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Primary Hypothesis: Repletion of mild zinc (Zn) & iron (Fe) deficiencies will improve neuropsychological performance of women. Design: This is a 16 week double-blind stratified randomized controlled treatment (Rx) trial with a Rx cross-over at 8 weeks. Subjects are 60 Zn & Fe deficient (D) & 20 normal (N) premenopausal women. Before baseline measurements, other potentially limiting nutrients are repleted. Rx includes micronutrients (M) alone, given to 20 D & 20 N subjects for 16 weeks with a pseudo-cross-over at 8 weeks; 30 mg Zn with M, and 30 mg Fe with M, each given to 40 D subjects for 8 weeks, 20 subjects at a time. Outcomes include Zn & Fe status, lean body mass (LBM), Zn kinetics, other indices that might be affected by Zn status, and neuropsychological performance. Results: Screening plasma zinc concentration in 73 subjects (since start) was (mean ± SD) 712 ± 85.1 µg/L. Hair zinc concentrations in 102 subjects (since start) were 174 ± 65.4 µg/g. Platelet , leukocyte and/or lymphocyte Zn concentrations were generally within the reference range. In contrast to previous findings plasma Zn disappearance and serum ferritin concentrations were not related. Chelation and ultrafiltration of 67Zn labeled plasma found plasma Zn fractions were not in equilibrium. Polyatomic isobaric interferences impaired ICP-MS assay of 64 & 70 Zn, but not of 66 67 & 68Zn in nonextracted serum. The 24 hour exchangeable Zn pool (EZP) and turnover rate (TR) were related to the number of days the subjects were repleted with M (r=0.88 and 0.85, p<0.0001 for both).

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H. Sandstead

PI - Signature Date

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Introduction

Subject: The relationship of zinc and iron nutriture to neuropsychological performance.

Purpose: This project tests the hypothesis: Repletion of mild zinc and iron deficiencies will improve neuropsychological performance of premenopausal women.

Scope: The project has three major components:

- Premenopausal women who are likely to be mildly deficient in zinc and iron will be identified through food frequency histories and measurement of serum ferritin concentrations.
- Zinc status will be confirmed by plasma, granulocyte, lymphocyte, platelet, urine, and hair zinc concentrations and zinc kinetics. Iron status will be confirmed by serum ferritin and iron, percent iron saturation of transferrin, hemoglobin and red blood cell indices.
- Effects of zinc and iron repletion on neuropsychological performance will be measured by computerized tasks, using a double-blind stratified randomized controlled cross-over design.

Statement of Work

This project tests the hypothesis: Repletion of mild zinc and iron deficiencies will improve neuropsychological performance of premenopausal women.

The design will be a double-blind stratified randomized controlled treatment trial with a cross-over of treatments. Sixty zinc and iron deficient subjects and 20 normal control subjects will be studied. Twenty of the deficient subjects and the 20 normal subjects will be given a mixture of micronutrients, based on the Recommended Dietary Allowances, to replete latent deficiencies and control for study effects. These subjects will undergo a pseudo-cross over after 8 weeks of treatment. Twenty of the deficient subjects will be given 30 mg zinc with micronutrients, and 20 will be given 30 mg iron with micronutrients for 8 weeks. These two groups will then be switched (cross-over) to the other treatment. To avoid interferences from other limiting micronutrients all subjects will be repleted with micronutrients before the baseline measurements. Neuropsychological performance will be measured at baseline and after 8 and 16 weeks of treatment. Individual and combined effects of zinc and iron, and unique effects related to the order of treatments will be determined. Secondary outcomes will include improvement of methods for measurement of Zn tracers by inductively couple plasma-mass spectroscopy (ICP-MS) and measurement of zinc kinetics, and relationships of zinc kinetics to body composition, general nutritional status, zinc and iron status, other aspects of metabolism, and neuropsychological performance outcomes.

Project Time Line (from the grant application, edited and status indicated):

0-90 days	Hire personnel Purchase initial equipment and supplies---------Minimal needs continue Advertise project; interview respondents----------616 total, 200 this year Screen respondents----------262 total, 112 this year
Year 1.	Enroll 30 subjects in the treatment trial----------5 subjects qualify ----------5 subjects enrolled ----------Great difficulty with recruitment and retention
Year 2	Enroll 30 subjects in the treatment trial----------50 subjects qualify ----------32 subjects enrolled ----------22 completed the first phase of the treatment trial ----------15 subjects completed the study ----------8 subjects resigned ----------Replaced administrative assistant, recruitment improved ----------Subject retention was impaired by the length of the protocol. Therefore, with permission, Zn kinetic studies were stopped after 52.
Year 3	Enroll 20 subjects in the treatment trial.----------52 subjects qualified ----------45 subjects enrolled ----------37 completed the first phase of the treatment trial ----------34 subjects completed the study ----------49 total subjects have completed the study ----------7 subjects are at present part way though the treatment trial ----------6 subjects resigned ----------New administrative assistant, good recruitment continues ----------Permission was granted for study continuation
Year 4	Enroll 24 subjects in the treatment trial Prepare final reports for publication

Subjects Time Line (with Notes):

d 1	Telephone interview respondents to advertisements -------200 this year
d 7	Screen respondents that qualify for the project ----------112 this year Medical history ----------by our staff Food frequency history ----------by our staff Physical Examination ----------by our staff Screening blood chemistries, CBC, serum ferritin ---------- ----------provided by the NIH sponsored Clinical Research Center
d 14	Select subjects ----------52 this year Treat latent deficiencies with micronutrients before baseline measurements of outcomes. The duration (about 7-10 days) of treatment depends on stage of menstrual cycle. Our previous findings in children indicate that the micronutrient mixture we are giving will little if any effect by itself on the subjects neuropsychological performance.

d 24	Orient the subjects to the neuropsychological tasks Randomize the subjects to the treatments Measure baseline bioelectrical Impedance, taste acuity and other indices related to Zn status
~d 30	Measure baseline neuropsychological outcomes on day 8-12 of menstrual cycle ----------45 subjects this year Begin treatment
~d 90	After 8 weeks of treatment repeat measurements of outcomes on day 8-12 of menstrual cycle ----------37 subjects this year Cross-over the zinc and iron treatment groups and pseudo-cross-over the control groups
~d 150	After 16 weeks of treatment measure outcomes on day 8-12 of menstrual cycle ----------34 subjects this year Thank subjects for participation Give subjects a copy of their medical evaluation Give subjects nutrition information Pay the final compensation
Later	Send subjects copies of published reports

Methods:

Subject Recruitment: Advertisements were posted on bulletin boards at UTMB and surrounding Colleges, in news letters and newspapers, and on the Internet. Respondents were screened by telephone interview. Potential candidates were invited for detailed evaluation by medical and dietary history, physical examination and laboratory assessment, and determination of iron status. Individuals with serum ferritin <20μg/mL or >30μg/mL who are otherwise normal were offered an opportunity to volunteer for the study.

Assessment of Zinc Status: Plasma zinc concentrations were measured by Atomic Absorption Spectrometry (AAS) (1). Granulocyte, lymphocyte and platelet zinc were measured by AAS (2, 3). Hair zinc was measured by AAS (4, 5).

Assessment of Iron Status: Iron status was assessed by serum ferritin (6, 7), serum iron, percent saturation of transferrin, and hemoglobin concentration (8).

Other Indices Possibly Affected by Zinc Nutriture: An electrogustometer (Rion TR-06) developed by Tomita et al (9) was used to measure the electrical threshold of taste buds. A circular, 5mm in diameter, stainless steel electrode was used. Subject were comfortably sitting and familiar with the difference between the electrical stimulation and the tactile sensation of the electrode. A neutral electrode was applied to the neck and the voltage adjusted to 10 dB above the expected threshold. Subjects were asked to identify when they experienced an electrical sensation by pressing a buzzer. Areas of the tongue, 2 cm from the midline, on the left and right side of the anterior and posterior tongue (circumvallate papillae) were assessed. Threshold was determined by increasing the stimulation current starting from zero. Taste acuity was measured before and after treatment. Taste acuity was also measured by the filter paper disc method. A

drop of a solution of either sweet (sucrose), salty (sodium chloride), sour (tartaric acid), and bitter (quinine hydrochloride) was placed on a small 5 mm paper disc on the same areas of the tongue as described for the electrogustometer, left for 3 seconds and then removed. The mouth was then rinsed with water. The subject was asked to identify the taste quality. The level at which the taste quality was correctly identified was the threshold. Previous experiments found Zn essential for taste (10-12).

β-hydroxybutyrate (13) was measured in serum using a kit (Procedure No. 310-UV, Sigma Diagnostics, PO Box 14508, St. Louis, MO). The purpose of these measurements was to determine if zinc nutriture, at the levels observed in these subjects, had a practical effect on fat metabolism, as was observed in zinc deprived rats (14).

Since the start of the project (primarily this year), and at not cost to the project, blood from 66 subjects (36 screening, 66 baseline, 38 after the first period of treatment, and 30 after the second period treatment) was assayed in plasma and erythrocyte folate microbiologically using *L. casei* (15, 16) by T. Tamura, MD, of the University of Alabama Department of Nutrition Sciences. He also measured plasma cobalamine concentrations using a Ciba-Corning (Medfield, MA) Radioassay kit, plasma pyridoxal-5-phosphate concentrations by the method of Camp et al. (17), and plasma homocysteine concentrations by the method of Tamura (18). The purpose of these measurements is to determine if zinc nutriture, at the levels present in our subjects, has a practical effect on these important indices, as was suggested by other experiments (19-22).

Since the start of the project amino acids have been measured by Richard Fritz, PhD of the Department of Human Biological Chemistry and Genetics at UTMB in serum and urine baseline and follow-up samples from 50 subjects. The purpose of these measurements was to determine if zinc nutriture, at the level observed in our subjects, has a practical effect on amino acid metabolism, as was observed in rats (23, 24).

Since the start of the project David Simmons, PhD of the UTMB Department of Orthopedics has measured 24 hr urine cross linked n-telopeptides for type I collagen in 28 baseline samples, 26 after the first period of treatment, and 25 after the second period of treatment; and serum osteocalcin in 34 samples from each phase of the repletion study. The purpose of these measurements is to determine if zinc nutriture, at the level observed in our subjects, has a practical effect on bone metabolism, as was suggested by other experiments (25-27).

Douglas Goeger, PhD, of the UTMB Department of Preventive Medicine and Community Health measured metabolism of the substrate chlorzoxazone to determine if zinc nutriture affects activity of a zinc mediated cytochrome P450 mixed function oxidase. After an over-night fast subjects were given 500 mg chlorzoxazone orally and the fasting continued for 2 hours. Then 5 mL of blood was drawn into an EDTA containing vacutainer and the plasma separated and stored at -20° C until analysis of the ratio of chlorzoxazone and 6-hydroxychlorzoxazone was determined (28). The ratio at this time point is significantly correlated to area under the curve ratios for 6-hydroxychlorzoxazone and chlorzoxazone and is less invasive than multiple blood

samples needed to determine area under the curve (29). This procedure has been used successfully for detecting changes in chlorzoxazone metabolism (29). The metabolism of chlorzoxazone to 6-hydroxychlorzoxazone can vary 4 to 5-fold between individuals and no age or sex-related effects are apparent (30). This study was intended to show if zinc nutriture, in the range observed in these subjects, has a practical effect on this important enzyme, as was suggested by experiments in rats (31).

Zinc Kinetics: Serum and urine collected for measurement of zinc kinetics were analyzed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) by modification of published methods (32). These measurements were done to determine if indices of zinc kinetics are related to serum ferritin concentrations, as our previous findings suggest (33); and if indices of zinc kinetics are related to indices of neuropsychological performance at baseline and/or after treatment.

Effect of Zinc and Iron Repletion on Outcomes: This phase of the project is "in progress". Neuropsychological performance indices are measured on the 8-12th day of the menstrual cycle before and after the experimental treatments.

Results and Discussion:

We requested and received permission to continue the study for one additional year (with no additional money).

Subject Recruitment: Two-hundred women responded to advertisements and were screened by telephone. One-hundred-twelve met the criteria for detailed screening. Fifty-two respondents met the criteria to be inclusion in the study. Fort-five subjects were enrolled in the treatment trial. Thirty-seven completed the phase of the study (baseline assessment, 8 weeks of treatment, and follow-up evaluation), and 34 completed the second phase of the study (cross over of treatment, 8 weeks of treatment, and follow-up evaluation). Six subjects resigned and 7 are currently receiving treatment.

Zinc Status: Since initiation of this project the fasting plasma zinc concentrations in 73 subjects at screening (mean ± SD, median, and range) were 712 ± 85.1, 720, 545-970 μg/L. The concentrations in 51 subjects after other potentially limiting nutrients were repleted were 752 ± 83.7, 745, 600-915 μg/L. The accepted lower limit of normal for fasting adult plasma is 700 μg/L (34). Thus even though plasma zinc is an insensative indicator of zinc status (35) nearly half of the subjects had plasma zinc concentrations in the low range, consistent with the selection criteria.

Leukocyte and platelet zinc concentrations were measured by the method of Beck (3) in baseline (after repletion of other potentially limiting nutrients) fasting whole blood samples (Table 1). Considerable variation was encountered in the yield of lymphocytes and granulocytes in spite of the fact that the isolation procedure is highly standardized and is reproducible at its various steps. Investigators at other institutions have found similar variations. The reference ranges cited are based on review of several reports. The wide range suggests there is no consensus.

Table 1. Leukocyte and Platelet Zinc Concentrations (in 1998)

	Platelets ($ug/10^{10}$ cells)	Lymphocytes ($ug/10^{10}$ cells)	Granulocytes ($ug/10^{10}$ cells)
n	43	34	28
Mean	3.9	164	124
SD	1.47	105.9	54
Median	3.9	127.4	94.2
Range	1.7-8.4	24.5-451	21.6-278
Reference values	3.0 - 6.6	45.0 - 218.0	37.8 - 117

The analysis of 24-hour urinary zinc excretion in all subjects is pending.

Since initiation of this project hair zinc concentrations were measured in 102 individuals. The mean ± SD, median, and range were 174±65.4, 162, and 65.9-413 μg/g. Thus most subjects were within the range considered normal (> 100 μg/g).

Iron Status: For 109 potential subjects the range and mean ± SD serum ferritin were 4.0-163.0 and 26.1±24.7 ng/mL.

Other Indices Possibly Affected by Zinc Nutriture:

Baseline taste acuity measured by electrogustometer found significant correlations ($r = 0.34$-0.36, $p < 0.02$-0.03) between plasma Zn concentration and the threshold for three of four tongue locations. No correlation was found with serum ferritin. Baseline taste acuity by filter paper disc found significant correlations between plasma Zn and taste acuity for salt, sour and bitter (0.31-0.40, $p<0.05$) on the right posterior tongue and bitter ($r= 0.33$, $p<0.05$) on the left posterior tongue. No correlation was found with serum ferritin.

Since initiation of the project plasma β-hydroxybutyrate concentrations were measured in 97 specimens stored at -70°C. All were within the reference range of 0-4.39 mg/dL.

Inter-assay coefficients of variation for homocysteine, folate, cobalamine and PLP determined on pooled plasma or control samples provided by the manufacturer were 8%, 10%, 8% and 11% respectively. Plasma homocysteine, plasma and erythrocyte folate and plasma cobalamine were within the normal range at screening and after repletion of other potentially limiting micronutrients. There was no change in the concentrations of these indices after repletion. In contrast at screening 18 of 36 subjects had plasma pyridoxal-5-phosphate (PLP) concentration <30 nmol/L (low) while the range was 8.6-150.0 nmol/L and the mean±SD was 36.5±26.2 nmol/L. The subsequent range of plasma PLP after 10 or more days of pyridoxine repletion was 9.1-124.7 nmol/L and the mean±SD was 38.2±24.7 nmol/L in 61 subjects. This failure of PLP to increases after repletion is consistent with decreased pyridoxal kinase activity. Pyridoxal kinase uses Zn-ATP as a substrate (21). Determination of the effects of Zn repletion on PLP will be determined at the end of the experiment, when the treatment code is opened.

The analysis of plasma and urine samples for amino acids is in progress.

The analysis of samples for assessment of bone metabolism is in progress.

No effects of zinc nutriture on metabolism of blood cytochrome P450 activity were detected in 52 subjects at baseline, 44 after the first period of treatment, and 29 after the second interval of treatment.

Zinc Kinetics:
Preliminary analysis did not find a relation between plasma Zn disappearance and serum ferritin. This is in contrast to our earlier finding of a highly significant inverse relationship (33). We are reevaluating the plasma Zn disappearance data and will also measure the ferritin iron. Recent findings by Herbert et al (36) indicate that ferritin protein can be increased by inflammation in subjects with low ferritin iron, giving an incorrect, by immunoassay of the protein, indication of iron stores. Measurement of ferritin iron will provide a better indicator of iron status to correlate with Zn kinetics. Additional findings are indicated in the attached abstracts.

Effects of Zn or Fe on Neuropsychological Performance:
The double blind randomized controlled repletion trial is progressing.

Conclusions:

Recruitment of subjects and accomplishment of the objectives was successful. Because this is a double blind randomized controlled trial the effects of treatments on outcomes continue to be unknown. Methods for analysis of Zn stable isotopes were improved. The randomized controlled trial is progressing satisfactorily.

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Presentations:

Abstracts presented to the American Society for Nutrition Sciences at Experimental Biology '98, San Francisco, California, April 18-22, 1998.

1)The Intake of Micronutrients Influences Zinc Kinetic Parameters. N. Egger, K. Yokoi, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead.

2)Polyatomics in Zinc Isotope Ratio Analysis of Plasma Samples by ICP-MS and Applicability of Nonextracted Samples for Zinc Kinetics. V.M. Sadagopa Ramanujam, K. Yokoi, N. Egger, H.H. Dayal, N.W. Alcock, H.H. Sandstead.

3)Measurement of Plasma Chelatable Zinc for Zinc Kinetic Studies in Humans. K. Yokoi, V.M. Sadagopa Ramanujam, N.W. Alcock, N. Egger, H.H. Dayal, H.H. Sandstead.

Publications:

The manuscript, "Polyatomics in Zinc Isotope Ratio Analysis of Plasma Samples by Inductively Coupled Plasma-Mass Spectrometry and Applicability of Nonextracted Samples for Zinc Kinetics" by V.M. Sadagopa Ramanujam, K. Yokoi, N.G. Egger, H.H. Dayal, N.W. Alcock, and H.H. Sandstead, was accepted by *Biological Trace Element Research.*

The manuscript, "Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics in Humans" by K. Yokoi, N.G. Egger, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead., was rejected by the *American Journal of Physiology.* It is being revised for submission to the *Journal of Laboratory and Clinical Medicine.*

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ABSTRACTS 1-3617
PART I

The American Physiological Society
American Society for Pharmacology and Experimental Therapeutics
American Society for Investigative Pathology
American Society for Nutritional Sciences
The American Association of Immunologists
American Association of Anatomists

The Biomedical Engineering Society
Society for Experimental Biology and Medicine
Chinese Physiological Society
The Microcirculatory Society
North American Vascular Biology Organization
Society for International Nutrition Research
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Association of Medical Laboratory Immunologists
International Society for Interferon and Cytokine Research
International Society for NeuroImmunoModulation

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MEASUREMENT OF PLASMA CHELATABLE ZINC FOR ZINC KINETIC STUDIES IN HUMANS K. Yokoi, V.M. Sadagopa Ramanujam, N.W. Alcock, N. Egger, H.H. Dayal, H.H. Sandstead. Univ. of Texas Med. Br., Galveston, TX 77555-1109 and Jichi Med School, Tochigi, Japan

Kinetic parameters are more informative than plasma Zn for assessment of zinc (Zn) status. However, all fractions of plasma Zn are assumed to be completely equilibrated in kinetic analyses. We compared isotope ratios (IR) in total (Tot) and chelatable (Chel) Zn in plasma measured by inductively coupled plasma-mass spectrometry (ICP-MS). Samples were collected from 5 men and 5 women at 5 min to 24 h after 2 mg ^{67}Zn iv as ZnCl. The Chel Zn was obtained using EDTA. The ultrafiltrate containing Chel Zn was directly measured by ICP-MS. Background counts in the reagent blank were lower for Chel Zn than for Tot Zn. After subtraction of baseline, the Zn IR was divided by the natural Zn IR to yield the normalized IR (NIR). The ratio of NIR for Tot Zn to Chel Zn was 0.883 to 0.975 (mean 0.937) and 0.877 to 1.159 (mean 1.023) at 5 min and at 1 day after iv ^{67}Zn, respectively. ^{67}Zn was spiked in vitro to plasma obtained from one subject. The ratio of NIR for Tot Zn to Chel Zn was 0.905 in spiked plasma and 0.919 in plasma from the same subject 5 min after iv ^{67}Zn. These results suggest that: 1) plasma Zn fractions are not in complete equilibrium; 2) measuring Chel Zn may be more suitable for Zn kinetic analysis since Chel Zn is chemically specified and iv ^{67}Zn is chelatable. *(Supported by a DOD grant, DAMDB 17-95-C-5112).*

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THE INTAKE OF MICRONUTRIENTS INFLUENCES ZINC KINETIC PARAMETERS. N. Egger, K. Yokoi, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead. Univ. of Texas Med. Br., Galveston, TX 77555-1109 and Jichi Med School, Tochigi-Ken 329-04, Japan

The readily exchangeable zinc pool (EZP) is responsible for the physiological functions of zinc (Zn). Studies suggested a positive relation between dietary Zn intake and EZP. To explore further relationships we studied 12 healthy females (age ranged from 24-39 years, body mass index from 19-35 kg/m^2). No substantial changes to the diets were made and one tablet, containing micronutrients without Zn or iron, was administered daily for 5-22 days. EZP was measured from the 24-h truncated exponential kinetic model and the 24-h spot plasma pool. The turn over rate (TR) was calculated as the product of plasma Zn and the initial slope of the disappearance curve after i.v. administration of ^{67}Zn. Zinc isotope ratios in nonextracted plasma samples were measured by inductively coupled plasma mass spectrometry and plasma Zn by atomic absorption spectrometry. EZP ranged from 102-240 mg, TR from 278-504 mg/day and plasma Zn from 640-876 μg/L. Highly significant positive correlations were found between EZP ($r=0.88, p<0.0001$), TR ($r=0.85, p=0.0001$) and the number of days on micronutrients. Plasma Zn was not related to micronutrient intake ($r=0.055$, $p=0.87$). These findings suggest that: 1) micronutrient supplementation mobilized Zn from a sequestered pool such as muscle or bone; 2) factors other than Zn nutriture can affect Zn kinetics. *(Supported by a Department of Defense grant, DAMDB 17-95-C-5112).*

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POLYATOMICS IN ZINC ISOTOPE RATIO ANALYSIS OF PLASMA SAMPLES BY ICP-MS AND APPLICABILITY OF NONEXTRACTED SAMPLES FOR ZINC KINETICS

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Inductively coupled plasma-mass spectrometry (ICP-MS) is rapidly displacing alternative inorganic MS techniques in nutrition research. The main disadvantage is the presence of polyatomic isobaric interferences at key masses. Isolation of zinc (Zn) from samples by an extraction procedure is recommended to avoid interferences due to polyatomic backgrounds. However, extraction increases the chances of contamination. The major matrix interferences in serum or plasma are Na, Cl^-, and S. There are no apparent major isobaric interferences for ^{66}Zn and ^{67}Zn although $^{32}S^{16}O_2$ and $^{32}S_2$ overlap ^{64}Zn. A series of mineral solutions "simulated human plasma" containing S, Na, Cl, K, P, and Ca were subjected to isotope ratio (IR) measurements by ICP-MS. The mixture of all mineral elements interfered only with ^{64}Zn (6.66 ng/mL) and ^{70}Zn (8.51 ng/mL). Interferences to ^{66}Zn, ^{67}Zn, and ^{68}Zn were minimal containing 0.90, 0.94, and 0.39 ng/mL, respectively. The mixture of S - Cl and Na - Cl reduced the interference with ^{67}Zn. The co-presence of Na or S affected the chemical reaction of Cl in argon plasma, and the major interferent was shifted from $^{35}Cl^{16}O_2$ to $^{35}Cl_2$. The normalized IRs from $^{66/67}Zn$ and $^{67/68}Zn$ in both the "extracted" and "nonextracted" samples agreed well (r^2= 0.976 and r^2=0.985, respectively) compared to those from other ratios (r^2=0.838 for $^{67/64}Zn$ and r^2=0.747 for $^{67/70}Zn$). *(Supported by a Department of Defense grant, DAMDB 17-95-C-5112).*

Appendix

Biological Trace Element Research

Managing Editor M. F. Flores-Arce

The Journal of the International Association of Bioinorganic Scientists

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August 6, 1998

Dear Dr. Ramanujam:

I am pleased to inform you that your manuscript number 98062: *"Polyatomics in Zinc Isotope Ratio Analysis of Plasma Samples by Inductively Coupled Plasma-Mass Spectrometry and Applicability of Nonextracted Samples for Zinc Kinetics,"* has been accepted for publication in Biological Trace Element Research.

You should be receiving proofs from the publisher as soon as they become available. Due to large volumes of material in process, average production times are upwards of eight months. Please remember that our Editorial Office is not involved in the printing process, so in case you have any further questions regarding galley proofs or the expected publication date, contact Ms. Madeleine Landaeta, The Humana Press, Inc., 999 Riverview Drive, Suite 208, Totowa, NJ 07512, phone (201) 256-1699, fax (201) 256-8341.

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POLYATOMICS IN ZINC ISOTOPE RATIO ANALYSIS OF PLASMA SAMPLES BY INDUCTIVELY COUPLED PLASMA - MASS SPECTROMETRY AND APPLICABILITY OF NONEXTRACTED SAMPLES FOR ZINC KINETICS

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Index Entries: Enriched ^{67}Zn isotope; extraction, nonextraction; inductively coupled plasma-mass spectrometry (ICP-MS); isotope ratios (IRs); Zn kinetics; Zn turnover rate (TR); exchangeable Zn pool (EZP)

ABSTRACT

Inductively coupled plasma-mass spectrometry (ICP-MS) is a powerful tool for both quantitative multielement analyses of inorganic elements and measurement of isotope ratios (IRs). The main disadvantage of this technique is the existence of polyatomic isobaric interferences at some key masses. Zinc has been investigated for such potential interferences in serum or plasma. The Zn isotopes, ^{66}Zn and ^{68}Zn, have no apparent interferences but $^{32}S^{16}O_2$ and $^{32}S_2$ are isobaric with ^{64}Zn. The possible effects of S and other major components of blood plasma - Na, K, Cl, P, Ca - on Zn IRs were investigated using a series of mineral solutions which simulated human plasma with respect to these elements. The mixture of all mineral elements interfered only with ^{64}Zn (6.66 ng/mL) and ^{70}Zn (8.51 ng/mL). Interferences to ^{66}Zn, ^{67}Zn, and ^{68}Zn were minimal containing 0.90, 0.94, and 0.39 ng/mL, respectively. The co-presence of Na or S shifted $^{35}Cl^{16}O_2$ (atomic mass 67 coming from Cl solution) to $^{35}Cl_2$ which reduced the contribution to ^{67}Zn. The hypothesis that Zn IRs obtained from plasma at various intervals after the intravenous administration of enriched ^{67}Zn to humans would reflect those obtained after extraction of Zn was therefore tested. To compare the two pretreatment methods, "extraction" versus "nonextraction", specimens were collected from 10 human subjects at intervals of 5 min to 24 hours post injection, and in 4 subjects from 5 min to 9 days post injection. Two separate aliquots of plasma from each time point were dried and digested with hydrogen peroxide, and the residue dissolved in nitric acid. One specimen was subjected to zinc extraction using ammonium diethyldithiocarbamate chelate followed by back extraction into nitric acid. The matching aliquot received no further pretreatment. The normalized IRs obtained from $^{67}Zn/^{66}Zn$ and $^{67}Zn/^{68}Zn$ in both the "extracted" and "nonextracted" samples agreed well (r^2=0.976 and r^2=0.985, respectively) compared to those from other ratios (r^2 = 0.838 for $^{67}Zn/^{64}Zn$ and r^2=0.747 for $^{67}Zn/^{70}Zn$). Considering the minimum possibility of isobaric interferences in plasma samples, $^{67}Zn/^{68}Zn$ obtained from "nonextracted" samples is sufficient for routine Zn kinetic analysis by ICP-MS.

INTRODUCTION

For the assessment of Zn nutriture, determinations of plasma or serum Zn concentrations are used extensively in clinical practice, with normal plasma or serum Zn concentrations generally considered to be 700-1200 μg/L. However, many physiological factors [1] influence plasma zinc. Plasma or serum zinc concentrations vary with stress conditions unassociated with Zn deficiency and the zinc content of accessible tissues does not provide a reliable index of its status. Isotopic techniques seem to provide an answer to this problem.

Radioisotopes of Zn have been used to develop complex mathematical models which describe Zn kinetics under various conditions in man and laboratory animals [2-5]. Such models have been used to identify sites of regulation of Zn metabolism and calculate the size and turnover rate of body Zn pools. A simpler model describing Zn kinetics over a short time period (90 minutes) has been developed using ^{65}Zn in the rat [6]. Using this model it was shown that a rapidly exchanging pool of Zn is responsive to changes in dietary Zn intake, becoming significantly depleted in animals maintained on a Zn deficient diet. ^{65}Zn has a biological half-life of 500 days [7], and hence its applicability in the study of Zn metabolism in animals or humans is limited.

A potential approach to the study of relationships between dietary Zn supply and body status is the measurement of plasma Zn kinetics following an intravenous injection (i.v.) of a nonradioactive stable Zn isotope. Stable isotopes offer an advantage over radioisotopes in that there is no radiation exposure of the subjects. Stable isotopes offer a clear advantage over radioisotopes in that they occur naturally and hence have been used to study various aspects of zinc metabolism in humans [8-12], although only Jackson et al [13, 14] used ^{67}Zn to examine Zn

turnover rates. Several instrumental techniques have been reported for the determination of isotope ratios (IR) of Zn including neutron activation analysis [4, 15-17] and mass spectral methods such as thermal ionization [18], fast atom bombardment [12, 19-21], and inductively coupled plasma [22-24].

Recently, we [25, 26] and others [6, 14, 23] have shown that stable isotopes of Zn can be successfully used to measure Zn turnover rates (TR) and exchangeable Zn pools (EZP) which are responsive to changes in Zn status. The availability of and ability to measure stable isotopes of Zn by mass spectrometry make this a viable technique for Zn metabolic studies. Studies from our laboratory indicate that inductively coupled plasma-mass spectrometry (ICP-MS) provides sensitive and reliable detection of the isotopes of Zn.

The stable isotope kinetics methodology for the determination of Zn status involves: (i) the use of intravenously administered Zn-67 stable isotope tracer, collection of blood at various time points, and (ii) determination of isotope ratios $^{67}Zn/^{64}Zn$, $^{67}Zn/^{66}Zn$, $^{67}Zn/^{68}Zn$ and $^{67}Zn/^{70}Zn$ at each time point using ICP-MS. The data are used to calculate Zn disappearance and turnover rates, and the exchangeable Zn pools after injection. The findings are related to biochemical and physiological indices of functions in order to establish zinc status.

Isolation of Zn from samples by an extraction procedure has usually been performed to obtain accurate results for mass spectrometric analysis [12, 22, 25, 26]. The extraction requires a number of procedural steps, which limits application to large numbers of specimens. Hence potential interferences from non-extracted specimens have been explored. The major matrix elements in human blood plasma or serum samples are sodium (Na, 3130-3370 mg/L), chloride (Cl^-, 2940-4120 mg/L), and sulfur (S, 1120-1270 mg/L) [27]. Theoretically there are no

apparent major isobaric interferences for ^{66}Zn and ^{68}Zn in blood plasma, although $^{32}S^{16}O_2$ and $^{32}S_2$ overlap ^{64}Zn. Hence, we wanted to test this theory by conducting a detailed study of comparing the isotope ratios obtained from several sets of "extracted" and "nonextracted" plasma samples. In this project we compared the four different isotope ratios obtained from Zn-extracted and Zn-nonextracted digested (using hydrogen peroxide) plasma samples in order to determine whether the simple "nonextraction" procedure is applicable for routine Zn isotope ratio analysis for kinetic studies in humans.

EXPERIMENTAL

Chemicals, Reagents and Supplies

The enriched stable isotope ^{67}Zn (as oxide, purity 93.11%) was purchased from Oak Ridge National Laboratory (Martin Marietta Energy Systems, Inc., Oak Ridge, TN, U.S.A.). Hydrogen peroxide (30%, ultrapure), nitric acid (ultrapure double-distilled from Vycor), ammonium hydroxide (high purity grade), hydrochloric acid (ACS grade) and sulfuric acid (ACS grade) were purchased from GFS Chemicals, OH, U.S.A. Hydrochloric acid (suprapure grade) was obtained from EM Science, Gibbstown, NJ.

Sodium nitrate, diammonium monohydrogenphosphate and calcium carbonate (Baker analyzed chemical grades) were purchased from Baker, Phillipsburg, NJ. Potassium nitrate (ACS grade) was obtained from Fisher Chemicals. Carbon tetrachloride (ACS grade) and 2,6-dinitrophenol, and Zn and yttrium reference standard solutions were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Diethyl ammonium diethyl dithiocarbamate was purchased from Tokyo Casei Co., Tokyo, Japan.

Deionized water was prepared using a Milli-Q System (Millipore Corp., Milford, MA, U.S.A.). Monovette syringes containing lithium heparin (10 U/mL blood) used for blood collections and polypropylene sterile tubes (29 x 114 mm size with red caps) free from Zn contamination used for plasma digestions were purchased from Sarstedt Inc., Newton, NC, U.S.A. Disposable Falcon polypropylene tubes (15 mL capacity) used for preparing the final ICP-MS digestate solutions and absolute ethanol used to dissolve the 2,6-dinitrophenol indicator were purchased from Fisher Scientific Co., Pittsburgh, PA, U.S.A. The carbon tetrachloride extraction of Zn from the digestates were carried out in hydrochloric acid (10%) washed borosilicate glass tubes (Kimax Inc., Toledo, OH, U.S.A.).

Human Subjects and Zinc Kinetics

Five healthy men and one woman living in Galveston, Texas were the subjects for the 9-day observation. Eleven women in apparent good health who were participating in a study of effects of zinc and iron status on brain function participated in a 24-hour observation study. This project was approved by the Institutional Review Board of the University of Texas Medical Branch (UTMB) and written consent was obtained from all subjects. The disappearance rate for ^{67}Zn from blood plasma, turnover rate, and the exchangeable Zn pool sizes were measured using the procedures well established in our laboratory [25, 26, 28-30].

Zinc kinetics were measured using ^{67}Zn (natural abundance 4.11%; enrichment, 93.11%) chloride which was prepared from ^{67}Zn oxide by dissolving 59.52 mg in a few drops of concentrated hydrochloric acid (ACS grade, GFS Chemicals, Columbus, OH), and heating it to dryness on a hot plate. The synthesized chloride was dissolved in saline (12 mL, corresponds

to 0.5 mL = 2 mg of ^{67}Zn), aliquots of 0.5 mL sterilized by passing the solution through Millipore filter (0.2 μM pore size) into glass vials containing 10.0 mL saline. Several of these vials (one per 10 vials) were randomly selected and tested for sterility (Department of Clinical Microbiology and Immunology, UTMB) and pyrogenicity (Scientific Associates Inc., St. Louis, MO).

Subjects were admitted to the General Clinical Research Center at UTMB for administration of ^{67}Zn and collection of blood samples. The diet was limited in bioavailable zinc. At 07:00 A.M., after the subject had fasted at least 12 hours, short Teflon catheters connected to a 3-way stop cock were placed in each antecubital vein and kept open by 0.9% saline solution. After 30 min, a blood sample was taken to establish the baseline $^{67}Zn/^{68}Zn$ ratio.

The ^{67}Zn tracer - 2 mg in 10.5 mL saline further diluted to 30 mL in saline - was then administered over 3 min (timed by stop watch). The line was flushed rapidly with saline for 30 seconds. Blood samples were then collected from the opposite arm starting 5 min after completion of the ^{67}Zn administration. Additional samples were collected at 5, 15, 30, 40, 50, 60, 90 minutes, and 2, 6, 12 hours, and 1, 2, 3, 5, 7, and 9 days later. The 9-day and 1-day samples were collected from 4 and 10 subjects, respectively. Blood samples were placed in an ice chest after collection and delivered to the laboratory for processing. The blood samples were centrifuged at 2000 rpm for 20 minutes and the plasma layers transferred to polypropylene tubes and stored in a freezer (-70°C) until ready to use.

Digestion of Plasma and Extraction of Zinc

Sample digestion was based on the method of Alcock [31]. Duplicate aliquots of plasma were measured out in 50 mL polypropylene tubes, kept overnight at -70°C, transferred to a freeze-drier and lyophilized overnight, further dried for 8 hours at 80°C in an oven, and digested with 30% hydrogen peroxide (2 aliquots of 5 and 7 mL, high purity grade, GFS Chemical Co.) for 2 days at 85-90°C. The white ash was dissolved in 1.5 mL 1.2 M Ultrapure nitric acid. Several (4 tubes/batch) hydrogen peroxide blanks run throughout the entire procedure were used to calculate ICP-MS IR blank subtractions.

"Extraction": The extraction of Zn was based on the method of Serfass et al [22] modified by Yokoi et al [26]. After digestion of the plasma, the ash was dissolved in 1.5 mL of 1.2 M nitric acid and the solution transferred to 20 mL acid-washed borosilicate tube using the Zn-free polyethylene transfer pipette followed by two washes with deionized water. One drop (40 uL) of 0.1% 2,6-dinitrophenol in 50% ethanol was added to the solution as a pH indicator. Dilute ammonium hydroxide was added in drops with shaking the tube to bring the pH to 2.5 (indicated by the color change to yellow). One mL of 0.25% diethyl ammonium diethyl dithiocarbamate in carbon tetrachloride was added, the tube closed tight with Teflon-lined cap, and the contents shaken vigorously for 2 minutes. Each tube was allowed to stand until separation of the acid and carbon tetrachloride layers was complete.

The carbon tetrachloride layer containing chelated zinc was transferred to another glass tube carefully using the acid-washed glass pasteur pipette, the Zn-chelate decomposed with 1 mL of 1.2 M nitric acid, and the Zn back-extracted into the acid by vigorously shaking the tube. The back-extraction of Zn was usually indicated by the transfer of yellow color into the acid layer

followed by its disappearance. If such a transfer did not occur immediately, the solution was allowed to stand for an hour and shaken again to complete the decomposition and transfer steps. The top acid layer was then transferred to another glass tube and the solution heated overnight at 80°C to remove traces of CCl_4, and made up to 10 mL with Milli-Q deionized water after adding yttrium internal standard (100 uL of 5 mg/L solution in 1% nitric acid). Batches of 12-20 tubes were processed at one time. Yttrium was chosen as the internal standard since it has only one natural isotope, 89 amu, which gives abundant ICP-MS signals and also closer to Zn isotopic masses.

"Nonextraction": After digestion of the plasma, the ash was dissolved in 1.5 mL of 1.2 M nitric acid and each solution transferred to a polypropylene falcon tube followed by two washes with Milli-Q deionized water. The yttrium internal standard and Milli-Q water were directly added to the digestate and made up to 10 mL with water.

Solutions for the Measurement of ICP-MS Interferents

Because the preparation of human plasma for isotope ratio ICP-MS analysis involves digestion by hydrogen peroxide and solubilization of the obtained white ash with nitric acid, the main added matrix elements in the well digested solution should only be hydrogen, oxygen and nitrogen. The polyatomics that interfere with zinc isotopes (64, 66, 67, 68 and 70 atomic mass units) are limited to the combination of this matrix (H, O and N), the plasma minerals (Na, Cl, S, K, P and Ca) and argon (Ar, used as a source for the inductively coupled plasma). Therefore, in order to investigate possible interferences, solutions were prepared which contained only hydrogen, oxygen and nitrogen except for the mineral elements cited.

Single mineral solutions that contained the respective mineral found in the "actual human plasma" were prepared by dissolving each salt or diluting each acid in Milli-Q water. Nitric acid (1.2 M) was prepared by diluting the concentrated nitric acid using Milli-Q water. Calcium carbonate (1250 mg) was dissolved by a few drops of nitric acid and the excess acid was evaporated by heating on a hot plate to obtain calcium nitrate. To start with, 3600 μg Na/mL as sodium nitrate, 3300 μg Cl/mL as hydrochloric acid, 1200 μg S/mL as sulfuric acid, 189μgK/mL as potassium nitrate, 141 μg P/mL as diammonium monohydrogen phosphate, and 99 μg Ca/mL as calcium nitrate were prepared. Finally, solutions containing one tenth of each mineral concentration that is usually found in the representative human plasma were prepared in 0.12 M nitric acid with 50 ng Y/mL as an internal standard. Single mineral solutions contained only one mineral element - Na, Cl, S, K, P, or Ca. Solutions which contained two different mineral elements were also prepared. The mixture of all minerals contained all the 6 mineral elements (Na, Cl, S, K, P and Ca).

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

A VG PlasmaQuad-1, upgraded to PlasmaQuad-2 plus status (VG Instruments, Winsford, England, U.K.) ICP-MS instrument was used for all isotope ratio measurements. Each solution was aspirated and nebulized (Meinhardt concentric type) into the argon plasma (8000-6000^{o} K) via a peristaltic pump with a flow rate of approximately 1 mL/min. The yttrium (mass 89) internal standard was used to correct errors due to instrumental drifts during data acquisitions. Isotope ratio analyses were performed using "Peak-Jump Acquire" IR data acquisition mode of the VG PlasmaQuad software. The peak-jump acquisition mode gave better

relative standard deviations (RSD 0.2 - 0.3%) compared to the scan acquisition mode (2-4%) and peak-jump mode is the highly recommended mode for IR measurements. The mass range scanned was 50-95 amu with 200 scan sweeps of 2048 channels, 160 µsec dwell time per channel, and 200 peak jump sweeps with 10240 µsec per peak jump sweep. These mass spectral acquisition parameters normally require about 9 mL of solution and 20 min acquisition time for ten replicate measurements of each sample. Instrument control, methods procedures and the data processing system, including calculations and statistics, were operated via a Compaq AT personal computer with version 3.2 of the VG PlasmaQuad software. All the four Zn isotope ratios ($^{67}Zn/^{64}Zn$, $^{67}Zn/^{66}Zn$, $^{67}Zn/^{68}Zn$, $^{67}Zn/^{70}Zn$) were measured in each sample. The mass discrimination among Zn isotopes was corrected by the frequent measurements of Zn standard solutions (125, 250, and 500 ng/mL) during the sequence of IR analysis.

Measurement of Polyatomic Interferents in the "Simulated Human Plasma" Solutions

After careful cleaning of the sampling/skimmer cones, torch, nebulizer, and the spray chamber, the various "simulated human plasma" mineral solutions prepared to quantify the ICP-MS interferents were introduced into the argon plasma and the counts at the desired atomic mass units (64, 66, 67, 68, 70 and 89) were recorded using "Peak-Jump Acquire" Isotope Ratio data acquisition mode as described earlier. The counts obtained at the desired atomic mass units were compared with the counts obtained from 250 ng Zn/mL and the equivalent concentrations of the interferents were calculated.

Calculations

Subtraction of the hydrogen peroxide mass spectral signal counts from each sample counts gave the blank-subtracted counts. Using the blank-subtracted signal counts, the four $^{67}Zn/^{64}Zn$, $^{67}Zn/^{66}Zn$, $^{67}Zn/^{68}Zn$, $^{67}Zn/^{70}Zn$ IR values were recalculated for each sample. The value obtained after subtraction of the baseline (zero time) IR from each IR value was divided by the natural Zn IR value to obtain the normalized IR (NIR) value. A data set of 4 normalized isotope ratios (67/64, 67/66, 67/68 and 67/70) x 163 time points x 2 treatments (extraction and nonextraction) obtained from 14 subjects after iv dose of ^{67}Zn was subjected to statistical analysis. All statistical analyses were carried out using the SYSTAT5 (version 5.2.1) Macintosh software (SYSTAT Inc., Evanston, IL).

RESULTS AND DISCUSSION

Inductively coupled plasma-mass spectrometry has become a powerful alternative for the determination of isotope ratio measurements along with other well established techniques such as neutron activation analysis and thermal ionization and fast atom bombardment mass spectrometry. However, when biological material is analyzed by ICP-MS, potential interferences from polyatomic ions must be considered. These interfering polyatomic ions originate mainly from argon, nitrogen, and/or oxygen in combination with Na, S, Cl, and Ca, which are present at approximate concentration ranges of 3130-3370, 1120-1270, 2940-4120, and 92-109 mg/L in human serum, respectively [27]. Zinc has five isotopes: 64, 66, 67, 68, and 70. The most abundant isotope, ^{64}Zn (48.9%), is interfered to a large extent by polyatomic ions containing sulfur, oxygen, and calcium.

Polyatomic Interferences During the Isotope Ratio Measurements of the "Simulated Human Plasma" Mineral Solutions

In order to accurately calculate the actual polyatomic background signals generated during the ICP-MS analysis of the digested plasma solutions, the various "simulated human plasma" mineral solutions were subjected to the IR analyses of the routine ICP-MS instrumental conditions. Table 1 shows the equivalent contributions of the interferents in the mineral solutions to naturally occurring Zn. Investigations of the mineral solutions were limited to the single mineral elements, and the mixture of two mineral elements and all mineral elements. The investigation of the interaction among three mineral elements or more were omitted because of the statistical difficulties. The mixture of all minerals was tested because it was the closest to the digested human plasma. Tenfold diluted solutions (in 0.12 M nitric acid) were chosen because we utilized similar dilutions in the on-going Zn nutritional study.

The tenfold dilution of the digested human plasma contains approximately 100ng Zn/mL. A careful study of the results from single element solutions in Table 1 indicates that the polyatomic interferences to ^{64}Zn by sulfur (3.61 ng/mL) and to ^{67}Zn by chlorine (2.58 ng/mL) alone are significant . On the other hand, the mixture of all mineral elements (S, Na, Cl, K, P and Ca) which is approximately equivalent to the digested human plasma, largely interfered only with ^{64}Zn (6.66 ng/mL) and ^{70}Zn (8.51 ng/mL). However, the interferences to ^{66}Zn, ^{67}Zn, and ^{68}Zn are minimal - 0.90, 0.94, and 0.39 ng/mL, respectively.

It is obvious from these results that interactions among mineral elements evoked the shift of the interferents from 67 to 70 of the atomic mass units. The mixture of Na - Cl, S - Cl, Cl - K

and Na - P evoked the interference with ^{70}Zn. The mixture of S - Cl, Na - Cl reduced the interference with ^{67}Zn. These results suggest that co-presence of Na or S affects the chemical reaction of Cl in argon plasma, and the major interferent is shifted from $^{35}Cl^{16}O_2$ (atomic mass 67 coming from Cl solution) to $^{35}Cl_2$. Table 2 summarizes the possible polyatomic species generated by the introduction of single and various combinations of mineral solutions to the inductively coupled argon plasma.

Comparison of Isotope Ratio Results from Human Plasma Samples - "Extraction" versus "Nonextraction"

Isotope ratios were compared from Zn "extracted" and "nonextracted" human plasma specimens. Table 3 lists the range of normalized isotope ratios (NIRs) for the four isotope ratios chosen. When Zn is extracted from a sample, Zn isotopes 64, 66 and 68 can be used as a denominator isotope to calculate the normalized isotope ratio. However, the low abundance and counts of ^{70}Zn does not allow an accurate measurement of normalized isotope ratio even after the extraction of Zn. As expected, all the NIR values were found to be the lowest after 9 days of intravenous administration of ^{67}Zn and highest at 5 minutes after injection. Negative values are irrational because all NIRs were obtained only after the administration of ^{67}Zn. The frequency of negative values for NIR was found to be very low; 2 out of 163 values (1.2%) for NIR-A $^{67}Zn/^{70}Zn$ and 7 out of 163 values (4.3%) for NIR-B $^{67}Zn/^{70}Zn$. Negative values were not observed for NIRs obtained from $^{67}Zn/^{66}Zn$ and $^{67}Zn/^{68}Zn$.

Figure 1 shows correlation plots of normalized isotope ratios of $^{67}Zn/^{68}Zn$ versus $^{67}Zn/^{66}Zn$ for extracted (A, $r^2 = 0.998$) and nonextracted (B, $r^2 = 0.992$) plasma samples. Only

at very low NIR values, the data points tend to deviate from linearity for the nonextracted samples. The value of $r^2 = 0.992$ obtained for nonextracted samples is very close to $r^2 = 0.998$ for extracted samples and acceptable for kinetics. Table 4 summarizes the correlations (r^2) between different NIRs obtained from extracted samples only using simple linear regression and double logarithmic (power function fitting) plots. As expected, the correlations are high for all the four isotopes, 64, 66, 67 and 68, due to the removal of the interfering polyatomic background ions during the extraction of Zn. Figure 2 shows the correlations of normalized isotope ratios for $^{67}Zn/^{68}Zn$ (A, $r^2 = 0.987$) and $^{67}Zn/^{66}Zn$ (B, $r^2 = 0.976$) for extracted versus nonextracted plasma samples.

Table 5 compares the normalized isotope ratios obtained from both the extracted (NIR_A) and nonextracted (NIR_B) samples using simple linear regression and double logarithmic (power function fitting) plots. NIR_B calculated from 67/68 and 67/66 agrees very well with NIR_A. As expected from the results of the detailed investigation of polyatomic interferences for ^{64}Zn and ^{70}Zn (Tables 1 and 2), the agreement between NIR_A and NIR_B calculated from 67/64 and 67/70 was poor. For $^{67}Zn/^{64}Zn$, $r^2 = 0.838$ and for $^{67}Zn/^{70}Zn$, $r^2 = 0.747$ (see Table 5). Due to sulfur and oxygen polyatomic backgrounds at ^{64}Zn mass (mostly $^{32}S^{16}O_2$ and $^{32}S_2$) and shifting of the major interferent $^{35}Cl^{16}O_2$(atomic mass 67 coming from Cl in the solution) to $^{35}Cl_2$ (atomic mass 70), in combination with very low natural abundance for ^{70}Zn, respectively, account for the poor agreement.

Ideally extraction of zinc as a purification step appears desirable. The Zn extraction procedure, however, involves many steps, a deterrent for large numbers of samples since some steps are susceptible to contamination of Zinc from the environment. In summary, the regression

analyses values (r^2, the slope "a", and the intercept "b") for NIR correlations from both the "extraction" and "nonextraction" methods show high correlations for $^{67}Zn/^{68}Zn$ and $^{67}Zn/^{66}Zn$. Such high correlations for "nonextracted" samples can be routinely achieved by: (a) keeping the resolution of the mass spectrometer less than unity peak width (0.8 - 0.9 amu), (b) cleaning the skimmer and sampling cones, torch, and the nebulizer prior to analysis of each batch of samples, and (c) passing nitric acid (1%) followed by Milli-Q water between the samples until the ^{89}Y(internal standard) signal reaches below 200 counts (the rate-meter reading approximately 2 at 1 KHz setting).

Considering the possibility of isobaric interferences generated during the ionization processes of the digested plasma samples inside the inductively coupled plasma of the ICP-MS coupled with this detailed investigation indicate that $^{67}Zn/^{68}Zn$ and $^{67}Zn/^{66}Zn$ NIRs with least possibility of polyatomic backgrounds obtained from "nonextracted" samples are sufficient for routine Zn kinetic analysis using ^{67}Zn enriched isotope. It should be pointed out that since the polyatomic backgrounds at atomic mass 67 are shifted to atomic mass 70, the "nonextraction" procedure may not be suitable for Zn kinetic analysis using ^{70}Zn enriched stable isotope.

ACKNOWLEDGEMENTS

We acknowledge the Department of the Army (DAMD 17-95-C-5112) for financial support for the project. The administration of ^{67}Zn stable isotope to subjects and collection of blood samples were conducted at the General Clinical Research Center, University of Texas Medical Branch at Galveston, funded by grant MO1 RR-00073 from the National Center for Research Resources, NIH, USPHS.

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Table 1. The equivalent concentrations (ng/mL) of the interferents in the mineral solutions to naturally occuring zinc.

	Mass number				
	64	66	67	68	70
Single mineral					
S	3.61	0.38	0.01	0.07	-1.45
Na	0.59	0.48	0.28	0.49	0.60
Cl	0.25	0.25	2.58	0.30	1.59
K	-0.08	-0.09	-0.15	-0.10	-0.47
P	-0.09	-0.11	-0.14	-0.11	-0.63
Ca	-0.08	-0.09	-0.15	-0.09	-0.58
Mixture of two minerals					
S - Na	5.53	0.55	0.22	0.10	0.00
S - Cl	5.08	0.65	0.94	0.29	6.82
S - K	4.19	0.17	-0.13	-0.19	-0.62
S - P	3.96	0.32	-0.02	-0.03	-0.58
S - Ca	3.45	0.13	-0.05	-0.15	-0.80
Na - Cl	1.02	0.63	1.00	0.65	13.35
Na - K	0.50	0.27	0.40	0.29	1.27
Na - P	0.74	0.38	0.67	0.40	2.15
Na - Ca	0.45	0.31	0.48	0.33	0.83
Cl - K	0.17	0.16	2.78	0.21	3.10
Cl - P	0.22	0.22	2.65	0.25	1.34
Cl - Ca	0.15	0.15	1.94	0.17	1.14
K - P	-0.08	-0.09	0.08	-0.08	-0.23
K - Ca	0.00	-0.01	0.11	0.00	-0.23
P - Ca	0.07	0.06	0.32	0.08	-0.23
Mixture of all minerals	6.66	0.90	0.94	0.39	8.51
Sum of the single minerals	4.19	0.82	2.43	0.57	-0.95

Each solution contains one tenth of the mineral concentration(s) found in the representative human plasma.
S: 120 µg/mL S as H_2SO_4. Na: 330 µg/mL Na as $NaNO_3$. Cl: 360 µg/mL as HCl. K: 18.9 µg/mL K as KNO_3. P: 14.1 µg/mL as $(NH_4)_2HPO_4$. Ca: 9.9µg/mL Ca as $Ca(NO_3)_2$.
All mineral solutions were prepared in 0.12 M HNO_3.

Table 2. Possible polyatomics generated by the introducion of the mineral solutions to inductively coupled argon plasma.

	Mass number				
	64	66	67	68	70
Single mineral					
S	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
Na	-	-	-	-	-
Cl	-	-	$^{35}Cl^{16}O_2$	-	-
K	-	-	-	-	-
P	-	-	-	-	-
Ca	-	-	-	-	-
Mixture of two minerals					
S - Na	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - Cl	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	$^{35}Cl_2$
S - K	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - P	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - Ca	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
Na - Cl	-	-	-	-	$^{35}Cl_2$
Na - K	-	-	-	-	-
Na - P	-	-	-	-	$^{23}Na^{31}P^{16}O$
Na - Ca	-	-	-	-	-
Cl - K	-	-	$^{35}Cl^{16}O_2$	-	$^{35}Cl_2$
Cl - P	-	-	$^{35}Cl^{16}O_2$	-	-
Cl - Ca	-	-	-	-	-
K - P	-	-	-	-	-
K - Ca	-	-	-	-	-
P - Ca	-	-	-	-	-
Mixture of all minerals	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	$^{35}Cl_2$
Sum of the single minerals	$^{32}S^{16}O_2$, $^{32}S_2$	-	$^{35}Cl^{16}O_2$	-	-

Each solution contained one tenth of the mineral concentration(s) found in the representative human plasma.
S: 120 µg/mL S as H_2SO_4. Na: 330 µg/mL Na as $NaNO_3$. Cl: 360 µg/mL as HCl. K: 18.9 µg/mL K as KNO_3. P: 14.1 µg/mL as $(NH_4)_2HPO_4$. Ca: 9.9 µg/mL Ca as $Ca(NO_3)_2$.

Table 3. The range of Normalized Isotope Ratios (NIRs)

	"Extracted" Samples			
	67/64	**67/66**	**67/68**	**67/70**
Minimum	0.04	0.06	0.06	-0.43
Median	0.78	0.75	0.72	0.68
Maximum	14.33	13.67	12.91	12.89
	"Nonextracted" Samples			
	67/64	**67/66**	**67/68**	**67/70**
Minimum	-0.160	0.05	0.06	0.05
Median	0.53	0.71	0.75	0.69
Maximum	12.42	13.23	12.69	12.15

Minimum NIR was found 9 days after i.v. dose of ^{67}Zn.
Maximum NIR was found 5 minutes after i.v. ^{67}Zn administration.
Negative values are irrational because all NIRs were obtained after administration of ^{67}Zn.

Table 4. Correlations (r^2) between different NIRs obtained from extracted samples using simple linear regression and double logarithmic (power function fitting) plots

	Simple Linear Regression Plot		
	67/66	**67/68**	**67/70**
67/64	0.996	0.994	0.887
67/66		0.999	0.889
67/68	-		0.802
	Double Logarithmic Plot		
	67/66	**67/68**	**67/70***
67/64	0.996	0.991	0.786
67/66		0.998	0.794
67/68			0.893

*Negative values were removed for calculation.

Table 5. Comparison of normalized Zn isotope ratios (NIRs) obtained from extracted (A batch, NIR_A) and nonextracted (B batch, NIR_B) samples using simple linear regression and power function fitting (double logarithmic) plots

	Simple Linear Plot		
NIR	**r2**	**a**	**b**
67/64	0.838	0.059	0.175
67/66	0.983	0.985	0.040
67/68	0.985	0.964	0.035
67/70	0.747	0.907	0.132

	Double Logarithmic Plot		
NIR	**r2**	**a**	**b**
67/64*	0.838	1.237	0.773
67/66	0.976	1.023	0.958
67/68	0.985	0.987	1.001
67/70*	0.747	0.966	0.903

Regression equation for the simple linear equation is: NIR_A = a NIR_B + b, where 'a' and 'b' are the slope and the intercept, respectively. For perfect correlations, 'a' should be equal to 1 and and 'b' should be zero.

Regression equation for the double logarithmic plot is: NIR_A = a NIR_B power b. If both the values completely agree, then 'a' and 'b' should each be equal to1.

*Negative values were removed because they do not allow fitting.

Legends for Figures

Figure 1. Correlation plots of normalized isotope ratios of $^{67}Zn/^{68}Zn$ versus $^{67}Zn/^{66}Zn$ for extracted (A) and nonextracted (B) plasma samples.

Figure 2. Correlation plots of normalized isotope ratios for $^{67}Zn/^{68}Zn$ (A) and $^{67}Zn/^{66}Zn$ (B) for extracted versus nonextracted plasma samples.

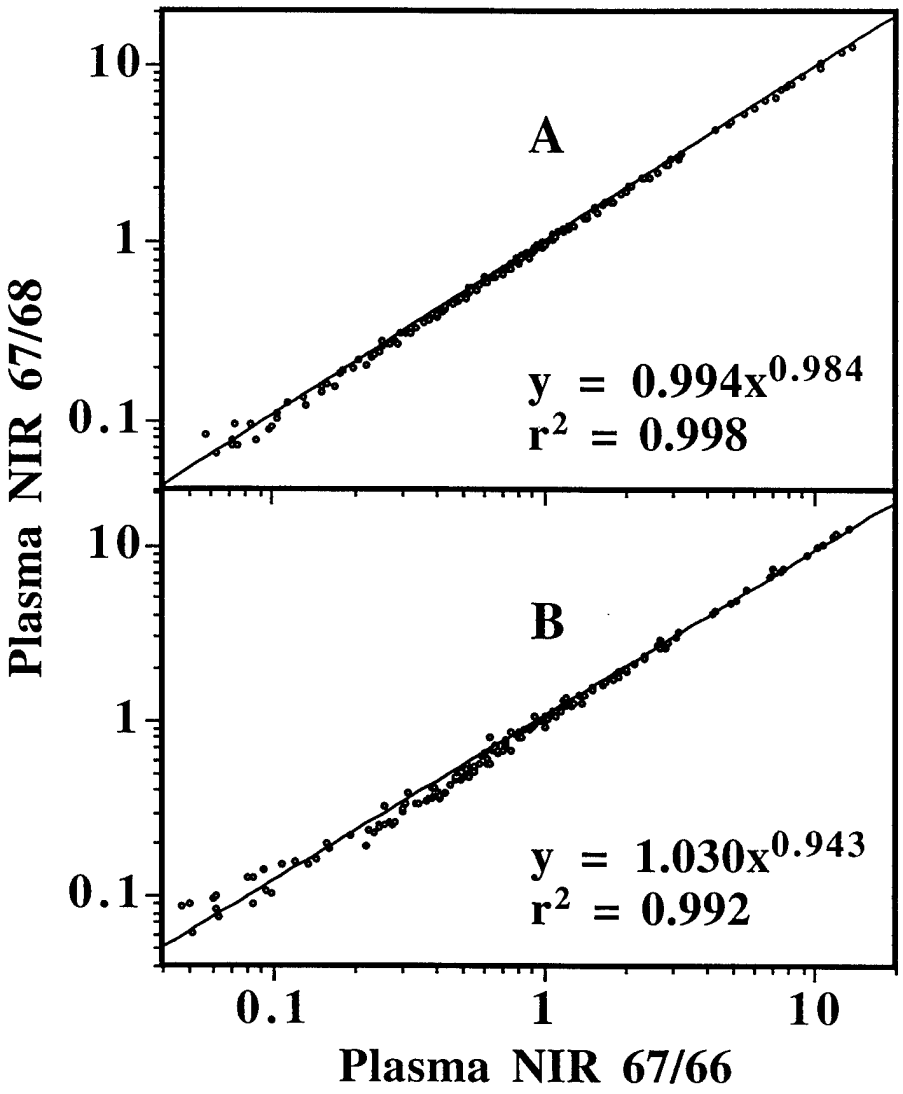
A
y = 0.994x^0.984
r^2 = 0.998
B
y = 1.030x^0.943
r^2 = 0.992
Plasma NIR 67/68
Plasma NIR 67/66
10
1
0.1
0.1
1
10

Figure 1

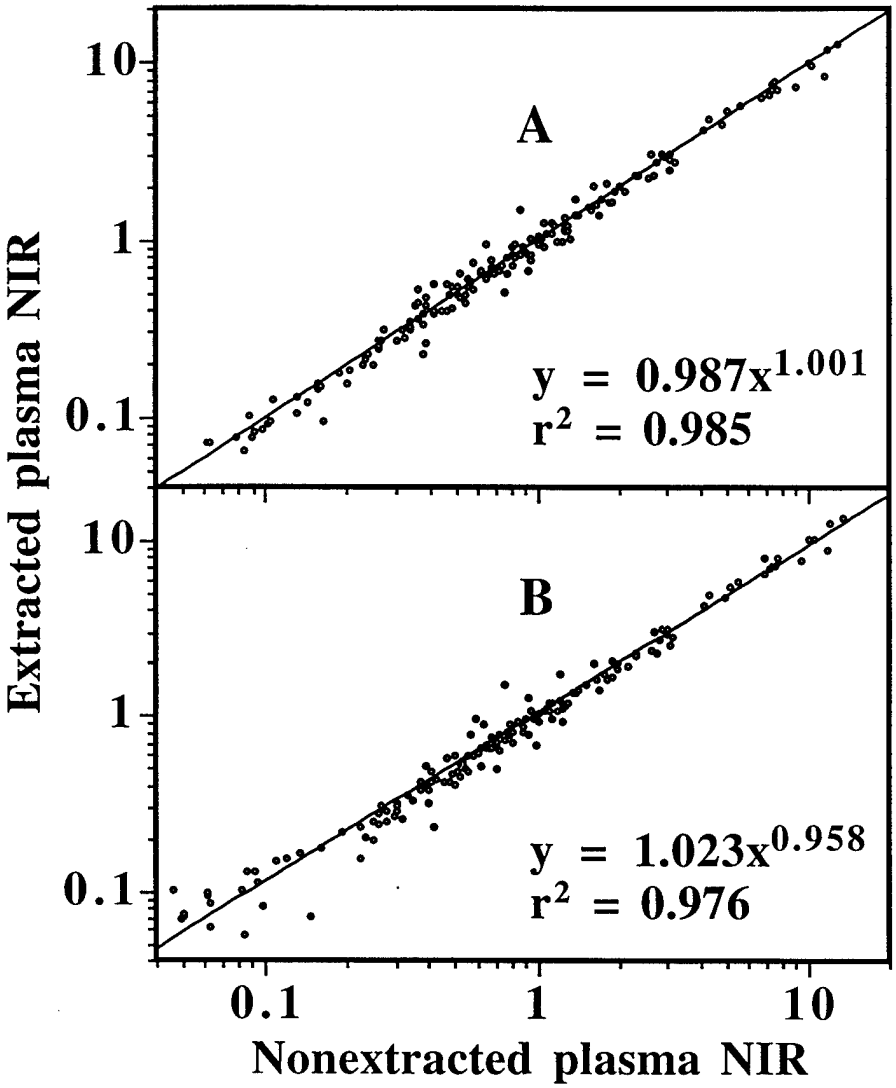
A
B
y = 0.987x^1.001
r^2 = 0.985
y = 1.023x^0.958
r^2 = 0.976
Extracted plasma NIR
Nonextracted plasma NIR
10
1
0.1
0.1
1
10

Figure 2

The University of Texas Medical Branch at Galveston

Division of Human Nutrition,
Department of Preventive Medicine and Community Health

January 15, 1998

Commander
US Army Research Institute of Environmental Medicine
Attn: MCMR-UE-RP/ Ms. Marie E. Stevens
Natick, MA 01760-5007

Subject: Contract No. DAMD 17-95-C-5112 Quarterly Report

Dear Ms. Stevens,

Attached is the Quarterly Report for September 22, 1997 to December 22, 1997 for Contract No. DAMD 17-95-C-5112. If you have questions or need further information, please contact me at 409 772-4661.

Thank you,

Harold H. Sandstead, MD
Professor

Attachments:

Experimental Biology '98 Abstract Forms on: N.E. Egger, K. Yokoi, H. Sandstead

Letters to: Dr. J.E. Pessin, Prof. Harry L. Pardue

Manuscript:

Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics in Humans

Department of Preventive Medicine and Community Health
Division of Human Nutrition
The University of Texas Medical Branch, 700 Harborside Dr.
Galveston, Texas 77555-1109
Phone: 409-772-4661 FAX: 409-772-6287

Principal Investigator: Sandstead, Harold H.
Contract No. DAMD17-95-C-5112

QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 1/15/98

3. Reporting Period from: 9/23/97 to 12/22/97

4. PI: Harold H. Sandstead 5. Telephone No. 409) 772-4661

6. Institution: The University of Texas Medical Branch

7. Project Title: Repletion of Zinc and Iron deficiencies improves cognition of premenopausal women.

8. Current Staff, with percent effort of each on project:

Harold H. Sandstead	15%	Nancy W. Alcock	10%
VM Sadagopa Ramanujam	25%	Hari H. Dayal	10 %
Norman G. Egger	90%	Michael Loftus	100 %
Jackie Curtis	75%		

9. Contract expenditures to date (as applicable):

	This Otr/Cumulative		This Otr/Cumulative
Personnel:	35,488/276,409	Travel:	0/4,299
Fringe Benefits:	8,260/62,070	Equipment:	760/2,443
Supplies:	8,906/68,583	Other:	0/0

This Otr/Cumulative

Subtotal: 54,174/413,804

Indirect Costs: 33,645/205,300

Fee: 0/0

TOTAL: 87,819/585,459

619,104

10. Administrative and logistical matters.

a) During this quarter, 51 women contacted us by phone and expressed their interest (467 since the start of the study); 34 respondents underwent detailed screening to determine eligibility (184 since the start of the study); 15 individuals completed assessment of zinc status by measurement of zinc concentrations in hair and in fasting plasma, platelets granulocytes and lymphocytes (since beginning 70 subjects have completed this phase of the study); 18 subjects were enrolled into the repletion trial (55 since the start of the study); 15 subjects completed the first phase of the repletion trial and started the second phase (37 since the start of the study); and 11 subjects completed the repletion trial (26 since the start of the study).

b) Enrollment was stable during most of this quarter except for the holiday season when enrollment was somewhat less. We continue to advertise the study in newspapers, at local health clubs, and local and regional Universities.

c) An error by the supplier of the nutritional supplements resulted in 7 subjects being given the wrong supplement during the interval before they started the repletion trial. These individuals were dropped from the study. The same error resulted in one subject being given the wrong supplement during the initial phase of the repletion trial. This subject was dropped from the study.

11. Scientific progress:

a) Experiments were done using ultrafiltration to develop an improved method for preparation of plasma samples for measurement of zinc kinetics. Zinc in plasma is bound to albumin, certain amino acids and to an alpha-2-macroglobulin. The latter zinc is not freely available for exchange with other zinc pools. Thus it dilutes the exchangeable plasma zinc and causes error in measurement of the true exchangeable zinc pool size. This error can be avoided by separation of the exchangeable zinc from the macroglobulin bound zinc. Plasma from 5 men and 5 women (subjects) that was collected at intervals from 5 minutes to 24 hours after intravenous injection of 2 mg ^{67}Zn. Zinc in the plasma sample was chelated with ethylene diamine tetra acetate (EDTA) to form Zn-EDTA. Zn-EDTA was isolated from the plasma by ultra-filtration. The ultrafiltrates were aspirated directly into the Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) for measurement of the isotope ratios (IR). After subtraction of baseline counts, the Zn IR was divided by the natural Zn IR to yield the normalized IR (NIR). The ratio of NIR for total Zn to chelated Zn was 0.883 to 0.975 (mean 0.937) and 0.877 to 1.159 (mean 1.023) at 5 min and 24 hours after intravenous administration of ^{67}Zn, respectively. These findings indicate that chelation of zinc with EDTA and separation by ultrafiltration is a potentially useful method for preparation of plasma for measurement by ICP-MS and determination of the exchangeable zinc pool.

b) Two research articles were submitted to journals for publication (see addendum):

"Simplified Pretreatment Method for the Analysis of Plasma Samples Applicable to Zinc Kinetics and Inductively Coupled Plasma-Mass Spectrometry". By V.M. Sadagopa Ramanujam, K. Yokoi, N.G. Egger, H.H. Dayal, N.W. Alcock, and H.H. Sandstead. Submitted to Analytica Chimical ACTA Journal.

"Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics in Humans". By K. Yokoi, N.G. Egger, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead. Submitted to the American Journal of Physiology.

c) Three abstracts were submitted for presentations at the Experimental Biology '98, American Society for Nutrition Sciences meeting in San Francisco, California, April 18-22, 1998 (see addendum):
:

The Intake of Micronutrients Influences Zinc Kinetic Parameters. N. Egger, K. Yokoi, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead.

Polyatomics in Zinc Isotope Ratio Analysis of Plasma Samples by ICP-MS and Applicability of Nonextracted Samples for Zinc Kinetics. V.M. Sadagopa Ramanujam, K. Yokoi, N. Egger, H.H. Dayal, N.W. Alcock, H.H. Sandstead.

Measurement of Plasma Chelatable Zinc for Zinc Kinetic Studies in Humans. K. Yokoi, V.M. Sadagopa Ramanujam, N.W. Alcock, N. Egger, H.H. Dayal, H.H. Sandstead.

EXPERIMENTAL BIOLOGY '98

ABSTRACT FORM

DO NOT FOLD

ABSTRACT AND $30 PROCESSING FEE* MUST BE RECEIVED IN SOCIETY OFFICE BY MONDAY DECEMBER 1, 1997

SEE OVER FOR COMPLETE INSTRUCTIONS

SEE PAGE 27 FOR ELECTRONIC SUBMISSIONS

MEASUREMENT OF PLASMA CHELATABLE ZINC FOR ZINC KINETIC STUDIES IN HUMANS

K. Yokoi, V.M. Sadagopa Ramanujam, N.W. Alcock, N. Egger, H.H. Dayal, H.H. Sandstead. Univ. of Texas Med. Br., Galveston, TX 77555-1109 and Jichi Med School, Tochigi, Japan

Kinetic parameters are more informative than plasma Zn for assessment of zinc (Zn) status. However, all fractions of plasma Zn are assumed to be completely equilibrated in kinetic analyses. We compared isotope ratios (IR) in total (Tot) and chelatable (Chel) Zn in plasma measured by inductively coupled plasma-mass spectrometry (ICP-MS). Samples were collected from 5 men and 5 women at 5 min to 24 h after 2 mg ^{67}Zn iv as ZnCl. The Chel Zn was obtained using EDTA. The ultrafiltrate containing Chel Zn was directly measured by ICP-MS. Background counts in the reagent blank were lower for Chel Zn than for Tot Zn. After subtraction of baseline, the Zn IR was divided by the natural Zn IR to yield the normalized IR (NIR). The ratio of NIR for Tot Zn to Chel Zn was 0.883 to 0.975 (mean 0.937) and 0.877 to 1.159 (mean 1.023) at 5 min and at 1 day after iv ^{67}Zn, respectively. ^{67}Zn was spiked in vitro to plasma obtained from one subject. The ratio of NIR for Tot Zn to Chel Zn was 0.905 in spiked plasma and 0.919 in plasma from the same subject 5 min after iv ^{67}Zn. These results suggest that: 1) plasma Zn fractions are not in complete equilibrium; 2) measuring Chel Zn may be more suitable for Zn kinetic analysis since Chel Zn is chemically specified and iv ^{67}Zn is chelatable. *(Supported by a DOD grant, DAMDB 17-95-C-5112).*

Blue lines are printer's cut lines; do not type on or outside of these lines.

MAILING ADDRESS OF FIRST AUTHOR
(Please print in black ink or type. Provide full

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STUDENT AWARDS

Check below the appropriate society for student award consideration. Abstracts will not be considered if submitted electronically. See instructions and application forms on pages 44-53.

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AAI _____

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Number Title
1. 5065-5 Trace Minerals
2. _____

PRESENTATION PREFERENCE

☐ Oral ☑ Poster ☐ Indifferent

AAI TOPIC CATEGORY

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Are you a Graduate Student?
Yes ☐ No ☑

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Submission of signed form indicates acceptance and compliance with "Rules for Submission of Abstracts." (see page 26)

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Harold A Sandstead
Member's Signature
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POLYATOMICS IN ZINC ISOTOPE RATIO ANALYSIS OF PLASMA SAMPLES BY ICP-MS AND APPLICABILITY OF NONEXTRACTED SAMPLES FOR ZINC KINETICS

V. M. Sadagopa Ramanujam, K. Yokoi, N. G. Egger, H. H. Dayal, N. W. Alcock and H. H. Sandstead. Univ. of Texas Med. Br. Galveston, TX 77555-1109 and Jichi Med. School, Tochigi-Ken 329-04, Japan.

Inductively coupled plasma-mass spectrometry (ICP-MS) is rapidly displacing alternative inorganic MS techniques in nutrition research. The main disadvantage is the presence of polyatomic isobaric interferences at key masses. Isolation of zinc (Zn) from samples by an extraction procedure is recommended to avoid interferences due to polyatomic backgrounds. However, extraction increases the chances of contamination. The major matrix interferences in serum or plasma are Na, Cl^-, and S. There are no apparent major isobaric interferences for ^{66}Zn and ^{67}Zn although $^{32}S^{16}O_2$ and $^{32}S_2$ overlap ^{64}Zn. A series of mineral solutions "simulated human plasma" containing S, Na, Cl, K, P, and Ca were subjected to isotope ratio (IR) measurements by ICP-MS. The mixture of all mineral elements interfered only with ^{64}Zn (6.66 ng/mL) and ^{70}Zn (8.51 ng/mL). Interferences to ^{66}Zn, ^{67}Zn, and ^{68}Zn were minimal containing 0.90, 0.94, and 0.39 ng/mL, respectively. The mixture of S - Cl and Na - Cl reduced the interference with ^{67}Zn. The co-presence of Na or S affected the chemical reaction of Cl in argon plasma, and the major interferent was shifted from $^{35}Cl^{16}O_2$ to $^{35}Cl_2$. The normalized IRs from $^{66/67}Zn$ and $^{67/68}Zn$ in both the "extracted" and "nonextracted" samples agreed well (r^2= 0.976 and r^2=0.985, respectively) compared to those from other ratios (r^2=0.838 for $^{67/64}Zn$ and r^2=0.747 for $^{67/70}Zn$). *(Supported by a Department of Defense grant, DAMDB 17-95-C-5112).*

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THE INTAKE OF MICRONUTRIENTS INFLUENCES ZINC KINETIC PARAMETERS. N. Egger, K. Yokoi, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead. Univ. of Texas Med. Br., Galveston, TX 77555-1109 and Jichi Med School, Tochigi-Ken 329-04, Japan

The readily exchangeable zinc pool (EZP) is responsible for the physiological functions of zinc (Zn). Studies suggested a positive relation between dietary Zn intake and EZP. To explore further relationships we studied 12 healthy females (age ranged from 24-39 years, body mass index from 19-35 kg/m^2). No substantial changes to the diets were made and one tablet, containing micronutrients without Zn or iron, was administered daily for 5-22 days. EZP was measured from the 24-h truncated exponential kinetic model and the 24-h spot plasma pool. The turn over rate (TR) was calculated as the product of plasma Zn and the initial slope of the disappearance curve after i.v. administration of ^{67}Zn. Zinc isotope ratios in nonextracted plasma samples were measured by inductively coupled plasma mass spectrometry and plasma Zn by atomic absorption spectrometry. EZP ranged from 102-240 mg, TR from 278-504 mg/day and plasma Zn from 640-876 µg/L. Highly significant positive correlations were found between EZP ($r=0.88, p<0.0001$), TR ($r=0.85, p=0.0001$) and the number of days on micronutrients. Plasma Zn was not related to micronutrient intake ($r=0.055$, $p=0.87$). These findings suggest that: 1) micronutrient supplementation mobilized Zn from a sequestered pool such as muscle or bone; 2) factors other than Zn nutriture can affect Zn kinetics. *(Supported by a Department of Defense grant, DAMDB 17-95-C-5112).*

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The University of Texas Medical Branch at Galveston

Division of Human Nutrition,
Department of Preventive Medicine and Community Health

December 30, 1997

Dr. J.E. Pessin, Editor
American Physiological Society,
AJP: Endocrinology and Metabolism
9650 Rockville Pike
Bethesda, Maryland 20814-3391

Reference Manuscript: **Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics in Humans.**
Authors: K. Yokoi[1,2], N.G. Egger[1], V. M. Sadagopa Ramanujam[1], H. H. Dayal[1], N. W. Alcock[1] and H. H. Sandstead[1]

Dear Dr. Pessin:

Please find enclosed 4 copies of the above mentioned manuscript to be considered for publication in AJP: Endocrinology and Metabolism, modeling in Physiology.

The manuscript contains 49 pages of text including the appendix. There are 8 tables in the text and 1 table in the appendix. The text and appendix have 2 and 3 figures, respectively.

Please find enclosed the mandatory submission form with the signatures of the authors. Dr. Yokoi's signature was faxed from Japan. Dr. Sandstead has read the manuscript before he went on vacation. His original signature will be mailed to you as soon after he returns.

There are currently no related manuscripts submitted elsewhere or in press. Part of this manuscript has been presented in abstract form in: 1) Journal of The American College of Nutrition, 15, page 530, Abstract 60, 1996 and in 2) The Faseb Journal, 11, page A407, Abstract 2355, 1997.

Sincerely,

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Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics in Humans.

K. Yokoi[1,2], N.G. Egger[1], V. M. Sadagopa Ramanujam[1], H. H. Dayal[1], N. W. Alcock[1] and H. H. Sandstead[1]

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Running Head: Plasma zinc kinetics in humans

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December 23, 1997

Professor Harry L. Pardue,
Purdue University,
1393 BRWN Bldg,
Department of Chemistry,
West Lafayette, IN 47907-1393,

Reference: SIMPLIFIED PRETREATMENT METHOD FOR THE ANALYSIS OF PLASMA SAMPLES APPLICABLE TO ZINC KINETICS AND INDUCTIVELY COUPLED PLASMA - MASS SPECTROMETRY
Authors: V. M. Sadagopa Ramanujam, K. Yokoi, N. G. Egger, H. H. Dayal, N. W. Alcock and H. H. Sandstead.

Dear Professor Pardue:

Please find enclosed 3 copies of the above mentioned manuscript to be considered for publication in Analytica Chimica Acta.

The manuscript contains 19 pages of text including the title page and references with 5 tables and two figures each printed on a separate page.

Sincerely,

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SIMPLIFIED PRETREATMENT METHOD FOR THE ANALYSIS OF PLASMA SAMPLES APPLICABLE TO ZINC KINETICS AND INDUCTIVELY COUPLED PLASMA - MASS SPECTROMETRY

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SUMMARY

The inductively coupled plasma-mass spectrometry (ICP-MS) is rapidly displacing alternative inorganic MS techniques in nutrition research. The main disadvantage is the existence of polyatomic isobaric interferences at some key masses. Isolation of zinc (Zn) from samples by an extraction procedure is recommended to avoid interferences due to polyatomic backgrounds during isotope ratio (IR) MS. However, extraction increases the chances of contamination since Zn is ubiquitous in the environment. The major matrix interference in human plasma or serum are sodium, chloride, and sulfur. Theoretically there are no apparent major isobaric interferences for ^{66}Zn and ^{68}Zn, although $^{32}S^{16}O_2$ and $^{32}S_2$ overlap ^{64}Zn. A series of "simulated human plasma" mineral solutions of single and mixtures of double and all possible mineral elements (S, Na, Cl, K, P, and Ca) were subjected to IR measurements by ICP-MS. The mixture of all mineral elements interfered only with ^{64}Zn (6.66 ng/mL) and ^{70}Zn (8.51 ng/mL). Interferences to ^{66}Zn, ^{67}Zn, and ^{68}Zn are minimal containing 0.90, 0.94, and 0.39 ng/mL, respectively. The mixture of S - Cl, Na - Cl reduced the interference with ^{67}Zn. The co-presence of Na or S affected the chemical reaction of Cl in argon plasma, and the major interferent was shifted from $^{35}Cl^{16}O_2$ (atomic mass 67 coming from Cl solution) to $^{35}Cl_2$. For the comparison of two pretreatment methods, extraction vs nonextraction, blood samples were collected from 10 human subjects 5 min to 24 h and four subjects 5 min to 9 days after injecting 2 mg ^{67}Zn intravenously. The blood plasma was digested by hydrogen peroxide and dissolved in nitric acid, and nebulized into the argon plasma directly for IR's in "nonextracted" samples. In other set, "extraction" of Zn in the digestate was done using diethyl ammonium diethyl dithiocarbamate chelate procedure followed by back extraction of Zn in nitric acid. The normalized IRs obtained

from $^{67}Zn/^{66}Zn$ and $^{67}Zn/^{68}Zn$ in both the "extracted" and "nonextracted" samples agreed well (r^2=0.976 and r^2=0.985, respectively) compared to those from other ratios (r^2=0.838 for $^{67}Zn/^{64}Zn$ and r^2=0.747 for $^{67}Zn/^{70}Zn$). Considering the minimum possibility of isobaric interferences in plasma samples, $^{67}Zn/^{68}Zn$ obtained from "nonextracted" samples is sufficient for routine Zn kinetic analysis.

INTRODUCTION

For the assessment of Zn nutriture, determinations of plasma or serum Zn concentrations are used extensively in clinical practice, with normal plasma or serum Zn concentrations generally considered to be 700 μg/L as a cut off value. However, these concentrations are known to be influenced by many physiological factors [1]. There is a need for improved techniques to assess Zn status and monitor Zn metabolism since the Zn content of accessible tissues does not appear to provide a reliable index of Zn status and plasma or serum zinc concentrations vary with stress conditions unassociated with Zn deficiency. Isotopic techniques seem to provide an answer to this problem.

Radioisotopes of Zn have been used to develop complex mathematical models which describe Zn kinetics under various conditions in man and laboratory animals [2-5]. Such models have been used to identify sites of regulation of Zn metabolism and calculate the size and turnover rate of body Zn pools. A simpler model describing Zn kinetics over a short time period (90 minutes) has been developed using ^{65}Zn in the rat [6]. Using this model it was shown that a rapidly exchanging pool of Zn is responsive to changes in dietary Zn intake, becoming significantly depleted in animals maintained on a Zn deficient diet. ^{65}Zn

has a biological half-life of 500 days [7], and hence its applicability in the study of Zn metabolism in animals or humans is limited.

A potential approach to the study of relationships between dietary Zn supply and body status is the measurement of plasma Zn kinetics following an intravenous injection (i.v.) of a nonradioactive stable Zn isotope. Stable isotopes offer an advantage over radioisotopes in that there is no radiation exposure of the subjects. Stable Zn isotopes offer a clear advantage over radioisotopes in that they occur naturally and hence have been used to study various aspects of zinc metabolism in humans [8-12], although only Jackson et al [13, 14] used ^{67}Zn to examine Zn turnover rates. Several instrumental techniques have been reported for the determination of isotope ratios of Zn including neutron activation analysis [4, 15-17] and mass spectral methods such as thermal ionization [18], fast atom bombardment [12, 19-21], and inductively coupled plasma [22-24].

Recently, we [25, 26] and others [6, 14, 23] have shown that stable isotopes of Zn can be successfully used to measure Zn turnover rates (TR) and exchangeable Zn pools (EZP) which are responsive to changes in Zn status. The availability of and ability to measure stable isotopes of Zn by mass spectrometry make this a viable technique for Zn metabolic studies. Studies from our laboratory indicate that inductively coupled plasma-mass spectrometry (ICP-MS) provides reliable detection of the isotopes of Zn.

The stable isotope kinetics methodology for the determination of Zn status involves: (i) the use of intravenously administered Zn-67 stable isotope tracer, collection of blood at various time points, and (ii) determination of isotope ratios $^{67}Zn/^{64}Zn$, $^{67}Zn/^{66}Zn$, $^{67}Zn/^{68}Zn$ and $^{67}Zn/^{70}Zn$ at each time point using ICP-MS. The data are used to calculate Zn disappearance and turnover rates, and the exchangeable Zn pools after injection. The

findings are related to biochemical and physiological indices of functions in order to establish zinc status.

Isolation of Zn from samples by an extraction procedure has usually been performed to obtain accurate results for mass spectrometric analysis [12, 22, 25, 26]. Unfortunately, the extraction also increases the chances of contamination since Zn is ubiquitous in the environment. The major matrix elements in human blood plasma or serum samples are sodium (Na, 3130-3370 mg/L), chloride (Cl^-, 2940-4120 mg/L), and sulfur (S, 1120-1270 mg/L) [27]. Theoretically there are no apparent major isobaric interferences for ^{66}Zn and ^{68}Zn in blood plasma, although $^{32}S^{16}O_2$ and $^{32}S_2$ overlap ^{64}Zn. Hence, we wanted to test this theory by conducting a detailed study of comparing the isotope ratios obtained from several sets of "extracted" and "nonextracted" plasma samples. In this project we compared the four different isotope ratios obtained from Zn-extracted and Zn-nonextracted digested plasma samples in order to determine whether the "nonextraction" procedure (with little or no contamination) is applicable for routine Zn isotope ratio analysis for kinetic studies in humans.

EXPERIMENTAL

Chemicals, Reagents and Supplies

The enriched stable isotope ^{67}Zn (as oxide, purity 93.11%) was purchased from Oak Ridge National Laboratory (Martin Marietta Energy Systems, Inc., Oak Ridge, TN, U.S.A.). Hydrogen peroxide (30%, ultrapure), nitric acid (ultrapure double-distilled from Vycor), ammonium hydroxide (high purity grade), hydrochloric acid (ACS grade) and sulfuric acid

(ACS grade) were purchased from GFS Chemicals, OH, U.S.A. Hydrochloric acid (suprapure grade) was obtained from EM Science, Gibbstown, NJ.

Sodium nitrate, diammonium monohydrogenphosphate and calcium carbonate (Baker analyzed chemical grades) were purchased from Baker, Phillipsburg, NJ. Potassium nitrate (ACS grade) was obtained from Fisher Chemicals. Carbon tetrachloride (ACS grade) and 2,6-dinitrophenol, and Zn and yttrium reference standard solutions were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Diethyl ammonium diethyl dithiocarbamate was purchased from Tokyo Casei Co., Tokyo, Japan.

Deionized water was prepared using a Milli-Q System (Millipore Corp., Milford, MA, U.S.A.). Monovette syringes containing lithium heparin (10 U/mL blood) used for blood collections and polypropylene sterile tubes (29 x 114 mm size with red caps) free from Zn contamination used for plasma digestions were purchased from Sarstedt Inc., Newton, NC, U.S.A. Disposable Falcon polypropylene tubes (15 mL capacity) used for preparing the final ICP-MS digestate solutions and absolute ethanol used to dissolve the 2,6-dinitrophenol indicator were purchased from Fisher Scientific Co., Pittsburgh, PA, U.S.A. The carbon tetrachloride extraction of Zn from the digestates were carried out in hydrochloric acid (10%) washed borosilicate glass tubes (Kimax Inc., Toledo, OH, U.S.A.).

Human Subjects and Zinc Kinetics

Five healthy men and one woman living in Galveston, Texas were the subjects for the 9-day observation. Eleven women in apparent good health who were participating in a study of effects of zinc and iron status on brain function participated in 24-hour observation study. This project was approved by the Institutional Review Board of the University of

Texas Medical Branch and written consent was obtained from all subjects. The disappearance rate for ^{67}Zn from blood plasma, turnover rate, and the exchangeable Zn pool sizes were measured using the procedures well established in our laboratory [25, 26, 28-30].

Zinc kinetics were measured using ^{67}Zn (natural abundance 4.11%; enrichment, 93.11%) chloride which was prepared from ^{67}Zn oxide by dissolving 59.52 mg in a few drops of concentrated hydrochloric acid (ACS grade, GFS Chemicals, Columbus, OH), and heating it to dryness on a hot plate. The synthesized chloride was dissolved in saline (12 mL, corresponds to 0.5 mL = 2 mg of ^{67}Zn), aliquots of 0.5 mL sterilized by passing the solution through Millipore filter (0.2 μM pore size) into glass vials containing 10.0 mL saline. Several of these vials (one per 10 vials) were randomly selected and tested for sterility (University of Texas Medical Branch, Department of Clinical Microbiology and Immunology) and pyrogenicity (Scientific Associates Inc., St. Louis, MO).

Subjects were admitted to the General Clinical Research Center at the University of Texas Medical Branch for administration of ^{67}Zn and collection of blood samples. The diet was limited in bioavailable zinc. At 07:00 A.M., after the subject had fasted at least 12 hours, short Teflon catheters connected to a 3-way stop cock were placed in each antecubital vein and kept open by 0.9% saline solution. After 30 min, a blood sample was taken to establish the baseline $^{67}Zn/^{68}Zn$ ratio.

Then the ^{67}Zn tracer - 2 mg in 10.5 mL saline further diluted to 30 mL in saline - was administered over 3 min (timed by stop watch). The line was flushed rapidly with saline for 30 seconds. Blood samples were then collected from the opposite arm starting 5 min after completion of the ^{67}Zn administration. Additional samples were collected at 5, 15,

30, 40, 50, 60, 90 minutes, and 2, 6, 12 hours, and 1, 2, 3, 5, 7, and 9 days later. The 9-day and 1-day samples were collected from 4 and 10 subjects, respectively. Blood samples were placed in an ice chest after collection and delivered to the laboratory for processing. The blood samples were centrifuged at 2000 rpm for 20 minutes and the plasma layers transferred to polypropylene tubes and stored in a freezer (-70°C) until ready to use.

Digestion of Plasma and Extraction of Zinc

Sample digestion was based on the method of Alcock [31]. Duplicate aliquots of plasma were measured out in 50 mL polypropylene tubes, kept overnight at -70°C, transferred to a freeze-drier and lyophilized overnight, further dried for 8 hours at 80°C in an oven, and digested with 30% hydrogen peroxide (2 aliquots of 5 and 7 mL, high purity grade, GFS Chemical Co.) for 2 days at 85-90°C. The white ash was dissolved in 1.5 mL 1.2 M Ultrapure nitric acid. Several (4 tubes/batch) hydrogen peroxide blanks run throughout the entire procedure were used to calculate ICP-MS IR blank subtractions.

"Extraction": The extraction of Zn was based on the method of Serfass et al [22] modified by Yokoi et al [26]. After digestion of the plasma, the ash was dissolved in 1.5 mL of 1.2 M nitric acid and the solution transferred to 20 mL acid-washed borosilicate tube using the Zn-free polyethylene transfer pipette followed by two washes with deionized water. One drop (40 uL) of 0.1% 2,6-dinitrophenol in 50% ethanol was added to the solution as a pH indicator. Dilute ammonium hydroxide was added in drops with shaking the tube to bring the pH to 2.5 (indicated by the color change to yellow). One mL of 0.25% diethyl ammonium diethyl dithiocarbamate in carbon tetrachloride was added, the tube closed tight with Teflon-lined cap, and the contents shaken vigorously for 2 minutes. Each

tube was allowed to stand until separation of the acid and carbon tetrachloride layers was complete.

The carbon tetrachloride layer containing chelated zinc was transferred to another glass tube carefully using the acid-washed glass pasteur pipette, the Zn-chelate decomposed with 1 mL of 1.2 M nitric acid, and the Zn back-extracted into the acid by vigorously shaking the tube. The back-extration of Zn was usually indicated by the transfer of yellow color into the acid layer followed by its disappearance. If such a transfer did not occur immediately, the solution was allowed to stand for an hour and shaken again to complete the decomposition and transfer steps. Then the top acid layer was transferred to another glass tube and the solution heated overnight at 80°C to remove traces of CCl_4, and made up to 10 ml with Milli-Q deionized water after adding yttrium internal standard (100 uL of 5 mg/L solution in 1% nitric acid). Batches of 12-20 tubes were processed at one time.

"Nonextraction": After digestion of the plasma, the ash was dissolved in 1.5 mL of 1.2 M nitric acid and each solution transferred to a polypropylene falcon tube followed by two washes with Milli-Q deionized water. The yttrium internal standard and Milli-Q water were directly added to the digestate and made up to 10 mL with water.

Solutions for the Measurement of ICP-MS Interferents

Because the preparation of human plasma for isotope ratio ICP-MS analysis involves digestion by hydrogen peroxide and solubilization of the obtained white ash with nitric acid, the main matrix elements in the well digested solution should only be hydrogen, oxygen and nitrogen. The polyatomics that interfere with zinc isotopes (64, 66, 67, 68 and 70 atomic mass units) are limited to the combination of the main matrix (H, O and N), the

plasma minerals (Na, Cl, S, K, P and Ca) and argon (Ar, used as a source for the inductively coupled plasma). Therefore the prepared mineral solutions contained only hydrogen, oxygen and nitrogen except for the focused mineral elements (Na, Cl, S, K, P and Ca) to satisfy these requirements.

Single mineral solutions that contained the respective mineral found in the "actual human plasma" were prepared by dissolving each salt or diluting each acid in Milli-Q water. Nitric acid (1.2 M) was prepared by diluting the concentrated nitric acid using Milli-Q water. Calcium nitrate prepared from calcium carbonate was used to make the calcium solution. Calcium carbonate (1250 mg) was dissolved by a few drops of nitric acid and the excess acid was evaporated by heating on a hot plate to obtain calcium nitrate. To start with, 3600 μg Na/ml as sodium nitrate, 3300 μg Cl/ml as hydrochloric acid, 1200 μg S/ml as sufuric acid, 189 μg K/ml as potassium nitrate, 141 μg P/ml as diammonium monohydrogenphosphate, and 99 μg Ca/ml as calcium nitrate were prepared. At the second step, the solutions containing one tenth of each mineral concentration that usually found in the representative human plasma were prepared in 0.12 M nitric acid with 50 ng Y/ml as an internal standard. Single mineral solutions contained only one mineral element from Na, Cl, S, K, P and Ca. The mixture of two minerals contained two different kinds of mineral elements. The mixture of all minerals contained all the 6 mineral elements (Na, Cl, S, K, P and Ca).

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

A VG PlasmaQuad-1, upgraded to PlasmaQuad-2 plus status (VG Instruments, Winsford, England, U.K.) ICP-MS instrument was used for all isotope ratio measurements.

Each solution was aspirated and nebulized (Meinhardt concentric type) into the argon plasma (8000-6000^{o} K) via a peristaltic pump with a flow rate of approximately 1 mL/min. The yttrium (mass 89) internal standard was used to correct errors due to instrumental drifts during data acquisitions. Isotope ratio analyses were performed using "Peak-Jump Acquire" Isotope Ratio data acquisition mode of the VG PlasmaQuad software. The peak-jump acquisition mode gave better relative standard deviations (RSD <1%) compared to the scan acquisition mode (2-4%). The mass range scanned was 50-95 amu with 200 scan sweeps of 2048 channels, 160 µsec dwell time per channel, and 200 peak jump sweeps with 10240 µsec per peak jump sweep. These mass spectral acquisition parameters normally require about 9 mL of solution and 20 min acquisition time for ten replicate measurements of each sample. Instrument control, methods procedures and the data processing system, including calculations and statistics, were operated via a Compaq AT personal computer with version 3.2 of the VG PlasmaQuad software. All the four Zn isotope ratios ($^{67}Zn/^{64}Zn$, $^{67}Zn/^{66}Zn$, $^{67}Zn/^{68}Zn$, $^{67}Zn/^{70}Zn$) were measured in each sample. The mass discrimination among Zn isotopes was corrected by the frequent measurements of Zn standard solutions (125, 250, and 500 ng/mL) during the sequence of IR analysis.

Measurement of Polyatomic Interferents in the "Simulated Human Plasma" Solutions

After careful cleaning of the sampling/skimmer cones, torch, nebulizer, and the spray chamber, the various "simulated human plasma" mineral solutions prepared to quantify the ICP-MS interferents were introduced into the argon plasma and the counts at the desired atomic mass units (64, 66, 67, 68, 70 and 89) were recorded using "Peak-Jump Acquire" Isotope Ratio data acquisition mode as described earlier. The counts obtained at

the desired atomic mass units were compared with the counts obtained from 250 ng Zn/ml and the equivalent concentrations of the interferents were calculated.

Calculations

Subtraction of the hydrogen peroxide mass spectral signal counts from each sample counts gave the blank-subtracted counts. Using the blank-subtracted signal counts, the four $^{67}Zn/^{64}Zn$, $^{67}Zn/^{66}Zn$, $^{67}Zn/^{68}Zn$, $^{67}Zn/^{70}Zn$ IR values were recalculated for each sample. The value obtained after subtraction of the baseline (zero time) IR from each IR value was divided by the natural Zn IR value to obtain the normalized IR (NIR) value. A data set of 4 normalized isotope ratios (67/64, 67/66, 67/68 and 67/70) x 163 time points x 2 treatments (extraction and nonextraction) obtained from 14 subjects after iv dose of ^{67}Zn was subjected to statistical analysis. All statistical analyses were carried out using the SYSTAT5 (version 5.2.1) Macintosh software (SYSTAT Inc., Evanston, IL).

RESULTS AND DISCUSSION

Inductively coupled plasma-mass spectrometry has become a powerful alternative for the determination of isotope ratio measurements along with other well established techniques such as neutron activation analysis and thermal ionization and fast atom bombardment mass spectrometry. However, when biological material is analyzed by ICP-MS, potential interferences from polyatomic ions must be considered. These interfering polyatomic ions originate mainly from argon, nitrogen, and/or oxygen in combination with Na, S, Cl, and Ca, which are present at approximate concentration ranges of 3130-3370, 1120-1270, 2940-4120, and 92-109 mg/L in human serum, respectively [27]. Zinc has five

isotopes: 64, 66, 67, 68, and 70. The most abundant isotope, ^{64}Zn (48.9%), is interfered to a larger extent by polyatomic ions containing sulfur, oxygen, and calcium.

Polyatomic Interferences During the Isotope Ratio Measurements of the "Simulated Human Plasma" Mineral Solutions

In order to accurately calculate the actual polyatomic background signals generated during the ICP-MS analysis of the digested plasma solutions, the various "simulated human plasma" mineral solutions were subjected to the IR analyses of the routine ICP-MS instrumental conditions. Table 1 shows the equivalent concentrations of the interferents in the mineral solutions to naturally occuring Zn. Investigations of the mineral solutions were limited to the single mineral elements, and the mixture of two mineral elements and the mixture of all mineral elements. The invetigation of the interaction among three mineral elements or more were omitted because of the statistical difficulties. The mixture of all minerals was tested because it was the closest to the digested human plasma. Ten times diluted solutions (in 0.12 M nitric acid) were chosen because we utilized similar dilutions in the on-going Zn nutritional study.

The ten times dilution of the digested human plasma contains approximately 100 ng Zn/ml. A careful study of the results from single element solutions in Table 1 indicates that the polyatomic interferences to ^{64}Zn by sulfur (3.61 ng/mL) and to ^{67}Zn by chlorine (2.58 ng/mL) alone are significant . On the other hand, the mixture of all mineral elements (S, Na, Cl, K, P and Ca) which is approximately equivalent to the digested human plasma, largely interfered only with ^{64}Zn (6.66 ng/mL) and ^{70}Zn (8.51 ng/mL). However, the interferences to ^{66}Zn, ^{67}Zn, and ^{68}Zn are minimal containing 0.90, 0.94, and 0.39 ng/mL, respectively.

It is obvious from these results that interactions among mineral elements evoked the shift of the interferents from 67 to 70 of the atomic mass units. The mixture of Na - Cl, S - Cl, Cl - K and Na - P evoked the interference with ^{70}Zn. The mixture of S - Cl, Na - Cl reduced the interference with ^{67}Zn. These results suggest that co-presence of Na or S affects the chemical reaction of Cl in argon plasma, and the major interferent is shifted from $^{35}Cl^{16}O_2$ (atomic mass 67 coming from Cl solution) to $^{35}Cl_2$. Table 2 summarizes the possible polyatomic species generated by the introduction of single and various combinations of mineral solutions to the inductively coupled argon plasma.

Comparison of Isotope Ratio Results from Human Plasma Samples - "Extraction" versus "Nonextraction"

Table 3 lists the range of normalized isotope ratios (NIRs) for the four isotope ratios chosen. When Zn in sample is extracted, Zn isotopes 64, 66 and 68 can be used as a denominator isotope to calculate normalized isotope ratio. However, the low abundance and counts of ^{70}Zn does not allow an accurate measurement of normalized isotope ratio even after the extraction of Zn. As expected, all the NIR values were found to be the lowest after 9 days of intravenous administration of ^{67}Zn and highest at 5 minutes after injection. Negative values are irrational because all NIRs were obtained only after the administration of ^{67}Zn. The frequency of negative values for NIR was found to be very low; 2 out of 163 values (1.2%) for NIR-A $^{67}Zn/^{70}Zn$ and 7 out of 163 values (4.3%) for NIR-B $^{67}Zn/^{70}Zn$. Negative values were not observed for NIRs obtained from $^{67}Zn/^{66}Zn$ and $^{67}Zn/^{68}Zn$.

Figure 1 shows correlation plots of normalized isotope ratios of $^{67}Zn/^{68}Zn$ versus $^{67}Zn/^{66}Zn$ for extracted (A, $r^2 = 0.998$) and nonextracted (B, $r^2 = 0.992$) plasma samples.

Only at very low NIR values, the data points tend to deviate from linearity for the nonextracted samples. The value of $r^2 = 0.992$ obtained for nonextracted samples is very close to $r^2 = 0.998$ for extracted samples and acceptable for kinetics. Table 4 summarizes the correlations (r^2) between different NIRs obtained from extracted samples only using simple linear regression and double logarithmic (power function fitting) plots. As expected, the correlations are high for all the four isotopes, 64, 66, 67 and 68, due to the removal of the interfering polyatomic background ions during the extraction of Zn. Figure 2 shows the correlaions of normalized isotope ratios for $^{67}Zn/^{68}Zn$ (A, $r^2 = 0.987$) and $^{67}Zn/^{66}Zn$ (B, $r^2 = 0.976$) for extracted versus nonextracted plasma samples.

Table 5 compares the normalized isotope ratios obtained from both the extracted (NIR_A) and nonextracted (NIR_B) samples using simple linear regression and double logarithmic (power function fitting) plots. NIR_B calculated from 67/68 and 67/66 agrees very well with NIR_A. The extent of agreement of A and B batches for 67/68 is followed by 67/66. As expected from the results of the detailed investigation of polyatomic interferences for ^{64}Zn and ^{70}Zn (Tables 1 and 2), NIR_B calculated from 67/64 and 67/70 poorly agreed with NIR_A. The correlations are poor for $^{67}Zn/^{64}Zn$ ($r^2 = 0.838$) and $^{67}Zn/^{70}Zn$ ($r^2 = 0.747$) (see Table 5) due to sulfur and oxygen polyatomic (mostly $^{32}S^{16}O_2$ and $^{32}S_2$) backgrounds at ^{64}Zn mass and shifting of the major interferent, $^{35}Cl^{16}O_2$ (atomic mass 67 coming from Cl in the solution), to $^{35}Cl_2$ (atomic mass 70) in combination with very low natural abundance for ^{70}Zn, respectively.

Ideally extraction of zinc as a purification step appears desirable. The Zn extraction procedure, however, involves many steps and some steps are susceptible to contamination. Zinc is ubiquitous in the environment and contamination of Zn from the environment is

inevitable during extraction. In summary, the regression analyses values (r^2, the slope "a", and the intercept "b") for NIR correlations from both the "extraction" and "nonextraction" methods show high correlations for $^{67}Zn/^{68}Zn$ and $^{67}Zn/^{66}Zn$. Such high correlations for "nonextracted" samples can be routinely achieved by: (a) keeping the resolution of the mass spectrometer between 0.8 and 0.9 amu instead of unit mass resolution, (b) cleaning the skimmer/sampling cones, torch, and the nebulizer prior to analysis of each batch of samples, and (c) passing nitric acid (1%) followed by Milli-Q water between the samples until the ^{89}Y (internal standard) signal reaches below 200 counts (<200 Hz at the rate-meter). The resolution of the mass spectrometer is crucial to reduce the unexpected backgrounds.

Considering the possibility of isobaric interferences generated during the ionization processes of the digested plasma samples inside the inductively coupled plasma of the ICP-MS coupled with this detailed investigation indicate that $^{67}Zn/^{68}Zn$ and $^{67}Zn/^{66}Zn$ NIRs with least possibility of polyatomic backgrounds obtained from "nonextracted" samples are sufficient for routine Zn kinetic analysis using ^{67}Zn enriched isotope. It should be pointed out that since the polyatomic backgrounds at atomic mass 67 are shifted to atomic mass 70, the "nonextraction" procedure may not be suitable for Zn kinetic analysis using ^{70}Zn enriched stable isotope.

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Table 1. The equivalent concentrations (ng/ml) of the interferents in the mineral solutions to naturally occurring zinc.

	Mass number				
	64	66	67	68	70
Single mineral					
S	3.61	0.38	0.01	0.07	-1.45
Na	0.59	0.48	0.28	0.49	0.60
Cl	0.25	0.25	2.58	0.30	1.59
K	-0.08	-0.09	-0.15	-0.10	-0.47
P	-0.09	-0.11	-0.14	-0.11	-0.63
Ca	-0.08	-0.09	-0.15	-0.09	-0.58
Mixture of two minerals					
S - Na	5.53	0.55	0.22	0.10	0.00
S - Cl	5.08	0.65	0.94	0.29	6.82
S - K	4.19	0.17	-0.13	-0.19	-0.62
S - P	3.96	0.32	-0.02	-0.03	-0.58
S - Ca	3.45	0.13	-0.05	-0.15	-0.80
Na - Cl	1.02	0.63	1.00	0.65	13.35
Na - K	0.50	0.27	0.40	0.29	1.27
Na - P	0.74	0.38	0.67	0.40	2.15
Na - Ca	0.45	0.31	0.48	0.33	0.83
Cl - K	0.17	0.16	2.78	0.21	3.10
Cl - P	0.22	0.22	2.65	0.25	1.34
Cl - Ca	0.15	0.15	1.94	0.17	1.14
K - P	-0.08	-0.09	0.08	-0.08	-0.23
K - Ca	0.00	-0.01	0.11	0.00	-0.23
P - Ca	0.07	0.06	0.32	0.08	-0.23
Mixture of all minerals	6.66	0.90	0.94	0.39	8.51
Sum of the single minerals	4.19	0.82	2.43	0.57	-0.95

Each solution contains one tenth of the mineral concentration(s) found in the representative human plasma.

S: 120 μg/ml S as H_2SO_4. Na: 330 μg/ml Na as $NaNO_3$. Cl: 360 μg/ml as HCl. K: 18.9 μg/ml K as KNO_3. P: 14.1 μg/ml as $(NH_4)_2HPO_4$. Ca: 9.9 μg/ml Ca as $Ca(NO_3)_2$.

All mineral solutions were prepared in 0.12 M HNO_3.

Table 2. Possible polyatomics generated by the introduction of the mineral solutions to inductively coupled argon plasma.

	Mass number				
	64	66	67	68	70
Single mineral					
S	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
Na	-	-	-	-	-
Cl	-	-	$^{35}Cl^{16}O_2$	-	-
K	-	-	-	-	-
P	-	-	-	-	-
Ca	-	-	-	-	-
Mixture of two minerals					
S - Na	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - Cl	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	$^{35}Cl_2$
S - K	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - P	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - Ca	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
Na - Cl	-	-	-	-	$^{35}Cl_2$
Na - K	-	-	-	-	-
Na - P	-	-	-	-	$^{23}Na^{31}P^{16}O$
Na - Ca	-	-	-	-	-
Cl - K	-	-	$^{35}Cl^{16}O_2$	-	$^{35}Cl_2$
Cl - P	-	-	$^{35}Cl^{16}O_2$	-	-
Cl - Ca	-	-	-	-	-
K - P	-	-	-	-	-
K - Ca	-	-	-	-	-
P - Ca	-	-	-	-	-
Mixture of all minerals	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	$^{35}Cl_2$
Sum of the single minerals	$^{32}S^{16}O_2$, $^{32}S_2$	-	$^{35}Cl^{16}O_2$	-	-

Each solution contains one tenth of the mineral concentration(s) found in the representative human plasma.

S: 120 μg/ml S as H_2SO_4. Na: 330 μg/ml Na as $NaNO_3$. Cl: 360 μg/ml as HCl. K: 18.9 μg/ml K as KNO_3. P: 14.1 μg/ml as $(NH_4)_2HPO_4$. Ca: 9.9 μg/ml Ca as $Ca(NO_3)_2$.

Table 3. The range of Normalized Isotope Ratios (NIRs)

	"Extracted" Samples			
	67/64	**67/66**	**67/68**	**67/70**
Minimum	0.04	0.06	0.06	-0.43
Median	0.78	0.75	0.72	0.68
Maximum	14.33	13.67	12.91	12.89

	"Nonextracted" Samples			
	67/64	**67/66**	**67/68**	**67/70**
Minimum	-0.160	0.05	0.06	0.05
Median	0.53	0.71	0.75	0.69
Maximum	12.42	13.23	12.69	12.15

Minimum NIR was found 9 days after i.v. dose of ^{67}Zn.

Maximum NIR was found 5 minutes after i.v. ^{67}Zn administration.

Negative values are irrational because all NIRs were obtained after administration of ^{67}Zn.

Table 4. Correlations (r^2) between different NIRs obtained from extracted samples using simple linear regression and double logarithmic (power function fitting) plots

Simple Linear Regression Plot

	67/66	67/68	67/70
67/64	0.996	0.994	0.887
67/66		0.999	0.889
67/68			0.802

Double Logarithmic Plot

	67/66	67/68	67/70*
67/64	0.996	0.991	0.786
67/66		0.998	0.794
67/68			0.893

*Negative values were removed for calculation.

Table 5. Comparison of normalized Zn isotope ratios (NIRs) obtained from extracted (A batch, NIR_A) and nonextracted (B batch, NIR_B) samples using simple linear regression and power function fitting (double logarithmic) plots

Simple Linear Plot			
NIR	**r^2**	**a**	**b**
67/64	0.838	0.059	0.175
67/66	0.983	0.985	0.040
67/68	0.985	0.964	0.035
67/70	0.747	0.907	0.132
Double Logarithmic Plot			
NIR	**r^2**	**a**	**b**
67/64*	0.838	1.237	0.773
67/66	0.976	1.023	0.958
67/68	0.985	0.987	1.001
67/70*	0.747	0.966	0.903

Regression equation for the simple linear equation is: NIR_A = a NIR_B + b, where 'a' and 'b' are the slope and the intercept, respectively. For perfect correlations, 'a' should be equal to 1 and and 'b' should be zero.

Regression equation for the double logarithmic plot is: NIR_A = a NIR_B power b. If both the values completely agree, then 'a' and 'b' should each be equal to1.

*Negative values are removed because they do not allow fitting.

Legends for Figures

Figure 1. Correlation plots of normalized isotope ratios of $^{67}Zn/^{66}Zn$ versus $^{67}Zn/^{68}Zn$ for extracted (A) and nonextracted (B) plasma samples.

Figure 2. Correlation plots of normalized isotope ratios for $^{67}Zn/^{68}Zn$ (A) and $^{67}Zn/^{66}Zn$ (B) for extracted versus nonextracted plasma samples.

FIGURE 1

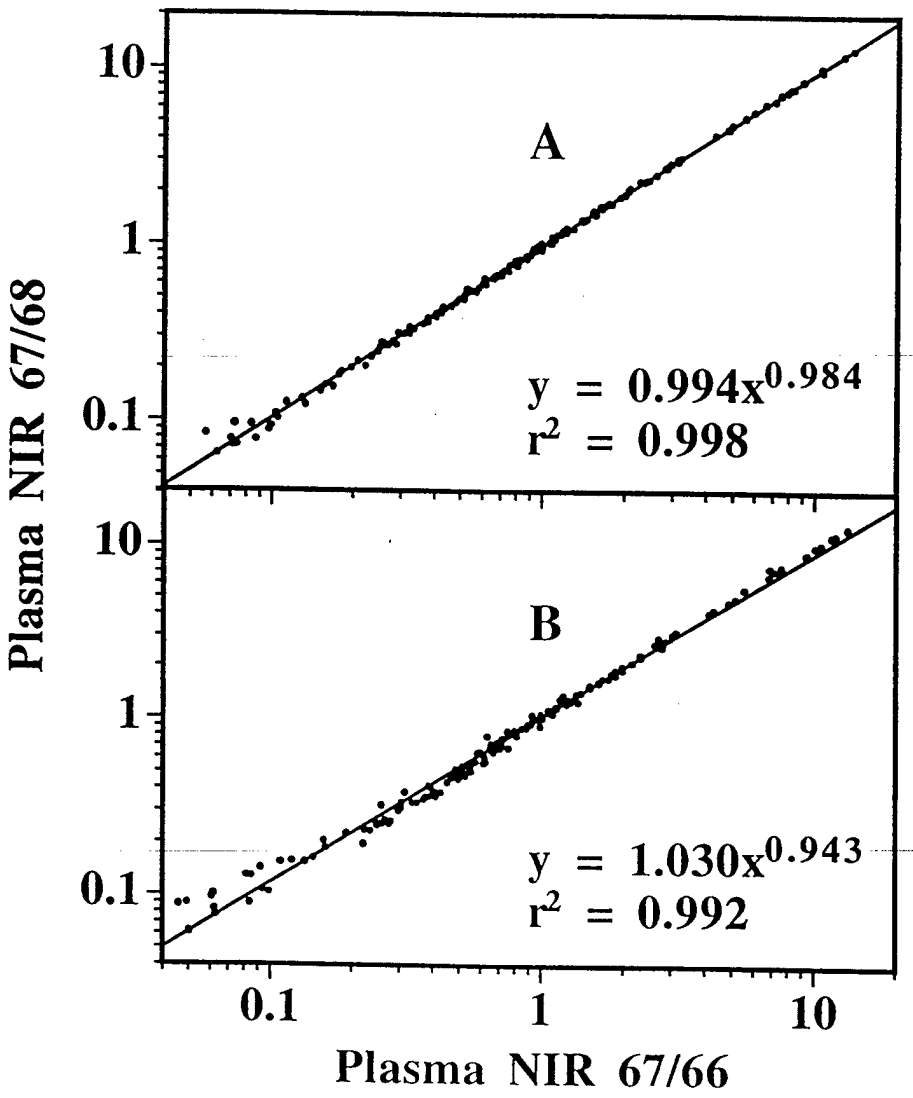
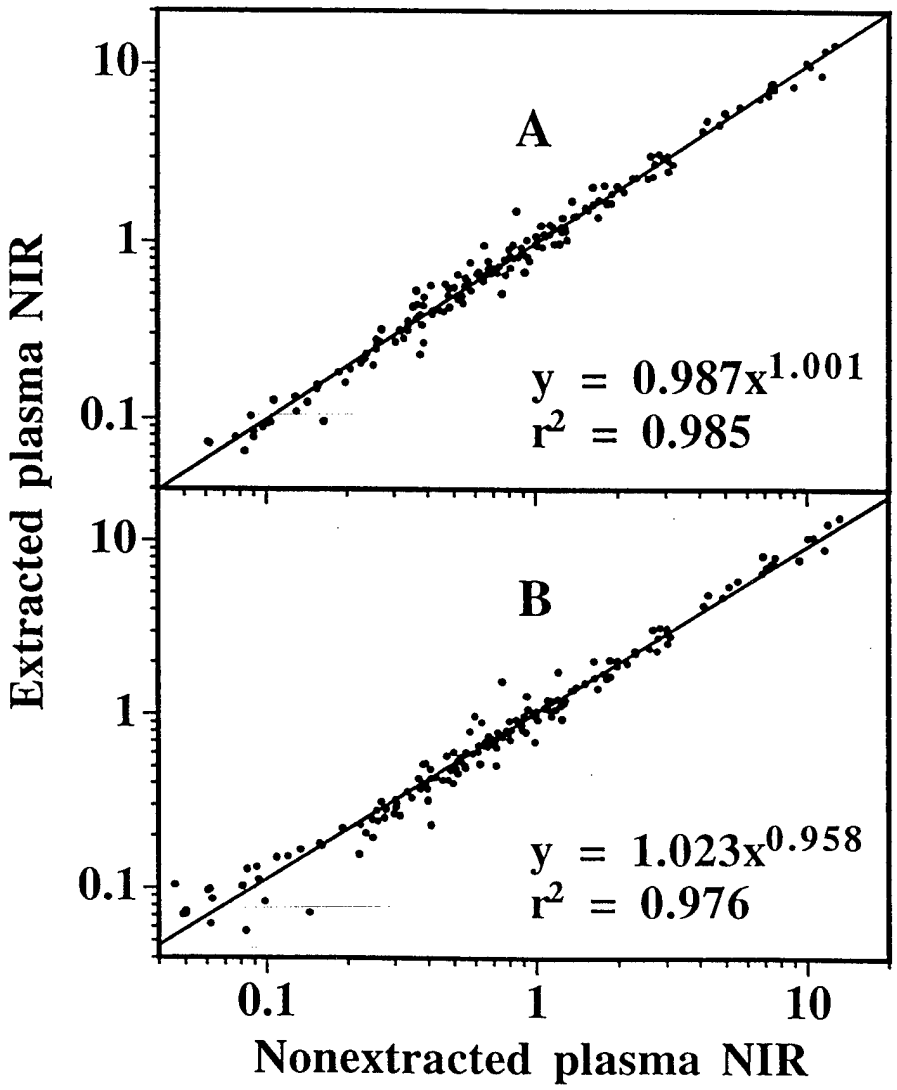

10
1
0.1
A
y = 0.994x^0.984
r^2 = 0.998
10
1
0.1
B
y = 1.030x^0.943
r^2 = 0.992
0.1
1
10
Plasma NIR 67/68
Plasma NIR 67/66

FIGURE 2


A
B
y = 0.987x^1.001
r^2 = 0.985
y = 1.023x^0.958
r^2 = 0.976
10
1
0.1
Extracted plasma NIR
Nonextracted plasma NIR

Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics in Humans.

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ABSTRACT

A practical method for zinc (Zn) kinetics based on a short observation period (24 hours) was developed and compared with an earlier described nine-day observation period. Plasma Zn isotope ratios (IR) were measured by inductively coupled plasma - mass spectrometry 0 to 9 days after iv administration of ^{67}Zn to six subjects (5 men and 1 woman). After baseline subtraction the plasma Zn IR was divided by the natural Zn IR to yield the normalized IR (NIR). The tri-exponential function explained NIR from 0 - 9 days ($r^2 = 0.99$). NIR from 0 - 24 hours was explained ($r^2 = 0.99$) by the above function when the last exponential term was replaced with the constant term. The calculated Zn pool, obtained from just one spot plasma sample 24 hr after isotope administration, was highly correlated ($r^2 = 0.974$) with the sum of three pools in the mammillary and catenary models. This suggests that this one-day spot plasma Zn pool was a practical indicator of the rapidly exchangeable Zn pool which is believed to be metabolically important. Turnover rate could be estimated from the initial two points (5 and 15 minutes) after iv administration of ^{67}Zn. These results indicate that our 24 hour observation period is concordant with the earlier described longer nine day observation period for calculation of Zn kinetics and therefore more practical.

Key words: plasma zinc kinetics, ^{67}Zn tracer, isotope ratio, exchangeable zinc pool, turnover rate

INTRODUCTION

Zinc (Zn) is essential for many biochemical functions including protein synthesis and nucleic acid metabolism. It serves as a catalytic component of over 300 enzymes and as a structural component of various proteins, hormones, and nucleotides (1). Human Zn deficiency occurs worldwide (2-4). Biochemical indices for evaluating Zn status are imperfect and the specificity of physiological indices is unknown. Zn kinetic parameters have been measured by others using radioactive Zn. Prasad et al (5) used ^{65}Zn to demonstrate rapid disappearance of plasma Zn in growth stunted adolescents. Aamodt et al (6, 7) observed changes in Zn kinetics after oral loading of 100 mg Zn using ^{69m}Zn and ^{65}Zn. Foster et al (8) and Wastney et al (9) developed an integrated Zn kinetic model using ^{65}Zn and ^{69m}Zn.

Stable Zn isotopes are alternatives. Wastney et al (10) compared the results obtained from ^{65}Zn and ^{70}Zn by neutron activation analysis. Miller et al (11) used stable Zn isotopes and fast atom bombardment mass spectrometry (12) for measuring Zn pools. Using a quadrapole inductively coupled plasma - mass spectrometry (ICP-MS), Lowe et al (13) analyzed the 120 min kinetics with ^{70}Zn and Yokoi et al (14-16) evaluated Zn disappearance from 30 to 60 min after an iv dose of ^{67}Zn. Fairweather-Tait et al (17) measured Zn pools using ^{70}Zn and thermal ionization mass spectrometry (TIMS). Scott and Turnlund (18) adapted Wastney's

approach (9) to ^{67}Zn and ^{70}Zn tracer using TIMS.

There are two mathematical approaches. 1. The deconvolution method (8, 9) which treats remaining Zn tracer in plasma as a forcing function in the convolution integral (19). 2. The conventional compartment method based on coefficients in the polyexponential function fitted to the remaining tracer in plasma (20). The kinetic parameters including the number of exponential terms depend on the observation intervals. We therefore investigated a short-term kinetic model concordant with the long-term kinetic model using ^{67}Zn and quadrapole ICP-MS.

The rapidly exchangeable zinc pool (EZP) is believed to represent metabolically active zinc which relates to its physiological function (9, 11). However, the definition of EZP is not clear. We report the mathematical equivalency of the sum of three pools between the mammillary and catenary models (21), indicating that this sum of pools is a well-defined invariant estimate of EZP.

METHODS

Human subjects

Five healthy men and 1 healthy woman living in Galveston, Texas were the subjects for the 9-day observation (Table 1). This study was approved by the Institutional Review Board of the University of Texas Medical Branch (UTMB) and written consent was obtained from each subject. The study was conducted in the

General Clinical Research Center at UTMB.

Vials containing 2 mg ^{67}Zn in saline were prepared as reported (16, 22). The solutions in the vials were tested for sterility (UTMB Department of Clinical Microbiology and Immunology) and pyrogenicity (Scientific Associates Inc., St. Louis, Missouri). After a 10 hour overnight fast short Teflon catheters were placed in both antecubital veins of the subjects. The catheters were attached to a slow drip of normal saline by a three-way-stop-cock. A baseline blood sample was taken for the $^{67}Zn/^{68}Zn$ ratio. Then 2 mg of sterile, pyrogen free, ^{67}Zn dissolved in normal saline was administered over three minutes through the stop-cock. This was followed by rapid drip of saline for 1 minute. Blood samples were taken from the other catheter at 5, 15, 30, 40, 50, 60 and 90 minutes, 2, 6, 12 and 24 hours, and (2), 3, 5, 7 and 9 days after the iv dose of ^{67}Zn. Amounts of blood taken at each time point were at least 10 mL. Before each blood collection, about 2 ml blood was taken in a plastic syringe to wash out remaining saline from the catheter. Blood samples were taken in a Monovette syringe containing lithium heparin (10 U/mL blood) obtained from Sarstedt. Blood samples were placed in an ice chest during the collection and promptly delivered to the laboratory for processing.

Laboratory wares and Reagents

The enriched ^{67}Zn (as oxide, purity 93%) was purchased from Oak Ridge National Laboratory, Martin Marietta Energy Systems, Inc., Oak Ridge, TN. Hydrogen peroxide (30%, ultrapure), nitric acid (ultrapure double distilled from Vycor),

ammonium hydroxide and hydrochloric acid (ACS grade) were purchased from GFS Chemicals, Ohio. Absolute ethanol was obtained from Fisher Scientific Co, Pittsburgh, PA. Carbon tetrachloride (ACS grade), 2, 6 - dinitrophenol, and Zn and yttrium reference standard solutions were purchased from Aldrich (St. Louis, Missouri, USA). Diethylammonium diethyldithiocarbamate was obtained from Tokyo Kasei, Co., Tokyo, Japan. Deionized water for dilution of the samples was prepared using a Milli-Q system (Millipore Corp, Milford, MA, USA). Argon gas (99.9% high purity grade) was provided to the ICP-MS from a liquefied argon cylinder (Tri-Gas Industrial Gases, Freeport, TX, USA) capable of delivering at least 20 liter/min at a pressure of 80 psi. The carbon tetrachloride extraction of Zn from the digestate was carried out in borosilicate glass tubes (Kimax, Owens-Illinois Inc., Toledo, Ohio, USA). Disposable Falcon polypropylene tubes (15 mL capacity) used for making multiple dilutions of the digestates were purchased from Fisher Scientific Co, Pittsburgh, Philadelphia, USA. Polypropylene sterile tubes (29 x 114 mm size with red caps) free from Zn contamination used for plasma digestion, were purchased from Sarstedt Inc., Newton, North Carolina, USA.

Chemical analysis

The analysis of isotope ratio (IR) $^{67}Zn/^{68}Zn$ in samples was performed as described in our previous studies (16, 22). Zn in the digestate was extracted into the carbon tetrachloride layer as a diethylammonium diethyldithiocarbamate chelate and back-extracted into the diluted nitric acid layer. The purified samples were

analyzed by ICP-MS.

Calculation of the normalized isotope ratio

After baseline subtraction, the plasma Zn IR was divided by the natural Zn IR to give the normalized IR (NIR). The spot pool size was calculated as follows:

Spot pool size = Dose of *iv* tracer / [(NIR).(Natural Abundance of ^{67}Zn)]

Mathematical analysis

Mathematical analysis of the data involved three phases. The initial phase was the development of a mathematical model to explain the disappearance of ^{67}Zn from plasma following a single iv administration during the restricted observation period. In short, this phase determined the number of exponential terms for the shorter observation period (24 h) concordant with the longer observation period (28 d). In addition, this phase estimated the stability of the nonlinear regression using the Monte Carlo simulation.

The second phase of the mathematical analysis involved the determination of invariant kinetic parameters against various connections of pools, i.e., mammillary and catenary models.

The third phase of the mathematical analysis involved the application of the model to the analysis of data obtained from human subjects.

All modeling was done on a Macintosh Powerbook 165C (Apple) using the SYSTAT 5 for Macintosh, version 5.2.1 software (SYSTAT, Inc., Evanston, IL). A logarithmic

transformation of the normalized isotope ratio was used to stabilize the random variation at fitting a polyexponential function to the disappearance data (23).

RESULTS

The first phase of the mathematical analysis

Formulation

The disappearance of Zn tracer from plasma is considered to be explained by a four-exponential function (5, 11) as follows:

$$\textit{Tracer in plasma} = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t} + K_4 e^{-g_4 t}$$
$$\textit{where} \quad g_1 > g_2 > g_3 > g_4$$

If $g_1 >> g_2 >> g_3 >> g_4$ as generally found in zinc kinetics (11), the truncated form of the polyexponential function (the bi- or tri-exponential function with a constant term) can be substituted for the complete form when the observation period is shorter than the half-life of the last term(s). For curve fitting, the truncated form should be used instead of the complete form to avoid hyper-parameterization. In extreme cases, hyper-parameterization often causes inconvergence found in nonlinear regression.

According to Miller et al's (11) model based on Wastney et al's report (9), g_1, g_2, g_3 and g_4 are 137.6, 3.564, 0.1106 and 0.00232, respectively. The corresponding half-lives are 7.25 min, 4.67 h, 6.26 d and 298.7 d. We therefore propose the truncated polyexponential model as follows:

For within 24 h, $Tracer\ in\ plasma = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3$

For within 28 d, $Tracer\ in\ plasma = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t} + K_4$

Table 2 shows the comparison of values in Miller et al's model and our estimated parameters from the 5 min - 24 h values using the bi-exponential function with a constant term. The estimates agreed well with the model values. This is correct with some caution because the above estimation is based on the calculated values from the model without analytical noise.

Table 3 shows the result of the Monte Carlo simulation. The simulation suggests that 1-2% CV in the measurement of the remaining tracer is acceptable to estimate the kinetic parameters if the Zn tracer disappears according to the four-exponential function described by Miller et al (11).

These investigators (11) developed a four-pool model based on Wastney et al's study (9). We also analyzed in plasma of normal subjects the mean remaining tracer after intravenous administration of ^{65}Zn reported by Wastney et al (9). For the data obtained from 0 to 28 days and 0 to 2 days, the truncated polyexponential functions were applied to fit the curve. Because Wastney et al did not report the data one day after administration of tracer, the two-day data were utilized. The value from 0 to 290 days was analyzed with the complete quadri-exponential function as a standard. The proportion parameters K_4 (for the term with 158 d of half-life) and

K_3 (6.66 d) were respectively predicted from the 28 day and 2 day observations, which were shorter than their corresponding half-lives (Table 4).

The second phase of the mathematical analysis

Calculation of the kinetic parameters in the mammillary model

Based on Landaw et al (24), the kinetic parameters in the mammillary model were calculated. Accepting the single outlet assumption as proposed by Miller et al (11), all parameters in the mammillary model are uniquely determined (See Figure 1; Appendix 1). If there are several outlets, only fluxes between pools are uniquely determined (24).

Definition of EZP

Jackson et al (25) and Miller et al (11) defined rapidly exchanging pools of zinc or rapidly exchangeable Zn pools (EZP) as a composite of pools of Zn that exchange completely with plasma within 2 days. Because the system is open to the outside, the system does not reach true isotopic equilibrium but can be in isotopic quasi-equilibrium for certain time intervals (Appendix 2). The extent of isotopic equilibrium (or tracer/tracee equilibrium in a broad sense), i. e., the extent of the mixing is evaluated using time vs the ratio of the isotopic enrichments between two pools. The ratio of isotopic enrichments in pool b to pool a (IER_b/IER_a) reaches a maximum value of 1.426 at t = 0.066 day and a 95 % maximum at t = 0.200 day.

The ratio of isotopic enrichments in pool c to a (IER_c/IER_a) reaches a maximum value of 1.135 at t = 2.752 day and a 95 % maximum at t = 1.130 day. The ratio of isotopic enrichments in the composite of pools a, b and c to pool a (IER_{a+b+c}/IER_a) takes a maximum value of 1.113 at t = 2.718 day and a 95 % maximum at t = 1.040 day. It is reasonable to conclude that pools b and c are 'completely' exchanged with pool a (plasma compartment) within 2 days. It is favored that at t = 1 day the ratio of isotopic enrichments in the composite of pool a, b and c to pool a (IER_{a+b+c}/IER_a) is 1.049 which is close to 1 (true complete exchange). The remaining tracer in the composite of pools a, b and c at t = 1 day is 81.2 %. This result supports the hypothesis that the 24 hour spot plasma pool gives a good estimation of EZP.

Possible methods estimating EZP

Obtaining the "true" EZP requires continuous monitoring of isotope ratios in plasma after tracer administration until infinity using the infinite number of terms in the polyexponential function. This method is ideal but impossible to realize. However, an estimation obtained from a long observation period and a polyexponential function with multiple terms approaches the true EZP (26, 27). Frequent initial sampling and longer observation periods impose a severe limitation on the application of the tracer technique to human zinc metabolism. The first point was discussed by Miller et al, who stated the limitation of the application to children. The second point limits the experimental design and does not allow any change of dietary regimen or another condition during the observation period for

the kinetic analysis, which presupposes the steady state. There is a need for a method for estimation of EZP from a shorter observation period and less sampling. The following is a proposed method to estimate EZP.

Method 1 (open three-pool): A sum of three pools in the open three-pool system (mammillary or catenary) calculated from the 9-day observation interval using a tri-exponential function model, which is considered as norm because it has the longest observation period and requires most frequent sampling.

Method 2 (closed three-pool): A pool calculated from the reciprocal of K_3 in the truncated exponential function model applied to the 24-hour observation period, which is equivalent to the sum of three pools in the closed three-pool system (mammillary or catenary).

Method 3 (constrained open-three pool): A sum of three pools in the open three-pool system (mammillary or catenary) with the parameter restriction that fixes g_3 at 0.120 (the empirical value) calculated from the 24-hour observation interval using a tri-exponential function model.

Method 4 (last term of tri-exponential function): A pool calculated from the reciprocal of K_3 of the third term in the tri-exponential function model.

Method 5 (simple extrapolation of Miller et al (11)): A pool calculated from the reciprocal of the intercept obtained from 3 to 9 day extrapolation to the time of the tracer administration ($t = 0$) using a simple exponential function.

Method 6 (one-day spot plasma pool): A pool calculated from the reciprocal of the

normalized isotope ratio in the spot plasma 24 hours after intravenous administration of tracer.

Considering the pool estimated from the open three-pool method (method 1) as norm, other methods approximate EZP. The restricted open-three pool method (method 3) is essentially a correction of the closed-three pool method (method 2) using the empirical average value of g_3. The restricted open three-pool method is tentative because g_3 might vary more than expected depending on the health condition of the subjects. The first and the second terms are negligible from 3 days after the administration of the tracer, because g_1 and g_2 are larger than g_3. The last term method (method 4) utilizes K_3 of the third term calculated from the 5 minutes to 9 days data. The simple extrapolation method (method 5) of Miller et al (11) approximates K_3 of the third term method from the 3 to 9 day data. The one-day spot plasma pool method (method 6) is the simplest method that requires just two samples, i.e., one day spot and baseline plasma and approximates the closed three-pool method.

Conserved parameters by matrix transformation of three pool systems of mammillary and catenary models

Because there is no theoretical or experimental basis that justifies the mammillary model as a "true" model, the catenary model is also possible for a three-pool system (Figure 1). Therefore, we investigated the invariant kinetic parameters over

different models based on Ramakrishnan's matrix transformation (21). His basic idea was derived from Berman's model (21). As was proven in the Appendix 3, the following kinetic parameters are invariant:

1. Pool size of the central compartment (plasma Zn pool)
2. Sum of the rate constant from the central compartment (initial slope)
3. Flux from the central compartment (plasma Zn turnover rate)
4. Sum of the pool sizes (so-called rapidly exchangeable Zn pool, EZP).

In the following section, we will limit the discussion within the invariant parameters.

The third phase of mathematical analysis: Application to the analysis of data from the human subjects

Figure 2 shows the illustration of curve fitting of the nine-day data using tri-exponential function model. Table 5 shows the determined coefficients for the tri-exponential model for the nine-day data and the truncated model (bi-exponential function with a constant) for 1 day data and the percent deviations of the coefficients of the truncated model from the tri-exponential model. The coefficient g_3 determined from the nine-day data using the tri-exponential model are less changeable compared to other coefficients (CV: 20 % for K_1; 18 % for g_1; 27 % for K_2;

13 % for g_2; 14 % for K_3; 11 % for g_3). Except for g_2, the determined coefficients from the different model (i.e., different observation period) were similar (Mean of the percent deviation: 2.7 % for K_1; 3.9 % for g_1; 8.9 % for K_2; 37.7 % for g_2; 4.8 % for K_3).

Table 6 shows the indicators describing quasi-equilibrium in the open mammillary system. The average values of the indicators that describe the quasi-equilibrium between central compartment and rapidly exchanging pools are similar to the indicators found in Miller's mammillary model, except for the time when IER_{1+2+3}/IER_1 reaches maximum. The notation of the subscripts '1, 2 and 3' of 'IER' for the subjects correspond to 'a, b and c' for Miller's mammillary model that uses four compartments rather than three compartments.

Table 7 shows the comparison of rapidly exchanging Zn pool (EZP) determined by various methods and the percent deviation of various calculated EZP estimates relative to Method 1. Considering EZP determined from Method 1 (open three-pool model), overestimation was obvious in the estimates of EZP from Method 2 (closed three-pool model), Method 4 (last term of tri-exponential function), Method 5 (3 - 9 day extrapolation) and Method 6 (one-day spot plasma pool). Method 2 that constrained g_3 as 0.120 in the open three-pool model corrected the overestimation found in Method 2, that constrained g_3 as 0 in the open three-pool model because the closed three-pool model is mathematically a special case of the open three-pool

model.

The mean percent deviation was 18 % for Method 2, -11 % for Method 3, 23 % for Method 4, 42 % for Method 5, 19 % for Method 6. Although Method 3 was aimed to correct the overestimation, the correction was too large. For Method 5 (3 to 9 day extrapolation) overestimation was larger than other methods. The percent deviation for subject 1 by Method 5 was much larger (66 %) than was observed in another subject. This might be due to the selected interval for simple regression analysis. When the data 2 days later were included (2 - 9 day extrapolation), estimated EZP was 242 mg and the slope was 0.107 day^{-1}. The slope was 0.086 day^{-1} for 3 - 9 day extrapolation, which was smaller than 0.1198 day^{-1} for g_3.

The correlation coefficient between EZP calculated by Method 1 as a norm and other methods were as follows (Table 7): 0.976 ($p = 0.0009$) for Method 2; 0.968 ($p = 0.002$) for Method 3; 0.962 ($p = 0.002$) for Method 4; 0.695 ($p = 0.12$) for Method 5; and 0.974 ($p = 0.001$) for Method 6.

Table 8 shows the plasma Zn turnover rate, i.e., the sum of flux from the central compartment. Since the turnover rate is determined by the initial slope and the intercept, the turnover rate determined from the three different models (open three-pool, closed three-pool and the constrained open three-pool models) agreed very well. The correlation coefficient between the TR estimated from the initial two

points (5 and 15 minutes) and the TR obtained from the open three-pool (as a norm) was 0.996 ($p = 0.00003$). The percent deviation for the estimate by the initial two points was -4.5 ± 2.4 % (Mean ± SD).

DISCUSSION

There are some limitations and demands of a kinetic study of zinc similar to other nutrients. 1: Chemical analysis of stable isotopes limits the observation period of tracer. 2: Frequency of blood sampling and observation period are limited by experimental design and convenience to the subjects. 3: The 'true' model of zinc kinetics is not established. 4: The model derived from the shorter observation period should be concordant with the model built based on the longer observation period.

Even in plasma or serum, Zn distributes in several compartments biochemically or chemically defined (28). Tissue Zn is likely distributed in several compartments rather than a single compartment. Most models assume that plasma Zn is in a single compartment (8, 9, 11, 17). When the number of subcompartments of plasma Zn derived from different chemical species and chemical equilibria among subcompartments are established, the analytical method of the plasma Zn disappearance data must be revised in the future. Some sophisticated multiple compartment modelings utilize tracer data obtained from excreta (urine and feces) and extracorporal detection (liver and thigh) without any knowledge of the chemical

speciation.

Based on Miller's model (11) and the analysis of Wastney's data (9), we have chosen a tri-exponential function to fit the disappearance curve of ^{67}Zn from plasma. As a possible three-pool model with all parameters uniquely determined, the mammillary and catenary models with a single outlet from the central compartment were considered. When the discussion is limited to the central compartment (plasma Zn compartment), EZP and TR, the mathematical analysis revealed that the type of model does not affect the results of parameter estimates. Until we will have the 'true' model or the appropriate approximation for the multi-compartment system, invariant parameters will avoid model-based biased comparisons.

It is impractical and unethical to check the adaptability of models using human subjects. We have rather chosen Monte Carlo simulation using Miller's model as the golden standard. Normal random errors were added using the 'Data' procedure of SYSTAT 5. Monte Carlo simulation revealed that the 2% random error is acceptable to estimate the coefficients in the tri-exponential model (Table 3). Therefore, routine ICP-MS analyses that produce measurement errors less than 1 % are suitable for Zn kinetic study.

Wastney's data (9) and ours demonstrate the appropriate number of terms for

various observation periods (Table 4) that allows concordance of the shorter to the longer observation period. Therefore, we investigated further the method for elucidating the practical indicators of the kinetic parameters determined from data based on several time points over 9 days.

During isotopic quasi-equilibrium, the ratio of isotope enrichment in the peripheral compartment to the central compartment is not monotonous and reaches a maximum at the specified time (Appendix 2). Therefore, we selected the time when the ratio reaches a maximum as a time of quasi-equilibrium, because it is uniquely determined (Table 2-1 in Appendix 2; Table 6).

As is shown in Table 5, the open mammillary system derived from 9 day data of ^{67}Zn disappearance from plasma reached 95 % of the maximum ratio of isotope enrichment in pool 1+2+3 (EZP) to pool 1 (plasma Zn or central compartment) 1.0 to 1.3 day after tracer administration, with an average of 1.1 day. These results guarantee that the method estimating EZP and TR developed by the analysis of Miller's mammillary model is valid. We suspect that the 'natural break point' at 2 days after iv dose of tracer proposed by Miller et al (11) may be a literal description of the quasi-equilibrium after 1 day.

The overestimation from various methods compared to the sum of pools turning over within 48 hours (i.e., the sum of pools 1, 2 and 3 for the open three-pool model)

is originated from the quasi-equilibrium and the loss of tracer from the system (Appendix 2; Table 7), as was described by Miller et al (11). Fortunately, the effect of the quasi-equilibrium is relatively small for estimation of EZP using one-day spot plasma pool because the IER_{1+2+3} / IER_1 at t = 1 day was close to 1 for the subjects. The average loss of the tracer from the system was about 19.7 %, that was similar to the overestimation of EZP by Method 6 (18.6 %). Therefore, the one-day spot plasma pool is a good and practical indicator although it is based on just a single time point.

As a definition, the turnover rate is a product of the initial slope of the time vs semilogarithmic plot of the isotope enrichment and the plasma Zn pool size calculated from the extrapolated intercept to t = 0. The contribution of the later time points is considered smaller than the initial points. The comparison among TR estimated from various methods revealed that the initial two points are enough to estimate TR (Table 8).

In conclusion, EZP and TR derived from the open three-pool model using 9-day data are invariant to the models (mammillary or catenary) and can be utilized as a norm for comparison among individual Zn kinetic parameters. Using the closed three-pool model for 1 day, concordant parameters to the longer observation period (9 days) are obtained. One-day spot plasma pool is a practical indicator of EZP. TR can be practically estimated from the initial two points (5 and 15 minutes) instead of a one-day or nine-day observation period.

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Legends for Figures in the Text

Figure 1. Mammillary and catenary models with three pools

M_1 corresponds to the central compartment in the mammillary model.

C_1 corresponds to the central compartment in the catenary model.

M_2 and M_3 are the peripheral pools.

C_2 and C_3 are the peripheral pools.

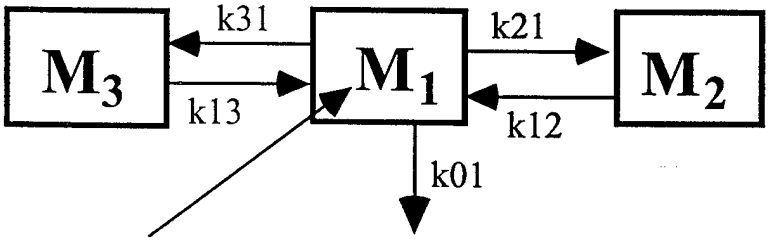
The Mammillary model is a linear kinetic system which has noncentral or peripheral pools, each separately connected to a central pool without interconnection among peripheral pools.

The Catenary model is a linear kinetic system which has several pools sequentially connected with each other in the chain form.

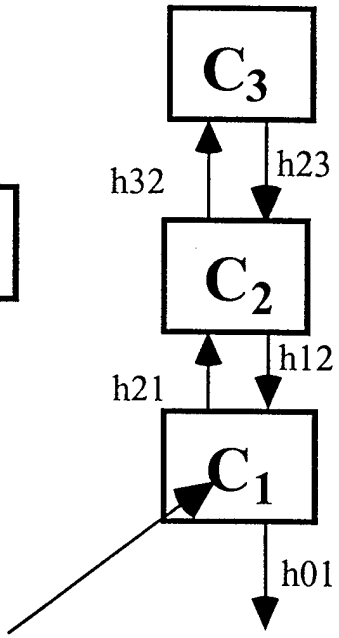
Figure 2. Illustration of the disappearance curve fitted to a triexponential function (Data were obtained from Subject No. 4). Simplex minimization of residual square by nonlinear regression of SYSTAT Software using the following model equation:

$$\text{Logarithm of normalized isotope ratio} = Log\left(K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t}\right)$$

Figure 1



Mammillary Model



Catenary Model

Figure 2

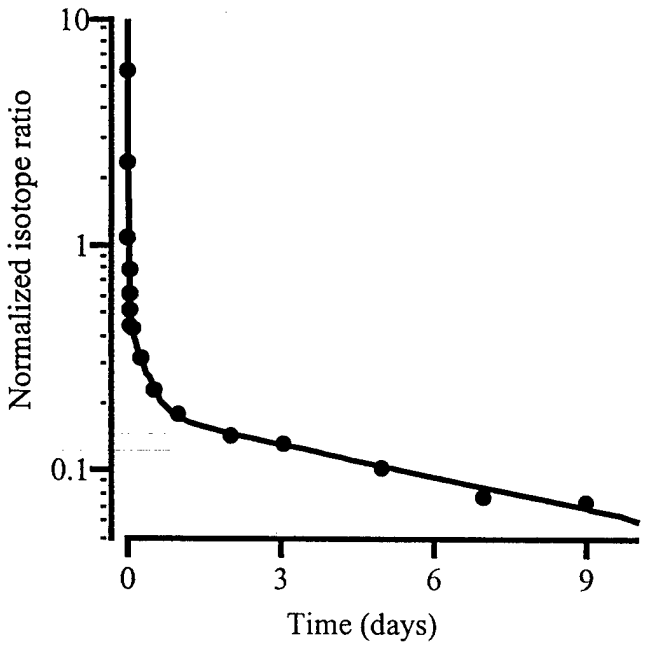


Table 1. Characteristics of the subjects

Subject	Sex	Age	Body height	Body weight	Body mass index
		years	m	kg	kg/m^2
1	Male	44	1.80	77.2	23.8
2	Male	65	1.73	93.2	31.2
3	Male	34	1.69	70.2	24.5
4	Male	42	1.73	78.4	26.3
5	Male	24	1.84	83.5	24.6
6	Female	24	1.76	71.9	23.3

Table 2. Parameter estimates from the 5 min - 24 h values using a bi-exponential function with a constant term according to Miller et al (11)

Parameter	Model value	Estimated parameters from the truncated exponential function	Asymptotic standard errors
K_1	0.9545	0.9538	0.0020
g_1	137.6	137.4	0.2
K_2	0.03046	0.03207	0.00006
g_2	3.564	3.322	0.0185
K_3	0.01443	0.01326	0.00004

The remaining tracer in plasma compartment from 5 min - 24 h was calculated from Miller et al's model.

g_3=0.1106, K_4=0.000628, g_4=0.00232.

These parameters were beyond estimation.

Tracer in plasma in Miller's model = $K_1e^{-g_1t} + K_2e^{-g_2t} + K_3e^{-g_3t} + K_4e^{-g_4t}$

Tracer in plasma in the truncated exponential = $K_1e^{-g_1t} + K_2e^{-g_2t} + K_3$

Table 3. Mean and SD of the estimated parameters from the Monte Carlo simulation with 100 trials using Miller et al's model (11)

	1% Random error given			2% Random error given		
Parameter	Mean	SD	CV	Mean	SD	CV
K_1	0.9523	0.0119	1.25%	0.9510	0.0237	2.49%
g_1	137.2	1.2	0.87%	137.1	2.5	1.82%
K_2	0.03201	0.00037	1.16%	0.03196	0.00074	2.32%
g_2	3.318	0.105	3.16%	3.314	0.210	6.34%
K_3	0.01326	0.00024	1.81%	0.01325	0.00049	3.70%

Estimation was based on the values from 5 min-24 h theoretical values given 1 or 2% noise (constant CV) using normal random numbers. SD is the standard deviation of the estimates. CV indicates the relative standard error of the estimates.

Table 4. Parameter estimates from various observation periods obtained from the analysis of Wastney et al's data (9)

Parameter	0 - 290 days	0 - 28 days	0 - 2 days
K_1	1.18	1.18	1.17
g_1	131	131	130
K_2	0.0433	0.0427	0.0451
g_2	4.50	4.66	4.19
K_3	0.0136	0.0143	0.0136
g_3	0.104	0.118	
K_4	0.00215	0.00228	
g_4	0.00439		

Table 5. Determined coefficients for the tri-exponential model and the truncated model (bi-exponential function with a constant)

Subject	R^2	K_1	g_1	K_2	g_2	K_3	g_3
Tri-exponential model							
1	0.996	9.86	101.4	0.6448	3.813	0.2143	0.1198
2	0.997	9.25	129.4	0.3238	2.953	0.2813	0.1383
3	0.996	12.04	105.5	0.6164	3.587	0.2893	0.1232
4	0.998	10.26	125.3	0.3865	3.530	0.2055	0.1100
5	0.996	10.98	145.1	0.4239	3.919	0.2387	0.1282
6	0.996	15.49	160.5	0.4538	2.825	0.2378	0.1029
Truncated model							
1	0.998	10.14	106.8	0.7092	5.260	0.2354	n.d.
2	0.996	9.39	132.4	0.3578	3.976	0.2773	n.d.
3	0.996	12.63	114.1	0.7237	6.181	0.3263	n.d.
4	0.997	10.33	126.4	0.4065	3.747	0.1953	n.d.
5	0.996	11.29	149.9	0.4533	5.119	0.2453	n.d
6	0.995	15.99	165.6	0.4696	4.086	0.2610	n.d.
Percent deviation of the truncated model from the tri-exponential model							
1		2.9	5.3	10.0	37.9	9.8	
2		1.5	2.3	10.5	34.6	-1.4	
3		4.9	8.2	17.4	72.3	12.8	
4		0.7	0.9	5.2	6.1	-5.0	
5		2.8	3.3	6.9	30.6	2.8	
6		3.3	3.2	3.5	44.6	9.8	

n.d. not defined.

Table 6. Indicators describing quasi-equilibrium in the open mammillary system found in the subjects

Subject	Time when $IER_{1+2+3} = IER_1$	Time when IER_{1+2+3}/IER_1 takes maximum	Maximum of IER_{1+2+3}/IER_1	Time when IER_{1+2+3}/IER_1 takes 95 % maximum	IER_{1+2+3}/IER_1 at t = 1 day	Remaining tracer in pools 1+2+3 at 1 day later
	day	day		day		%
1	0.810	6.9	1.146	1.085	1.068	77.4
2	0.657	8.2	1.176	1.141	1.094	82.3
3	0.823	6.1	1.119	1.058	1.051	79.0
4	0.844	4.6	1.101	1.035	1.040	81.4
5	0.757	6.9	1.097	1.091	1.056	80.2
6	1.035	7.8	1.109	1.304	0.990	81.6
Mean	0.821	6.8	1.125	1.119	1.050	80.3
SD	0.124	1.3	0.031	0.097	0.035	1.8

Table 7. Comparison of rapidly exchanging Zn pool (EZP) by various methods (mg)

	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Subject	Open three-pool*	Closed three-pool ($g_3 = 0$)	Constrained open three-pool ($g_3 = 0.120$)	Last term of tri-exponential	Simple extrapolation	One day spot plasma pool
	5 min - 9 d	5 min - 1 d	5 min - 1 d	5 min - 9 d	3 - 9 d	1 d
1	169	202	148	222	281	206
2	143	172	133	169	178	175
3	131	146	113	164	199	153
4	191	244	178	232	242	233
5	166	194	151	200	244	194
6	162	182	137	200	218	182
Percent deviation from Method 1						
1		19.5	-12.3	31.4	66.3	21.9
2		20.0	-7.2	18.3	24.2	22.1
3		11.3	-13.8	25.5	52.1	16.7
4		27.7	-6.9	21.5	26.7	22.0
5		16.9	-8.8	20.5	47.0	16.9
6		12.3	-15.5	23.5	34.6	12.3

Table 8. Comparison of plasma Zn turnover rate (TR) determined from various models (mg/day)

Models	Open three-pool	Closed three-pool ($g_3 = 0$)	Constrained open three-pool ($g_3 = 0.120$)	Initial two points (5 and 15 minutes)
Interval	5 min - 9 d	5 min - 1 d	5 min - 1 d	5 and 15 min
1 male	415	421	415	406
2 male	587	589	592	557
3 male	361	367	361	358
4 male	520	521	520	488
5 male	561	562	559	528
6 female	452	450	451	421
Mean	483	485	483	460
SD	88	86	89	77
CV	18	18	18	17

Appendix 1.

Solving the mammillary model

Using Landaw et al's (24) algorithm

$$k_{11} = k_{01} + k_{21} + k_{31} = \frac{\sum_{i=1}^{3} K_i g_i}{\sum_{i=1}^{3} K_i}$$

Roots of the numerator after Laplace transformation of tri-exponential function give: $k_{22} = k_{12} + k_{21}$ and $k_{33} = k_{13} + k_{31}$.

$$\gamma_j = k_{ij} k_{ji} = \frac{\sum_{i=1}^{3} K_i}{\sum_{i=1}^{3} \frac{K_i}{\left(k_{jj} - g_i\right)^2}}$$

For the single outlet or closed model, Q_2, Q_3, and 'k's are uniquely determined:

$$k_{22} = k_{12}$$
$$k_{33} = k_{13}$$
$$Q_1 = \text{Dose of tracer (mmol)} / \left(K_1 + K_2 + K_3\right)$$
$$Q_2 = \gamma_2 / k_{12}\ Q_1$$
$$Q_3 = \gamma_3 / k_{13}\ Q_1$$
$$\text{Rapidly Exchangeable Zn Pool (EZP)} = Q_1 + Q_2 + Q_3$$

where Q1 is the plasma Zn compartment (central compartment).

Appendix 2

Quasi-equilibrium in Miller's mammillary model

Tracer / tracee ratios in the respective compartments in Miller's mammillary model can be solved by both the numerical and analytical methods.

Numerical solution of Miller's Mammillary model

The following is the "Mathematica" statement that describes the numerical solution of Miller's mammillary model (open four-pool/single outlet).

NDSolve[{q_a'[t] == 0.92 q_c[t] + 0.0064 q_d[t] + 8.9 q_b[t]

- (2.4 + 40 + 4 + 85) q_a[t],

q_b'[t] == 85 q_a[t] - 8.9 q_b[t],

q_c'[t] == 40 q_a[t] - 0.92 q_c[t],

q_d'[t] == 4 q_a[t] - 0.0064 q_d[t],

q_a[0] == 1, q_b[0] == 0, q_c[0] == 0, q_d[0] == 0},

{q_a, q_b, q_c, q_d}, {t,0.001,10}]

where q_a [t], q_b [t], q_c [t] and q_d [t] correspond to the amount of tracer at time t in the respective pools (a, b, c and d) in Figure 2-1.

Analytical solution of Miller's mammillary model

$$q_a(t) = 0.95449e^{-137.55t} + 0.030457e^{-3.56365t} + 0.0144254e^{-0.110596t} + 0.000628113e^{-0.00231993t}$$
$$q_b(t) = -0.630638e^{-137.55t} + 0.485133e^{-3.56365t} + 0.139504e^{-0.110596t} + 0.0060004e^{-0.00231993t}$$
$$q_c(t) = -0.279438e^{-137.55t} + 0.460832e^{-3.56365t} + 0.712891e^{-0.110596t} + 0.0273783e^{-0.00231993t}$$
$$q_d(t) = -0.0277582e^{-137.55t} + 0.0342478e^{-3.56365t} + 0.55378e^{-0.110596t} + 0.615786e^{-0.00231993t}$$
$$IER_a(t) = q_a(t)/Q_a = q_a(t)/0.037$$
$$IER_b(t) = q_b(t)/Q_b = q_b(t)/0.35$$
$$IER_c(t) = q_c(t)/Q_c = q_c(t)/1.6$$
$$IER_d(t) = q_d(t)/Q_d = q_d(t)/23$$

where q_a [t], q_b [t], q_c [t] and q_d [t] correspond to the amount of tracer at time t in the respective pools (a, b, c and d); IER_a, IER_b, IER_c and IER_d are the isotopic enrichment in pools a, b, c and d in Figure 2-1.

Figure 2-2 and 2-3, and Table 2-1 show the result of the calculation regarding the quasi-equlibrium in Miller's mammillary model.

Table 2-1. Indicators describing quasi-equilibrium in Miller's mammillary model

Pool	Time when $IER_x = IER_a$	Time when IER_x / IER_a takes maximum	Maximum of IER_x / IER_a	Time when IER_x / IER_a takes 95 % maximum	IER_x / IER_a at t = 1 day
	day	day		day	
b	0.029	0.066	1.426	0.200	1.061
c	0.866	2.752	1.135	1.130	1.047
d	34.575	718.608	1.577	56.512	0.013
a+b+c	0.822	2.718	1.113	1.040	1.049

[1] a,b,c and d represent pools in Miller's mammillary model.

[2] a+b+c represents composite of pools a, b and c.

[3] x represents a, b, c or d.

[4] IER represents isotopic enrichments.

Legends for Figures in Appendix 2

Figure 2-1. Miller's mammillary model

The unit for pool sizes are mmol. The unit for the rate constant is day^{-1}.

Figure 2-2. Isotopic enrichment in the compartment of Miller's mammillary model

Figure 2-3. The ratio of isotopic enrichment in pool x to pool a (IER_x/IER_a).

x represents b, c and a+b+c. a+b+c represents the composite of pools a, b and c. About one day after the intravenous administration of tracer, pool b and the composite of pools a, b and c reach 95 % of maximum and are in the state of the quasi-equilibrium. Overshoot was observed in IER_b/IER_a.

Figure 2-1

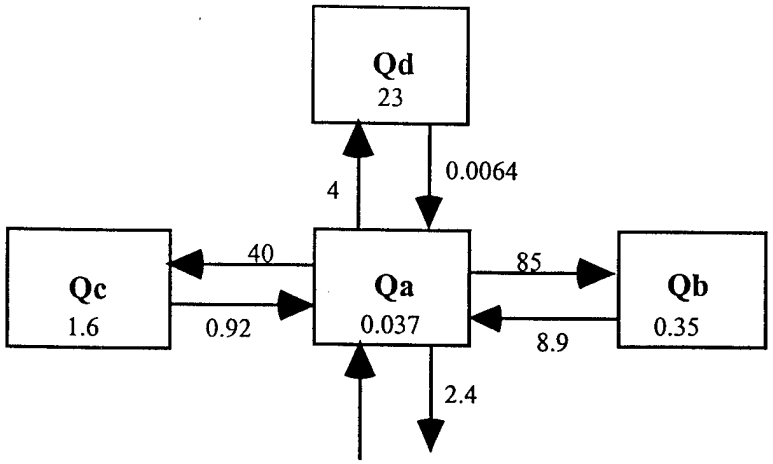


Figure 2-2

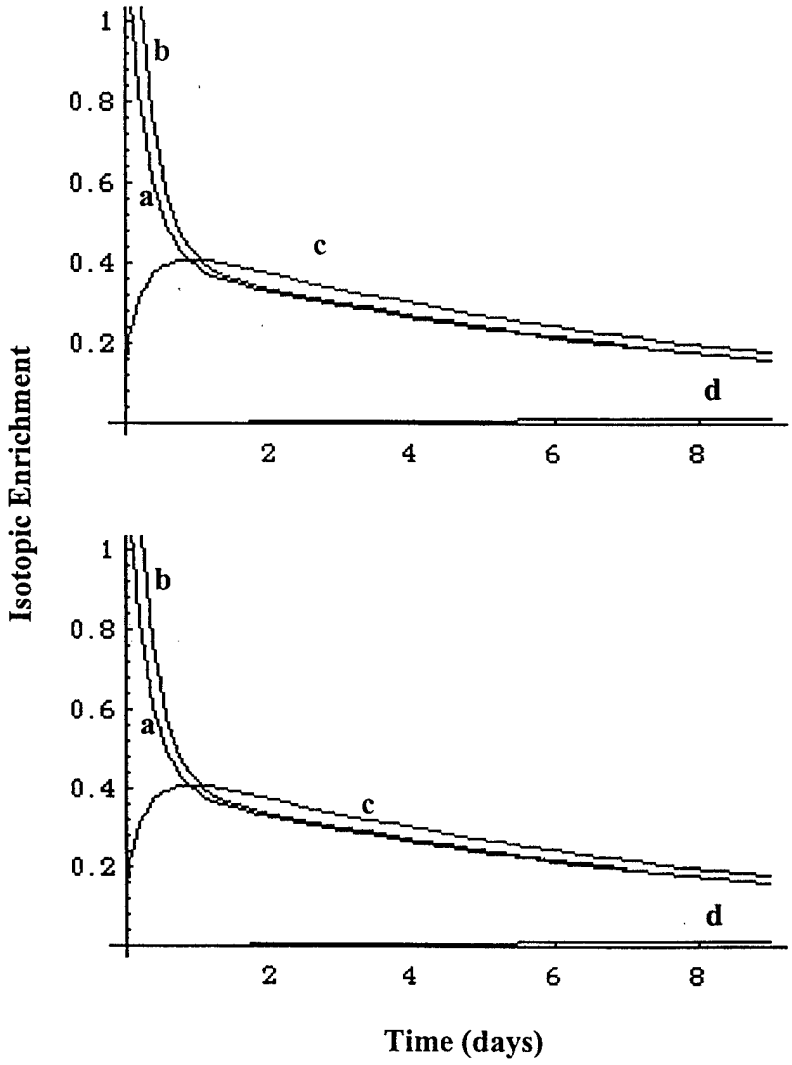
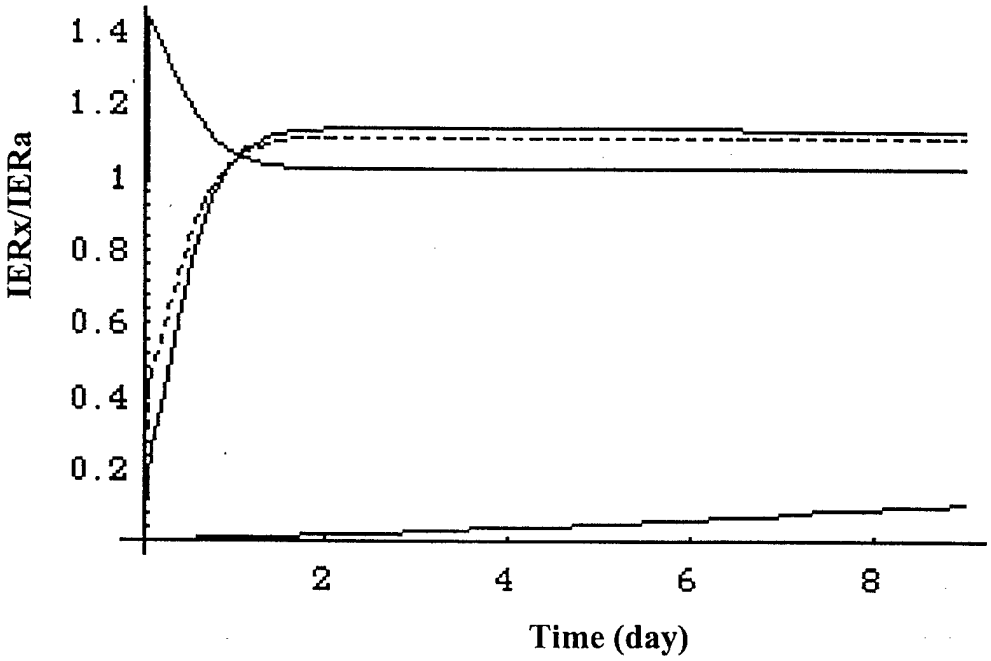


Figure 2-3



Appendix 3.

Conserved parameters by matrix transformation of three pool systems of mammillary and catenary models

When the disappearance curve is described by the tri-exponential function as shown in Figure 1, we have:

$$q_1(t) = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t}$$

where $q_1(t)$ is enrichment in the central Zn compartment, 'K's are linear coefficients, 'g's are exponential coefficient and t is time in days. To give the order to the terms, $g_1 > g_2 > g_3$ is defined without loosing the generality.

Since the pool size of the central compartment is given by the iv dose (D, mmol) divided by the extrapolated enrichment.

$$q_1(0) = K_1 + K_2 + K_3 \quad : Extrapolated enrichment\ at\ t = 0.$$

$$M_1 = \frac{D}{q_1(0)}$$

$$C_1 = \frac{D}{q_1(0)}$$

$$\therefore M_1 = C_1$$

where D is the intravenous dose of the tracer.

The sum of the rate constants from the central compartments is the initial slope of the disappearance curve divided by $q_1(0)$.

$$k_{11} = k_{01} + k_{21} + k_{31}$$

$$= \frac{\sum_{i=1}^{3}(K_i g_i)}{\sum_{i=1}^{3}(K_i)}$$

$$h_{11} = h_{01} + h_{21}$$

$$= \frac{\sum_{i=1}^{3}(K_i g_i)}{\sum_{i=1}^{3}(K_i)}$$

$$\therefore k_{11} = h_{11}$$

$$F_{m11} = k_{11} M_1$$

$$F_{c11} = h_{11} C_1$$

$$\therefore F_{m11} = F_{c11}$$

where F_{m11} and $F_{c11}$is the flux from the central compartment of the mammillary model and the catenary model, respectively.

Ramakrishnan (1984) reported the 'indistinguishability' between the mammillary and catenary models and reported the matrix transformation.

For the mammillary model,

$$A = \begin{bmatrix} -k_{11} & k_{21} & k_{31} \\ k_{12} & -k_{22} & 0 \\ k_{13} & 0 & -k_{33} \end{bmatrix} = \begin{bmatrix} -(k_{01}+k_{21}+k_{31}) & k_{21} & k_{31} \\ k_{12} & -k_{12} & 0 \\ k_{13} & 0 & -k_{13} \end{bmatrix}$$

$$q_m(0) = \begin{pmatrix} q_1(0) & 0 & 0 \end{pmatrix}$$

$$\frac{dq_m}{dt} = q_m A$$

At steady state,

$$\frac{dM_1}{dt} = F_{m10} + k_{12}M_2 + k_{13}M_3 - k_{11}M_1 = 0$$

$$\frac{dM_2}{dt} = k_{21}M_1 - k_{12}M_2 = 0$$

$$\frac{dM_3}{dt} = k_{31}M_1 - k_{13}M_3 = 0$$

$$M_2 = \frac{k_{21}M_1}{k_{12}}$$

$$M_3 = \frac{k_{31}M_1}{k_{13}}$$

$$M_1 + M_2 + M_3 = \left(1 + \frac{k_{21}}{k_{12}} + \frac{k_{31}}{k_{13}}\right)M_1$$

For the catenary model transformed from the mammillary model,

$$B = \begin{bmatrix} -h_{11} & h_{21} & 0 \\ h_{12} & -h_{22} & h_{32} \\ 0 & h_{23} & -h_{33} \end{bmatrix} = \begin{bmatrix} -h_{11} & h_{21} & 0 \\ h_{12} & -(h_{12}+h_{32}) & h_{32} \\ 0 & h_{23} & -h_{23} \end{bmatrix}$$

$$q_c(0) = (q_1(0) \quad 0 \quad 0)$$

$$\frac{dq_c}{dt} = q_c\, B$$

$$= q_c\, T^{-1} A T$$

$$Select\ T^{-1} = P = \begin{bmatrix} 1 & 0 & 0 \\ 0 & \dfrac{k_{21}}{k_{31}+k_{21}} & \dfrac{k_{31}}{k_{31}+k_{21}} \\ 0 & \dfrac{k_{13}}{k_{13}-k_{12}} & \dfrac{k_{12}}{k_{12}-k_{13}} \end{bmatrix}$$

$$\frac{dq_c}{dt} = q_c\, PAP^{-1}$$

Matrix transformation from the mammillary model to the catenary model is as follows:

$$B = PAP^{-1}$$

$$= \begin{bmatrix} -k_{11} & k_{31}+k_{21} & 0 \\ \dfrac{k_{31}k_{13}+k_{21}k_{12}}{k_{31}+k_{21}} & -\dfrac{\left(k_{31}k_{13}^2+k_{21}k_{12}^2\right)}{k_{31}k_{13}+k_{21}k_{12}} & \dfrac{k_{21}k_{31}(k_{12}-k_{13})^2}{(k_{21}+k_{31})(k_{31}k_{13}+k_{21}k_{12})} \\ 0 & \dfrac{k_{12}k_{13}(k_{31}+k_{21})}{k_{31}k_{13}+k_{21}k_{12}} & -\dfrac{k_{12}k_{13}(k_{31}+k_{21})}{k_{31}k_{13}+k_{21}k_{12}} \end{bmatrix}$$

At steady state,

$$\frac{dC_1}{dt} = {}_cF_{10} + h_{12}C_2 - h_{11}C_1 = 0$$

$$\frac{dC_2}{dt} = h_{21}C_1 + h_{23}C_3 - (h_{12} + h_{32})C_2 = 0$$

$$\frac{dC_3}{dt} = k_{32}C_2 - k_{23}C_3 = 0$$

$$C_2 = \frac{h_{21}C_1}{h_{12}}$$

$$= \frac{(k_{21} + k_{31})^2}{k_{12}k_{21} + k_{13}k_{31}} M_1$$

$$C_3 = \frac{h_{32}C_2}{h_{23}}$$

$$= \frac{(k_{12} - k_{13})^2 k_{21}k_{31}}{k_{12}k_{13}(k_{12}k_{21} + k_{13}k_{31})} M_1$$

$$\therefore C_1 + C_2 + C_3 = \left(1 + \frac{k_{21}}{k_{12}} + \frac{k_{31}}{k_{13}}\right) M_1$$

$$= M_1 + M_2 + M_3$$

Summary of the conserved parameters

1. Pool size of the central compartment (plasma Zn pool)
2. Sum of the rate constant from the central compartment
3. Flux from the central compartment (plasma Zn turnover rate)
4. Sum of the pool sizes (so-called rapidly exchangeable Zn pool, EZP)

The University of Texas Medical Branch at Galveston

Division of Human Nutrition,
Department of Preventive Medicine and Community Health

April 15, 1998

Commander
US Army Research Institute of Environmental Medicine
Attn: MCMR-UE-RP/ Ms. Marie E. Stevens
Natick, MA 01760-5007

Subject: Contract No. DAMD 17-95-C-5112 Quarterly Report

Dear Ms. Stevens

Attached is the Quarterly Report for December 23, 1997 to March 22, 1998 for Contract No. DAMD 17-95-C-5112. I apologize for the delay in submitting this report. Subject recruitment has increased this quarter and we have been very busy. If you have questions or need further information, please contact me at 409 772-4661.

Thank you,

Harold H. Sandstead, MD
Professor

Department of Preventive Medicine and Community Health
Division of Human Nutrition
The University of Texas Medical Branch, 700 Harborside Dr.
Galveston, Texas 77555-1109
Phone: 409-772-4661 FAX: 409-772-6287

Principal Investigator: Sandstead, Harold H.
Contract No. DAMD17-95-C-5112

QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 4/15/98

3. Reporting Period from: 12/23/97 to 03/22/98

4. PI: Harold H. Sandstead 5. Telephone No. 409) 772-4661

6. Institution: The University of Texas Medical Branch

7. Project Title: Repletion of Zinc and Iron deficiencies improves cognition of premenopausal women.

8. Current Staff, with percent effort of each on project:

Harold H. Sandstead	15%	Nancy W. Alcock	10 %
VM Sadagopa Ramanujam	25%	Hari H. Dayal	10%
Norman G. Egger	90%	Michael Loftus	100%
Jackie Curtis	75%		

9. Contract expenditures to date (as applicable):

	This Otr/Cumulative		This Otr/Cumulative
Personnel:	33,242/309,651	Travel	0/4,299
Fringe Benefits:	8,648/70,718	Equipment:	0/2,443
Supplies:	7,014/75,597	Other:	0/0

	This Otr/Cumulative
Subtotal:	48,904/462,708
Indirect Costs:	24,452/229,752
Fee:	0/0
TOTAL:	**73,356/692,460** ✓

10. Administrative and logistical matters.

a) During this quarter, 31 women contacted us by phone and expressed their interest (498 since the start of the study); 18 respondents were screened to determine eligibility (202 since the start of the study); 9 subjects completed assessment of zinc status by measurement of zinc concentrations in hair, fasting plasma, platelets, granulocytes and lymphocytes (79 since the start of the study) and were enrolled into the double-blind repletion trial (64 since the start of the study); 5 subjects completed the first eight weeks of treatment and cross over to the second treatment (42 since the start of the study); and 10 subjects completed the study (36 since the start of the study).

b) Enrollment was slower during this quarter, in part because of the holidays. We continue to advertise the study in newspapers (Galveston Daily, Houston Chronicle), at local health clubs, and local and regional University campuses.

c) The error in the treatment preparation that occurred last quarter was corrected. Subjects who were dropped were offered an opportunity to re-enroll after a 3 month was-out interval.

11. Scientific progress:

a) Initial findings from the laboratory of our collaborator at the University of Alabama School of Medicine, Dr. T Tamura, indicate that of 144 screened individuals 6% had low in plasma and red blood cell folate concentrations; 3% had low concentrations of plasma B12 and 46% had low concentrations of plasma B6. The plasma homocysteine were low.
b) The leukocyte and platelet isolation was performed on blood from 9 subjects. Zinc analysis was completed on 6. Platelet count ranged from 242 - 2,097 x $10^3/\mu l$. Zinc concentration was below the reference range of 3.0 - 6.6 $\mu g/10^{10}$ cells, in 2 of the 6 samples. Lymphocyte counts ranged from 1.77 to 13.28 x $10^3/\mu l$ in the 9 subjects.

The zinc content of the 6 specimens was 93, 136, 182, 216, and 232 $\mu g/10^{10}$ cells (reference range is 45 - 218 $\mu g/10^{10}$ cells). Granulocyte isolation and purity continued to present the greatest variability, as noted by others (Beck, personal communication). Manual differential count on the isolated cells showed poor yield of granulocytes in all but one specimen. The cell count of that specimen was 10.3 x $10^3/\mu l$, and the zinc concentration was 64.1 $\mu g/10^{10}$ cells (reference range 38 - 117 $\mu g/10^{10}$ cells).

c) Serum beta hydroxybutyrate was measured in specimens stored at -70 degrees C, from 14 subjects. No specimen had a value outside the reference range.

d) Analysis of samples of plasma previously collected for measurements of zinc kinetics continues using Inductively Coupled Plasma-Mass Spectrometer (ICP-MS).

e) The two research articles (below), that were submitted to journals have been reviewed and returned for revisions.

"Simplified Pretreatment Method for the Analysis of Plasma Samples Applicable to Zinc Kinetics and Inductively Coupled Plasma-Mass Spectrometry". By V.M. Sadagopa Ramanujam, K. Yokoi, N.G. Egger, H.H. Dayal, N.W. Alcock, and H.H. Sandstead.

"Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics in Humans". By K. Yokoi, N.G. Egger, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead.

f) The three abstracts submitted to Experimental Biology '98, American Society for Nutrition Sciences meeting in San Francisco, California, April 18-22, 1998 were accepted for presentation.

The Intake of Micronutrients Influences Zinc Kinetic Parameters. N. Egger, K. Yokoi, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead.

Principal Investigator: Sandstead, Harold H.
Contract No. DAMD17-95-C-5112

Polyatomics in Zinc Isotope Ratio Analysis of Plasma Samples by ICP-MS and Applicability of Nonextracted Samples for Zinc Kinetics. V.M. Sadagopa Ramanujam, K. Yokoi, N. Egger, H.H. Dayal, N.W. Alcock, H.H. Sandstead.

Measurement of Plasma Chelatable Zinc for Zinc Kinetic Studies in Humans. K. Yokoi, V.M. Sadagopa Ramanujam, N.W. Alcock, N. Egger, H.H. Dayal, H.H. Sandstead.

The University of Texas Medical Branch at Galveston

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School of Allied Health Sciences
School of Nursing

Marine Biomedical Institute
Institute for the Medical Humanities
UTMB Hospitals and Clinics

Preventive Medicine & Community Health
Human Nutrition Division

July 2, 1998

Commander, U.S. Army Research Institute of Environmental Medicine
ATTN: MCMR-UE-RP/Ms. Marie E. Stevens
Natick, MA 01760-5007

Re: Contract No. DAMD 17-95-C-5112 Quarterly Report

Dear Ms. Stevens:

Attached is the Quarterly Report for March 23, 1998 to June 22, 1998 for Contract No. DAMD 17-95-C-5112. If you have questions or need further information, please contact me at 409/772-4661.

Thank you,

Harold H. Sandstead, M.D.
Professor

700 HARBORSIDE DRIVE, EWING HALL 3.102 301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-1109
(409) 772-4661 FAX (409) 772-6287

QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 7/2/98

3. Reporting Period from: 3/23/98 to 6/22/98

4. PI: Harold H. Sandstead 5. Telephone No. (409) 772-4661

6. Institution: The University of Texas Medical Branch

7. Project Title: Repletion of Zinc and Iron deficiencies improves cognition of premenopausal women.

8. Current Staff, with percent effort of each on project:

Harold H. Sandstead	15%	Nancy W. Alcock	10 %
VM Sadagopa Ramanujam	25%	Hari H. Dayal	10 %
Norman G. Egger	90%	Michael Loftus	100 %
Jackie Curtis	75%		

9. Contract expenditures to date (as applicable):

	This Qtr/Cumulative		This Qtr/Cumulative
Personnel:	$32,508/342,5159	Travel:	$0/4,299
Fringe Benefits:	$7,950/78,668	Equipment:	$0/2,443
Supplies:	$4,064/79,661	Other:	$0/0

	This Qtr/Cumulative
Subtotal:	$44,522/507,229
Indirect Costs:	$16,393/246,145
Fee:	$0/0
TOTAL:	**$60,915/753,374**

10. Administrative and logistical matters.

a) During this quarter, 43 women contacted us by phone and expressed their interest (541 since the start of the study); 20 respondents were screened to determine eligibility ((222 since the start of the study); 9 subjects completed assessment of zinc status by measurement of zinc concentrations in hair, fasting plasma, platelets, granulocytes and lymphocytes (88 since the start of the study) and were enrolled into the double-blind repletion trial (73 since the start of the study); 8 subjects completed the first eight weeks of treatment and cross over to the second treatment (50 since the start of the study); and 4 subjects completed the study (40 since the start of the study).

b) Enrollment continues at about the same pace in spite of efforts to speed up the process. We continue to advertise the study in newspapers (Galveston Daily, Houston Chronicle), at local health clubs, local and regional University campuses, and in the local internet announcements the University schedule.

c) The study coordinator resigned to accept a similar, but higher paying position at MD Anderson Cancer Center in Houston. A replacement has been identified. She will transfer to this position for elsewhere in the University in early July.

11. Scientific progress:

a) The two research articles cited in last quarters report have been revised. One "Simplified Pretreatment Method for the Analysis of Plasma Samples Applicable to Zinc Kinetics and Inductively Coupled Plasma-Mass Spectrometry". By V.M. Sadagopa Ramanujam, K. Yokoi, N.G. Egger, H.H. Dayal, N.W. Alcock, and H.H. Sandstead has been submitted to "Biological Trace Element Research." The other, "Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics in Humans". By K. Yokoi, N.G. Egger, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead will be returned to the American J Physiology when all authors indicate agreement with the revisions.

b) Three abstracts (below) were presented as posters at the annual meeting of the American Society for Nutrition Sciences in San Francisco, California, April 18-22, 1998.

The Intake of Micronutrients Influences Zinc Kinetic Parameters. N. Egger, K. Yokoi, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead.

Polyatomics in Zinc Isotope Ratio Analysis of Plasma Samples by ICP-MS and Applicability of Nonextracted Samples for Zinc Kinetics. V.M. Sadagopa Ramanujam, K. Yokoi, N. Egger, H.H. Dayal, N.W. Alcock, H.H. Sandstead.

Measurement of Plasma Chelatable Zinc for Zinc Kinetic Studies in Humans. K. Yokoi, V.M. Sadagopa Ramanujam, N.W. Alcock, N. Egger, H.H. Dayal, H.H. Sandstead.

c) Plasma samples from 10 subjects were analyzed by Isotope Ratio ICP-MS for determination of zinc kinetics.
d) Cell isolations were performed on 13 subjects. The mean yield of lymphocytes and granulocytes was 64% and 70% respectively. Yield of platelets averaged 973 x 10-3 / µl. Zinc analysis of the three fractions is pending. Plasma zinc was measured in 133 subjects. The plasma samples are from various stages of the project. The plasma zinc concentrations in 15 of 30 subjects at initial screening was less than 70 µg/dL (reference range 70 - 120 µg/dL). In 10 of 34 subjects at baseline (start of the intervention trial), 14 of 38 after 8 weeks of randomized double-blind treatment (treatments were placebo, micronutrients only, zinc plus micronutrients, and iron plus micronutrients) and 14 of 23 from crossover to the new treatment and after 8 weeks of supplementation were less than 70 µg/dL. We are currently measuring the zinc in 24-hour urine specimens that were collected before nutritional repletion. We are also examining correlations of plasma and urine zinc concentrations with serum ferritin concentrations.

QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 11/6/98

3. Reporting Period from: 6/23/98 to 9/22/98

4. PI: Harold H. Sandstead 5. Telephone No. (409) 772-4661

6. Institution: The University of Texas Medical Branch

7. Project Title: Repletion of Zinc and Iron deficiencies improves cognition of premenopausal women.

8. Current Staff, with percent effort of each on project:

Harold H. Sandstead	15%	Nancy W. Alcock	10 %
VM Sadagopa Ramanujam	25%	Hari H. Dayal	10 %
Norman G. Egger	90%	Michael Loftus	100 %
Renee Galloway	75%		

9. Contract expenditures to date (as applicable):

	This Qtr/Cumulative		This Qtr/Cumulative
Personnel:	$30,200/372,359	Travel:	$0/4,299
Fringe Benefits:	$7,355/86,023	Equipment:	$0/2,443
Supplies:	$10,600/90,261	Other:	$0/0

	This Qtr/Cumulative
Subtotal:	$48,155/555,385
Indirect Costs:	$24,077/270,222
Fee:	$0/0
TOTAL:	**$72,232/825,606**

10. Administrative and logistical matters.

    a) During this quarter, 75 women contacted us by phone and expressed their interest (616 since the start of the study); 40 (262 since the start of the study) were screened during an outpatient visit and 19 had zinc status determined (107 since the start of the study); 9 were enrolled in the intervention part of the study (82 since the start of the study); 9 subjects completed the first phase of the intervention and started the second phase (59 since the start of the study); and 9 completed the study (49 since the start of the study).

    b) Enrollment was satisfactory this quarter.

    c) The administrative secretary accepted another post and was replaced.

11. Scientific progress.

    a) Plasma samples collected from 14 subjects for determination of zinc kinetics at the following times: baseline, 5, 15, 30, 40, 50, 60, 90, 120 min, and 24 hours were analyzed by isotope Ratio Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS). Before ICP-MS analysis 4 sets of plasma were digested with hydrogen peroxide and the zinc extracted, and 10 sets of plasma were digested with hydrogen peroxide but not extracted. Zinc kinetics were calculated for 31 subjects using data from non-extracted plasma samples. The applicability of 'non-extracted' samples for the determination of zinc kinetic parameters using the isotopic mass ratio 67/68 was established.

    b) The use of a spot urine sample 24 hours after intravenous administration of ^{67}Zn tracer for determination of 24 hour exchangeable zinc pool was determined in 29 women (ages 19-39 years, weight 47-85.5 kg, height 1.49-1.77 m, body mass index 17-36 kg/m^2). The 24-h spot plasma pool was 103-240 mg, the 24-h spot urine pool was 79-222 mg, and the plasma zinc was 625-939 ng/mL. The 24-h spot urine pool correlated with the 24-h spot plasma pool ($r=0.91$, $p<0.0001$), weight ($r=0.66$, $p<0.001$), and lean body mass ($r=0.73$, $p<0.0001$). The ratio of the 24-h spot urine pool to the 24-h spot plasma pool was 0.64-0.98. The findings suggest the 24-h spot urine can be used to estimate the 24 hour exchangeable zinc pool.

c) Two manuscripts were submitted for publication. One, Simplified Pretreatment Method for the Analysis of Plasma Samples Applicable to Zinc Kinetics and Inductively Coupled Plasma-Mass Spectrometry by V.M. Sadagopa Ramanujam, K. Yokoi, N.G. Egger, H.H. Dayal, N.W. Alcock, and H.H. Sandstead, was accepted by *Biological Trace Element Research*. The other, Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics in Humans by K. Yokoi, N.G. Egger, V.M. Sadagopa Ramanujam, H.H. Dayal, N.W. Alcock, H.H. Sandstead, was rejected by the American Journal of Physiology and is being revised for submission to the Journal of Laboratory and Clinical Medicine.