1[d]
36	593±17[e]	0.1294	0.7	94±1
48	624±23[e]	0.1061	0.8	95±1
60	601±22[e]	0.0906	0.8	93±1
72	595±25[e]	0.0808	0.8	94±1
84	604±12[e]	0.0728	0.4	95±1[d]

[a]See Table V. All except 84-hr milking were oxytocin-stimulated.
[b]Data are means±SD; triplicate samples.
[c]Means of 8-13 measurements (5).
[de]Means with different superscripts differ significantly ($p<0.05$).

Discussion. Investigators of bovine zinc distribution have relied upon radioisotopic ^{65}Zn administration to bull calves (7) or upon the feeding of markedly different zinc intakes to lactating animals (8). They have shown that the same overall tissue zinc distribution is achieved less expensively with intravenous than with oral tracer and that changes in concentration of zinc in milk are neither as pronounced nor as rapid as changes in zinc concentration of blood plasma.

Our results are the first documentation of the feasibility of using stable isotopes to study zinc kinetics in lactating animals and to label milk intrinsically for zinc bioavailability experiments. Stable isotopes are safer than radioisotopes, but often they cannot be considered true tracers, to the extent that pool sizes and distribution are affected. This limitation particularly applies to small pools that turn over rapidly, as is evident from the increases (up to 76%) in plasma zinc concentration that we observed. The increases in plasma zinc after a single intravenous dose did not last longer than 24 hr. Similar increases in plasma zinc concentration of lactating cows have been maintained for long periods through the feeding of high-zinc rations (8) without effects on milk production or signs of toxicity.

Zinc administration to the second and third cows affected zinc concentration of milk. The 15% increase that resulted from a single intravenous dose (Table VI) varied less over time than that from smaller, multiple doses (Table IV). The increase brought the zinc concentration of the dried skim milk nearer to reference concentrations (6) than it had been before zinc administration. Our interpretation is that the pool of zinc in the mammary gland is large and turns over slowly but that mixing within the pool is so rapid that zinc distribution between casein and whey is not materially altered by a modest increase in pool size. This interpretation is supported by the data on zinc distribution in milk. Pellet zinc (casein fraction), expressed as a percentage of the sum of zinc in the pellet and supernatant (whey) fractions, was within normal range at all milkings (Tables IV and VI) and did not change very much over time, although some of the differences are statistically significant.

Our results support the use of ^{70}Zn rather than ^{67}Zn for intrinsic labeling. Despite the much higher cost of ^{70}Zn, its use is justified by the greater intrinsic enrichment that can be achieved without undue perturbation of zinc concentration and distribution in the milk, provided that precision and accuracy of the isotope ratio measurements are acceptable. This proviso is important; analytical precision is harder to obtain for the rarer ^{70}Zn than for ^{67}Zn.

Janghorbani et al. (9) have calculated the error function pertinent to zinc bioavailability studies and have shown for ^{70}Zn that dietary enrichment

of 100 atom % excess is sufficient if overall precision of 1% can be obtained for intake and excretory measurements. The mean %RSD of 0.8% (range 0.4 to 1.1%) for the ratio determinations on milk in Table VI is precise and is illustrative of what can be achieved with a commercially available instrument in our laboratories (5). The results in Tables I through IV are not as precise and were obtained by using a prototypic instrument of different design and operating parameters(4). Given the precision and accuracy of the Zn isotope ratio determinations currently achievable by using ICP/MS, milk zinc enrichments of 200 atom % excess of ^{70}Zn should be sufficient for conducting intrinsic zinc bioavailability studies in infants, for whom half of the dietary zinc can be provided as labeled infant formula. The degree of ^{70}Zn enrichment reported here is sufficient for studies in infants. Where other food sources of zinc predominate, or in subjects such as adults, where there is more intestinal mixing of labeled zinc with unlabeled zinc from subsequent meals, 600-800% enrichment of milk zinc would be useful.

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